Xanthine oxidase promotes hepatic lipid accumulation through high fat absorption by the small intestine
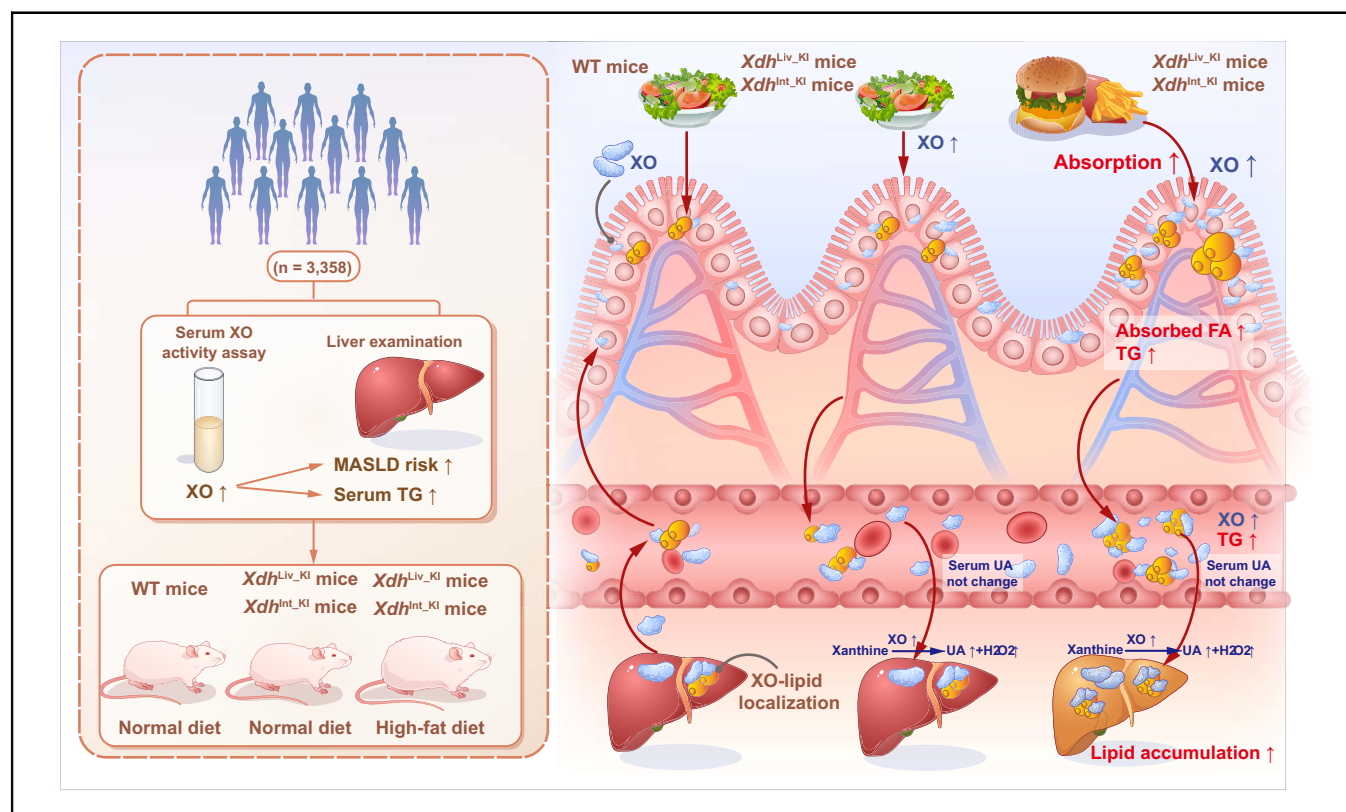
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Lin Liu, Yuntao Zhang, Xuanyang Wang, Hongxue Meng, Yan He, Xiaoqing Xu, Huan Xu, Chunbo Wei, Xuemin Yan, Xinmiao Tao, Keke Dang, Pingnan Ma, Xiaoyu Guo, Sen Yang, Jiemei Wang, Ying Li

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Graphical abstract

Highlights

- Serum XO activity is closely associated with the development of MASLD.

- *Xdh*[Liv-KI] mice displayed rapid obesity in the context of a high-fat diet.

- XO overexpression enhances the absorption of excess dietary fat.

Impact and implications

Using a prospective population-based cohort and various animal models, we have identified novel mechanisms by which xanthine oxidase regulates lipid metabolism. Our findings indicate that xanthine oxidase overexpression promotes lipid accumulation by increasing the absorption of excess dietary fat and possibly facilitating lipid transport *in vivo*. These results could be important for the development of therapies to treat diseases associated with lipid metabolism disorders.

Xanthine oxidase promotes hepatic lipid accumulation through high fat absorption by the small intestine

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Background & Aims: There are no studies investigating the direct effects of elevated xanthine oxidase (XO) on lipid metabolism disorders. Here, we aimed to clarify the role of XO in lipid metabolism in a prospective cohort study and elucidate the underlying mechanisms.

Methods: The association between serum XO activity and metabolic associated steatotic liver disease (MASLD) was examined in Cox proportional hazard models in a population-based cohort of 3,358 participants (20–75 years) at baseline. In addition, mouse models were used to investigate the underlying mechanism for the association between overexpression of XO and the lipid metabolism disorders.

Results: After an average 5.8 years of follow up, we found elevated serum XO activity was associated with an increased risk of developing MASLD (hazard ratio [HR]: 2.08; 95% CI: 1.44–3.01; p-trend <0.001). Moreover, serum XO activity was significantly associated with serum triglyceride levels (r = 0.68, p <0.001). We demonstrated that hepatic XO expression increased in liver samples from patients with MASLD. Using tissue-specific *Xdh* knockin mice, we observed rapid lipid metabolism disorders under a high-fat diet rather than a normal chow diet. We found that XO overexpression promotes the absorption of excess dietary fat in the small intestine. Inhibition of XO also significantly reduced the absorption of fat in mice fed a high-fat diet.

Conclusions: Our study clarified the association between serum XO activity levels and the development of MASLD in a large population-based prospective cohort study. Furthermore, our mouse models demonstrated that XO overexpression promotes lipid accumulation through mechanisms involving excessive fat absorption by the small intestine.

Impact and implications: Using a prospective population-based cohort and various animal models, we have identified novel mechanisms by which xanthine oxidase regulates lipid metabolism. Our findings indicate that xanthine oxidase overexpression promotes lipid accumulation by increasing the absorption of excess dietary fat and possibly facilitating lipid transport *in vivo*. These results could be important for the development of therapies to treat diseases associated with lipid metabolism disorders.

Introduction

Lipid metabolism disorders are often associated with the development of various major diseases, including hypertriglyceridemia, obesity, metabolic associated steatotic liver disease (MASLD), and other metabolic disease.[1–3] It has been observed that hyperuricemia frequently coexists with MASLD, leading to speculation that serum uric acid (SUA) may play a role in the development of MASLD.[4,5] However, the causal relationship between hyperuricemia and MASLD remains uncertain. A comprehensive analysis that includes Mendelian randomization studies has indicated that uric acid levels are only associated with gout and kidney stones, but not with fatty liver disease.[6] Additionally, numerous studies

have suggested that hyperuricemia may serve as a biomarker for metabolic disease rather than being a causal factor itself.[7,8] Consequently, there is still a need to understand the connection between hyperuricemia to MASLD.

