

# 1 **Early but not late exercise training in mice exacerbates hepatic** 2 **inflammation in early NAFLD**

3

4 Artemiy Kovynev<sup>1,2,\*</sup>, Zhixiong Ying<sup>1,2,\*</sup>, Joost Lambooi<sup>3</sup>, Bruno Guigas<sup>3</sup>, Patrick C.N.  
5 Rensen<sup>1,2</sup> and Milena Schönke<sup>1,2</sup>

6 *<sup>1</sup>Division of Endocrinology, Department of Medicine, Leiden University Medical Center, Leiden, The*  
7 *Netherlands; <sup>2</sup>Eindhoven Laboratory for Experimental Vascular Medicine, Leiden University Medical*  
8 *Center, Leiden, The Netherlands; <sup>3</sup>Department of Parasitology, Leiden University Medical Center,*  
9 *Leiden, The Netherlands; \*These authors contributed equally.*

10

## 11 **Abstract**

12 Exercise effectively prevents obesity-related disorders, but it is unclear whether the  
13 beneficial health effects of exercise are restricted to unique circadian windows.  
14 Therefore, we aimed to study whether timing of exercise training differentially  
15 modulates the development and progression of non-alcoholic fatty liver disease  
16 (NAFLD), a disease currently estimated to affect over two billion people worldwide.  
17 We endurance-trained high fat-high cholesterol-fed NAFLD-prone male APOE\*3-  
18 Leiden.CETP mice five times per week for eight weeks either in the early (ZT13) or in  
19 the late (ZT22) active phase and assessed the NAFLD score (histology) and hepatic  
20 inflammation compared to sedentary mice. Exercise training prevented an increase in  
21 body fat mass and fasting plasma glucose as expected, but neither early nor late  
22 training affected liver triglyceride or cholesterol content compared to sedentary mice,  
23 likely due to a very early stage of hepatic steatosis. In line, hepatic expression of de  
24 novo lipogenesis genes (e.g., Fasn, Srebp1c) was similarly downregulated by early  
25 and late training. However, exercise had a distinct time-dependent effect on hepatic  
26 inflammation, as only early training promoted an influx of pro-inflammatory cells into the  
27 liver paired with increased expression of the pro-inflammatory cytokines (e.g. Tnfa,  
28 Il1b). This data suggests that the timing of exercise is a critical factor for the effect on  
29 cardiometabolic disease development.

30

31

32 Two billion people worldwide are estimated to have non-alcoholic fatty liver disease  
33 (NAFLD), defined by excess hepatic fat. Commonly, the disease progression into non-  
34 alcoholic steatohepatitis (NASH) is characterized by the onset of liver inflammation  
35 following worsening steatosis [1]. This suggests that a reduction of liver inflammation  
36 – e.g. through lifestyle interventions such as exercise training – may only come  
37 secondary to a reduction of advanced steatosis. However, both the metabolic and  
38 inflammatory processes involved in NAFLD development are under circadian control  
39 and could hence respond differently to exercise at different times of day [2]. To  
40 investigate the time-of-day dependent effect of exercise training on NAFLD  
41 amelioration in the early disease stages we trained high-fat high-cholesterol (HFHC)-  
42 fed APOE\*3-Leiden.CETP mice early or late in their active period. For this, animals  
43 were trained on a treadmill (17 m/min) for 1 hour at either *Zeitgeber* time (ZT)13 (E-  
44 RUN) or ZT22 (L-RUN) five days per week. Corresponding sedentary animals (E-SED  
45 and L-SED) were put into empty cages without bedding at the same time to control for  
46 the experienced stress. This mouse model was chosen due to its humanized lipid  
47 metabolism and its ability to develop all hallmarks of human NAFLD upon HFHC  
48 feeding [3].

