nature portfolio

Peer Review File

A Mouse Model for Metabolic Dysfunction-associated Steatotic Liver Disease and Hepatocellular Carcinoma

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I read with great interest the paper entitled: "A mouse model for metabolic dysfunction - associated steatotic liver disease".

The paper analyzes a model that recapitulates the disease progression of Metabolic Associated Steatotic Liver Disease (MASLD) up to carcinogenesis.
The findings of the paper are very interesting and support the need to better unravel disease progression in MASLD and the underlying mechanisms. The analysis of dietary and pharmacological intervention throughout the disease progression is interesting.

I have some comments:
Minor points
o Given the new nomenclature has been introduced in the field, the authors could consider to update it throughout the manuscript (Rinella et al, Hepatology 2023).
o In the text abbreviations should be consistently explained, please check.
o Page 7, lines 125-126: the sentence is not clear, in particular with which timepoint were compared the changes at 32 weeks (C3, C4)?
o Fig 2 i: the authors should clarify better the early and late state: is it based on fibrosis? Or presence of NASH? Or both? And the advanced fibrosis is irrespective of the presence of NASH?
o Page 18: the human cohort should better characterized. What do the authors mean for NAFLD? Is that NAFL (Non alcoholic fatty liver)? For NASH fibrosis do the authors mean significant fibrosis (F2-F3) or any?
Major points
• In the study design, the authors include a longitudinal observation of the time course of the disease and use the early weeks as controls. It is not clear, however, if they include, however, age matched controls for the control diet and they do not include high fat diet arm. Those controls are mentioned in the methods but the results are important to include.
• The timeline of development of NASH is not clear 20 weeks, 34 weeks? The description is confusing as the authors describe in the text NAS score increase by 32 weeks, NASH from 20 weeks… They should report the histological findings better: when NASH occurs and when fibrosis occurs and if/when they see cirrhosis. Furthermore, do the authors should describe better in which histological background they find the tumors. In this regard a arm of control diet and high fat diet should have been useful to compare with respect to carcinogenesis.
• Would it be plausible that the group 3 tumors are less differentiated and therefore are similar to other etiologies? (page 10)
• Page 11: the authors claim they did not find differences in hepatic fibrosis after 6 week diet reversal. How extended was fibrosis at 26 weeks? May there was no much fibrosis at 26 weeks. Is could be that with a longer reversal fibrosis could have improved as well.
• Furhtermore, why the reversal period was different in the two experiments? (early and late stage). Fibrosis and n tumors (when appropriate should be included in the figure).
• In the HFD to SCD 51 wk mice there is no reversal in fasting glucose and insulin levels. This is an interesting finding. Did the authors explore this aspect at transcriptomic level?

Reviewer #2 (Remarks to the Author):

This manuscript communicates a modified version of "STAMTM" mouse model of STZ-HFD-induced NAFLD, NASH, and HCC which the authors claim better recapitulates phenotypic, histologic, and transcriptomic characteristics of NAFLD/NASH patients. Using the model, temporal therapeutic windows were tested for dietary and the GLP-1 receptor agonist tirzepatide interventions.

Strengths:
The study is based on quite extensive characterization of temporal phenotypic, metabolic, histologic, and transcriptomic changes in the model.

The modified method resulted in phenotypic advantages of reproducing hallmarks of NAFLD metabolic dysregulation including obesity, high fasting glucose, visceral adiposity, and dyslipidemia. This is a strength albeit descriptive in nature.

Similarities of histologic, biochemical, transcriptomic changes of the STZ-HFD model to NAFLD patients, support the model's translational relevance.

Temporal progression of NAFLD-associated liver pathology to NASH and HCC is characterized well. This allowed appropriate designs for testing of therapeutic windows for dietary and pharmacologic manipulations, yielding interesting differential effects based on the timing of the treatment initiation, as well as the profound tumor suppressive effect of tirzepatide even when given at the late stage.

Weaknesses:
The modified STZ-HFD model described in this manuscript is conceptually based on the original STAMTM STZ-HFD model previously published and commercialized for MASH research reported in >70 publications to date. Thus, the conceptual novelty of the STZ-HFD method is limited.

All numerical parameters are compared between STZ-HFD vs. STZ-SCD at the specified time points and in reference to pre-STZ injection base line. To best characterize how these two groups of mice phenotypically and metabolically behave as compared to SCD-fed normal mice at the same age, the data from these age-matched control mice need to be shown for the key time-points.

