LETTER

# Time-Restricted Feeding Is Not Effective in Modulating Fibrosis in a Male MASH Model

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*Abstract* Time-restricted feeding (TRF), a dietary intervention that consolidates food intake to specific hours of the day, ameliorates key metabolic risk factors for metabolic-associated steatohepatitis (MASH), including adiposity, insulin resistance, and liver steatosis. However, whether TRF can directly mitigate steatohepatitis or fibrosis remains uncertain. Moreover, whether the protective effects of TRF against MASH-related complications, such as inflammation and fibrosis, depend exclusively on improvements in insulin sensitivity or involve additional mechanisms remains unknown. Here, we examine the impact of 8-hour TRF on the development of fibrosis and steatohepatitis using a streptozotocin/high-fat diet (STAM/HFD) model, which recapitulates key MASH characteristics, including steatohepatitis and fibrosis, in an insulin-deficient context. TRF does not prevent the development of MASH in STAM/HFD male mice where insulin signaling is impaired. Unlike diet-induced obesity models, which exhibit greatly perturbed feeding and circadian behaviors under HFD conditions, STAM/HFD mice did not develop obesity and maintained regular or less-pronounced disruptions to circadian behaviors. This may explain why TRF failed to produce beneficial effects in this model. These findings indicate that intact insulin signaling is likely essential for TRF to effectively protect against MASH.

*Keywords* insulin resistance, steatosis, metabolic rhythms, feeding patterns, circadian rhythms

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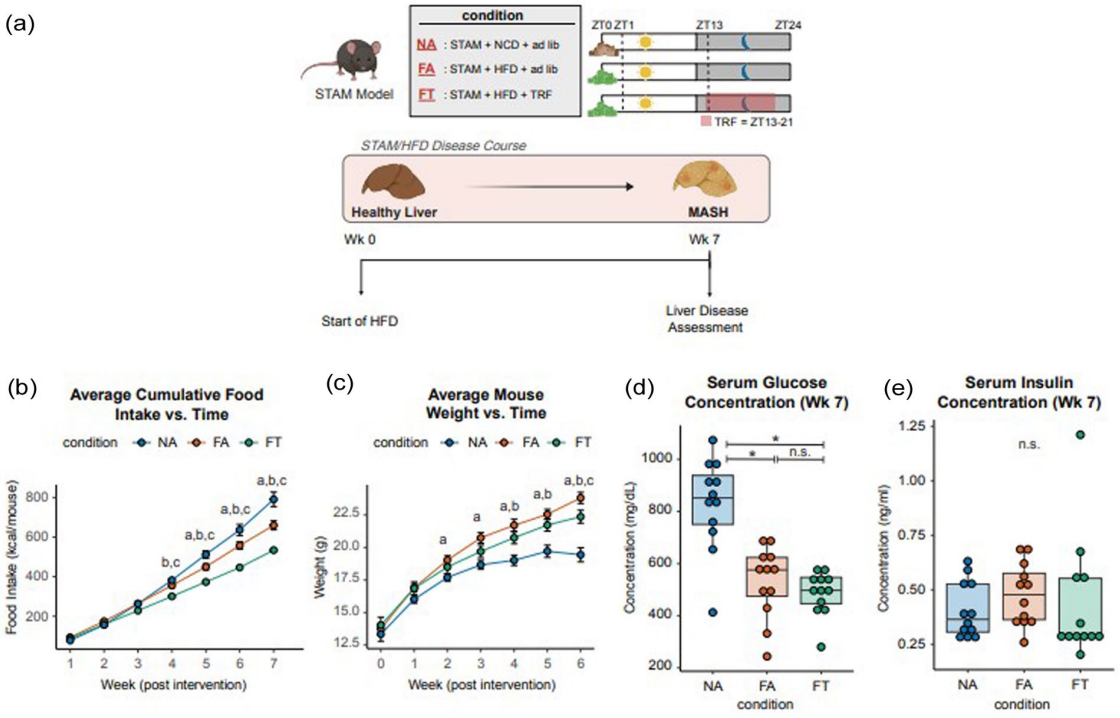