49 Following 8 weeks of training, all mice had a similar body weight (Fig. 1A) and lean  
50 body mass (Fig. S1A), but both exercising groups had gained less fat mass than their  
51 sedentary counterparts, which only reached statistical significance in the comparison  
52 of the early groups (Fig. 1B), indicating a measurable exercise effect. Fasting plasma  
53 glucose levels, independently positively associated with the risk to develop NAFLD [4],  
54 were unchanged among the groups (Fig. 1C). Simultaneously, no differences in  
55 hepatic steatosis, NAFLD activity score and liver weight were observed between any  
56 of the groups (Fig. 1D-F), likely due to an overall limited treatment potential of early  
57 steatosis. Accordingly, liver lipid levels (total cholesterol, triglycerides and  
58 phospholipids) were unchanged between the exercising and sedentary groups  
59 regardless of the time of training (Fig. 1G-I), and so were the levels of plasma  
60 triglycerides and cholesterol (Fig. S1B-C).

61 Surprisingly, however, exercise training had a time-of-day specific impact on liver  
62 inflammation, challenging the notion that hepatic inflammation can only be modulated  
63 later on in the disease through the reduction of steatosis. In livers collected at the  
64 same circadian timepoint (4 hours into the dark phase at ZT16; 17 and 26 hours after

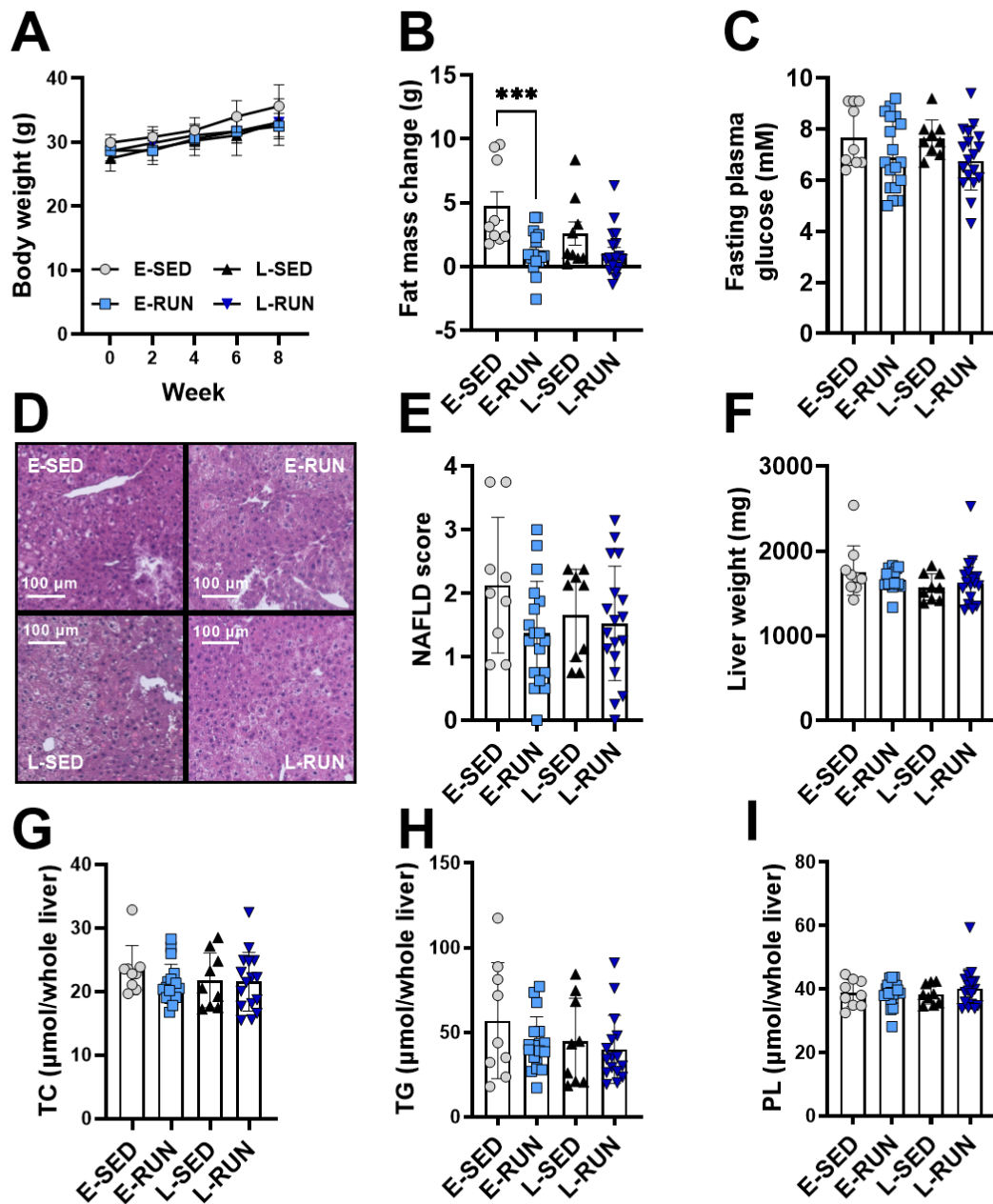
65 the last exercise bout for L-RUN and E-RUN, respectively), flow cytometry of the  
66 hepatic immune cells revealed a significant and unexpected increase in the number of  
67 leukocytes, neutrophils and monocytes with early exercise training (Fig. 2A-C). Late  
68 exercise, on the other hand, had no effect on liver immune cell populations. The  
69 increase of these cell populations particularly with early exercise may signify disease  
70 acceleration as infiltrating neutrophils are associated with early NAFLD development  
71 and progression into NASH [5, 6]. In line, infiltrating monocytes, that are recruited to  
72 the liver through hepatocyte-derived stress signals (such as IL-1 $\beta$  and TNF $\alpha$ ), promote  
73 NASH development once they differentiate into lipid-associated macrophages [7].  
74 Interestingly, early exercise training also increased the number of natural killer (NK)  
75 cells in the liver (Fig. 2D). The contribution of these cells that are specialized on killing  
76 infected and tumor cells to NAFLD development and progression to NASH remains  
77 controversial, but they produce large quantities of pro-inflammatory cytokines such as  
78 IFN $\gamma$  [8]. Taken together, early training leads to an inflammatory response in the liver  
79 characterized by an increase of pro-inflammatory and lipid damage-related cell  
80 populations.