Composition of three types of tumors identified in the model needs to be depicted in the tumor incidence bar graph in Fig. 1m to show time frame of a shift toward more malignant HCC development.

For the dietary and pharmacologic interventions, it is important to clarify if the treatment initiated at the different time points reversed the pathologic phenotype or halted the progression.

Tizrepatide, the GLP-1 receptor agonist, expectedly improved NAFLD due to its insulinotropic action at two earlier time points (21-32 wk and 28-38 wk) but not the later 41-52 wk while tumor development was convincingly suppressed in both early and late periods, dissociating NAFLD/NASH and tumorigenesis as also noted by the authors (page 16 line 316-320). This profound tumor suppression effect in sustained NAFLD environment, will deserve mechanistic exploration and insights.

New nomenclatures have been adopted by the international community in 2023 and the authors may follow this guideline (e.g., NAFLD > MASLD).

Response to reviewers

We thank the reviewers for their careful review of our manuscript. We found the comments and suggestions helpful, and we have made the requested revisions, which are highlighted in red color in the revised manuscript. Regarding the major issues raised by the reviewers, we have addressed them by including data on age-matched standard and high-fat diet controls without streptozotocin treatment. Additionally, we have provided mechanistic insight into how tirzepatide might suppress tumor development through additional analysis of hepatic transcriptomics. We have also clarified the effects of interventions. Furthermore, we have responded to the reviewer's other comments, including the updated nomenclature as requested. We have outlined those changes below.

Reviewer 1

I read with great interest the paper entitled: "A mouse model for metabolic dysfunction - associated steatotic liver disease". The paper analyzes a model that recapitulates the disease progression of Metabolic Associated Steatotic Liver Disease (MASLD) up to carcinogenesis. The findings of the paper are very interesting and support the need to better unravel disease progression in MASLD and the underlying mechanisms. The analysis of dietary and pharmacological intervention throughout the disease progression is interesting. I have some comments:

Comment 1) Given the new nomenclature has been introduced in the field, the authors could consider to update it throughout the manuscript (Rinella et al, Hepatology 2023).

We appreciate the reviewer's constructive suggestion. According to the reviewer's suggestion, we applied the new nomenclature in the revised manuscript. Specifically, we replaced NAFLD with MASLD, NASL with MASL, and NASH with MASH.

Comment 2) In the text abbreviations should be consistently explained, please check.

We appreciate the reviewer's valuable comment. In the revised manuscript, we have ensured consistency in the explanation of abbreviations.

Comment 3) Page 7, lines 125-126: the sentence is not clear, in particular with which timepoint were compared the changes at 32 weeks (C3, C4)?

We thank the reviewer for their careful review. In the revised manuscript, we have clarified the meaning of the relevant sentence more thoroughly (refer to Page 8, Lines 136-138).

Comment 4) Fig 2 i: the authors should clarify better the early and late state: is it based on fibrosis? Or presence of NASH? Or both? And the advanced fibrosis is irrespective of the presence of NASH?

We agree the reviewer's comment. In the revised manuscript, we imparted detailed descriptions in the figure legends to specify the comparisons made between mice and humans for the early and late progression of MASLD (refer to Page 36, Lines 776-781).

Comment 5) Page 18: the human cohort should better characterized. What do the authors mean for NAFLD? Is that NAFL (Non alcoholic fatty liver)? For NASH fibrosis do the authors mean significant fibrosis (F2-F3) or any?

We appreciate the reviewer's constructive comment. In the revised manuscript, we have provided a more comprehensive characterization of the human cohort, especially the MASLD patients, in the Methods section (refer to Page 19, Lines 397-403).

Comment 6) In the study design, the authors include a longitudinal observation of the time course of the disease and use the early weeks as controls. It is not clear, however, if they include, however, age matched controls for the control diet and they do not include high fat diet arm. Those controls are mentioned in the methods but the results are important to include.