**Figure 1. (a) Experimental design and sample-collection protocol. (b) Average cumulative food intake and (c) body weight post intervention (n = 12 mice/condition; 2-way ANOVA, LSD post hoc test, $p < 0.05$; a = NA vs FA, b = NA vs FT, c = FA vs FT). Serum (d) glucose and (e) insulin concentrations at the time of sacrifice (7 weeks post intervention, one-way ANOVA, LSD post hoc test, $^*p < 0.05$, n.s. = not significant). ANOVA = analysis of variance; STAM = streptozotocin; NCD = normal chow diet; HFD = high-fat diet; TRF = time-restricted feeding; ad lib = ad libitum.**

Time-restricted feeding (TRF), a behavioral strategy that confines food intake to the active phase, has emerged as a promising approach to mitigate key metabolic risk factors associated with metabolic-associated steatohepatitis (MASH), such as insulin resistance and hepatic steatosis (Hatori et al., 2012; Petersen et al., 2022; Saran et al., 2020; Zarrinpar et al., 2016). Although the mechanisms of TRF are not well understood, accumulating evidence suggests that its therapeutic effects may involve the restoration of circadian rhythms (Chaix et al., 2014; Hatori et al., 2012; Saran et al., 2020; Zarrinpar et al., 2014). To determine whether TRF can protect against fibrosis and steatohepatitis, which are key features of MASH, we used the streptozotocin/high-fat diet (STAM/HFD) mouse model. Streptozotocin is toxic to pancreatic β-cells, significantly diminishing host insulin secretion. When combined with a HFD, these mice rapidly develop steatohepatitis, fibrosis, and even hepatocellular carcinoma, making them a favorable model for studying advanced stages of metabolic-associated fatty liver disease that other models either fail to reproduce or require substantially longer periods to develop (Fujii et al., 2013). Moreover, the hyperglycemic and insulin-deficient state of STAM/HFD mice allows us to examine TRF's impact in conditions where insulin signaling is impaired.

In this study, STAM male mice were fed either ad libitum normal chow (NA), ad libitum HFD (FA), or 8-hour TRF HFD (FT) for the course of 7 weeks when the disease developed (Figure 1a). At 7 weeks post intervention, we observed that, on average, mice with ad libitum food access (FA and NA) consumed significantly more kcal than mice under TRF (FT; 2-way analysis of variance [ANOVA] with LSD post hoc test, $p < 0.05$, Figure 1b). This difference likely reflects the hyperphagic behavior of the ad libitum STAM mice due to their impaired insulin signaling. Mice in the NA group exhibited the highest caloric intake (Figure 1b). Although NA mice consumed the most calories, they gained the least weight compared to the mice on HFD (2-way ANOVA with LSD post hoc test, $p < 0.05$, Figure 1c) likely because their ability to use glucose from carbohydrates was impaired. At 6 weeks post intervention, there were weight differences between FA and FT mice, with the latter gaining less weight (Figure 1c). This weight difference may reflect the reduced caloric intake of the TRF mice due to a hyperphagia in the ad libitum conditions and the restricted access to diet in the TRF condition.

Mice in the NA group had nearly double the serum glucose than that of the two HFD conditions (one-way ANOVA with LSD post hoc test, NA vs FA and NA vs FT: $p < 0.05$, Figure 1d). In contrast, the mice in the two HFD conditions maintained comparable serum glucose measures between groups (Figure 1d), likely because their primary energy source—dietary lipids—does not rely on insulin for absorption and storage to the same extent as carbohydrates. We observed similar differences when stratifying the data by ZT1 and ZT13 (two-way ANOVA with LSD post hoc test, Supplementary Figure S1a). As expected for this model, mice in all conditions had low serum insulin at 7 weeks post intervention (one-way ANOVA with LSD post hoc test, Figure 1e). Stratifying by ZT revealed some condition-specific differences at ZT1 and ZT13, but overall insulin levels remained negligible regardless of fasted or fed state (two-way ANOVA with LSD post hoc test, Supplementary Figure S1b). Thus, these results show MASH/HFD mice under TRF weighed less than their ad libitum control counterparts and consumed significantly fewer calories than mice in the other two conditions.