81 An overall increase in liver inflammation with early exercise training was also  
82 confirmed by gene expression analyses in the isolated liver immune cells via  
83 quantitative polymerase chain reaction (qRT-PCR). The expression of the pro-  
84 inflammatory markers *Tnfa* and *Il-1 $\beta$*  and *Tnfa* was increased only with early but not  
85 with late exercise training (Fig. 2E-F). Similarly, the expression of the macrophage  
86 marker *Adgre1* (F4/80) tended to be increased and *Tim4*, a marker of monocyte-  
87 derived Kupffer cells [9], was increased only with early training (Fig. 2G-H). In line, in  
88 whole liver tissue early exercise training also increased the expression of *Tnfa*, *Il-1 $\beta$*   
89 and *Adgre1* (Fig. S1D-F).

90 From these findings, it is unclear whether the observed increase of liver inflammation  
91 in early NAFLD with early exercise training is beneficial or detrimental. Possibly, by  
92 stimulating liver inflammation, early exercise training activates an earlier immune  
93 response that contributes to disease resolution. On the other hand, it has been shown  
94 that early exercise can acutely worsen metabolic diseases as seen in people with  
95 obesity and type 2 diabetes where early high intensity cycling elicited unfavorable  
96 blood glucose spikes that did not occur with late exercise [10]. Accordingly, our  
97 findings could indicate that early exercise training accelerates disease progression

98 while late exercise potentially only affects liver steatosis and inflammation at a later  
99 disease stage. However, while not affecting liver lipid levels, the hepatic gene  
100 expression of *Srebp1c*, the mediator of insulin-induced fatty acid synthesis, was  
101 similarly downregulated with early and late training (Fig. S1G), suggesting that the  
102 regulation of metabolic and inflammatory disease drivers may not be synchronized.  
103 Future studies need to investigate how this immuno-modulatory exercise effect in early  
104 steatosis translates to advanced disease stages and to human NAFLD and NASH.  
105 Notably, while it is believed that the initiation of inflammation happens after the  
106 worsening of steatosis, we observe distinct inflammatory modulation already at an  
107 early stage of the disease with a low NAFLD score and low grade steatosis. This may  
108 present a previously underappreciated inflammation-targeted treatment opportunity in  
109 a large part of the population at risk for NASH.