Xanthine oxidase (XO) is the rate-limiting enzyme of the uric acid metabolic pathway, catalyzing the production of xanthine from hypoxanthine and uric acid from xanthine, accompanied by the formation of reactive oxide species (ROS), such as superoxide anions and H_2O_2.[9] XO is found in various tissues and organs in mammals, with the highest expression in the liver and intestinal tissues.[10] It is also present in biological fluids such as blood and milk.[11] Although previous research has suggested that serum XO activity, rather than SUA levels, may be a predictor of cardiovascular events in chronic kidney disease patients,[8] direct evidence linking XO to lipid metabolism in the general population is lacking. Excessive activation of XO can lead to oxidative stress and tissue injury, which may result in lipid metabolism disorders.[12] Mouse model studies have demonstrated that inhibiting XO with allopurinol, a XO inhibitor, can protect against lipid

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accumulation caused by high-fat diets.[13] These findings indicate that XO may play a significant role in oxidative stress damage that connects hyperuricemia and MASLD. However, the mechanisms underlying the impact of XO on lipid metabolism are still not well understood.

Here, we examined the association between serum XO activity and lipid metabolism outcomes, including the development of MASLD and serum lipid levels, in a large population-based prospective cohort study (n = 3,358). In addition, we investigated the mechanisms by which XO affects lipid metabolism in various animal models.

Materials and methods
Detailed methods are provided in the Supplementary material.

Study population
The Harbin Cohort Study on Diet, Nutrition, and Chronic Non-communicable Diseases (HDNNCDS) is a multi-stage stratified random cluster sampling health survey conducted in Harbin, China. Detailed information has been previously provided.[14] The baseline survey of the HDNNCDS was conducted in 2012 with 9734 recruited for the study. The present prospective cohort consisted of 3,358 participants (aged 20–75 years at baseline) who participated in the follow-up survey (2015–2018) and met the inclusion criteria. The mean follow-up period was 5.8 years. A total of 9,734 participants from the original cohort were excluded for the following conditions at baseline survey: (1) self-report of diagnosed MASLD and no self-report of liver condition; (2) hepatitis, liver cirrhosis, liver cancer, type 2 diabetes, cardiovascular disease, or other metabolic disease; (3) alcohol consumption of >20 g/day (females) or >30 g/day (males); and (4) taking medication for gout or diuretics. In addition, 321 participants who were lost to follow up were also excluded. The selection of participants is presented in a flowchart (Fig. S1A).

This study was approved by the Ethics Committee of Harbin Medical University and written informed consent was obtained from all participants.

Mice models
All animals were housed under pathogen-free conditions and maintained on a 12 h light/12 h dark cycle at a constant temperature of 24 °C. Mice were fed with AIN93M or XTHF60 (based on AIN93M 60 kCal%) (Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd., Jiangsu, China). The Medical Ethics Committee of Harbin Medical University (Harbin, China) approved all the animal experimental procedures.

Fatty acid uptake imaging
Mice were fasted for 4 h and then received gavage with corn oil (10 ml/g body weight) containing BODIPY 500/510 C1, C12 FAs (2 mg/g body weight; Molecular Probes #D3823) for 2 h. The jejunum was excised and frozen at the optimum cutting temperature. Subsequently, the nuclei of mucosal epithelial cells of the small intestine were localized with DAPI and observed with fluorescence microscopy together with lipid droplets.

Statistical analysis
For the population-based study, the mean (SD) and median (IQR) for continuous variables and frequencies for categorical variables were used to describe the baseline characteristics of the entire study cohort. Log-transformed serum XO activity was divided

into quartiles with equal frequencies. Univariate differences between the groups were tested using general linear models for continuous variables and chi-square tests for categorical variables. Cohen's kappa test was used to measure the agreement between the results of ultrasound and MRI examinations (kappa >0.6 was considered substantial). Cox proportional hazard models were used to evaluate the association between baseline serum XO activity and MASLD morbidity at follow up. Time duration was described as the years between the interview dates of the baseline and follow-up surveys. The proportional hazards assumption of Cox models was tested using the Schoenfeld partial residuals method, and values of $p > 0.05$ were considered to be consistent with the null hypothesis. Trends in continuous variables across XO subgroups were tested using linear regression models. Non-linear association of MASLD morbidity with serum XO activity was conducted using restricted cubic splines to visualize the shape of the dose–response relationship and to investigate whether the relationship should be judged as linear. All data were analyzed using R (version 3.5.3, R Foundation for Statistical Computing, Vienna, Austria), and p values were two-tailed at a significance level of $\alpha = 0.05$.

Results
Long-term prospective cohort study observed the close relationship between serum XO activity and incidence of MASLD
Of the original cohort of 3,679 eligible participants, 1,143 males and 2,215 females who completed the follow-up survey were included in the analysis (Fig. S1A). The median age of the participants at baseline was 49 years (Table S2). Grouping participants according to quartiles of serum XO activity, we observed significant increasing trends in baseline BMI, waist circumference, serum triglycerides (TG), total cholesterol (TC) and LDL-c concentration, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and malondialdehyde (MDA) levels with elevated serum XO activity (all $p < 0.001$) (Fig. 1A). Moreover, serum HDL-c concentration and superoxide dismutase (SOD) enzyme activity decreased with increasing serum XO activity ($p < 0.001$) (Fig. 1A). SOD is widely recognized as an enzyme that participates in the antioxidant enzyme system reaction, whereas MDA is considered a byproduct of membrane lipid peroxidation. Consequently, a decrease in SOD levels and an increase in MDA levels are indicative of elevated levels of oxidative stress in the body. In addition, higher baseline XO activity was associated with significantly increasing trends in follow-up BMI, waist circumference, blood pressure, SUA, TC, TG, LDL-c, AST, and ALT levels (Fig. 1A and Table S3). Similarly, serum XO activity was significantly positively correlated with serum TG, TC, and LDL-c ($r = 0.68$, $p < 0.001$; $r = 0.16$, $p < 0.001$; $r = 0.05$, $p = 0.003$); and negatively correlated with serum HDL-c at follow-up ($r = -0.32$, $p < 0.001$) (Fig. 1C–F).

