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Platelet GPIbα is a mediator and potential interventional target for NASH and subsequent liver cancer

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SUMMARY

Non-alcoholic fatty liver disease ranges from steatosis to non-alcoholic steatohepatitis (NASH), potentially progressing to cirrhosis and hepatocellular carcinoma (HCC). Here, we show that platelet-number, platelet-activation and platelet-aggregation are increased in NASH, but not in steatosis or insulin resistance. Antiplatelet therapy (APT; Aspirin/Clopidogrel, Ticagrelor) but not NSAID treatment with Sulindac prevented NASH and subsequent HCC development. Intravital microscopy showed that liver colonization by platelets depended primarily on Kupffer cells at early and late stages of NASH, involving hyaluronan-CD44 binding. APT reduced intrahepatic platelet-accumulation and frequency of platelet-immune cell interaction, thereby limiting hepatic immune-cell trafficking. Consequently, intrahepatic cytokine/chemokine release, macrovesicular steatosis and liver damage were attenuated. Platelet-cargo, platelet-adhesion and platelet-activation but not platelet-aggregation were identified as pivotal for NASH and subsequent hepatocarcinogenesis. In particular, platelet-derived GPIbα proved critical for development of NASH and subsequent HCC, independent of its reported cognate ligands vWF, P-Selectin or Mac-1, offering a potential target against NASH.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in high-income countries[1] and is on trajectory to become the most common indication for liver transplantation in the United States[2,3]. NAFLD ranges from simple steatosis (non-alcoholic fatty liver (NAFL)) to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis and ultimately hepatocellular carcinoma (HCC)[4,5]. HCC is the third most common cause of cancer-related death worldwide and is the fastest rising cancer in the United States and Europe[6-8]. Major risk factors for NASH include metabolic syndrome, abdominal obesity, insulin resistance, glucose intolerance or type 2 diabetes mellitus and dyslipidaemia[4,5,9,10].

We previously developed a pre-clinical model of human metabolic syndrome, NASH and NASH-induced HCC[11]. In this model, intrahepatic influx of metabolically activated $CD8^+$ T- and NKT-cells triggers metabolic reprogramming of hepatocytes, NASH and HCC development through cytokine-mediated cross-talk with hepatocytes. However, the mechanisms underlying immune cell recruitment during NASH and its consequences for NASH-to-HCC transition have remained unclear.

Platelets, produced by megakaryocytes in the bone marrow, play a fundamental role in hemostasis[12], but are also crucial for pathophysiological conditions like thrombosis, obesity, atherosclerosis, metastasis and stroke[13-15]. In addition, a growing body of evidence highlights platelets as active players in liver disease and inflammation[13,16,17]. Notably, it has been reported that activated platelets contribute to cytotoxic T lymphocyte (CTL)-mediated liver damage in a model of viral hepatitis[18,19]. Moreover, blocking platelet activation and aggregation by Aspirin-Clopidogrel (Asp-Clo) abrogates hepatic T cell influx, subsequent liver damage and tumorigenesis without affecting peripheral T cell function in viral hepatitis[19,20]. Additionally, in a

143   recent study of NAFL (but not NASH) patients, anti-platelet therapy (APT) lowered

144   serum markers of obesity and liver damage[21].

145   There is an unmet need for efficacious, low-risk therapies against NASH and NASH-

146   to-HCC transition. Although several drugs (e.g. decreasing blood sugar levels) are in

147   phase 2 and 3 development[22,23], currently no approved pharmacological therapies

148   are available which can prevent NASH or related pathologies. Further, the role of

149   platelets in NASH and HCC development is not well characterized. Thus, we

150   investigated whether APT and molecules involved in platelet function might prevent

151   NASH and NASH-induced HCC development.

152

RESULTS

Hepatic accumulation of activated platelets in NASH

To test whether platelets contribute to NASH development, we investigated platelet number and distribution in livers of C57Bl/6 mice fed a choline-deficient, high-fat diet (CD-HFD). Platelets numbers (**Fig 1a**) and aggregate size (**Fig 1b**) were significantly increased compared to age-matched normal chow diet (ND)-fed controls (**Fig. 1a**), whereas platelet counts in peripheral blood remained normal (**Supplementary Fig. 1a**). Although fibrinogen levels and prothrombin time (PT) remained unchanged, activated partial thromboplastin time (aPTT) was significantly reduced (**Supplementary Fig. 1a**). *Ex vivo* analyses of circulating platelets revealed no significant differences in activation/aggregation responses in CD-HFD-fed compared to ND-fed mice (**Supplementary Fig. 1b**). We next analyzed other dietary and genetic murine NASH models (**Fig. 1c-e; Supplementary Fig. 1c,d**), including high-fat, high-fructose, high-cholesterol, "Western-style" diets[24,25] with or without trans-fat (WD-HTF; WD-NTF), a "Western-style" diet with fructose (WD-FSDW)[26], a methionine/choline-deficient diet (MCD) and an inducible knock-in mouse expressing the human unconventional prefoldin RPB5 interactor (URI) in hepatocytes (hURI-tetOFFhep)[27]. All models induced NASH with varying degrees of NAS and fibrosis (**Supplementary Fig. 1e,f**), a primary determinant of outcome in NASH[28,29], and displayed a significant increase in intrahepatic platelet numbers compared to controls (**Fig. 1a,c-e; Supplementary Fig. 1c,d**). Further, human NAFLD/NASH patients displayed a significant increase in intrahepatic platelets in liver compared to healthy controls (**Fig. 1f; Table S1**).

In contrast, mice fed a 45% kcal high-fat diet (HFD), displaying only steatosis, or mice fed a 60% kcal HFD with low sucrose experiencing only simple steatosis and

178   insulin resistance[30], lacked any significant increase in intrahepatic platelet numbers,

179   despite increased body weight, NAS, liver damage and insulin resistance (HFD-

180   60%+LS) (**Fig. 1g,h; Supplementary Fig. 1g-i**). Intrahepatic platelet activation in

181   NASH was confirmed by electron microscopy (EM) (**Supplementary Fig. 1j**). Thus,

182   increased intrahepatic platelet number, platelet aggregation and platelet activation

183   were specific to NASH.

184   Asp-Clo treatment is an APT currently used in several diseases (e.g. to prevent

185   coronary stent thrombosis)[31]. We first addressed whether CD-HFD-fed mice would

186   respond to APT. Compared to untreated CD-HFD-fed mice, Asp-Clo-treated mice

187   displayed significantly lower intrahepatic platelet numbers (**Supplementary Fig. 1k-**

188   **m**), as well as reduced platelet aggregation and activation (**Supplementary Fig. 1n**).

189   To investigate the effects of APT on human NAFLD, we performed a pilot case-study

190   (German Clinical Trials Register (DRKS) 587/2016BO2) with patients at risk for

191   NAFLD (BMI>30 and/or diabetes mellitus type II) undergoing a heart catheter

192   procedure. Depending on the outcome of the catheter procedure, one of three

193   outcomes was carried out: (1) Patients received dual antiplatelet therapy (DAPT) for

194   at least 6 months if a coronary stent was implanted. (2) ASA monotherapy was given

195   for at least 6 months if coronary artery disease (CAD) was present but stent

196   implantation was not indicated. (3) No anti-aggregation therapy was given if coronary

197   artery disease was absent. None of the patients included received long-term

198   treatment with ASA or P2Y12 before study inclusion (**Supplementary Fig. 1o; Table**

199   **S2**). In this pilot case-study of 24 individual patients/observations, platelet function

200   analyses revealed that patients generally responded well to APT (**Supplementary**

201   **Fig. 1p**), although serum total cholesterol, LDL- and HDL- cholesterol levels

202   remained unchanged (**Supplementary Fig. 1p,q**). For control, we investigated

203   patients without APT (**Table S2**). NAFLD patients underwent liver MRI, liver

204 ultrasound and serum analysis at study inclusion and after 6 months of follow-up.

205 APT-treated NAFLD patients showed significantly reduced liver volume and liver fat

206 mass (**Supplementary Fig. 1r-t**). Patients who received a new therapy with ASA or

207 DAPT were diagnosed with CAD. The standard treatment in patients with CAD

208 includes statins. Hence, patients who received a new anti-aggregation therapy were

209 usually treated with a new statin treatment if statins were not already administered on

210 a regular basis and if contra-indications were absent. Thus, statins might present a

211 significant confounder in the actual pilot case-study, which we tried to address by

212 performing a linear regression analysis. Since the study collective was small, we

213 included age, gender, anti-aggregation and new statin treatment into the regression

214 analysis. For the course of liver fat, we found the strongest associations for anti-

215 aggregation (b=-0.334, 95% CI -4.719-1.197, p=0.226), whereas initiation of a new

216 statin treatment showed weaker associations (b=-0.095, 95% CI -3.551-2.526,

217 p=0.726). For the course of liver volume, we found associations for anti-aggregation

218 (b=-0,454, 95% CI -0.170-0.006, p=0.066) stronger compared to initiation of a new

219 statin treatment (b=0.047, 95% CI -0.084-0.101, p=0.847). This indicated that effects

220 of anti-aggregation were stronger than statin administration. Notably, n=4 patients

221 showed significant reductions in liver fat content and/or liver volume without new

222 statin therapy. However, there was a trend towards effect of anti-aggregation without

223 being statistically significant. Higher BMI did not significantly affect liver fat or liver

224 volume.