Despite consuming fewer calories and weighing less than FA mice, TRF failed to protect FT mice against STAM/HFD-induced MASH (Fisher's exact test, NA vs FA: $p = 0.0047$, NA vs FT: $p = 0.0074$, FA vs FT: $p = 0.57$, Figure 2a). Over 50% of mice in both the FA and FT conditions had a liver histological NAFLD activity score (NAS) of 3 or higher, indicating MASH, whereas all NA mice had a NAS score of 2 or lower, indicative of non-MASH. In addition, 50% or more of FA and FT mice developed fibrosis (Fisher's exact test, NA vs FA: $p = 0.014$, NA vs FT: $p = 0.0013$, FA vs FT: $p = 0.83$) and steatosis (Fisher's exact test, NA vs FA and NA vs FT: $p < 0.001$, FA vs FT: $p = 1.0$), whereas NA mice did not (Figure 2b-c). Interestingly, hepatic triglyceride measurements showed that TRF moderately reduced triglyceride levels compared to FA (one-way ANOVA with LSD post hoc test, $p < 0.05$, Figure 2d). Nonetheless, these results show that TRF did not prevent MASH development in a model with impaired insulin signaling.

To understand why TRF did not affect MASH development, we examined the behavioral and metabolic rhythms of the mice in different conditions. Typically in diet-induced obesity (DIO), FA mice display erratic and dysregulated diurnal behaviors (e.g. sleep, activity) compared to NA mice (Chaix et al., 2014; Hatori et al., 2012; Kohsaka et al., 2007; Zarrinpar et al., 2014). Consistent with these previous observations, behavioral rhythms, like cycling of motor activity and sleep (a period of immobility lasting at least 40 seconds), were lost in FA mice but maintained in NA and FT mice (MetaCycle, $q < 0.05$; Figure 2e-f). However, this disruption in FA mice was less pronounced. For instance, motor activity differed significantly between NA and FA mice only during ZT9-ZT13 (two-way ANOVA with LSD post hoc test, $p < 0.05$, Figure 2e). Similarly, FA mice slept less than NA mice only during ZT9-ZT13 and ZT21-ZT1—two windows corresponding to light transitions ($p < 0.05$, Figure 2f). Interestingly, sleep duration also differed between FT mice and both NA and FA mice, suggesting that FT mice generally slept more than those in other conditions.

Beyond behavioral rhythms, we also examined metabolic parameters to assess whether TRF might have exerted its effects through restoring metabolic cycling. We observed no differences in rhythmicity between NA and FA mice for mean respiratory exchange ratio (RER), energy expenditure, or food consumption (MetaCycle, $q < 0.05$; Figure 2g-i). None of the conditions exhibited rhythmicity in RER; however, NA mice displayed higher RER during the dark phase than the HFD-fed mice (two-way ANOVA with LSD post hoc test, $p < 0.05$; Figure 2g). Energy expenditure was rhythmic in all three groups but only differed between NA and FA during ZT21 to ZT1 ($p < 0.05$; Figure 2h). For food consumption, rhythmicity was absent in both NA and FA mice but present in the FT group. However, this rhythm in FT reflects the imposed feeding window rather than endogenous diurnal control (Figure 2i). In addition, FA mice consumed fewer calories than NA mice during the dark phase, with no differences observed during the light phase—contrary to previous DIO studies, which typically show increased food intake across all ZTs. These findings suggest that TRF may have failed to ameliorate MASH in this insulin-deficient model due to the lack of pronounced disruptions in feeding and metabolic rhythms, leaving little for TRF to correct.