After an average 5.8 years of follow up, 357 (10.6%) participants developed MASLD, as shown in Fig. 1B. Moreover, increased baseline serum XO activity was associated with a higher risk of MASLD after adjustment for lipid parameters and oxidative indicators (Model 1, Q4 HR: 2.67, 95% CI: 1.88–3.80, P-trend <0.001; Model 2, Q4 HR: 2.29, 95% CI: 1.59–3.28, p-trend <0.001). In addition, the association between XO activity and MASLD was only slightly attenuated when further adjusted for SUA (Model 3, Q4 HR: 2.08, 95% CI: 1.44–3.01, p-trend <0.001; restricted cubic spline P-linear <0.001, P-non-linear = 0.064,
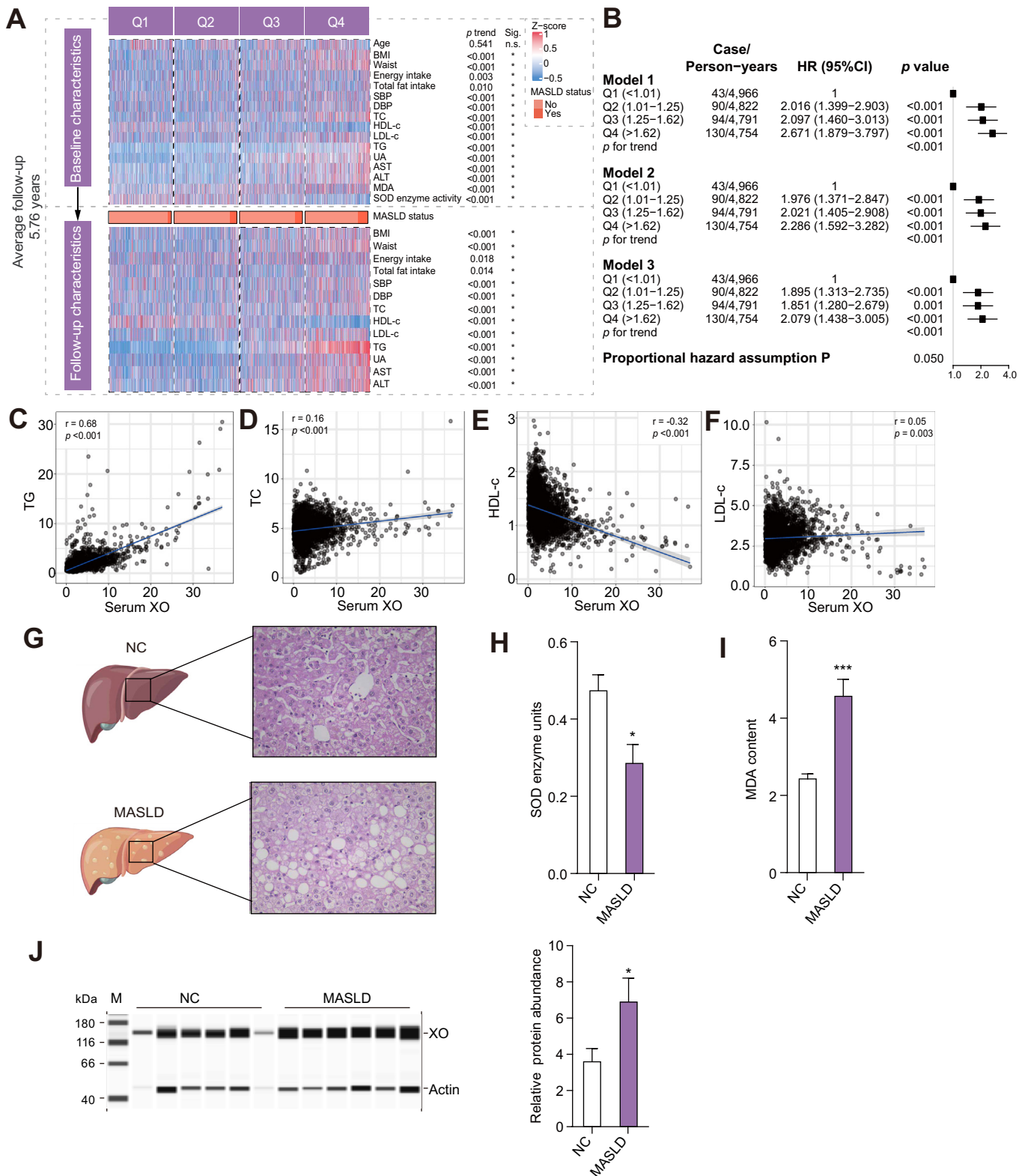
Fig. 1. Association between XO activity and the development of MASLD in a population study. (A) Heatmap of baseline and follow-up characteristics according to quartiles of baseline XO activity. Z-score showed the 95% range of each variable. Follow-up endpoint MASLD status was described as 'YES' and 'NO'. (B) Forest plot of HRs for association between XO activity and MASLD were performed using Cox regression analysis. Q1 to Q4 was calculated according to quartiles of XO activity. Model 1 was adjusted for age, sex, BMI, alcohol consumption, current smoking status, regular exercise, and dietary energy intake; Model 2 was further adjusted serum TC, TG, HDL-c, LDL-c, MDA, and SOD enzyme activity; Model 3 was further adjusted SUA. (C–F) Pearson correlation of serum XO levels with lipid parameters including TG, TC, HDL-c, and LDL-c. (G) H&E staining of human liver samples (scale bar, 100 μm). (H) SOD enzyme units in the liver. (I) MDA content in the liver. (J) Relative expression of XO protein using a Wes approach. All data are presented as mean ± SEM, *p <0.05, **p <0.01, ***p <0.001 (bars represent mean, bold lines on boxes represent standard errors, Student t test). HR, hazard ratio; MASLD, metabolic associated steatotic liver disease; MDA, malonaldehyde; NC, normal control; SOD, superoxide dismutase; SUA, serum uric acid; TC, total cholesterol; TG, triglyceride; XO, xanthine oxidase.
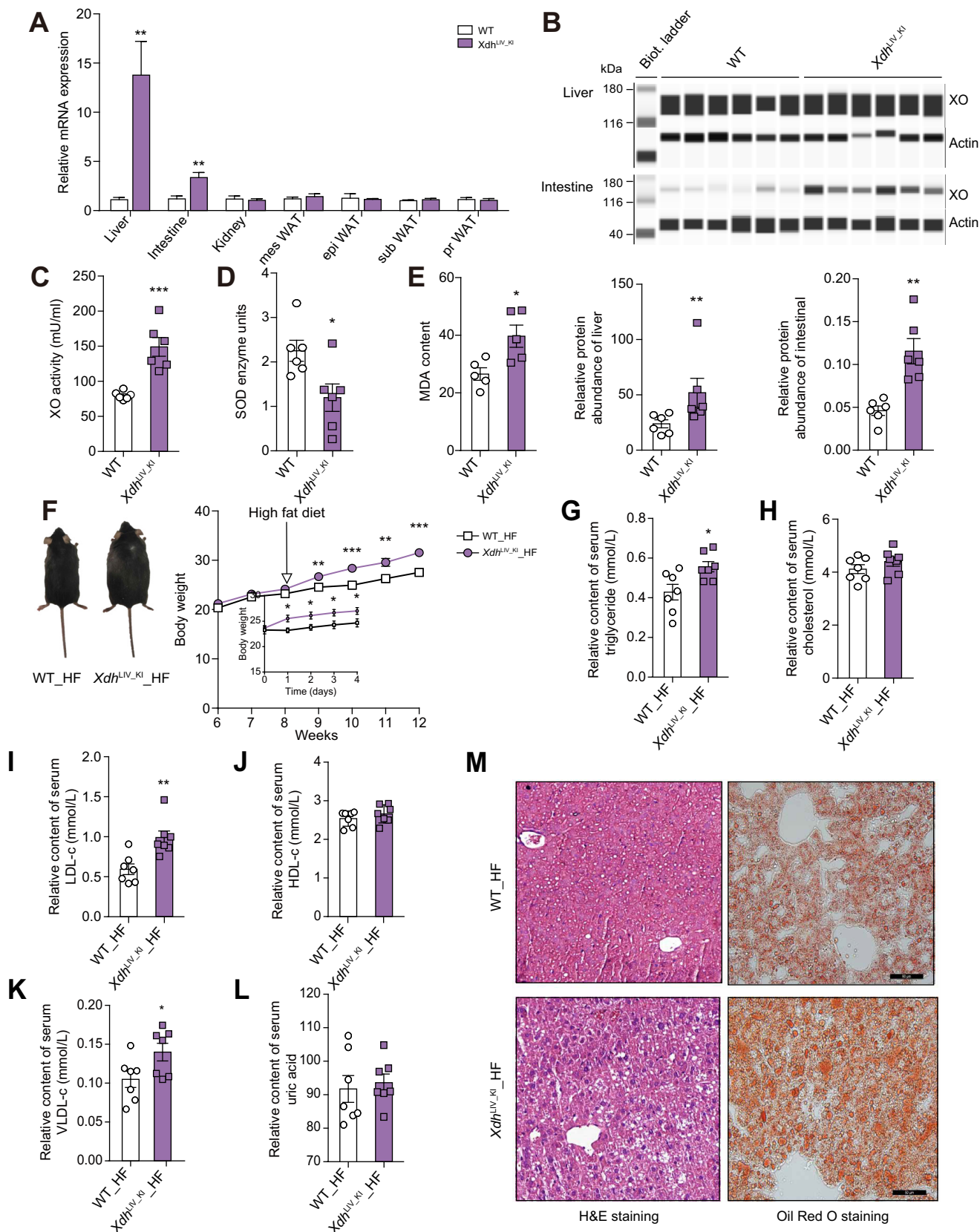
Fig. 2. Phenotype of _Xdh_^{Liv-KI} mice fed on normal chow diet and high-fat diet. (A) Relative expression of _Xdh_ mRNA in various tissues. (B) Relative abundance of XO protein in the liver and intestine. (C) XO activity in two groups of mice. (D) SOD enzyme units measurement in the serum. (E) MDA content in the serum. (F) Changes in body weight between WT mice and _Xdh_^{Liv-KI} mice under a high-fat diet. (G–K) Relative content of serological indicators including TG, TC, LDL-c, HDL-c, and VLDL-c. (L) UA content in the serum. (M) H&E staining (scale bar, 100 μm) and Oil Red O staining (scale bar, 50 μm) of liver from WT mice and _Xdh_^{Liv-KI} mice