We appreciate the reviewer's valuable comment. In response to the reviewer's suggestion, we included age-matched control data in the revised manuscript. These experiments comprised investigations into hepatic and metabolic phenotypes as well as hepatic transcriptomics in age-matched groups of mice (standard-chow diet (SCD) fed mice without

STZ treatment (SCD-only), STZ+SCD mice, HFD-fed mice without STZ treatment (HFD-only), and STZ+HFD mice at 20, 32, and 50-56 weeks) (**Supplementary Fig. 3, 4, and 6**). Our data supported the observation that STZ+HFD mice exhibited a rapid progression of MASLD along with metabolic disturbances. Compared to STZ+SCD mice or HFD-only mice, STZ+HFD mice showed significantly higher NAS score at 20 and 32 weeks, and more severe hepatic fibrosis at 32 weeks. Moreover, while more than 75 % of STZ+HFD mice developed hepatic tumor, STZ+SCD mice and HFD-only mice did not develop hepatic tumor in 50-56 weeks. Additionally, analysis of hepatic transcriptomics among the four groups of mice at 32 weeks revealed that signaling pathways associated with Wnt and Notch signaling, as well as the cell cycle pathway, were highly activated in STZ+HFD mice compared to the other groups. This suggests the potential contribution of these pathways to hepatic tumorigenesis in STZ+HFD mice.

Comment 7) The timeline of development of NASH is not clear 20 weeks, 34 weeks? The description is confusing as the authors describe in the text NAS score increase by 32 weeks, NASH from 20 weeks… They should report the histological findings better: when NASH occurs and when fibrosis occurs and if/when they see cirrhosis. Furthermore, do the authors should describe better in which histological background they find the tumors. In this regard a arm of control diet and high fat diet should have been useful to compare with respect to carcinogenesis.

We understand the reviewer's concern. In the revised manuscript, we have provided a clearer description of the ages at which MASH, significant fibrosis, and advanced fibrosis, including cirrhosis, occur in STZ+HFD mice (refer to Page 6 Lines 94-100). Additionally, we have modified **Supplementary Table 2** to offer a more detailed histologic background of HCC occurrence in STZ+HFD mice. As previously mentioned (in response to Comment 6), we conducted additional experiments comparing the hepatic phenotypes of four groups of mice (SCD-only, STZ+SCD, HFD-only, and STZ+HFD mice) at 50-56 weeks (**Supplementary Fig. 3**). Notably, STZ+HFD mice exhibited a hepatic tumor incidence above 75%, while STZ+SCD mice and HFD-only mice showed no hepatic tumors at 50-56 weeks.

Comment 8) Would it be plausible that the group 3 tumors are less differentiated and therefore are similar to other etiologies? (page 10)

We appreciate the reviewer's constructive comment. To address this, we conducted further analysis of our hepatic transcriptomics data, which has been included in the revised manuscript (**Supplementary Fig. 8a, b**). We compared gene sets representing various molecular subtypes of human HCC from publicly available data sources, including Desert et al. (revised manuscript reference 25), Hoshida et al. (revised manuscript reference 26), and Boyault et al. (revised manuscript reference 27), using Gene Set Enrichment Analysis, against hepatic tumor groups in STZ+HFD mice. Our analysis revealed that the group 1 tumors were enriched in signatures of well-differentiated periportal-type HCC, whereas groups 2 and 3 tumors were enriched in signatures associated with the HCCs exhibiting high proliferation rate (Group 2; Boyault's G1 and G2 subclasses) or signatures related to HCCs with *CTNNB1* mutation (Group3; Boyault's G6 subclass, Hoshida's S2 subclass, and peri-venous type HCC). This new analysis suggested that group 1 tumors were well differentiated compared to groups 2 and 3 tumors. Consistent with these findings, the relative proportion of the groups 2 and 3 tumors tended to increase as metabolic stress accumulated with advancing age in STZ+HFD mice.

In comparing tumor groups 2 and 3, we think that group 2 represents a specific type of MASLD-related HCC with a lower correlation to HCCs from other etiologies, while group 3 exhibits transcriptomic characteristics similar to HCCs from other etiologies, probably due to commonly shared features of *CTNNB1* mutation.

Comment 9) Page 11: the authors claim they did not find differences in hepatic fibrosis after 6 week diet reversal. How extended was fibrosis at 26 weeks? May there was no much fibrosis at 26 weeks. Is could be that with a longer reversal fibrosis could have improved as well.