Collectively, our results show that TRF does not protect against STAM/HFD-induced MASH, where insulin signaling is perturbed. While TRF modestly reduced hepatic triglycerides levels, it was insufficient to prevent the histological progression of MASH. These results indicate that weight loss and reduced caloric intake alone are insufficient to prevent MASH in the absence of functional insulin signaling or perturbed metabolic behaviors. TRF's effectiveness appears to be limited by the impaired insulin response in STAM/HFD mice, suggesting that effective insulin signaling is necessary for reversing steatohepatitis and fibrosis. This may explain why tirzepatide has been effective in reversing these features of MASH (Harrison et al., 2024). Thus, interventions targeting both insulin pathways and caloric restriction may be required to address the later complications of MASH.

Perhaps the most surprising result from our study was that, in the absence of normal insulin signaling of

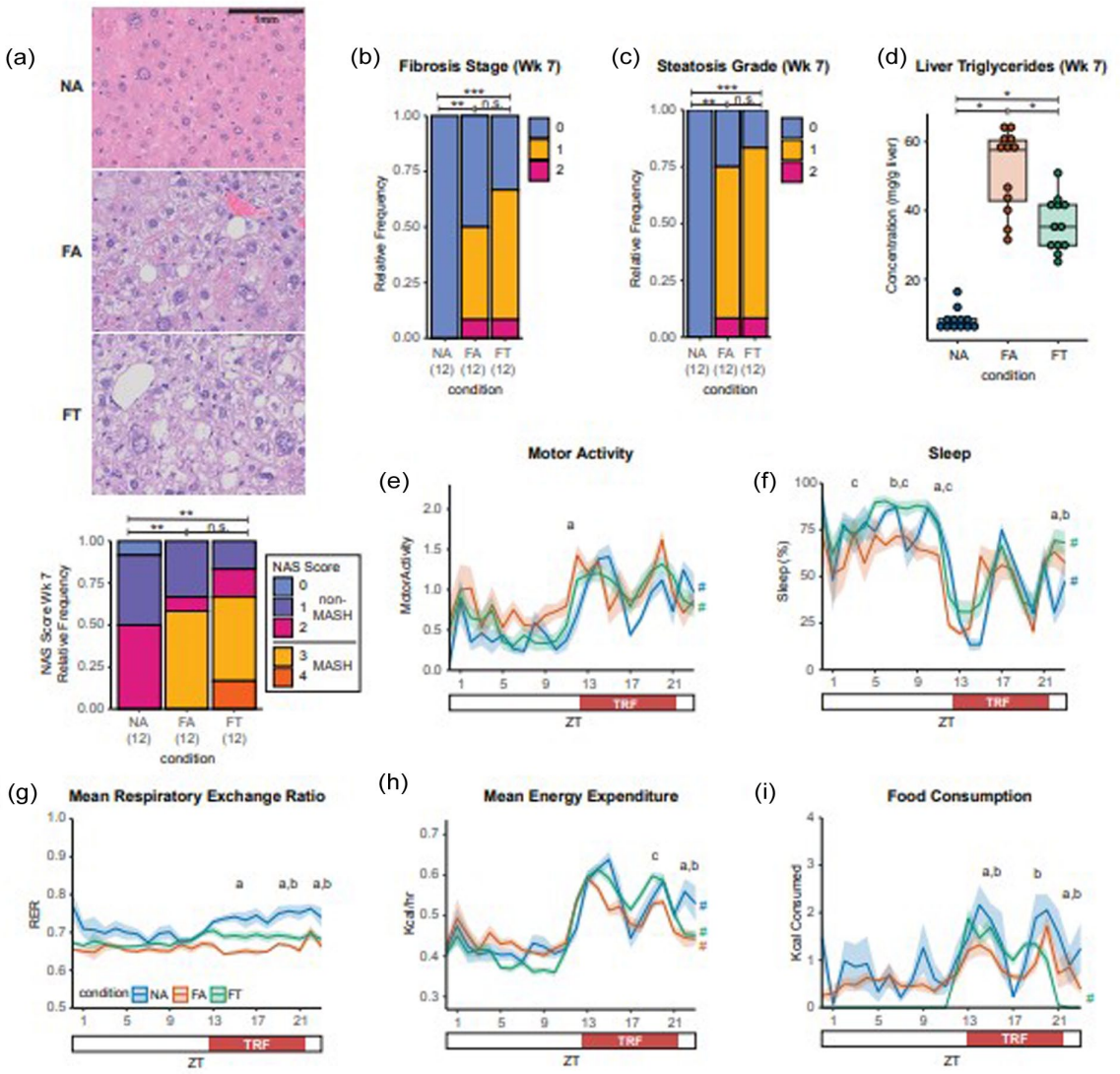


**Figure 2. Liver histology (a) representative images by condition (top) and NAFLD Activity Score (NAS) (bottom), (b) fibrosis score, and (c) steatosis grade at the time of sacrifice (n = 12 mice/condition; Fisher's exact test, $p < 0.05$). (d) Liver triglyceride concentrations at the time of sacrifice (n = 12, one-way analysis of variance (ANOVA), LSD post hoc test, $^*p < 0.05$, n.s. = not significant). (e) Motor activity, (f) sleep, (g) mean respiratory exchange (RER) ratio, (h) mean energy exchange ratio, and (i) food consumption averaged over 48 hours in 1-hour bins (n = 3-8 mice/condition, MetaCycle, #q < 0.05; two-way ANOVA, LSD test, $p < 0.05$, a = NA vs FA, b = NA vs FT, c = FA vs FT). FT mice had their feeding consolidated from ZT13-ZT21 (red).**

the STAM/HFD model, feeding patterns were not profoundly disrupted under ad libitum conditions, though other diurnal behaviors were. That is, unlike previous studies with wild-type mice on HFDs, the STAM/HFD mice did not redistribute their caloric intake across day and night but maintained rather normal feeding behavior, with the bulk of calories consumed in the nocturnal period. This contrasts with many studies on wild-type mice with normal insulin signaling, where HFD-fed mice typically exhibit disrupted diurnal feeding rhythms and spread their food intake throughout the day and night (Chaix et al., 2014; Hatori et al., 2012; Zarrinpar et al., 2014). The unchanged feeding patterns in STAM/HFD mice suggest that TRF may have limited efficacy in models with minimally disrupted diurnal rhythms, as there are no aberrant patterns for TRF to correct.