p-test of Proportional Hazards (PH) = 0.708) (Fig. 1B and S1B). In summary, we conclude that serum XO activity might be an independent risk factor for the development of MASLD in our cohort study.

Hepatic XO expression also significantly increased in patients with MASLD in the case-control study

Liver samples were collected from patients with and without MASLD to further investigate the relationship between hepatic XO expression and lipid metabolism. H&E staining revealed a significant lipid accumulation in the livers of patients with MASLD (Fig. 1G). Concurrently, oxidative stress levels, as indicated by SOD enzyme activity and MDA content, were also altered (Fig. 1H and I). Moreover, hepatic XO expression was found to be significantly increased in patients with MASLD (Fig. 1J). These findings support the conclusion that XO activity and expression are significantly elevated in patients with MASLD.

Xdh knockin mice showed different responses in lipid metabolism when exposed to a normal chow diet and a high-fat diet

To validate the relationship observed in population studies and determine if a similar relationship exists between XO and lipid metabolism disorders *in vivo*, we created a mouse model of XO overexpression. As XO is predominantly expressed in the liver and intestine, we initially generated mice with a liver-specific *Xdh* knockin (*Xdh*[Liv-KI] mice), as depicted in Fig. S2A. To confirm tissue-specific *Xdh* knockin, we assessed *Xdh* gene expression in various tissues from *Xdh*[Liv-KI] mice. Our findings revealed that *Xdh* mRNA expression was upregulated in the liver and intestine, but not in peripheral adipose tissue and kidney (Fig. 2A). Additionally, XO protein levels increased in the liver and intestine (Fig. 2B). Moreover, XO activity in the serum of *Xdh*[Liv-KI] mice showed a 1.92-fold increase (Fig. 2C), suggesting the secretion properties of XO. To further investigate the role of XO in lipid disorders, *Xdh*[Liv-KI] mice were fed either a normal chow diet or a high-fat diet for 4 weeks. However, no significant lipid disturbances were observed at 12 weeks of age when *Xdh*[Liv-KI] mice were fed a normal chow diet. There were also no notable differences in body weight or serum lipid levels, including serum TG, TC, HDL-c, LDL-c, and SUA levels, between *Xdh*[Liv-KI] and wild-type (WT) mice (Fig. S2B–G). H&E results indicated no pathological changes in the liver (Fig. S2H). Furthermore, *Xdh*[Liv-KI] mice exhibited a decrease in serum SOD activity and an increase in serum MDA content, indicating a significant rise in oxidative stress levels (Fig. 2D and E). Overall, in our animal models fed a normal chow diet, XO overexpression did not result in any significant disturbances in lipid metabolism, despite the presence of elevated oxidative stress levels.

When fed a high-fat diet (60 kcal% fat), *Xdh*[Liv-KI] mice displayed rapid weight gain, with a daily weight gain approximately twice that of WT mice (Fig. 2F). To investigate this response, we initially examined the food intake of *Xdh*[Liv-KI] and WT mice, finding no difference in their daily calorie intake (Fig. S2I). Corresponding to the change in body weight, *Xdh*[Liv-KI] mice exhibited a greater distribution of peripheral fat, particularly in epididymis fat, subcutaneous fat, and mesenteric fat (Fig. S2J).

Furthermore, serum TG levels significantly increased compared with WT mice, whereas serum TC levels did not show a significant difference (Fig. 2G and H). LDL-c and VLDL-c levels demonstrated a significant increase, whereas HDL-c did not show a significant difference (Fig. 2I–K). Moreover, *Xdh*[Liv-KI] mice displayed notable accumulation of hepatic lipid droplets after being fed a high-fat diet, as evidenced by H&E and Oil Red O staining (Fig. 2M). However, there was no significant difference in SUA levels between *Xdh*[Liv-KI] mice and WT mice fed the high-fat diet (Fig. 2L). In conclusion, XO overexpression resulted in lipid accumulation with adequate dietary fat intake independent of SUA levels.

XO overexpression did not regulate directly the lipid synthesis and catabolism

To investigate the mechanisms underlying the rapid onset of obesity in *Xdh*[Liv-KI] mice when fed a high-fat diet, RNA sequencing was performed on the liver and small intestine of *Xdh*[Liv-KI] and WT mice. The results revealed co-enrichment of many lipid metabolic pathways in both the liver and intestine. Fatty acid metabolism pathways were more commonly observed in the liver, whereas the transport of dietary fatty acids was more frequently observed in the small intestine as a result of the distinct roles of these organs in lipid metabolism (Fig. 3, S3A and B). However, there was no clear evidence that overexpression of XO directly regulates lipid synthesis and catabolism, as *Xdh*[Liv-KI] mice did not respond positively to a normal chow diet. To further explore this, we examined the expression of lipid metabolism genes in the livers of *Xdh*[Liv-KI] and WT mice fed a normal chow diet. Interestingly, most of the key lipid metabolism genes that showed significant changes under a high-fat diet became less significant under the normal chow diet (Fig. 4A and B). *In vitro* studies using AML12 cells overexpressing XO also demonstrated that it did not affect the expression of key proteins involved in lipid synthesis and catabolism (Fig. S3C). Therefore, it is likely that the impact of XO overexpression on lipid disorders under a high-fat diet is mediated through its influence on other lipid metabolism processes, rather than on lipid synthesis and catabolism.