We appreciate the reviewer's valuable comment. As pointed out by the reviewer, the 6-week dietary change from HFD to SCD during the 20-26 week period may not have been suitable for observing histological differences in fibrosis between STZ+HFD mice and HFD→SCD 26-week mice. However, in the revised manuscript, we observed a decrease in the expression of fibrosis-related genes, such as those involved in ECM receptor interaction, in HFD→SCD 26-week mice (**Supplementary Table 4**). Based on this finding, we concur with the reviewer's opinion that a longer period of dietary change could have led to improved histological assessment of hepatic fibrosis. Since our data demonstrated that an 18-week dietary

change from HFD to SCD during the 33-51 week period was sufficient to improve fibrosis (**Figure 4n, o**), we did not conduct an additional mice experiment regarding dietary change starting at 20 weeks.

Comment 10) Furthermore, why the reversal period was different in the two experiments? (early and late stage). Fibrosis and n tumors (when appropriate should be included in the figure).

We thank the reviewer for their careful review. In the second cohort, where dietary change from HFD to SCD occurred during the 33-51 week period, we extended the diet reversal period to better evaluate the effects of dietary changes on advanced fibrosis and hepatic carcinogenesis. In the STZ+HFD mice, advanced fibrosis was observed around 44 weeks (**Figure 1e, g**), and more than half of the mice developed hepatic tumors around 50 weeks (**Figure 1m, n**). Therefore, we conducted a dietary change from HFD to SCD for 18 weeks starting at 33 weeks.

Comment 11) In the HFD to SCD 51 wk mice there is no reversal in fasting glucose and insulin levels. This is an interesting finding. Did the authors explore this aspect at transcriptomic level?

We appreciate the reviewer's constructive comment. In response to the reviewer's suggestion, we conducted additional comparisons of gene expression related to gluconeogenesis and glycogenolysis in the liver, as outlined in the revised manuscript (**Supplementary Fig. 9s, t**). However, we did not observe significant differences in overall expression changes.

Reviewer 2

This manuscript communicates a modified version of "STAMTM" mouse model of STZ-HFD-induced NAFLD, NASH, and HCC which the authors claim better recapitulates phenotypic, histologic, and transcriptomic characteristics of NAFLD/NASH patients. Using the model, temporal therapeutic windows were tested for dietary and the GLP-1 receptor agonist tirzepatide interventions. Strengths: The study is based on quite extensive characterization of temporal phenotypic, metabolic, histologic, and transcriptomic changes in the model. The modified method resulted in phenotypic advantages of reproducing hallmarks of NAFLD metabolic dysregulation including obesity, high fasting glucose, visceral adiposity, and dyslipidemia. This is a strength albeit descriptive in nature. Similarities of histologic, biochemical, transcriptomic changes of the STZ-HFD model to NAFLD patients, support the model's translational relevance. Temporal progression of NAFLD-associated liver pathology to NASH and HCC is characterized well. This allowed appropriate designs for testing of therapeutic windows for dietary and pharmacologic manipulations, yielding interesting differential effects based on the timing of the treatment initiation, as well as the profound tumor suppressive effect of tirzepatide even when given at the late stage.

Comment 1) The modified STZ-HFD model described in this manuscript is conceptually based on the original STAMTM STZ-HFD model previously published and commercialized for MASH research reported in >70 publications to date. Thus, the conceptual novelty of the STZ-HFD method is limited.

We understand the reviewer's concern. While our STZ+HFD mice model may seem conceptually similar to the previous STAMATM model, there are substantial differences in the time point and amount of STZ administration, leading to significant variations in hepatic and metabolic phenotypes. As detailed in the Discussion section (refers to Page 14 Line 294 – Page 15 Line 311), the STAMTM model exhibited limitations such as the predominance of microvesicular steatosis over macrovesicular steatosis, absence of hepatocyte ballooning, and lack of metabolic dysfunction phenotypes including obesity and insulin resistance. In contrast, our STZ+HFD mice showed macrovesciaulr steatosis and hepatocyte ballooning, along with metabolic dysfunction characterized by increased fat mass, insulin resistance, dyslipidemia, and visceral adipocyte hypertrophy and inflammation.