Future studies should investigate the role of insulin signaling in MASH and TRF's effects using models like liver-specific insulin receptor knockout (LIRKO) mice or insulin antagonists. Furthermore, additional dietary models such as choline-deficient, L-amino acid-defined, high-fat diet (CDA-HFD)

could help assess TRF's effectiveness in preventing steatohepatitis and fibrosis. These approaches will clarify whether TRF can serve as a standalone therapy or be used in combination with treatments aimed at improving insulin sensitivity. By integrating TRF with therapies that address insulin dysfunction, we may unlock new strategies to manage MASH and other metabolic liver diseases across a broader spectrum of patients. Recent studies further support the importance of microbiota rhythmicity and circadian alignment in the pathogenesis of metabolic liver disease. For example, restoration of rhythmic oscillations in gut microbiota and microbial metabolites by TRF alleviates MASH, highlighting microbial rhythmicity as a potential mediator of TRF's beneficial metabolic effects (Xia et al., 2023). In addition, studies show significant inter-individual variation in the severity of liver pathology induced by circadian disruption, emphasizing the protective role of robust circadian rhythms against metabolic liver diseases (Koike et al., 2024). These observations align closely with our findings, suggesting that intact insulin signaling and pronounced disruptions in diurnal rhythms may critically influence the efficacy of TRF interventions. While our study was limited to male mice since STAM/HFD female mice do not develop MASH (Fujii et al., 2013), future studies could test whether the lack of TRF effect we observed in males parallels the resistance seen in females. Collectively, these data underscore the complex interactions among dietary timing, circadian biology, microbial dynamics, and host metabolic responses, revealing new opportunities for tailored therapeutic approaches in MASH and associated metabolic disorders.

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## AUTHOR CONTRIBUTIONS

Conceptualization—KAF, AZ; Methodology—KAF, SFR, AZ; Formal Analysis—SFR, KAF; Investigation—KAF, SFR, VBM, JH, MH, WZ; Resources—AZ; Data Curation—SFR, KAF; Writing (Original Draft)—SFR, KAF; Writing (Review & Editing)—SFR, AZ, RL; Visualization—SFR; Supervision—AZ; Project Administration—AZ; Funding Acquisition—AZ.