225

226 **Asp-Clo treatment attenuates NASH and NASH-associated conditions**

227 We next investigated whether Asp-Clo (adjusted to the body weight) affects NASH or

228 HCC development in mice receiving ND, CD-HFD or CD-HFD with Asp-Clo for 12

10

229 months. Weight gain over time was significantly higher in CD-HFD and CD-HFD/Asp-

230 Clo-fed mice compared to ND-fed controls (**Fig. 2a**). Similar to body weight,

231 epididymal fat (eWAT) weight was not different to CD-HFD in CD-HFD/Asp-Clo-fed

232 mice (**Supplementary Fig. 2a**). Low platelet numbers were found in eWAT and

233 remained unaltered (**Supplementary Fig. 2b,c**). CD3^{+} T-cell infiltration was

234 significantly reduced by Asp-Clo (**Supplementary Fig. 2c**). RNAseq analysis of

235 eWAT from ND- and CD-HFD-fed mice revealed a different gene expression pattern

236 resulting in the spatial separation in the PCA plot. Analysis of the most differentially

237 expressed genes between diets showed upregulation of pathways involved in

238 immune signaling, cell-cell interactions and extracellular matrix regulation in eWAT

239 from CD-HFD-fed mice. In contrast, eWAT of CD-HFD fed-mice showed a significant

240 downregulation in pathways involved in metabolism and antioxidant response

241 (**Supplementary Fig. 2d**). ALT, AST, liver/body weight ratio, platelet numbers and

242 aggregation state were significantly lower in livers of mice fed CD-HFD/Asp-Clo for 6

243 and 12 months (**Fig. 2b; Supplementary Fig. 1k-n; Supplementary Fig. 2e,f**). Asp-

244 Clo significantly improved glucose tolerance (**Fig. 2c**), reduced liver triglycerides

245 (**Fig. 2d**), and attenuated serum total cholesterol, LDL- and HDL- cholesterol levels

246 (**Fig. 2e; Supplementary Fig. 2g,h**). Several genes involved in fatty acid β-oxidation,

247 lipolysis and cholesterol metabolism are dysregulated during NASH development[11].

248 Asp-Clo treatment prevented downregulation of genes from all three groups (**Fig. 2f**).

249 CD-HFD/Asp-Clo-fed mice lacked statistically significant changes in oxygen

250 consumption, respiratory exchange ratio (RER), physical activity or in food and water

251 intake compared to CD-HFD-fed mice (**Fig. 2g,h; Supplementary Fig. 2i**). These

252 data were corroborated in WD-HTF fed mice (**Supplementary Fig 2. j,k**).

253 To analyze platelet activation, P-selectin, a marker of α-granule release, and integrin

254 αIIbβ3 activation were analyzed by flow cytometry. In Asp-Clo-treated CD-HFD mice,

circulating platelets showed markedly reduced integrin αIIbβ3 activation and P-selectin exposure compared with ND and CD-HFD platelets in response to all tested agonists (**Supplementary Fig. 2l**), suggesting that Asp-Clo treatment effectively reduced platelet activation. Levels of major platelet surface glycoproteins were unchanged (**Supplementary Fig. 2m**).

MRI-analysis revealed subcutaneous/abdominal fat accumulation in CD-HFD and CD-HFD/Asp-Clo treated mice, but not in ND-fed controls (**Fig. 2i**). However, liver steatosis was ameliorated or even prevented by Asp-Clo treatment in CD-HFD mice (**Fig. 2i**). In contrast, untreated CD-HFD-fed mice displayed histopathological features of NASH, including liver fat deposition (Sudan red$^+$ areas), fibrosis, damaged hepatocytes and lobular inflammation including satellitosis (**Fig. 2j, 2k, Supplementary Fig. 2n,o**). We concluded that Asp-Clo treatment effectively prevented NASH development.

Asp-Clo treatment abrogates intrahepatic immune-cell infiltration and inhibits NASH-induced HCC

In addition to hepatic infiltration of CD3$^+$CD8$^+$ T cells, CD11b$^+$ MHCII$^+$ myeloid cells and Ly-6G$^+$ granulocytes are increased in CD-HFD-fed mice, similar to human NASH patients[11]. Immune cell infiltration was reduced in 6-month and 12-month CD-HFD/Asp-Clo-treated mice (**Fig. 3a**). Flow cytometry demonstrated strong reduction in total number, effector differentiation (CD8$^+$CD62L$^-$CD44$^+$CD69$^+$) and proportion of CD4$^+$/CD8$^+$ and NKT-cells (**Fig. 3b,c**). B-cells remained unchanged in 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (**Supplementary Fig. 3a,b**).

Analyses of several potentially carcinogenic inflammatory signaling pathways activated under CD-HFD were dampened by Asp-Clo (**Fig. 3d** and **Supplementary**

279   **Fig. 3c**). Asp-Clo significantly reduced CD11b$^+$F4/80^{hi} Kupffer cells (KCs) in WD-HTF

280   livers (**Supplementary Fig. 3d-k**). An unbiased t-distributed stochastic neighbor

281   embedding (t-SNE)-based clustering approach identified 9 myeloid sub-clusters

282   (**Supplementary Fig. 3h,l** and **Table S3**). Asp-Clo significantly reduced the

283   abundance of cluster 6, characterized by a high expression of CD11b, F4/80 and

284   Gr1, closely resembling CD11b$^+$F4/80$^+$ monocyte-derived macrophages (MoMFs).

285   Further, a multiplex gene expression analysis for FACS-isolated Ly6C$^+$ MoMFs,

286   LY6C$^-$ MoMFs and KCs was performed. Principal component analysis of 561 genes

287   revealed similar Ly6C$^+$ MoMFs and Ly6C$^-$ MoMFs in NASH-diet-fed mice with and

288   without Asp/Clo treatment. However, KCs from WD-HTF/Asp-Clo livers clustered

289   more closely to ND KCs than WD-HTF (**Supplementary Fig. 3h-k**). This indicates

290   that Asp-Clo influences the KC compartment. Taken together, these data indicate

291   that Asp-Clo treatment attenuated KC activation, alongside reduced inflammatory

292   myeloid cell infiltration in the injured liver. Altogether, Asp-Clo prevented NASH,

293   reduced intrahepatic immune cell influx and dampened pathways potentially

294   supporting hepatocarcinogenesis [32].

295   Next, we studied the effects of Asp-Clo treatment on CD-HFD-induced HCC[11]. 13 out

296   of 51 CD-HFD livers (~25%) displayed macroscopically visible tumors by 12 months

297   (**Fig. 3e-g**). In contrast, CD-HFD/Asp-Clo-treated mice lacked macro- and

298   microscopically visible liver tumors (**Fig. 3e-g; Supplementary Fig. 3l**). CD-HFD-fed

299   mice treated with a lower dose of Asp-Clo (according to the initial body weight and

300   not further adjusted to diet/age-related weight gain) developed significantly fewer

301   HCC (3/52) compared to untreated CD-HFD-fed mice (13/51) (**Supplementary Fig.**

302   **3m**). Therefore, the dose of Asp-Clo continuously adjusted to the body weight is

303   critical to fully prevent HCC.

Asp/Clo dampens hepatic cytokine expression, platelet-liver endothelium and platelet-immune cell interaction

Gene expression and signaling pathway analyses of ND, CD-HFD and WD-HTF livers revealed a significant induction in gene expression profiles involved in platelet activation, aggregation and degranulation (**Extended Data-Fig. 1a-i**). Moreover, NASH-related enrichment of genes was associated with expression of TNF-superfamily members, cytokine/chemokine production and chemotaxis[11,27] (**Extended Data-Fig. 1a-i**). Asp-Clo treatment significantly attenuated the latter, some of which are also released from activated platelets (e.g. CCL5, TGFβ) (**Extended Data-Fig. 1j**)[33].

Coupling high-resolution confocal microscopy and 3D-reconstruction of liver sinusoids enabled visualization and quantification of platelet interactions with the liver endothelium and immune cells. Asp-Clo reduced NASH-related increased interaction of platelets with the liver endothelium, T-cells and innate immune cells (**Extended Data-Fig. 1k-q**).

To exclude COX2-dependent effects of Asp-Clo, we used another NSAID/COX1/2 inhibitor, Sulindac. CD-HFD/Sulindac-treated mice exhibit obesity, no significant changes in liver/body weight ratio, hepatic triglycerides, glucose tolerance, no significant alteration in gene expression involved in metabolism, severe steatosis and increased liver damage comparable to CD-HFD treated mice (**Extended Data-Fig. 2a-f**). Thus, Asp/Clo-mediated effects on NASH are COX-independent.

To corroborate our results with another platelet inhibitor, CD-HFD-fed mice were treated with Ticagrelor (CD-HFD/Ticagrelor), an FDA-approved direct and reversible

328   antagonist of the platelet P2Y12 receptor[34] for coronary artery disease used in the

329   clinic (e.g. with ASA after coronary stent implantation in patients presenting with an

330   acute coronary syndrome without increased bleeding risk)[35]. Intrahepatic platelet

331   number, platelet/liver endothelium coverage, platelet aggregation, liver damage, liver

332   triglycerides and serum cholesterol levels were significantly reduced in Ticagrelor-

333   treated mice, whereas VLDL secretion was not affected (**Extended Data-Fig. 3a-h**).

334   Expression of genes involved in cholesterol metabolism, ´*de novo*´ lipogenesis and

335   lipid storage was unchanged by the treatment, while downregulation of several

336   genes involved in fatty acid oxidation and lipolysis that occurs during CD-HFD

337   (**Extended Data-Fig. 3i,j**) as well as histopathological features of NASH were

338   prevented by Ticagrelor (**Extended Data-Fig. 3k-m**). Total numbers, activation and

339   proportion of $CD4^+/CD8^+$, $CD8^+CD62L^-CD44^+CD69^+$ and $CD3^+NK1.1^+$ cells were also

340   reduced (**Extended Data-Fig. 3n,o**). In contrast to liver tissues, increased

341   expression of genes involved in inflammation and fibrosis was found in eWAT from

342   CD-HFD or WD-HTF mice receiving Asp-Clop or Ticagrelor (**Extended Data-Fig.**

343   **3p**). This indicate that APT is beneficial specifically to the liver, affecting not only

344   intrahepatic platelets and immune-cell infiltration, but also improving hepatic steatosis

345   by maintaining normal expression of genes involved in lipid catabolism.