In addition to these strengths of our model, we further characterized the histologic

subtypes of the hepatic tumors in STZ+HFD mice and demonstrated their transcriptomic similarity to MASLD-related HCCs from patients. Concerning the pathogenesis of tumorigenesis, the STAMTM model results in a high proportion of mutation signatures associated with alkylating agents compared to HCCs in humans (2~3 mutations per megabase pair (Mbp)), leading to a very high mutation burden (~122.56 mutations per Mbp, PMID: 30287485). Therefore, it is challenging to claim that it accurately mimics MASLD-related HCC in humans. However, in our ongoing collaborative research using this model, whole-genome sequencing results have shown that the mutation burden in HCCs of STZ+HFD mice (0.62 single nucleotide variants per Mbp) is more similar to that of humans (**Reviewer only data 1**). Furthermore, major driver mutations of HCC in humans, such as *CTNNB1* mutations related to the Wnt/β-catenin pathway, are also frequently observed in our model (**Reviewer only data 2a**). We also observed that the *Ctnnb1* mutations in hepatic tumors of STZ+HFD mice exactly matched the recognized *CTNNB1* mutation hotspots in human tumors (**Reviewer only data 2b**). Based on these findings, we believe that this model not only better mimics human MASLD in terms of histologic and metabolic phenotype compared to the previous STAMTM model, but also serves as a more suitable model for tumorigenesis induced by metabolic stress.

Comment 2) All numerical parameters are compared between STZ-HFD vs. STZ-SCD at th e specified time points and in reference to pre-STZ injection base line. To best characterize how these two groups of mice phenotypically and metabolically behave as compared to SCD - fed normal mice at the same age, the data from these age-matched control mice need to be shown for the key time-points.

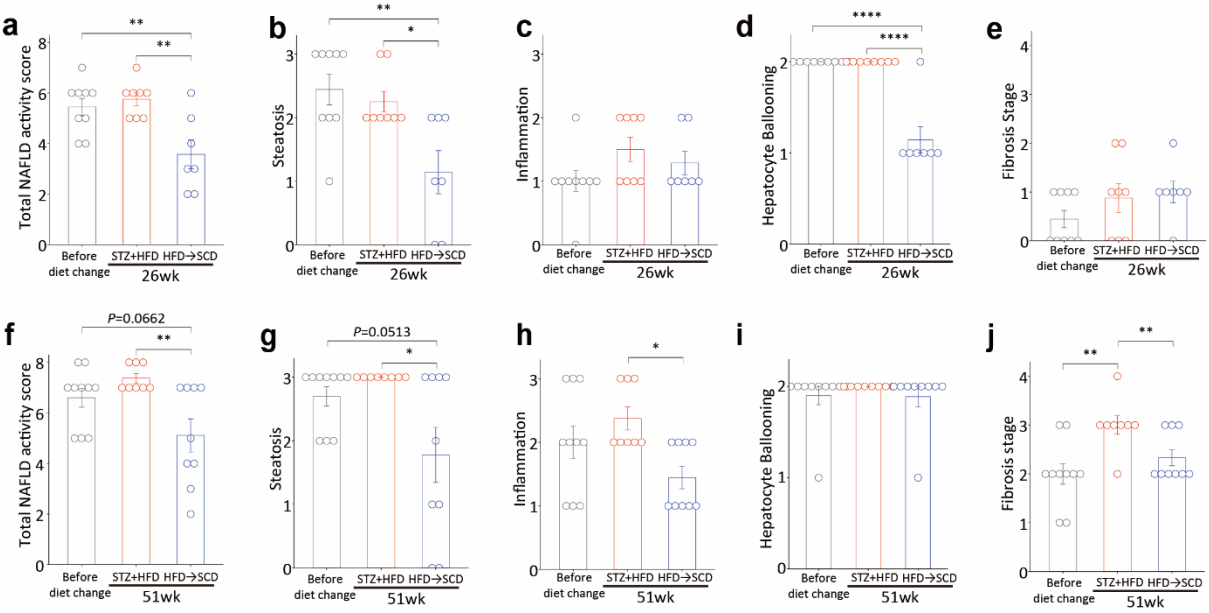
We agree with the reviewer's comment. In response to the reviewer's suggestion, we included age-matched control data in the revised manuscript. These experiments comprised investigations into hepatic and metabolic phenotypes as well as hepatic transcriptomics in age-matched groups of mice SCD fed mice without STZ treatment (SCD-only), STZ+SCD mice, HFD fed mice without STZ treatment (HFD-only), and STZ+HFD mice at 20, 32, and 50-56 weeks) (**Supplementary Fig. 3, 4, and 6**). Our data supported the observation that STZ+HFD mice exhibited a rapid progression of MASLD along with metabolic disturbances. Compared to STZ+SCD mice or HFD-only mice, STZ+HFD mice showed significantly higher NAS at 20 and 32 weeks, and more severe hepatic fibrosis at 32 weeks. Moreover, while more than 75 % of STZ+HFD mice developed hepatic tumor, STZ+SCD mice and HFD-only mice did not develop hepatic tumor in 50-56 weeks. Additionally, analysis of hepatic transcriptomics among the four groups of mice at 32 weeks revealed that signaling pathways associated with Wnt and Notch signaling, as well as the cell cycle pathway, were highly activated in STZ+HFD mice compared to the other groups. This suggests the potential contribution of these pathways to hepatic tumorigenesis in STZ+HFD mice.