## CONFLICT OF INTEREST STATEMENT

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: AZ is a co-founder and equity-holder in Endure Biotherapeutics. RL serves as a consultant to Aardvark Therapeutics, Altimmune, Arrowhead Pharmaceuticals, AstraZeneca, Cascade Pharmaceuticals, Eli Lilly, Gilead, Glympse bio, Inipharma, Intercept, Inventiva, Ionis, Janssen Inc., Lipidio, Madrigal, Neurobo, Novo Nordisk, Merck, Pfizer, Sagimet, 89 bio, Takeda, Terns Pharmaceuticals, and Viking Therapeutics. RL has stock options in Sagimet biosciences. In addition, his institution received research grants from Arrowhead Pharmaceuticals, Astrazeneca, Boehringer-Ingelheim, Bristol-Myers Squibb, Eli Lilly, Galectin Therapeutics, Gilead, Intercept, Hanmi, Intercept, Inventiva, Ionis, Janssen, Madrigal Pharmaceuticals, Merck, Novo Nordisk, Pfizer, Sonic Incytes, and Terns Pharmaceuticals. He is a co-founder of LipoNexus Inc.

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## NOTE

Supplementary material is available for this article online.

## REFERENCES

Chaix A, Zarrinpar A, Miu P, and Panda S (2014) Time-restricted feeding is a preventative and therapeutic intervention against diverse nutritional challenges. Cell Metab 20:991-1005.

Fujii M, Shibazaki Y, Wakamatsu K, Honda Y, Kawauchi Y, Suzuki K, Arumugam S, Watanabe K, Ichida T,

Asakura H, et al. (2013) A murine model for non-alcoholic steatohepatitis showing evidence of association between diabetes and hepatocellular carcinoma. Med Mol Morphol 46:141-152.

Harrison SA, Bedossa P, Guy CD, Schattenberg JM, Loomba R, Taub R, Labriola D, Moussa SE, Neff GW, Rinella ME, et al. (2024) A phase 3, randomized, controlled trial of resmetirom in NASH with liver fibrosis. N Eng J Med 390:497-509.

Hatori M, Vollmers C, Zarrinpar A, DiTacchio L, Bushong EA, Gill S, Leblanc M, Chaix A, Joens M, Fitzpatrick JAJ, et al. (2012) Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab 15:848-860.

Kohsaka A, Laposky AD, Ramsey KM, Estrada C, Joshu C, Kobayashi Y, Turek FW, and Bass J (2007) High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell Metab 6:414-421.

Koike N, Umemura Y, Inokawa H, Tokuda I, Tsuchiya Y, Sasawaki Y, Umemura A, Masuzawa N, Yabumoto K, Seya T, et al. (2024) Inter-individual variations in circadian misalignment-induced NAFLD pathophysiology in mice. iScience 27:108934.

Petersen MC, Gallop MR, Flores Ramos S, Zarrinpar A, Brouss ard JL, Chondronikola M, Chaix A, and Klein S (2022) Complex physiology and clinical implications of time-restricted eating. Physiol Rev 102:1991-2034.

Saran AR, Dave S, and Zarrinpar A (2020) Circadian rhythms in the pathogenesis and treatment of fatty liver disease. Gastroenterology 158:1948-1966.e1.

Xia J, Guo W, Hu M, Jin X, Zhang S, Liu B, Qiu H, Wang K, Zhuge A, Li S, et al. (2023) Resynchronized rhythmic oscillations of gut microbiota drive time-restricted feeding induced nonalcoholic steatohepatitis alleviation. Gut Microbes 15:2221450.

Zarrinpar A, Chaix A, and Panda S (2016) Daily eating patterns and their impact on health and disease. Trends Endocrinol Metab 27:69-83.

Zarrinpar A, Chaix A, Yooseph S, and Panda S (2014) Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. Cell Metab 20:1006-1017.