XO overexpression in the small intestinal villus enhanced the absorption of excess dietary fat

Given the differential responses of *Xdh*[Liv-KI] mice to various dietary patterns, with no significant difference in food uptake compared with WT mice, we aimed to investigate whether the observed differences were attributable to increased absorption in *Xdh*[Liv-KI] mice. To address this, we administered corn oil containing BODIPY-labeled fatty acids to both *Xdh*[Liv-KI] and WT mice fed a normal chow diet. After a 2-h period, we isolated the jejunum to quantify lipid absorption in enterocytes. As expected, we observed a higher presence of fluorescently labeled lipid droplets in the small intestine of *Xdh*[Liv-KI] mice compared with WT mice, indicating an increase in fatty acid absorption (Fig. 4C). Furthermore, *Xdh*[Liv-KI] mice exhibited a significant reduction in TG content in feces under a high-fat diet, further supporting the notion of increased lipid absorption ability (Fig. 4D). Additionally, we investigated the expression and distribution of XO in the

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under a high-fat diet. All data are presented as mean ± SEM, *p <0.05, **p <0.01, ***p <0.001 (bars represent mean, bold lines on boxes represent standard errors, Student *t* test). epi WAT, epididymal adipose tissue; MDA, malonaldehyde; mes WAT, mesentery adipose tissue; pr WAT, perirenal adipose tissue; SOD, superoxide dismutase; sub WAT, subcutaneous adipose tissue; TC, total cholesterol; TG, triglyceride; UA, uric acid; WT, wild type; XO, xanthine oxidase.
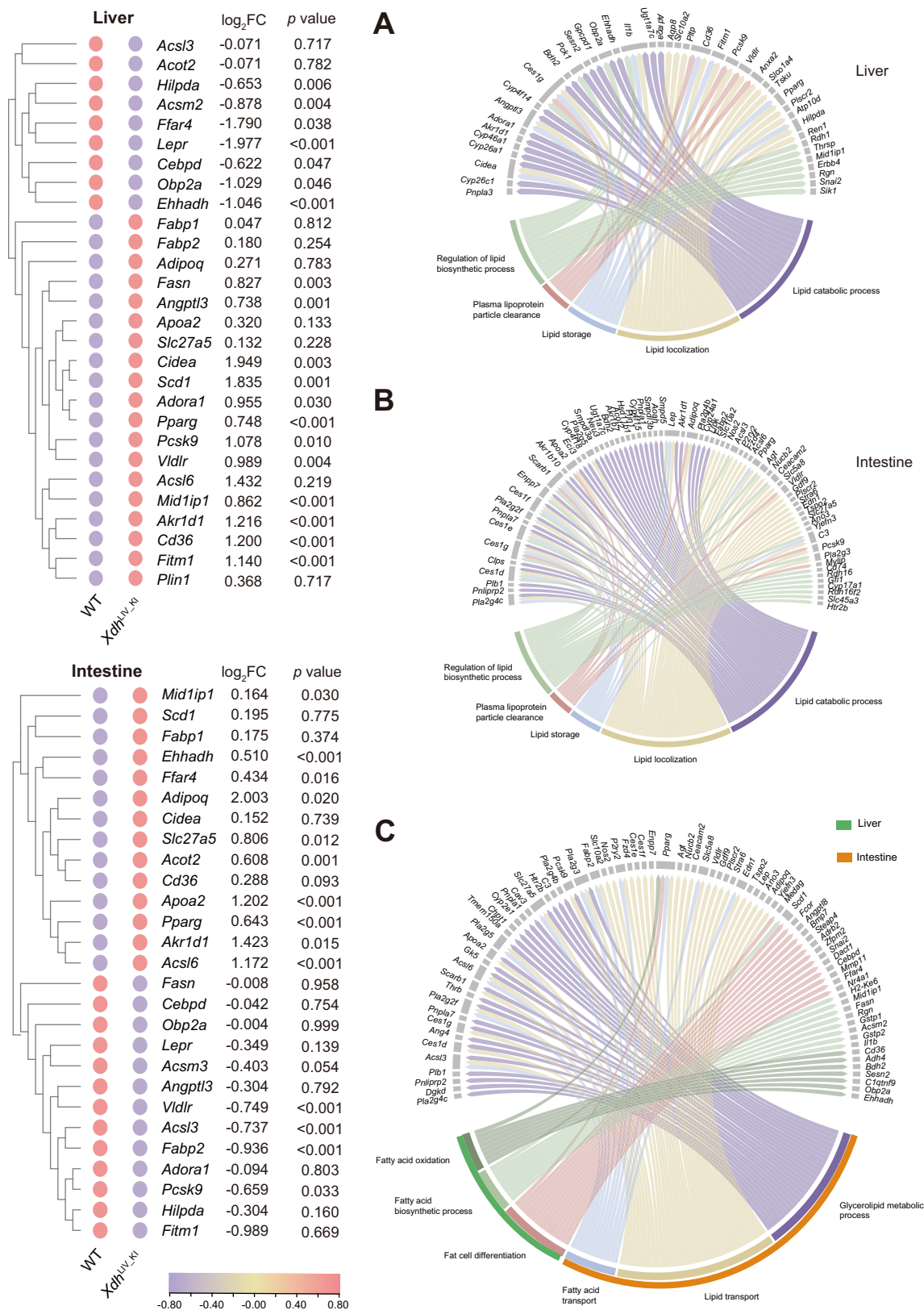
Fig. 3. Chord diagrams showed RNA sequencing results of liver and intestine. (A,B) Differential genes in common pathways analyzed for GO enrichment in the liver and intestine from *Xdh*^Liv-KI mice and WT mice under a high-fat diet. (C) Differential genes in unique pathways analyzed for GO enrichment in two organs. The length of the brick for each gene corresponds to the sum of |log$_2$(FC)| in different lipid metabolic pathways. The length of the brick for each pathway corresponds to the sum of |log$_2$(FC)| in one or more genes. The list on the left showed the expression of differential genes related to lipid metabolism in the two organs, respectively. GO, gene ontology; WT, wild type.
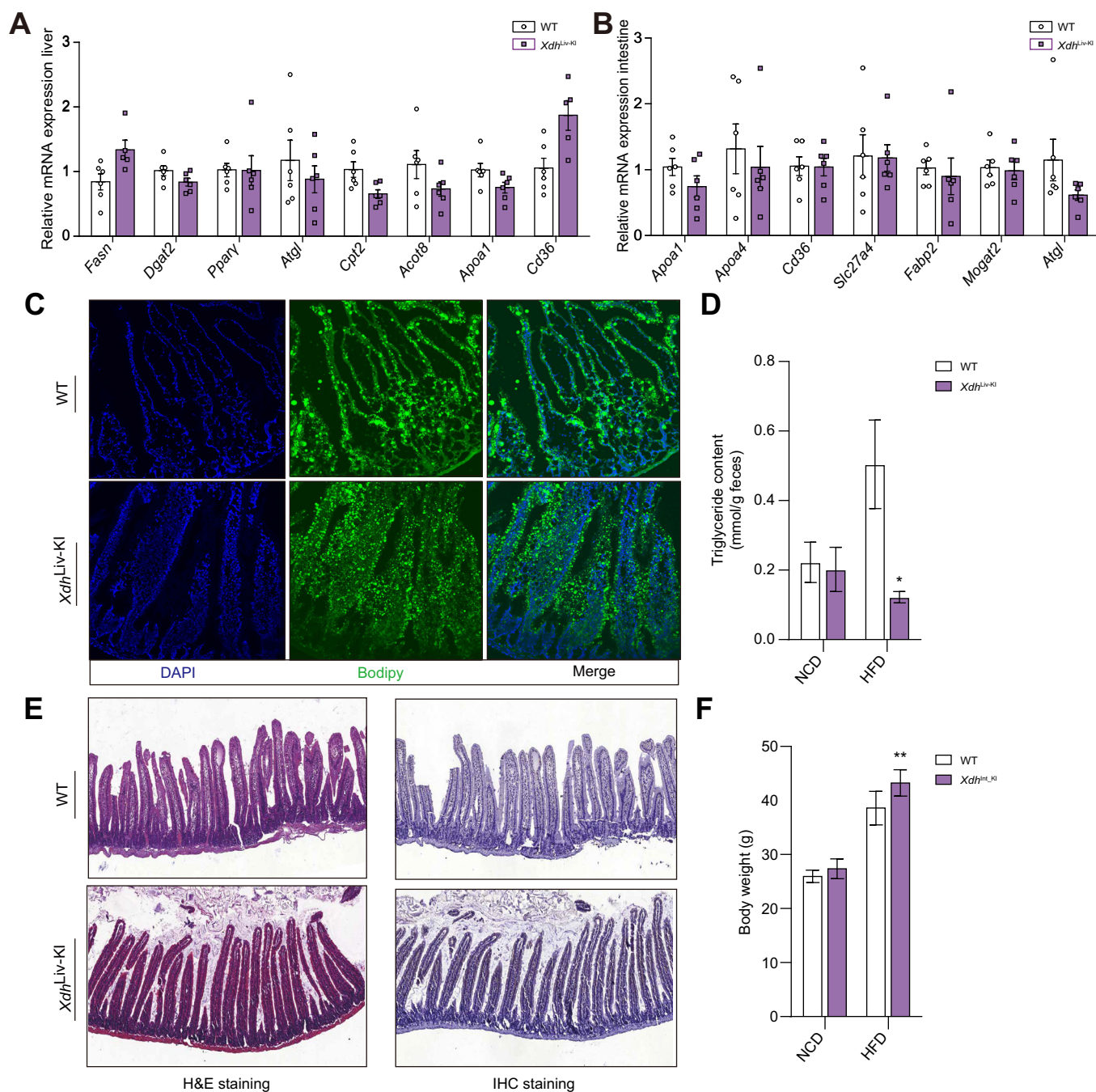
Fig. 4. High expression of XO in the small intestinal villus increased the absorption of dietary fat. (A) Relative mRNA expression of liver from WT mice and Xdh^{Liv-KI} mice under a normal chow diet feeding. (B) Relative mRNA expression of intestine from WT mice and Xdh^{Liv-KI} mice under a normal chow diet feeding. (C) Fluorescence images of small intestine of WT mice and Xdh^{Liv-KI} mice after oral gavage with corn oil containing BODIPY-labeled fatty acids (scale bar, 100 μm). (D) TG content of feces from WT mice and Xdh^{Liv-KI} mice. (E) H&E staining (scale bar, 100 μm) and IHC staining (scale bar, 100 μm) of intestine from WT mice and Xdh^{Liv-KI} mice. (F) Body weight between WT mice and Xdh^{Int-KI} mice under a normal-chow diet and high-fat diet. All data are presented as mean ± SEM, $^*p <0.05$, $^{**}p <0.01$, $^{***}p <0.001$ (bars represent mean, bold lines on boxes represent standard errors, Student t test). IHC, immunohistochemical; TG, triglyceride; WT, wild type; XO, xanthine oxidase.