346

347   We next investigated whether Ticagrelor would affect NASH-HCC transition.

348   Compared to untreated CD-HFD mice, Ticagrelor treatment significantly reduced

349   HCC development. One tumor was detected in 29 livers of CD-HFD/Ticagrelor-

350   treated mice (**Extended Data-Fig. 3q**). The therapeutic potential of Ticagrelor was

351   tested in CD-HFD mice with fully established NASH (4 months CD-HFD), followed by

352   8-weeks of Ticagrelor treatment with CD-HFD. Intrahepatic platelet numbers, NAS,

353   liver damage were reduced, and no difference in glucose tolerance was found

(**Extended Data-Fig. 3r,s**), or effect on the blood immune cells populations was observed (**Extended Data-Fig. 3t**). Similar results were obtained with therapeutic Ticagrelor treatment in the context of a WD-HTF including of fibrosis; no difference in whole body metabolism was found upon Ticagrelor treatment in CD-HFD or WD-HTF (**Extended Data-Fig. 3u-x**).

Early platelet recruitment in fatty liver correlates with liver damage, hepatocyte swelling and reduced sinusoidal diameter

To understand the dynamics of intrahepatic platelet recruitment/attachment during the initial events of NAFL preceding NASH, we performed intravital microscopy in CD-HFD or WD-HTF-fed mice over 4, 5, 6 and 8 weeks post-diet induction (pdi) (**Fig. 4a; Supplementary Fig. 4a-c**). Platelets were the first non-resident cell type to populate the liver at ≤4 weeks pdi in both CD-HFD and WD-HTF (**Fig. 4a-d; Supplementary Fig. 4a-c**). Platelets progressively aggregated and increased in number in liver sinusoids over 8 weeks in the absence of significantly elevated CD3$^+$ T-cells or Ly6G$^+$ granulocytes (**Fig. 4a-d; Supplementary Fig. 4a-c**). Even at this early stage, mild steatosis, reduced sinusoidal diameter and hepatocyte swelling were observed (**Fig. 4e,f; Supplementary Fig. 4e**). NAS, liver triglycerides were increased in CD-HFD and WD-HTF (**Fig. 4g,h; Supplemental Fig. 4d-f**). Platelets interacted primarily with Kupffer cells, as determined by 3D high-resolution reconstruction (**Fig. 4i**). Although intrahepatic granulocyte numbers remained unaltered in the first 8 weeks pdi, granulocytes might still support intrahepatic platelet recruitment and NAFL/NASH induction. Administration of anti-Ly6G antibodies via osmotic pumps for 8 weeks to CD-HFD-fed mice did not reduce intrahepatic platelet numbers but successfully reduced granulocytes. Experiments revealed no significant

role of granulocytes in the early development of NAFL and borderline NASH (**Supplementary Figure 4g-k**).

We next screened for possible adhesion molecules/danger markers responsible for early platelet attachment/recruitment. We found progressive induction of the extracellular matrix component hyaluronan (HA) co-localizing with hepatocytes, Kupffer cells and, to lesser degree, on LSECs (**Fig. 4j**).

Kupffer cell-dependent platelet recruitment involving hyaluronan-CD44 binding supports early and advanced stages of NAFL including NASH

To investigate the functional role of Kupffer cells and molecules involved in platelet-LSEC/immune-cell interaction (e.g. hyaluronan, CD44) in borderline NASH, CD-HFD or WD-HTF-fed mice were treated with clodronate liposomes (CLL), control liposomes (CL), hyaluronidase (HYAL), HYAL/CLL (double treatment), CD44-binding/HA-blocking AB (clone KM81) or CD44-binding/HA-non-blocking AB (clone IM7), for control (**Fig. 5a, Supplementary Fig. 5a,b**). CLL and CLL/HYAL, but not CL treatment reduced Kupffer cell numbers in CD-HFD- and WD-HTF-fed mice (**Fig. 5b, Supplementary Fig. 5c,d**). CLL and HYAL reduced NAS significantly in CD-HFD and WD-HTF-fed mice (**Fig. 5b, Supplementary Fig. 5d**). CLL and HYAL treatments significantly reduced intrahepatic platelets (**Fig. 5c**), a significant reduction in liver damage was found following HYAL but not CLL (**Fig. 5d**). Combined treatment with both CLL/HYAL did not result in further decrease of platelet numbers (**Supplementary Fig. 5f).** Similar data (platelet reduction, NAS reduction) with CLL treatment were obtained using WD-HTF mice, although ALT levels remained elevated (**Supplementary Fig. 5d,e**). In addition, treatment with a CD44-binding/HA-

403 blocking, but not with a CD44-binding/HA non-blocking antibody led to a reduction in

404 NAS and liver damage (**Fig. 5e-g**).

405 Notably, significantly reduced NAS, platelet accumulation, triglycerides, and liver

406 damage by CLL-treatment in mice fed CD-HFD for 6 months demonstrate a potential

407 therapeutic effect of CLL even in a short treatment scheme (**Fig. 5 h,i;**

408 **Supplementary Fig. 5h,i**). These data imply that Kupffer cells, hyaluronan and

409 CD44 are important players in the early and advanced stages of NAFL, including

410 NASH.

411 **Platelet cargo is indispensable for NASH development**

412 Platelets release bioactive factors from intracellular granules in response to cellular

413 activation. During thrombo-inflammatory reactions, the mostly proteinous

414 components of α-granules are essential for immune cell recruitment and tissue

415 damage[36]. Nbeal2 knockout mice (*Nbeal2*$^{-/-}$), which lack α-granules in platelets and

416 are thus protected from thrombosis and thrombo-inflammatory tissue damage[37-39]

417 were on CD-HFD for 6 months. CD-HFD-fed *Nbeal2*$^{-/-}$ displayed no significant

418 difference in intrahepatic platelet number or aggregation and gained weight similarly

419 to CD-HFD-fed controls (**Fig. 5j; Supplementary Fig. 5j**). Still, significantly lower

420 serum ALT and AST levels were found in CD-HFD-fed *Nbeal2*$^{-/-}$ mice compared to

421 CD-HFD-fed mice (**Fig. 5k**). This was paralleled by a significant decrease in liver

422 triglycerides, lower serum cholesterol (**Fig. 5l,m**), and improved glucose tolerance in

423 CD-HFD-fed *Nbeal2*$^{-/-}$ mice (**Supplementary Fig. 5k**). Deregulation of lipid

424 metabolism genes found in CD-HFD livers was partially prevented in CD-

425 HFD/*Nbeal2*$^{-/-}$ (**Supplementary Fig. 5l**). CD-HFD *Nbeal2*$^{-/-}$ livers lacked steatosis,

426 NASH, and displayed a reduced NAS (**Fig. 5n; Supplementary Fig. 5m**).

427 Suppression of NASH in *Nbeal2*$^{-/-}$ mice on CD-HFD was corroborated by significant

428   diminution of lipid content (**Fig. 5o**). In line, decreased T-cell infiltration, neutrophil

429   accumulation and macrophage activation were found in *Nbeal2*$^{-/-}$ mice on CD-HFD

430   (**Supplementary Fig. 5n**). Together, these results indicated that platelet α-granule

431   components contribute to NASH.

432   CD-HFD-induced NASH could not be rescued in mice lacking the GPIIb subunit of

433   the platelet fibrinogen receptor GPIIb/IIIa (integrin $\alpha2\beta3$; *Itga2b*$^{-/-}$ mice) which harbors

434   activatable platelets unable to aggregate (**Extended Data-Fig. 4**). This is in

435   agreement with a recent study indicating that deletion of the platelet integrin $\alpha2\beta3$

436   binding motif of fibrinogen did not alter NASH[40].

437   Moreover, mice lacking the activating platelet collagen receptor glycoprotein VI

438   (GPVI; *Gp6-/-*)[41], the platelet-derived C-type lectin-like receptor 2 (*Clec-2*$^{-/-}$) or the

439   hematopoietic-specific podoplanin (*Pdpn*$^{-/-}$) all developed NASH and NASH-

440   associated conditions upon CD-HFD feeding (**Extended Data-Fig. 5-6**).

441   Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) was recently

442   discovered as an important player in NASH[42]. Animals with genetic inactivation of

443   MAdCAM-1 presented lower NAS and less liver damage after 6-month of WD-HTF[42].

444   We analyzed livers of WD-HTF-fed mice lacking MAdCAM-1 (*MAdCAM-1*$^{-/-}$), L-

445   selectin (*L-sel*$^{-/-}$), integrin beta7 ($\beta7^{-/-}$) and both L-selectin and integrin beta7 (*L-*

446   *sel/$\beta7$*$^{-/-}$). *MAdCAM-1*$^{-/-}$ mice displayed significantly reduced intrahepatic platelet

447   numbers correlating with a partial protection from NASH (**Extended Data-Fig. 7a,b**).

448   In contrast, deletion of MAdCAM-1 ligands integrin beta7, L-selectin and L-

449   selectin/integrin beta7 did not affect intrahepatic platelet numbers or platelet

450   aggregation and did not or only partially prevent NASH (**Extended Data-Fig. 7a-b**).

451

Platelet GPIbα and α-granules are required to induce NASH

Our data demonstrate that intrahepatic interaction of platelets with Kupffer cells, involving hyaluronan/CD44 binding and platelet cargo-function, but not platelet aggregation contribute to NASH. Platelet-derived GPIbα has been implicated in platelet attachment and activation[43]

We thus hypothesized that GPIbα might mediate early platelet-trafficking/activation in NASH livers[43]. We first analyzed the interaction of GPIbα with parenchymal and non-parenchymal liver cells (LSECs; Kupffer cells etc.) in NASH. 3D reconstruction revealed that the most abundant interactions of GPIbα[+] platelets were with Kupffer cells and less so with LSECs in mouse and human NASH samples (**Fig. 6a,b; Supplementary Fig. 6a**).