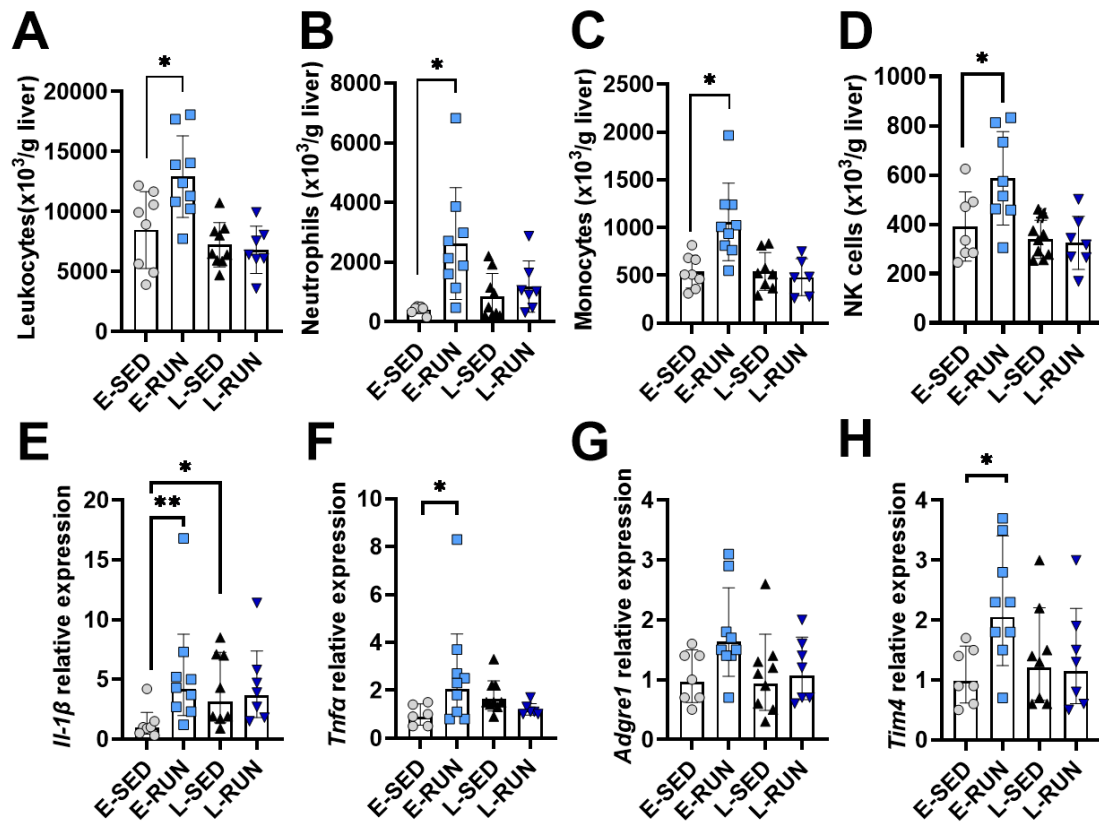
110 In summary, we demonstrate that early and late exercise training in a mouse model of  
111 NAFLD differently influenced liver inflammation in early steatosis. While both early and  
112 late exercise prevented a gain of body fat mass in comparison to sedentary animals,  
113 an unexpected increase in liver inflammation was observed with early exercise  
114 training.



115

116 **Figure 1. Early and late exercise training both improve body composition but do**  
 117 **not alter early liver steatosis in APOE\*3-Leiden.CETP mice.** Over 8 weeks of  
 118 treadmill training, body weight (A) and changes of fat mass (B) were monitored and  
 119 fasting plasma glucose was measured after 8 weeks (C). Representative images of  
 120 H&E-stained liver sections are shown (D) that were used to assess the NAFLD score  
 121 (E). Liver weight (F), total liver cholesterol (G), triglyceride (H) and phospholipid (PL)  
 122 content (I) were assessed after 8 weeks. \*\*\*P<0.001 in one-way ANOVA, n=9-18.