Comment 3) Composition of three types of tumors identified in the model needs to be depicted in the tumor incidence bar graph in Fig. 1m to show time frame of a shift toward more malignant HCC development.

We appreciate the reviewer's constructive comment. In accordance with the reviewer's suggestion, we conducted further analysis on the relative proportion of hepatic tumor groups in STZ+HFD mice based on age, as detailed in the revised manuscript. Our findings revealed that the proportion of the groups 2 and 3 tumors tended to increase as metabolic stress accumulated with advancing age in STZ+HFD mice (**Supplementary Fig. 8b**).

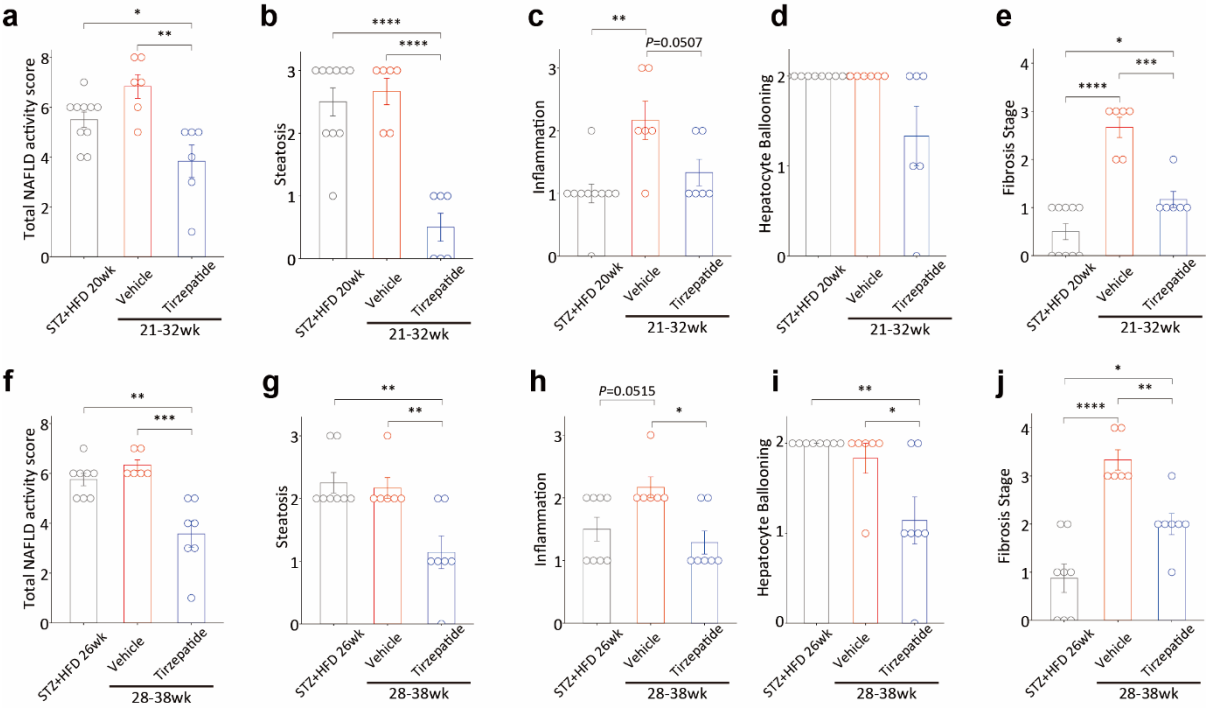
Comment 4) For the dietary and pharmacologic interventions, it is important to clarify if the treatment initiated at the different time points reversed the pathologic phenotype or halted the progression.