Thus, we blocked the major ligand binding domain of GPIbα in 6-months CD-HFD-fed mice therapeutically using Fab fragments of the anti-GPIbα antibody, pop/B[44] for 5 weeks. Notably, this short therapeutic treatment significantly reduced intrahepatic platelet accumulation in the presence of CD-HFD (**Fig. 6c,d**). Consequently, steatosis, NAS, liver damage, liver triglycerides and intrahepatic immune-cell infiltration were reduced; fibrosis was dampened (**Fig. 6c-h**; **Supplementary Fig. 6b-d**). In addition, anti-GPIbα antibody treatment reduced intrahepatic protein expression of several pro-inflammatory and homeostatic cytokines/chemokines, including CCL5, CCL6, ICAM-1, P-selectin and CD40, linking intrahepatic platelet activation and Nbeal2-dependent cargo release with mediators of inflammation **(Supplementary Fig. 6e)**.

We next tested whether therapeutic anti-GPIbα antibody treatment would prevent fatty liver to NASH transition in early disease progression (e.g. after 6 week CD-HFD;

476   see also Figure 4). However, these treatments did not ameliorate NAFL/ borderline

477   NASH - most likely due to lack or low expression of yet unidentified GPIbα ligands at

478   early disease stages (**Supplementary Fig. 6f,g**). These data highlight distinct

479   mechanisms of platelet recruitment in early versus established NAFL/borderline

480   NASH – still involving Kupffer cells at both disease stages.

481   To corroborate the above data in a genetic model, we fed transgenic mice expressing

482   an IL-4rα/GPIbα fusion-protein in a *GPIbα^{-/-}* background[45] in which the ligand-binding

483   ectodomain of GPIbα is replaced by the α-subunit of the human IL-4 receptor

484   (*hIL4rα/GPIbα*-Tg)[45] with a CD-HFD for 6 months. Remarkably, platelet aggregate

485   size, platelet area and platelet-liver endothelium coverage were significantly lower in

486   CDHFD-fed *hIL4Rα/GPIbα*-Tg mice compared to CD-HFD-fed C57Bl/6 controls

487   (**Supplementary Fig. 6h-j**). Both *hIL4rα/GPIbα*-Tg and C57Bl/6 mice gained weight

488   similarly when fed CD-HFD (**Supplementary Fig. 6k**). Serum cholesterol, liver

489   triglycerides, serum ALT and AST levels were significantly lower in CD-

490   HFD/*hIL4rα/GPIbα*-Tg mice (**Fig. 6i,j; Supplementary Fig. 6l**), accompanied by less

491   LDL- and HDL-cholesterol (**Supplementary Fig. 6l**). Similarly, dysregulated mRNA

492   expression of lipid metabolism-related genes in CD-HFD-fed C57BL/6 livers was

493   prevented in CD-HFD/*hIL4rα/GPIbα*-Tg livers (**Supplementary Fig. 6m**). We also

494   observed strong and significant reductions in intrahepatic CD8^{+} T- and NKT-cells by

495   flow cytometry analysis (**Fig. 6k**). In line, CD3^{+} and reduced macrophage -

496   influx/activation were observed by immunohistochemistry (**Supplementary Fig. 6n-**

497   **o**). CD-HFD/*hIL4rα/GPIbα*-Tg mice showed lower intrahepatic protein expression of

498   several pro-inflammatory and homeostatic cytokines/chemokines, including CCL5,

499   CD40 and TNFRSF11B (**Supplementary Fig. 6p**), mechanistically linking platelet

500   attachment/activation, cytokine/chemokine expression to efficient intrahepatic

501   immune-cell attraction. CD-HFD/hIL4rα/GPIbα-Tg mice lacked histological features of

502   NASH, paralleled by a reduction in lipid accumulation and absence of macro-

503   vesicular steatosis analyzed by H/E and Sudan red staining (**Fig. 6l-o**).

504   Notably, mice lacking the major platelet adhesion receptors P-selectin (*Selp*$^{-/-}$)[46], von-

505   Willebrand-factor (*vWF*$^{-/-}$)[47] or Mac-1 (*Mac1*$^{-/-}$)[48], the known ligands of GPIbα,

506   displayed full-blown NASH after six months of CD-HFD **(Extended Data-Fig. 8-10**).

507   Finally, we investigated whether hIL4rα/GPIbα-Tg mice would develop HCC upon

508   long-term CD-HFD. Of note, *hIL4rα/GPIbα*-Tg mice receiving CD-HFD for 12 months

509   displayed significantly lower fibrosis, serum ALT levels, and lacked any macro- or

510   microscopical evidence of HCC (**Fig. 6p-u**).

511

DISCUSSION

It is becoming increasingly clear that beyond their central role in hemostasis and wound repair after vascular injury[49], platelets are key players in multiple pathophysiological conditions[16-18,20], including cytotoxic T lymphocyte (CTL)-mediated liver damage and associated pathologies[18]. Here, we identified Kupffer cells as key players in intrahepatic platelet recruitment in early and advanced stages of NAFL/borderline NASH and NASH. In early NAFL/borderline NASH, hyaluronan and CD44 binding are also involved. In late NASH, GPIbα expressed by platelets appeared to be primarily involved in the interaction of platelets with Kupffer cells and in the maintenance of NASH. Thus, Kupffer cells play distinct roles in intrahepatic platelet recruitment at different NAFLD stages.

We found no evidence for a role of platelet-derived GPIIb/IIIa (*Itga2b*^−/−^ mice) in NASH, suggesting platelet activation and adhesion are important, whereas platelet aggregation is dispensable.

What is the function of platelets recruited to the liver? Our results indicate a major contribution of the platelet-cargo function (α-granule components) in NASH progression, exemplified by the marked protection of *Nbeal2*^−/−^ mice. The exact nature of platelet-derived α-granule constituents is currently unclear. However, several chemokines/cytokines were reduced upon therapeutic anti-GPIbα antibody treatment in the liver. This suggests that the number and activation state of intrahepatic platelets directly or indirectly correlates with an increase in immune-cell attracting chemokines/cytokines.

The key role of GPIbα in NASH identified in this study parallels a similarly vital function of this receptor in the development of experimental autoimmune encephalomyelitis (EAE), where it orchestrates the recruitment of leukocytes to the

537 inflamed CNS[50]. Our results, similar to the EAE study, argue against a key role of the

538 three known cognate interaction partners of GPIbα: P-selectin, vWF and Mac-1

539 [48,51,52] and point to a GPIbα-activation-dependent pro-inflammatory function of α-

540 granules in intrahepatic immune-cell attraction.

541 Selectins have also been shown to be dispensable for leukocyte recruitment in

542 inflamed liver microvasculature[53]. Other interaction partners might be involved (e.g.

543 coagulation factors XI, XII). It is also conceivable that GPIbα exerts its function in

544 disease development independent of an ectopic ligand[54]. Moreover, due to the

545 complex pathogenesis underlying NASH, it is plausible that GPIbα is not the only

546 molecule involved. Mice lacking MAdCAM-1 (*MAdCAM-1*^{-/-}) also showed significantly

547 reduced intrahepatic platelet infiltration, but the exact mechanisms by which this

548 occurs is unknown.

549 Cholesterol also plays a pivotal role during NASH pathogenesis by inducing hepatic

550 lipotoxicity[55]. Notably, different Western diets (e.g., HTF/NTF) with high cholesterol

551 recapitulated platelet-dependent phenotypes (increased intrahepatic platelets and

552 responsiveness to therapeutic APT) described in the CD-HFD; however, with a more

553 pronounced NASH phenotype with higher NAS and fibrosis.

554 Of note, platelets and platelet-derived GPIbα are potential therapeutic targets of

555 NASH and subsequent HCC development. Although GPIbα-antibody treatment

556 induces prolonged bleeding-time, it does not induce spontaneous bleeding [44], as only

557 one GPIbα epitope is blocked, leaving receptor function largely intact. Thus, GPIbα

558 antibody is a potentially safe new treatment modality against a metabolic disease of

559 major public health burden[4,9]. So far, there is no drug treatment available specifically

560 targeting NASH. The use of pioglitazone (most promising drug so far/off-label outside

561 T2DM because of side-effects) or vitamin E (better safety/tolerability in the short-

562 term) or combination thereof could be used for NASH treatment[4,56]. Other agents

563 such as obeticholic acid have also improved histological features of NASH, although

564 data with respect to their long-term benefits are still being awaited[57]. Combined

565 therapies for NASH treatment are currently being pursued, as only a subset of

566 patients responds well to monotherapies[5]. This might be overcome by stratifying

567 more responsive patients to a given monotherapy or by utilizing multi-target drugs. A

568 viable approach might be a metabolic target in combination with an anti-inflammatory

569 or anti-fibrotic drug, such as APT and statins, as described in our pilot case-study.

570 Nevertheless, given that APT or anti-GPIbα treatment attenuated metabolic

571 dysfunction, inflammation and fibrosis, these constitute potential monotherapies

572 against NASH. Although a risk for bleeding cannot be excluded for both, which

573 warrants careful monitoring, a single substance therapy is expected to harbor a lower

574 risk of side effects compared to combined therapies.

575 Given that only a proportion of patients with NAFLD progress to NASH and HCC, the

576 question remains at which stage of NAFLD such treatments should be initiated.

577 Future studies are needed not only to identify novel druggable targets for

578 NASH/HCC, but also to identify non-invasive biomarkers for detection of early

579 NAFLD and NASH, when it is still amenable to therapeutic intervention. In line, it was

580 recently demonstrated that higher serum ALT correlated with HCC development not

581 only in a mouse model of chronic liver cell damage, but also in patients with chronic

582 liver disease of diverse etiology including NASH[11, 58].

583 Prophylactic APT, commonly used for acute and long-term treatment of coronary

584 artery disease[59], and Ticagrelor attenuate NASH and NASH-induced HCC[16]. In

585 contrast, Sulindac did not prevent NASH in mice. Thus, rather than NSAIDs in

25

general, therapies that specifically block intrahepatic platelet accumulation/platelet function seem to be required to prevent NASH and NASH-associated conditions.

For NASH, we also observed a therapeutic effect of APT in preventing NASH-triggered HCC (although potential therapeutic effects of APT in the context of pre-existing HCC was not tested)[60]. Similarly, we have observed a therapeutic anti-NASH effect of anti-GPIbα antibody treatment. Remarkably, in both cases therapeutic treatment partially dampened fibrosis.