123



124

125 **Figure 2. Early exercise promotes distinct changes to liver immune cell**  
 126 **populations and inflammatory markers in early steatosis.** The number of liver  
 127 leukocytes (A), neutrophils (B), monocytes (C) and NK cells (D) was determined after  
 128 8 weeks of treadmill training using flow cytometry. The gene expression of *Il-1β* (E),  
 129 *Tnfa* (F), *Adgre1* (G) and *Tim4* (H) was assessed in the isolated liver immune cells  
 130 and shown relative to the expression levels of E-SED. \*P<0.05, \*\*P<0.01 in one-way  
 131 ANOVA, n=7-9.

132

### 133 References

- 134 1. Friedman, S.L., et al., *Mechanisms of NAFLD development and therapeutic*  
 135 *strategies*. Nature Medicine, 2018. **24**(7): p. 908-922.
- 136 2. Mazzoccoli, G., S. De Cosmo, and T. Mazza, *The Biological Clock: A Pivotal*  
 137 *Hub in Non-alcoholic Fatty Liver Disease Pathogenesis*. Frontiers in  
 138 Physiology, 2018. **9**.



- 139 3. van den Hoek, A.M., et al., *Beneficial effects of elafibranor on NASH in*  
140 *E3L.CETP mice and differences between mice and men*. Scientific Reports,  
141 2021. **11**(1).
- 142 4. Zou, Y., M. Yu, and G.T. Sheng, *Association between fasting plasma glucose*  
143 *and nonalcoholic fatty liver disease in a nonobese Chinese population with*  
144 *normal blood lipid levels: a prospective cohort study*. Lipids in Health and  
145 Disease, 2020. **19**(1).
- 146 5. Bertola, A., et al., *Hepatic Expression Patterns of Inflammatory and Immune*  
147 *Response Genes Associated with Obesity and NASH in Morbidly Obese*  
148 *Patients*. Plos One, 2010. **5**(10).
- 149 6. Hoogerland, J.A., B. Staels, and D. Dombrowicz, *Immune-metabolic*  
150 *interactions in homeostasis and the progression to NASH*. Trends Endocrinol  
151 Metab, 2022. **33**(10): p. 690-709.
- 152 7. Daemen, S., et al., *Dynamic Shifts in the Composition of Resident and*  
153 *Recruited Macrophages Influence Tissue Remodeling in NASH*. Cell Rep,  
154 2021. **34**(2): p. 108626.
- 155 8. Martinez-Chantar, M.L., T.C. Delgado, and N. Beraza, *Revisiting the Role of*  
156 *Natural Killer Cells in Non-Alcoholic Fatty Liver Disease*. Frontiers in  
157 Immunology, 2021. **12**.
- 158 9. Scott, C.L., et al., *Bone marrow-derived monocytes give rise to self-renewing*  
159 *and fully differentiated Kupffer cells*. Nat Commun, 2016. **7**: p. 10321.
- 160 10. Savikj, M., et al., *Afternoon exercise is more efficacious than morning exercise*  
161 *at improving blood glucose levels in individuals with type 2 diabetes: a*  
162 *randomised crossover trial*. Diabetologia, 2019. **62**(2): p. 233-237.

163

## 164 **Acknowledgements**

165 We thank Trea Streefland and Reshma Lalai (Div. of Endocrinology, Dept. of Medicine,  
166 LUMC, Leiden, the Netherlands) for their excellent technical assistance. We  
167 furthermore thank Lars Hoeve, Sjahnaaz Bholai and Jack Brouwer for their technical  
168 contributions. This study was financed by a grant from the Novo Nordisk Foundation  
169 to M.S. (NNF18OC0032394). Z.Y. was supported by a full-time PhD scholarship from  
170 the China Scholarship Council.