We appreciate the reviewer's valuable comment. In response, we compared the differences in NAS and hepatic fibrosis stage between pre- and post-intervention states. In experiments regarding dietary changes, we observed a decrease in NAS in HFD→SCD 26wk mice and a tendency towards a decrease in HFD→SCD 51wk mice, while fibrosis showed no significant difference between pre- and post-intervention states (**Reviewer only data 3**). Based on these results, we conclude that dietary change reversed the pathologic phenotype. In experiments involving tirzepatide administration, we observed a decrease in NAS but an increase in hepatic fibrosis stage between pre- and post-intervention states (**Reviewer only data 4**). Consequently, we think that tirzepatide administration halted the progression of MASLD. From a translational perspective, considering that clinical guidelines recommend pharmaceutical therapy alongside lifestyle modification for MASLD, we anticipate that conducting both dietary change and tirzepatide administration will lead to the reversal of MASLD progression.



Reviewer only data 3. NAFLD activity score and fibrosis stage grading using the NASH CRN scoring system before and after dietary changes. **(a-e)** Detailed NAS grading and fibrosis stage with the NASH CRN scoring system before and after dietary changes during 20-26 weeks; *n* = 7-9 per group. **(f-j)** Detailed NAS grading and fibrosis stage with the NASH CRN scoring system before and after dietary changes during 33-51 weeks; *n* = 8-10 per group. Data are

expressed as means ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, compared with one-way ANOVA with post-hoc Tukey's test or Welch's ANOVA with post-hoc Games-Howell test according to homogeneity of variance among groups.



Reviewer only data 4. NAFLD activity score and fibrosis stage grading using the NASH CRN scoring system before and after tirzepatide treatment. **(a-e)** Detailed NAS grading and fibrosis stage with the NASH CRN scoring system before (STZ+HFD 20 weeks) and after tirzepatide treatment during 21-32 weeks; *n* = 6-10 per group. **(f-j)** Detailed NAS grading and fibrosis stage with the NASH CRN scoring system before (STZ+HFD 26 weeks) and after tirzepatide treatment during 28-38 weeks; *n* = 6-8 per group. Data are expressed as means ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, compared with one-way ANOVA with post-hoc Tukey's test or Welch's ANOVA with post-hoc Games-Howell test according to homogeneity of variance among groups.

Comment 5) Tirzepatide, the GLP-1 receptor agonist, expectedly improved NAFLD due to its insulinotropic action at two earlier time points (21-32 wk and 28-38 wk) but not the later 41-52 wk while tumor development was convincingly suppressed in both early and late periods, dissociating NAFLD/NASH and tumorigenesis as also noted by the authors (page 16 line 316-320). This profound tumor suppression effect in sustained NAFLD environment, will deserve mechanistic exploration and insights.

We agree with the reviewer's comment. In response, we conducted further analysis on the transcriptomics data from the four groups of mice at 32 weeks (SCD-only, STZ+SCD, HFD-only, and STZ+HFD mice) and vehicle- and tirzepatide-administered mice, as outlined in the revised manuscript (**Supplementary Fig. 6, 11**). As previously mentioned (in response to Comment 2), analysis of hepatic transcriptomics among the four groups of mice at 32 weeks revealed that signaling pathways associated with Wnt and Notch signaling, as well as the cell cycle pathway, were more activated in STZ+HFD mice compared to the other groups (**Supplementary Fig. 6**), suggesting the potential contribution of these pathways to hepatic tumorigenesis in STZ+HFD mice. Interestingly, we observed a decrease in the expression of several important genes associated with the Wnt signaling pathway in the tirzepatide treatment group, regardless of the timing of administration (**Supplementary Fig. 11**). Considering that previous studies have linked Wnt signaling activation with MASLD-related HCC development (PMID: 27556491, 28671671), downregulation of Wnt signaling pathway in the liver may have contributed to the tumor suppressive effect of tirzepatide in the sustained MASLD environment.

Comment 6) New nomenclatures have been adopted by the international community in 2023 and the authors may follow this guideline (e.g., NAFLD > MASLD).

We appreciate the reviewer's constructive suggestion. According to the reviewer's suggestion, we applied the new nomenclature in the revised manuscript. Specifically, we replaced NAFLD with MASLD, NASL with MASL, and NASH with MASH.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors adressed the questions and the comments that were rised. The paper is improved.

Reviewer #2 (Remarks to the Author):

The revision has adequately addressed the critiques raised and incorporated additional data in improving the manuscript. No further comments.