Furthermore, in a small prospective human cohort study, APT reduced liver volume and liver fat accumulation in NAFLD patients, supporting the results of our murine *in vivo* experiments. The results of this pilot case-study, being a starting point for further studies have to be taken with caution as it is currently underpowered. Although a multivariate analysis on a small patient cohort inherently warrants careful interpretation, our data have shown that effects of anti-aggregation were stronger than those of statin administration.

In our study, NASH prevention (e.g. by two distinct APTs: Ticagrelor and Asp/Clo; in hIL4rα/GP1bα-Tg mice) ultimately suppressed subsequent HCC formation, mostly because the pro-carcinogenic NASH-related environment (e.g. intrahepatic inflammation, signaling, hepatocyte damage) was lacking. Moreover, treatment with anti-GPIbα antibody or depletion of Kupffer cells abrogated NASH therapeutically. These approaches might have an important impact for chemo-preventive strategies, which still are lacking against NASH. Thus, our findings provide a rationale for APT, P2Y12 antagonists or reagents directly blocking platelet-derived GPIbα or related pathways as possible therapeutic approaches for NASH patients not only to prevent/revert NASH but also to prevent NASH to HCC transition.

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742
743

METHODS

745 Methods, including statements of data availability and any associated accession

746 codes and references are available in the online version of the paper.

747

748 **Mice, diets and treatments**

749 4- to 5-week-old mice (C57BL/6JOlaHsd) were purchased from ENVIGO. P-selectin

750 knockout mice ($Selp^{-/-}$) were purchased from Jackson Laboratories (mouse strain 002289).

751 Knockout mice for Nbeal2$^{-/-}$, GPVI ($Gp6^{-/-}$)[61], GPIIb/IIIa ($GpIIb^{-/-}$; $Itga2b^{-/-}$), von Willebrand

752 factor ($vWF^{-/-}$) and also transgenic mice, lacking functional GPIbα, hIL4rα/GPIbα-Tg, all on

753 the background of C57Bl/6J were kindly provided by Professor Bernhard Nieswandt and

754 Prof. Jerry Ware (University Hospital and Rudolf Virchow Center, University of Würzburg,

755 Würzburg, Germany; Department of Physiology and Biophysics, University of Arkansas for

756 Medical Sciences, 4301 West Markham Street, Little Rock, Arkansass 72205, USA).

757 Inducible knock-in mice expressing the human unconventional prefoldin RPB5 interactor

758 (URI) in hepatocytes (hURI-tetOFFhep) were received from Prof. Dr. Nabil Djouder, CNIO,

759 Madrid. *Pdpn*fl/flx Vav1-iCre mice (obtained from Jackson Laboratories) and *Clec1b*fl/flx Pf4-

760 Cre mice are described elsewhere[62]. All strains of genetically-altered mice were on a

761 C57BL/6J background. Control mice were negative for cre recombinase and matched by

762 genetic background, age and sex. Mice were housed at the University Hospital Zurich (USZ),

763 the Technical University Munich/Helmholtz Zentrum Munich, the Biomedical Services Unit at

764 University of Birmingham or University of Newcastle, the University of Calgary and the

765 German Cancer Research Center (DKFZ). All animal work was conducted under the

766 approval of the Swiss Veterinary Office (136/2014), according to German Law (G7/17 or

767 55.2-1-54-2532-39-2015), the UK Animals Scientific Procedures Act of 1986, with project

768 licence approval granted by the UK Home Office, the local biomedical research ethics

769 committee approval and the University of Calgary Animal Care Committee (protocol AC16-

770 0148) in accordance with the Canadian Council for Animal Care Guidelines. Animals were

771 maintained under specific pathogen-free conditions and experiments were performed in

772   accordance to the guidelines of the respective institution and were in accordance with ethical

773   regulations and humane endpoints.

774   Five-week-old mice were fed *ad libitum:* normal diet (ND) (Provimi Kliba) or choline-deficient

775   high-fat diet (CD-HFD) (Research Diets; D05010402) for 6 or 12 months; Western diet with

776   trans-fat (WD-HTF) (Research Diets; D09100301 - 40 kcal % fat (Primex shortening), 20 kcal

777   % fructose, 2% cholesterol) for 6 months; methionine-choline-deficient diet (MCD) (MP

778   Biomedicals) for 4 weeks and control mice were fed a calorifically matched control diet;

779   Western diet with fructose in drinking water (WD-FSDW) (Custom Research Diet TD.06303;

780   Harlan Laboratories in conjunction with fructose-supplemented drinking water - 55% fructose,

781   45% glucose by weight at a concentration of 42 g/l *ad libitum* for 6 and 9 months. Control

782   animals received ND and non-supplemented drinking water.

783

784   Cohorts of mice fed CD-HFD or WD-HTF were in addition treated with either Aspirin (through

785   food pellets containing 7.5 mg per kg of food) in combination with Clopidrogel high (40 µg/ml

786   drinking water; ~3 mg/kg/day) or low dose (20 µg/ml drinking water; ~1.5 mg/kg/day), with

787   Sulindac (200 µg/ml drinking water; ~20 mg/kg/day) or with Ticagrelor (40 µg/ ml drinking

788   water; ~3 mg/kg/day).

789

790   For interventional studies five-week-old male mice were fed normal diet (ND) or choline-

791   deficient high-fat diet (CD-HFD) (Research Diets; D05010402) or Western Diet containing

792   trans-fat (WD-HTF) (Research Diets, D09100301) for 3.5 weeks and then treatment started

793   for 2.5 weeks, 2x per week i.v. in 100 µl PBS of either 20µg/mouse anti-CD44 antibody

794   (clone KM81, Cedarlane), 100 µg/mouse anti-CD44 (clone IM7, Bioxcell), 100µl/mouse

795   Clodrosome® (Liposomal Clodronate), 100µl/mouse Encapsome® (Control Liposomes),

796   100µg/mouse anti-GPIbα or 100 µg/mouse Fab-Rat IgG (kindly provided by Prof. Bernhard

797   Nieswandt - University Hospital and Rudolf Virchow Center, University of Würzburg,

798   Würzburg, Germany) or i.p 20 U/gr murine hyaluronidase (HYAL). In late treatment regimes,

799   mice were fed CD-HFD for 6 months and treated with the same protocol for indicated time

800   points.

801   For osmotic Pump experiment five-week-old male mice were fed choline deficient high fat

802   diet (CD-HFD) (Research Diets; D05010402) for 12 weeks. 4 weeks after the diet (Mini-

803   pumps (Alzet, model 2004) were implanted subcutaneously into the mice to deliver 30 µg per

804   day of Ly6G (clone 1A8) neutrophil depleting antibody or Rat IgG2a (clone 2A3) (BioXCell)

805   for a further 8 weeks. At the end of the experiment, animals were culled, and the liver, fat and

806   serum harvested for analysis.

807

808   **Human material**

809   Specimens were obtained from formalin-fixed, paraffin-embedded non-diseased, NAFLD, or

810   NASH diagnosed human liver tissue, retrieved from the archives and the biobank of the

811   Department of Pathology and Molecular Pathology, University Hospital Zurich, University

812   Hospital Würzburg and in the setting of the HEP-CAR consortium (Institute for Research in

813   Biomedicine (IRB) Barcelona). Tissues were examined by certified liver pathologists (Prof.

814   Prof. Achim Weber, Prof. Josep M. Llovet.). These studies were approved by the local ethics

815   committee ("Kantonale Ethikkommission Zürich", application numbers StV26/2005 and KEK-

816   ZH-Nr. 2013-0382) or the Institute for Research in Biomedicine (IRB) Barcelona, Hospital

817   Clinic HCB/2015/0789. In line with the regulation of KEK, individual informed consent from all

818   patients was not required for this kind of retrospective analysis on patients' material.

819

820   The prospective clinical trial has been listed at the German Clinical Trials Register (DRKS),

821   evaluated and accepted by the local ethical authorities (Ethik-Kommission

822   Universitätsklinikum Tübingen) with the Nr.587/2016BO2 - under the name: "Platelet

823   inhibition to recues formation of non-alcoholic steatohepatitis in cardiovascular patients"

824   (Prometheus - prospective, monocenter, observational study).

825

826

827

828

Prospective trial, ethics, MRI and ultrasound analyses

830 The case study of 24 individual observations prospective trial was approved by the

831 institutional ethics committee (587/2016BO2) and comply with the declaration of Helsinki and

832 the good clinical practice guidelines[63-66].

833 For the prospective, ongoing case study, we have included only patients (n=23) with

834 diabetes mellitus type II and/or BMI>30, two risk factors, that are highly correlated with

835 development of NAFLD/NASH. All patients underwent cardiac cathererization due to

836 suspected coronary artery disease (CAD). Patients received no antiplatelet treatment if CAD

837 was excluded. Patients received antiplatelet therapy with 100mg acetylsalicylic acid (ASA)

838 once daily if CAD was present but coronary stent implantation was not indicated. Patients

839 received dual antiplatelet therapy with ASA 100 mg once daily and P2Y12 inhibitor

840 (Clopidogrel 75mg once daily, Ticagrelor 90mg twice daily or Prasugrel 10mg once daily)

841 depending on acuity and severity of CAD (e.g. myocardial infarction vs. stable CAD). None of

842 the patients included received long-term treatment with ASA or P2Y12 before study

843 inclusion.

844

845 All patients remained on the respective treatment regimens until time point of follow-up. All

846 patients underwent liver MRI and liver ultrasound at baseline (within 4 weeks after hospital

847 discharge) and after 6 months of follow up. The serum from all patients was analyzed for

848 classic liver damage parameters like AST, ALT, Bilirubin, GGT etc. Additional measurements

849 were performed to exclude other than NAFLD reasons for liver damage, e.g. immune-

850 hepatitis, viral hepatitis, AFLD. All patients underwent the Michigan Alcoholism Screening

851 Test to evaluate drinking habits. Patients with left ventricular ejection fraction <45% or severe

852 valve diseases were not included into the study due to the risk of liver congestion.