small intestine through immunochemistry staining and found that XO was highly expressed in the small intestinal villus of Xdh^{Liv-KI} mice (Fig. 4E). These findings suggest that the upregulation of XO in the small intestine may play a role in promoting lipid absorption.

To confirm the role of XO in the intestine, we subsequently generated intestine-specific Xdh knockin mice (Xdh^{Int-KI} mice). We then examined Xdh gene expression in various tissues from Xdh^{Int-KI} mice to confirm tissue-specific Xdh knockin. Our results showed that mRNA expression of Xdh is upregulated in the small

intestine, but not in the liver and peripheral adipose tissue (Fig. S4A). Consistent with these results, Xdh^{Int-KI} mice also exhibited weight gain when fed a high-fat diet, but not when fed a normal-chow diet (Fig. 4F). Additionally, Xdh^{Int-KI} mice also showed increased lipid absorption. When we administered corn oil containing BODIPY-labeled fatty acids to Xdh^{Int-KI} and WT mice fed a normal chow diet via gavage, we observed more fluorescently labeled lipid droplets in the small intestine of Xdh^{Int-KI} mice (Fig. S4B). Based on these findings, we concluded that XO overexpression enhances the absorption of excess dietary fat by the small intestine, and the absorbed excess fat is stored as raw material for subsequent lipid metabolism. This explains why Xdh^{Liv-KI} and Xdh^{Int-KI} mice fed a high-fat diet exhibited lipid metabolism disorders, whereas those fed a normal chow diet did not.

Co-localization between XO and lipid droplets indicated that XO might have the ability to transport lipids

In addition to the above processes, we investigated the potential involvement of XO in lipid transport. We utilized pymol software (an open source molecular visualisation software written by Warren Lyford DeLano) to analyze the structure of the XO protein, revealing that the majority of hydrophilic amino acids were located on the surface of XO, whereas hydrophobic amino acids were predominantly found internally (Fig. 5A). This distribution pattern of amino acids indicated that XO might possess a structural foundation for lipid binding. Subsequently, we examined the spatial relationship between XO and lipid droplets in the liver. Immunofluorescence results demonstrated that XO was highly expressed in the livers of MASLD patients and co-localized with lipid droplets, irrespective of the presence of MASLD (Fig. S4C). Similarly, comparable outcomes were observed in the livers of Xdh^{Liv-KI} and WT mice subjected to a high-fat diet (Fig. 5B). Furthermore, Nanolive 3D Cell Explorer (Nanolive,

Lausanne, Switzerland) revealed that XO fluorescence signal was present in conjunction with lipid droplets in AML12 cells, exhibiting continuous movement alongside the lipid droplets (Video and Fig. S5). Additionally, we observed the same phenomenon in both human and mouse serum, with immunofluorescence staining confirming the co-localization of XO with serum lipids (Fig. S6). Moreover, lipid transport-related genes were significantly upregulated in Xdh^{Liv-KI} mice under a high-fat diet (Fig. 5C). In summary, morphological evidence strongly suggested a high correlation between XO and lipid levels, both *in vivo* and *in vitro*. Collectively, these findings indicated that overexpression of XO might be implicated in lipid transport, resulting in the rapid accumulation of lipids and subsequent disruption of lipid metabolism under high-fat diet conditions.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jhepr.2024.101060.

XO knockdown mice protected from high-fat-diet induced lipid metabolic disorders

To verify the role of XO in lipid disorders, we developed a high-fat fed mouse model with *Xdh* knockdown using adeno-associated virus (AAV). The expression of XO protein in the liver samples was confirmed by Western blot (Fig. S7A). Our findings revealed that XO knockdown reduced weight gain and triglyceride accumulation in both the serum and liver compared with mice on a high-fat diet alone (Fig. 6A and B and S7B). Furthermore, XO knockdown led to increased levels of HDL-c and decreased levels of LDL-c in both the serum and liver tissues of high-fat fed mice, with no significant difference in TC levels (Fig. 6C–E and S7C–E). Liver histology as determined by H&E staining, also showed less fatty or vesicular degeneration in the liver of HF_AAV mice (Fig. S7G). To gain insight into the underlying mechanism, we identified differential gene sets associated with lipid metabolism in the transcriptomic results of the liver
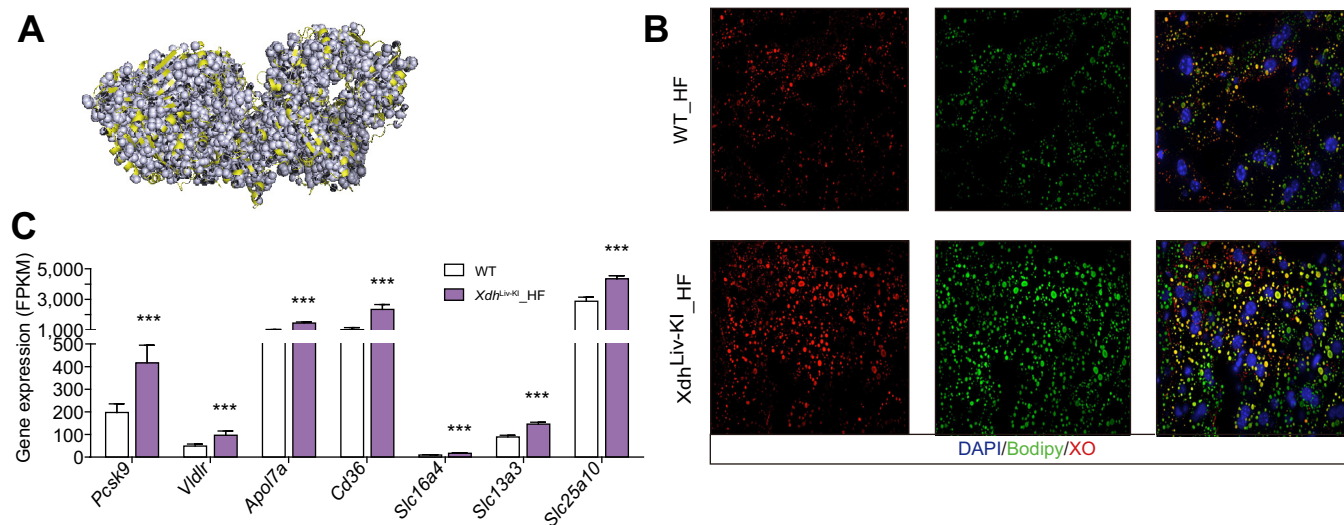

Fig. 5. Co-localization between XO and lipid droplets indicated that XO might have the ability to transport lipids. (A) The positions of hydrophobic and hydrophilic amino acids of XO proteins were annotated with pymol software. The gray spheres represent hydrophilic amino acids and the yellow spheres represent hydrophobic amino acids. (B) XO protein was stained with anti-xanthine oxidase (red), lipid droplet was detected by Bodipy staining (green), and counterstained with DAPI (blue). Scale bar, 20 μm. (C) Relative expression of lipid transport-related genes in liver from WT mice and Xdh^{Liv-KI} mice under a high-fat diet. All data are presented as mean ± SEM, *p <0.05, **p <0.01, ***p <0.001 (bars represent mean, bold lines on boxes represent standard errors, Student t test). FPKM, fragments per kilobase of exon model per million mapped fragments; WT, wild type; XO, xanthine oxidase.
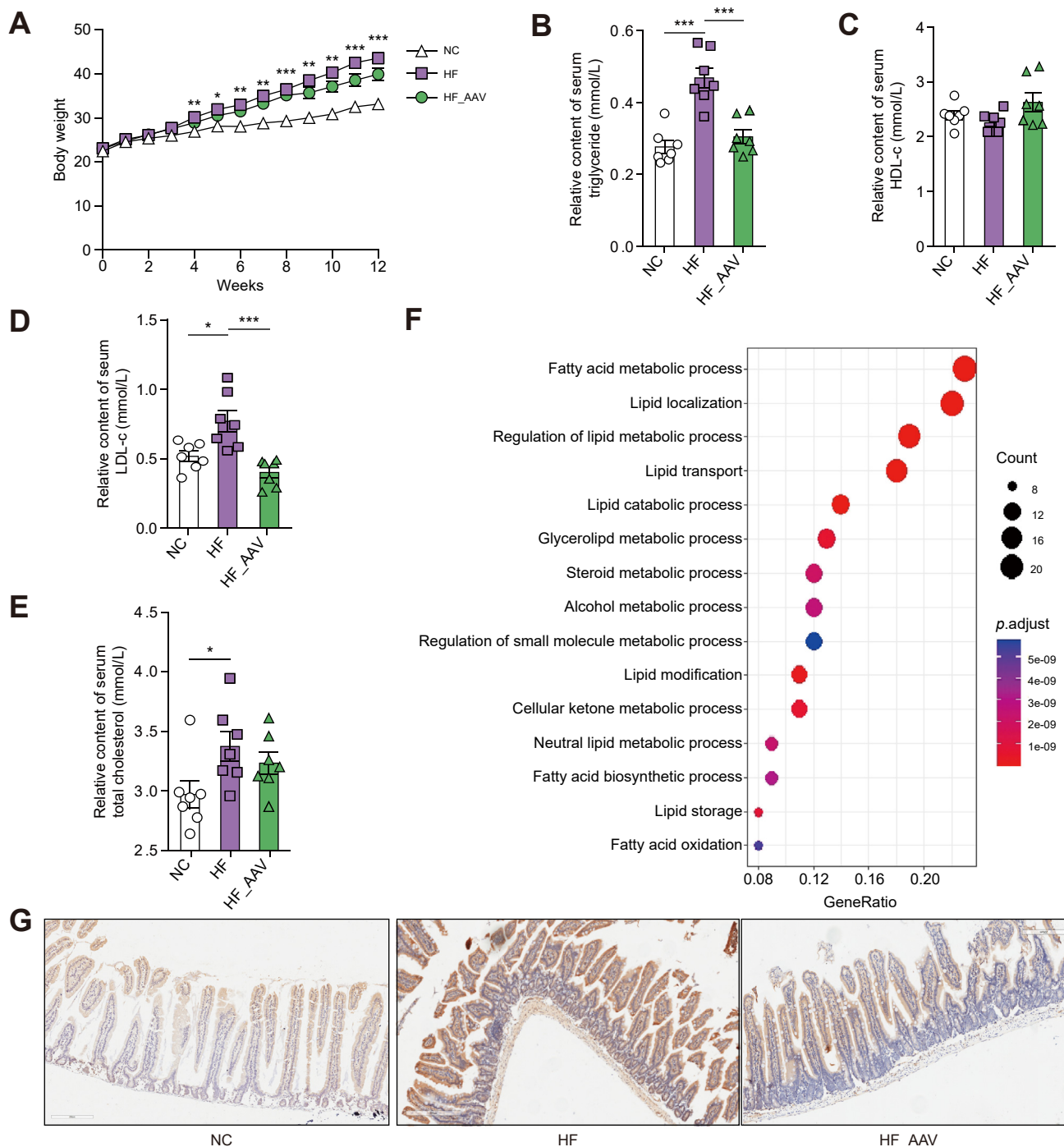
Fig. 6. XO knockdown mice protected from high-fat-diet induced lipid metabolic disorders. (A) Change of body weight among three groups, including NC, HF, and HF_AAV mice. (B–E) The relative content of TG, HDL-c, LDL-c, and TC in serum, respectively. (F) GO pathway analysis. (G) IHC staining of intestine (scale bar, 200 μm). All data are presented as mean ± SEM, *p <0.05, **p <0.01, ***p <0.001 (bars represent mean, bold lines on boxes represent standard errors, Student t test). GO, gene ontology; HF, high-fat; HF-AAV, high-fat adeno-associated virus; IHC, immunohistochemical; NC, normal control; TC, total cholesterol; TG, triclyceride; XO, xanthine oxidase.

and performed pathway enrichment analysis. These results further supported our conclusion that XO inhibition primarily affected pathways related to fatty acid metabolic processes and lipid transport (Fig. 6F). To visually explore the relationship between XO and lipid metabolism, we conducted immunohistochemistry to detect XO expression in the intestine. The results showed a decrease in XO distribution in the small intestinal villus after XO inhibition (Fig. 6G). Additionally, we

observed a significant increase in TG content in feces after XO inhibition (Fig. S7F), indicating a decrease in lipid absorption in XO knockdown mice. Moreover, the co-localization between XO and lipid droplets decreased in the liver following XO inhibition (Fig. S8). In summary, our findings demonstrate that XO inhibition effectively reduces lipid accumulation in the liver, primarily by downregulating lipid absorption and transport.