853   The hepatic lipid accumulation was quantified by 3 T MRI (Skyra, Siemens Healthcare,

854   Erlangen Germany) at the Department of Diagnostic and Interventional Radiology of the

855   University Hospital of Tübingen, Germany. A commercially available multi-echo Dixon

856   sequence (LiverLab, Siemens Healtcare, Erlangen Germany) was used, allowing the

857   computation of proton density fat fraction maps and inline segmentation of the liver.

858   Ultrasound examinations in patients were performed twice, at the time point of patient

859   recruitment and six months after patient recruitment. In order to identify patients presenting

860   with signs of liver steatosis or with signs of liver cirrhosis, bright hepatic echos, increased

861   hepatorenal echogenicity, presence of nodular liver morphology and signs of portal

862   hypertension (e.g. splenomegaly, ascites or intra-abdominal varices) were analyzed using an

863   Aplio 500/T1 (Toshiba) / Ultrasonic transducer 3-5 MHz.

864

865   The multivariate analysis (age, gender, antiaggregation and new statin treatment) to identify

866   statin administration as a possible confounder of the observed effects, patients were

867   differentiated into groups, that received statin treatment prior to hospital admission and those

868   that received new statin treatment at hospital discharge prior to the first MRI examination.

869   We performed a linear regression analysis.

870   For further details regarding the human samples, please refer to the Supplementary Material

871   (Table 1a, Table 1b, Table 2).

872

873   **Cholesterol measurements**

874   Hyperlipidemia is defined as baseline LDL cholesterol ≥160 mg/dl and triglycerides ≥200

875   mg/dl. Interestingly, only 3 of the patients of the prospective cohort offer triglyceride values

876   ≥200mg/dl. However, all patients of the prospective cohort show significantly elevated liver

877   fat accumulation indicating that patients might not need elevated serum lipid levels to

878   develop fatty liver disease. As previously mentioned, according to our measurements, only 3

879   patients of the prospective trial fulfill the diagnosis hyperlipidemia. However, we decided to

880   stay with pre-existing conditions e.g. diagnosed by family doctors because serum lipid levels

881   undergo dynamic regulations. This explains the discrepancy between hyperlipidemia and

882   measured lipid levels in baseline Table 2.

883

884   **Isolation of liver leukocytes for in depth myeloid characterization**

885   Livers were perfused once with PBS followed by collagenase digestion at 37°C for 40

886   minutes (collagenase type IV, Worthington, UK). Additionally, 0.1 mg/ml DNase I was added

887   after 20 min. Single cell suspension was obtained by filtering the digested liver through a 70

888   µm mesh. Hepatocytes were removed by low speed centrifugation (50 rcf, 1 min) and density

889   gradient centrifugation was performed using 18 % Nycodenz dissolved in Gey`s Balanced

890   Salt Solution (GBSS) as previously described.[67]

891

892   Cells were then stained for CD206 (C068C2), CX3CR1 (SA011F11), CD3 (17A2), MHC-II

893   (M5/114.15.2) (all Biolegend); CD11b (M1/70), F4/80 (BM8) (all Invitrogen); CD86 (GL-1,

894   eBioscience); CD45 (30-F11), Ly6C (RB6-8C5), Ly6C (1A8) CD11c (HL3), CD31 (MEC13.3),

895   NK1.1 (PK136), TIM-4 (RMT4-54) (all BD Bioscience) followed by multi-color flow cytometry

896   using an LSR-Fortessa (BD Biosciences). Analysis was done using FlowLogic (v7.2, Inivai,

897   Australia) and FlowJo (v10.4, BD Biosciences). Additionally, liver MoMF and KC for

898   NanoString gene expression analysis were sorted using an Aria-II (BD Biosciences).

899   For the details regarding the definition of violin-plot elements of Supplemental Figure 3i,

900   please, refer to the Supplementary Material (Table 3).

901

902   **t-distributed stochastic neighbor embedding (t-SNE)**

903   Liver leukocytes were pregated for living (7-AAD-), CD45+, Ly6G-, CD31-, F4/80+ cells by

904   using FlowJo (v10.4, BD Biosciences), followed by t-SNE based clustering as described

905   previously,[68] by using the Rtsne package for R. Clusters were identified by hierarchical

906   clustering and projected onto the two dimensional t-SNE plot, while relative protein

907   expression was displayed for each cluster by violin plots using R (v3.5.1).

908

nCounter gene expression analysis

Gene expression analysis of 561 selected gene targets of liver MoMF and KC was done by using NanoString assays (nCounter Mouse Immunology Kit, NanoString Technologies). Analysis was done by using the nSolver Software (v2.0, NanoString Technologies). Principal component analysis (PCA) was done by using the prcomp package for R (v3.5.1).

Measurement of serum parameters

Serum was isolated from mice and liver enzymes AST and ALT were quantified by Roche Modular System (Roche Diagnostics) with a commercially available automated colorimetric system at the Institute of Clinical Chemistry at the University Hospital Zurich using a Hitachi P-Modul (Roche). Total cholesterol was measured in a 96-well format using CHOL or TG GPO-PAP substrates (Roche Diagnostics).

Measurement of liver triglycerides

Liver specific triglyceride levels were analyzed from snap-frozen liver tissue samples. Liver tissue samples were homogenized in sodium chloride (0.9 % NaCl) and liver resident lipids were precipitated using ethanolic potassium hydroxide (0.5 M KOH) and solubilized in magnesium sulphate (0.15 M MgSO4). The concentration of hepatic triglycerides was then measured using triglycerides GPO-PAP from Roche Diagnostics on a spectrophotometer at 505 nm.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance tests (IPGTTs) were carried out on mice fasted overnight for 16 h. Basal blood glucose concentrations were determined for each mouse prior to glucose (2 mg/g body weight) administration using a hand-held glucose analyzer (FreeStyle Freedom Lite; Abbott). Each mouse then received glucose via intraperitoneal (i.p.) injection and blood glucose concentrations were subsequently re-measured 15, 30, 60 and 120 min post glucose administration.

938

939

940

Intraperitoneal insulin tolerance test and fasting insulin determination

Intraperitoneal insulin tolerance tests were carried out on mice mild fasted for 6 h. Basal blood glucose concentrations were determined for each mouse prior to insulin (1U/g lean mass determined by EchoMRI analyis) administration using a hand-held glucose analyzer (FreeStyle Freedom Lite; Abbott). Each mouse then received Insulin via intraperitoneal (i.p.) injection and blood glucose concentrations were subsequently re-measured 15, 30, 60 and 120 min post glucose administration. Fasted insulin levels were measured in mildly fasted (8h) using ALPCO mouse insulin ELISA kit.

Immunoblot analysis

Liver homogenates were prepared in a pH 7.4 lysis buffer containing 1% NP-40 (Sigma-Aldrich, Gillingham, UK), 50 mmol/L Tris, 10% glycerol, 0.02% NaN3, 150 mmol/L NaCl, and a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich, Gillingham, UK), and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's manual. Liver homogenates were prepared using a homogenizer. Tissue preparation was performed with gentleMACS™ Octo Dissociator (Miltenyi Biotec GmbH). 20–80 µg of proteins were separated under reducing conditions (2.5% β-mercaptoethanol) by gel electrophoresis (Mini Protean Gels, Bio Rad) and blotted by semi-dry blotting (Trans-Blot Turbo Transfer, Bio Rad) onto nitrocellulose membranes (Bio Rad). Membranes were blocked in 5% milk/PBS-T for at least 1 hr at RT. Primary antibodies against P-p38MAPK ((Thr180/Tyr182) D3F9), p38 MAPK (D13E1), P-p65 (Ser536), p65 (D14E12), COX1 (D5H5), GAPDH (14C10) (all Cell Signaling) were incubated at 4°C overnight under shaking conditions. Incubation with the secondary antibody (HRP-anti rabbit IgG, 1:5000; Promega) was performed under shaking conditions for 1 hr. Detection was

965 achieved using Clarity Western ECL Substrate (Bio Rad) using Stella 3200 imaging system

966 (Raytech).

967

968

969 **Flow cytometry**

970 For flow cytometry analysis, antibodies against CD8-α (53-6.7), CD3-ε (500A2), IgM (RMM-

971 1), CD19 (6D5), NK1.1 (PK136), were purchased from BioLegend. Antibodies against CD4

972 (RM4-5) and Foxp3 (FJK-16s) were purchased from eBioscience. Viability was assessed by

973 LIVE/DEAD Fixable Aqua or ZombieDyeNIR (Life Technologies). TA99 was labeled with an

974 Alexa Fluor 647 NHS Ester (Life Technologies) to generate TA99–647. Further antibodies

975 that were used include CD44: Clone IM7, Biolegend, diluted 1:200, CD69: Clone H1.2F3,

976 Biolegend, diluted 1:200, CD62L: Clone MEL-14, Biolegend, diluted 1:200. Intracellular

977 cytokine staining (ICS) was performed as described previously[11]. Peptides used for

978 restimulation were 10 µg/ml of the relevant antigen: Trp2180–188 (SVYDFFVWL),

979 Her2/Neu66–74 (TYVPANASL), Tyrp-1455–463native (TAPDNLGYA), or gp10025–33native

980 (EGSRNQDWL). Cells were analyzed using BD FACS LSR II, BD FACS LSR Fortessa, BD

981 FACSCanto, Sony spectral analyzer SP6800 flow cytometers, and data were analyzed using

982 FlowJo.

983

984 **Histology, immunohistochemistry, scanning and automated analysis**

985 Liver samples were fixed in 4% paraformaldehyde and paraffin-embedded at the University

986 Hospital Zürich, Department of Pathology and Molecular Pathology Core, at the Technical

987 University of Munich (TUM), or at the DKFZ, Department of Chronic Inflammation and

988 Cancer (Heidelberg) as described[11]. Briefly, 2µm sections from FFPE and cryo-preserved

989 tissues were prepared and stained with Hematoxylin/Eosin or IHC antibodies. Incubation in

990 Ventana buffer and staining was performed on a NEXES immunohistochemistry robot

991 (Ventana Instruments) using an IVIEW DAB Detection Kit (Ventana) or on a Bond MAX

992 (Leica). For Sudan Red staining, cryo sections (5 µm) were cut and stained with Sudan Red

993   (0.25% Sudan IV in ethanolic solution). Slides were scanned with a Nano Zoomer

994   (Hamamatsu, Japan).

995

996   Antibodies that were used include: anti-MHCII, rat (clone M5/114.15.2) 1:500, anti-CD3,

997   rabbit (clone SP7) 1:250, anti-F4/80, rat, BioLegend Cat# 123105; 1:50. Further antibodies

998   used include: Collagen IV, rabbit 1:50, Cedarlane, clone CL50451AP-1; Ki67, rabbit 1:200,

999   Thermo Scientific, clone RM-9106-S1; B220, rat 1:3000, BD, clone 553084; Ly6G rat 1:600,

1000   BD, clone 551459, PERK1/2, rabbit, 1:400, Cell Signaling, clone 4370; Glutamine

1001   Synthethase (GS) rabbit 1:500, abcam, clone ab16802, PSTAT3, rabbit, 1:100, Cell signaling

1002   clone 9145.

1003

1004   For quantification of stains, slides were scanned using a SCN400 slide scanner (Leica) and

1005   analyzed using Tissue IA image analysis software (4.0.6 Slidepath, Leica). For quantification

1006   of platelet staining on human (CD61) and mouse (CD42b) tissue software based analysis

1007   and counting at the screen (CD42b; 100 high-power fields; 40x) was performed.

1008   For Sudan Red$^+$ liver area, data are presented as Sudan Red positive area in percent of total

1009   tissue area. Three random liver tissue areas of approximately 6-8 mm² (87000 µm^2) were

1010   selected in DIH (digital image hub, Leica), submitted to analysis and merged.

1011   NASH activity score (NAS) was applied to murine livers.

1012

1013   **Electron microcopy**

1014   For electron microscopy, sections from epon-embedded, glutaraldehyde-fixed liver samples

1015   were cut and stained with toluidine blue. The tissue was trimmed and ultrathin cross sections

1016   of the liver were cut and treated with uranyl acetate and lead citrate as described

1017   previously[69]. Electron micrographs were analyzed for cell composition and localization using

1018   the analySIS Docu System (Soft Imaging System GmbH).

1019
1020   **Preparation of mouse platelets**
1021

1022 Blood was obtained from the tail of mice and was drawn into citrate tubes at a 1:10 ratio.

1023 Platelet-rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterward,

1024 platelet-rich plasma was centrifuged at 640 g for 5 minutes to pellet the platelets. After 2

1025 washing steps, the pellet of washed platelets was resuspended in modified Tyrode-HEPES

1026 buffer (pH 7.4, supplemented with 1mM CaCl2).

1027

1028 **Platelet aggregometry**

1029 Washed platelets were adjusted to a concentration of 150×10^3 platelets/µl in Tyrode-HEPES

1030 buffer (pH 7.4 supplemented with 1mM CaCl2). Aggregation was estimated from light

1031 transmission determined with a luminoaggregometer model 700 (ChronoLog, Havertown,

1032 PA, USA). Following calibration, agonists including Adenosine 5′-diphosphate (ADP),

1033 U46619 (U46), thrombin (Thr), collagen-related peptide (CRP), rhododcytin (RC) were added

1034 at the indicated concentrations and aggregation was measured for 10 minutes with a stir

1035 speed of 1000 rpm at 37 °C. Afterwards analysis was performed using the aggrolink8

1036 software (ChronoLog).

1037
1038 **Analysis of platelets and flow cytometry of platelets**
1039
1040 Mice were bled under isoflurane anesthesia. Blood was collected in a tube containing 20

1041 U/ml heparin, and PRP was obtained by 2 cycles of centrifugation at 300 g for 6 min at room

1042 temperature (RT). For preparation of washed platelets, PRP was washed twice at 800 g for 5

1043 minutes at RT and the pellet was re-suspended in modified Tyrodes-HEPES (N-2-

1044 hydroxyethyl-piperazone-N′2-ethanesulfonic acid) buffer (134 mM NaCl, 0.34 mM Na2HPO4,

1045 2.9 mM KCl, 12 mM NaHCO3, 5 mM HEPES, 1 mM MgCl2, 5 mM glucose, 0.35% BSA, pH

1046 7.4) in the presence of prostacyclin (0.1 µg/ml) and apyrase (0.02 U/ml).

1047 Apyrase grade III (Sigma-Aldrich), prostacyclin (PGI2), ADP (Sigma-Aldrich), U-46619 (Enzo

1048 Life Sciences), thrombin (Roche), (Sigma-Aldrich), rabbit anti-human VWF (DAKO), rabbit

1049 anti-human fibrinogen (DAKO) were purchased as indicated. CRP and Rhodocytin were

1050 generated and isolated as previously described[36]. The antibody against the activated form of

1051　integrin αIIbβ3 (JON/A-PE) was from Emfret Analytics. Other antibodies we received were

1052　generated in the laboratory of Prof. Nieswandt as following:

1053

Antibody	Clone	Isotype	Antigen	Reference
JON/A	4H5	IgG2b	αIIbβ3	70
WUG 1.9	5C8	IgG1	P-selectin	71
JAQ1	98A3	IgG2a	GPVI	72
LEN1	12C6	IgG2b	α2	73
ULF1	96H10	IgG2a	CD9	74
p0p4	15E2	IgG2b	GPIb	74
p0p6	56F8	IgG2b	GPIX	74
JON2	14A3	IgG2b	αIIbβ3	74
DOM2	89H11	IgG2a	GPV	74
INU1	11E9	IgG1	CLEC-2	75

1054

1055　**RNA isolation and quantitative real-time PCR**

1056　Total RNA was isolated from snap-frozen liver tissues according to the manufacturer's

1057　protocol using RNeasy Mini Kit (Qiagen). The quantity and quality of the RNA was

1058　determined spectroscopically using a Nanodrop analyser (Thermo Scientific). 1 µg of purified

1059　RNA was subsequently transcribed into cDNA using Quantitect Reverse Transcription Kit

1060　(Qiagen) according to the manufacturer's protocol. Quantitative RT-PCR was performed

1061　using Fast Start SYBR Green Master Rox (Roche). Primers were custom made by

1062　Microsynth as previously described[11]. For mRNA expression analysis quantitative real-time

1063　PCR was performed in duplicates in 384-well plates using Fast Start SYBR Green Master

1064　Rox (Roche) on a 7900 HT qRT-PCR system (Applied Biosystems, Life Technologies

1065　Darmstadt, Germany). Relative mRNA levels were calculated according to the ΔΔCt relative

1066   quantification method and were normalized to a house-keeping gene (GAPDH or ROTH2)

1067   levels. The data were normalized to the expression of housekeeping gene and analyzed

1068   using the GraphPad Prism software version 7.03 (GraphPad Software). For the analysis of

1069   whole liver homogenates by microarray (Agilent) the following genes were selected,

1070   analyzed and shown. For a list of all used primers for RT-qPCRs please refer to

1071   Supplementary Material Table 4.

1072   **Gene expression profiling**

1073   Transcriptional profiling was performed using SurePrint G3 Mouse Gene Expression 8x60k

1074   microarrays (Agilent Technologies, AMADID 28005) according to the manufacturer's

1075   protocol. 75 ng of total RNA was used in labeling using the Low Input Quick Amp Labeling Kit

1076   (one-color, Agilent Technologies). Raw gene expression data were extracted as text files

1077   with the Feature Extraction software 11.0.1.1 (Agilent Technologies). The expression

1078   microarray data were uploaded to ArrayExpress (www.ebi.ac.uk/arrayexpress/) and the data

1079   set is available under the accession number E-MTAB-6073, entitled "Transcriptomic

1080   differences in livers of mice fed with normal diet and choline-deficient high-fat diet".

1081   All data analysis was conducted using the R statistical platform (version 3.2.2, www.r-

1082   project.org). Data quality assessment, filtering, preprocessing, normalization, batch

1083   correction based on nucleic acid labeling batches and data analyses were carried out with

1084   the Bioconductor R-packages limma, Agi4x44PreProcess and the ComBat function of the

1085   sva R-package. All quality control, filtering, preprocessing and normalization thresholds were

1086   set to the same values as suggested in Agi4x44PreProcess R-package user guide. Only

1087   HGNC annotated genes were used in the analysis. GSEA was conducted on calculated log2-

1088   expression values of all array probes using the „GSEA Pre-ranked" function with default

1089   settings[76].

1090

RNA sequencing

Library preparation for bulk 3'-sequencing of poly(A)-RNA was done as described previously[77]. Briefly, barcoded cDNA of each sample was generated with a Maxima RT polymerase (Thermo Fisher) using oligo-dT primer containing barcodes, unique molecular identifiers (UMIs) and an adapter. 5' ends of the cDNAs were extended by a template switch oligo (TSO) and after pooling of all samples full-length cDNA was amplified with primers binding to the TSO-site and the adapter. cDNA was tagmented with the Nextera XT kit (Illumina) and 3'-end-fragments finally amplified using primers with Illumina P5 and P7 overhangs. In comparison to Parekh S *et. al.* 2016, the P5 and P7 sites were exchanged to allow sequencing of the cDNA in read1 and barcodes and UMIs in read2 to achieve a better cluster recognition. The library was sequenced on a NextSeq 500 (Illumina) with 75 cycles for the cDNA in read1 and 16 cycles for the barcodes and UMIs in read2.