Discussion

In a population-based prospective cohort study, we observed that elevated serum XO activity was significantly associated with the risk of the development of MASLD. Interestingly, subsequent functional and morphological experiments showed that XO overexpression promotes lipid accumulation through mechanisms involving increased absorption of excess dietary fat and possibly facilitated the lipid transport *in vivo* in a high-fat context. Moreover, XO inhibition also reduced lipid absorption and co-localization in mice fed a high-fat diet. Our studies also highlight a novel mechanism for the regulation of lipid metabolism by XO *in vivo*.

Previous research has suggested that XO may be involved in metabolic diseases through its role in increasing oxidative stress.[12,13] However, our animal model findings indicate that *Xdh* knockin mice did not exhibit any disturbances in lipid metabolism when fed a normal chow diet despite increased oxidative stress levels. This suggests that compensatory mechanisms within the body may counteract the effects of XO-generated ROS, preventing such disturbances. Moreover, ROS signaling plays a key role in regulating physiological processes such as cellular homeostasis and adaptation to metabolic dysfunction/inflammation, which suggests that oxidative stress may not always be detrimental.[15,16] Overall, our data suggests that the XO-induced dysregulation of lipid metabolism was independent of oxidative stress levels.

Lipid metabolism is a complex process that involves various steps such as fat absorption, transport, lipogenesis, and lipolysis. One surprising finding from our study is that XO overexpression did not induce lipid accumulation or affect the expression of genes directly involved in lipid synthesis and catabolism when mice were fed a normal chow diet condition. However, under high-fat feeding, XO overexpression regulates the expression of several MASLD susceptibility genes, such as *CD36* and *Cidea*. It is essential to acknowledge that MASLD is not solely governed by monogenic factors; susceptibility genes do play a role and their effects may be revealed by environmental exposure. For instance, *CD36* is responsible for the transmembrane transport of extracellular long-chain fatty acids across the cell membrane into the cell for lipid synthesis and catabolism.[17] *Cidea* is located on the surface of the lipid droplet and is involved in the fusion of the lipid droplet.[18] Taken together, these two genes are highly dependent on the lipid environment for their function. In conclusion, the MASLD susceptibility genes regulated by XO overexpression may only play a role in regulating lipid metabolism when induced by a high-fat diet. Additionally, the levels of residual triglycerides in the feces of mice with XO overexpression were similar to those of WT mice on a normal chow diet. However, both *Xdh*[Liv-KI] and *Xdh*[Int-KI] mice responded strongly to high-fat diets (60 kcal%

fat) and fecal residual TG levels in XO overexpressing mice significantly decreased compared with WT mice. In addition, XO overexpressing mice showed a high level of XO expression in the villi of the small intestine. These findings suggest that high expression of XO in the small intestine may play a crucial role in facilitation of dietary lipid absorption.

It is well known that, under normal circumstances, the mean coefficients of fat absorption were up to 96%.[19] The enzymes in the small intestine itself are sufficient to absorb dietary fat adequately. Thus, XO overexpressing mice made a limited contribution to fat absorption on a normal chow diet. However, the ability to absorb fat was significantly enhanced when XO overexpressing mice received fatty acid gavage or a high-fat diet, thus leading to a subsequent increased lipid synthesis and rapid lipid metabolism disorders. Of note, we found that AAV-mediated XO inhibition protected against high-fat diet-induced lipid metabolism disorders. Furthermore, the XO inhibition mice had higher fecal residual TG levels without differences in food intake. This reflects a reduction in lipid absorption, which may be the main mechanism by which XO inhibition reduces fat accumulation caused by a high-fat diet. Moreover, these results are in line with previous studies in which allopurinol intervention prevented fat accumulation in mice caused by a high-fat diet[20] and reduced serum triglyceride levels in patients with hyperuricemia combined with dyslipidemia.[21] In conclusion, the abundant expression of XO promoted lipid accumulation by enhancing the absorption of excess dietary fat in the small intestine.

Our study showed that overexpression of XO not only increases serum TG levels, but also promotes lipid accumulation in enterocytes, liver, and adipose tissue. It is widely recognized that lipids are absorbed by the small intestine and subsequently enter the bloodstream for distribution across all organs.[22] Therefore, it is speculated that via the bloodstream, the over-absorbed lipids are transported from the intestine to the liver and adipose tissue, leading to fat deposition in both liver and adipose tissue. XO is a secretory protein that can be released from the liver and transported to various tissues and organs via the bloodstream.[23] Additionally, it can bind to vascular endothelial cells via glycosaminoglycan and has a widespread effect on circulation.[24] XO was initially discovered and purified from bovine milk[25,26] and is a major protein component of the milk fat globule membrane.[27] Previous studies have shown that XO is involved in packaging and secretion of milk fat globules in mammary epithelial cells.[28] Molecular docking and kinetic simulation experiments have further indicated that XO has a hydrophobic structure, suggesting a potential structural basis for lipid transport.[29,30] We found a significant correlation between serum XO activity and serum lipid levels. Furthermore, we observed the co-localization of XO and lipid droplets in both human and mouse samples. Taken together, these findings suggest that XO may also play a critical role in lipid transport, potentially leading to fat deposition in many tissues, including liver and adipose tissue.

Strengths and limitations of the study

The current study has several strengths. Firstly, it is the first large prospective cohort study to establish a causal relationship between serum XO activity and the development of MASLD. Sec-

ondly, an ingenious experimental design demonstrated that XO-induced lipid metabolism disorders are independent of both SUA and oxidative stress. However, it is important to acknowledge the limitations of this study. Although morphological experiments observed co-localization of XO with lipid droplets, the difficulty in obtaining pure XO proteins has greatly hindered our understanding of the specific mechanisms by which XO interacts with lipid droplets.

Abbreviations

AAV, adeno-associated virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FPKM, fragments per kilobase of exon model per million mapped fragments; HDL-c, HDL cholesterol; HDNNCDS, Harbin Cohort Study on Diet, Nutrition and Chronic Non-communicable Diseases; HR, hazard ratio; IHC, immunohistochemistry; GO, gene ontology; LDL-c, LDL cholesterol; MASLD, metabolic associated steatotic liver disease; MDA, malonaldehyde; NC, normal control; OCT, optimum cutting temperature; PH, proportional hazards; ROS, reactive oxide species; SOD, superoxide dismutase; SUA, serum uric acid; TC, total cholesterol; TG, triglyceride; VLDL-c, VLDL cholesterol; WT, wild type; XO, xanthine oxidase.

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

Conception and design: YL, LL. Acquisition of data: LL, YZ, XW, XG, SY, JW. Analysis and interpretation of data: LL, YZ. Animal feeding: LL, XT, KD, PM. Morphological experiment: LL, HM, YH. Drafting and critical revision of the manuscript: YL, LL, YZ, XX, HX, CW, XY. Guarantor of this study with full access to the database and responsibility for the integrity of the data and accuracy of the data analysis: YL.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101060.

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