RNASeq analysis

Gencode gene annotations version M18 and the mouse reference genome GRCm38.p6 were derived from the Gencode homepage (https://www.gencodegenes.org/). Dropseq tools v1.12[78] was used for mapping the raw sequencing data to the reference genome. The resulting UMI filtered countmatrix was imported into R v3.4.4. Prior differential expression analysis with DESeq2 1.18.1[79], dispersion of the data was estimated with a parametric fit including the dietary status of the mice as explanatory variable in the model. The Wald test was used for determing differentially regulated genes between the high fat and normal diet group and shrunken log2 foldchanges were calculated afterwards with setting the type argument of the lfcShrink function to 'normal'. A gene was determined to be differentially regulated if the absolute log2 foldchange| was greater than 1 and the adjusted p-value was below 0.05. Gene set enrichment analysis was conducted with EnrichR[80] within the Reactome database. Raw sequencing data is available under the accession number MTAB-7625.

MRI analysis for mice

Steatosis was analyzed in 6-month-old B6 mice fed with ND, CD-HFD or CD-HFD/Asp-Clop treated groups. A Pharmascan 7T MRI (Bruker) with Paravision 5.1 software was used in FLASH scan mode without fat suppression using an echo-time of 2.2 ms for out-phase and 2.9 ms for in-phase based on previous reports[81,82].

Calorimetric TSE analysis

Mice were individually housed for indirect calorimetry in PhenoMaster (TSE systems). Mice were allowed to acclimate to the new environment for at least 2-3 days followed by recording of metabolic parameters such as food and water intake, O_2 consumption, CO_2 production, respiratory exchange ratio and total activity. All parameters were measured for at least four consecutive days with 5 measurement values every hour. Analysis of covariance (ANCOVA) was first conducted to ensure that body weight does not play a significant effect on the measured parameters.

Immunofluorescence microscopy of liver sections

To achieve rapid fixation after euthanasia, livers were fixed with paraformaldehyde–lysine–periodate (PLP) for 4–8 h, rehydrated in 30% sucrose solution for 48 h and snap frozen in OCT (Cell Path). Thick (5 µm) cryosections were obtained using a Leica Cryostat and the Cryojane tape transfer system (Leica Microsystems).

Generation of liver slices for confocal microscopy

The generation of the liver slices for microscopy was performed and adapted from a previously described protocol[83]. Briefly, livers were collected and processed as described above. OCT-embedded frozen livers were iteratively sectioned using a cryostat. Samples were then reversed and the procedure was repeated on the opposite face of the liver until a 30 µm thick slice of liver was obtained. OCT freezing medium covering the sample was removed, and slices were washed with PBS and blocked overnight at 4 °C in blocking

1147 solution (0.2% Triton/1%BSA/10% donkey serum/PBS). Liver slices were stained with Rat

1148 anti-CD41, goat anti-CD105, rabbit anti-Col IV and rabbit anti-CD8 for 3 days in blocking

1149 solution, washed overnight in PBS and stained with DyLight488 donkey anti-rabbit IgG and

1150 DyLight549 or DyLight649 donkey anti-rat IgG. Stained slices were then washed in PBS and

1151 incubated overnight in FocusClear (CelExplorer Lab). For observation under the confocal

1152 microscope, liver slices were embedded in FocusClear and mounted on glass slides.

1153

1154 **Immunofluorescence staining**

1155 Livers were perfused with PBS trough inferior vena cava, harvested and fixed in 4%

1156 paraformaldehyde for 16 h, then dehydrated in 30% sucrose prior to embedding in OCT

1157 freezing media (Sakura). 25 micrometer sections were cut on a HM550 cryostat

1158 (ThermoFisher) and adhered to Superfrost Plus slides (Thermo Scientific). Sections were

1159 then permeabilized and blocked in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and

1160 10% FBS followed by staining in the same blocking buffer. The following primary Abs were

1161 used for staining: rabbit anti-collagen IV (1:200 Abcam-10808); CD41 PE (1:100 MWReg-30

1162 Biolegend), rat anti-F4/80 APC (1:100 BM8 thermofisher), rabbit ant-CD3 (1:100 DAKO

1163 A0445229-2), B220 AF 647 (1:100 RA3-6B2 Biolegend), CD11b APC (1:100 M1/70

1164 Biolegend). Stained slides were mounted with fluorescent mounting medium (DAKO) and

1165 images were acquired on an inverted Leica microscope (TCS STED CW SP5, Leica

1166 Microsystems) with a motorized stage for tiled imaging.

1167

1168 To minimize fluorophore spectral spillover, we used the Leica sequential laser excitation and

1169 detection modality. The bleed-through among sequential fluorophore emission was removed

1170 applying simple compensation correction algorithms to the acquired images. The

1171 semiautomatic surface-rendering module in Imaris (Bitplane) was used to create 3D

1172 volumetric surface objects corresponding either to individual cells or to the liver sinusoids.

1173 Signal thresholds were determined using the Imaris Surface Creation module, which

1174   provides automatic threshold identification and value-based visual surface thresholding

1175   around the positively stained objects.

1176

1177   For the semi-quantitative analysis of platelet adhesion to liver sinusoids, high resolution

1178   confocal xyz stacks of 30xy sections (1024 x 1024 pixel) sampled with 0,5 µm z spacing

1179   were acquired to provide image volumes of 388 x 388 x 30 μm^3. The confocal z stacks were

1180   imported into Imaris software (Bitplane) and platelets were reconstructed as 3D volumes by

1181   means of the semiautomatic surface-rendering module with a seed point diameter of 2.08 µm

1182   (the mean platelet diameter [84,85]). The split touching objects option of the module supports

1183   the separation of two or more objects that are identified as one, enabling the splitting of

1184   aggregates into single components. All analysis was performed at least on 12 random FOV.

1185

1186   **Confocal imaging analysis**

1187   For platelet aggregate size analysis, the surface and volume of each single platelet

1188   aggregate (containing more than 2 single platelet) from at least 12 different FOV was

1189   automatically obtain from the surface-rendering module (imaris, Bitplane); The

1190   PLT/endothelium coverage was derived from the same FOV as percentage of the total

1191   sinusoidal surface (calculated in each FOV) covered by PLT.

1192   For the quantification of the immune cells PLT interaction the number of immune cells (CD3^{+},

1193   CD11b^{+} and B220^{+}) adjacent and non-adjacent to PLT (see supplementary movie S1 and

1194   S2) was manually counted in 12 FOV. The interaction was expressed as total number

1195   adjacent cell as well as percentage of cell adjacent to PLT over the total number of cells.

1196   The CD3^{+} cells/PLT interaction was further analyzed by calculating, for each cells, the

1197   contact surface area between them.

1198

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Multiplex ELISA

Cytokines and chemokines in whole liver of ND, CD-HFD and Asp-Clo treated CD-HFD mice (12 months old) were evaluated using a laser bead technology based Mouse Cytokine Array / Chemokine Array 31-Plex (MD31) by Eve Tech (Eve Technologies, Calgary, AB, Canada).

Cytokine Profiler

Cytokines and chemokines in whole liver of ND, CD-HFD (ctrl ab) and CD-HFD (anti-GPIbα antibody) mice (6 months old) or in CD-HFD and CD-HFD/hIL4Rα/GPIbα-Tg mice (12 months old) were assessed using the Mouse XL Cytokine Array Kit by R&D Systems.

Spinning-disc microscopy

All imaging experiments were performed using 7 – 12 weeks old male mice. All mice were co-housed and bread in a specific pathogen-free facility at the University of Calgary with a 12 hour light/dark cycle and access to food and water ad libitum.

Preparation for intravital Microscopy

Multichannel spinning-disk confocal microscopy was used to image the liver as previously described (Surewaard and Kubes 2017). Briefly, mice were anesthetized by intraperitoneal injection of ketamine (200 mg/kg body weight; Bayer Animal Health) and xylazine (10 mg/kg body weight; Bimeda-MTC). A catheter was inserted into the tail vein to allow for administration of fluorescently conjugated antibodies, proteins and additional anesthetics. A midline and lateral abdominal incision were made, and the abdominal wall was partly removed to access the liver. The mouse was placed in a right lateral position on a heating plate to maintain body temperature at 37°C. The liver was exteriorized onto a glass coverslip and covered with moisturized laboratory tissues to restrict movement and breathing artefacts. Abdominal organs were covered with saline-soaked gauze to prevent dehydration.

Intravital microscopy

Image acquisition of the liver was performed using an inverted spinning-disk confocal microscope (IX81; Olympus), equipped with a focus drive (Olympus) and a motorized stage (Applied Scientific Instrumentation). The microscope was fitted with a motorized objective turret equipped with 43/0.16 UPLANSAPO, 103/0.40 UPLANSAPO, and 203/0.70 UPLANSAPO objective lenses. The microscope was linked with a confocal light path (WaveFx; Quorum Technologies) based on a modified CSU-10 head (Yokogawa Electric Corporation). Cells of interest were visualized using fluorescently conjugated antibodies. Volocity software (Perkin Elmer) was used to drive the confocal microscope and for acquisition and analysis of images.

Antibodies for intravital imaging

Antibodies for intravital imaging were as follows: Alexa Fluor (AF) 750–conjugated anti–mouse F4/80 (2 µg/mouse; clone BM8; AbLab), Alexa Fluor (AF) 647-conjugated anti-mouse CD49b (3 µg/mouse; clone HMa2; BioLegend), PE-conjugated anti-mouse Ly6G (3 µg/mouse; clone 1A8; BioLegend) and FITC-conjugated anti-mouse CD3e (2µg(mouse; clone 145-2c11; eBioscience). HAPB (Sigma Aldrich) was fluorescently (Alexa Fluor 555; Invitrogen) conjugated and injected intravenously to detect intrahepatic HA.

In vivo treatments

Kupffer cell depletion was performed by intravenous injection of 100µl Clodronate (17 mM) i.v. twice a week for two weeks. Hyaluronidase (Sigma-Aldrich) was given by intraperitoneal injection twice a week at a dose of 20U/g per mouse for two weeks. The last dose was given 3 hours before intravital imaging.

1258 **In vivo image analysis**

1259 All videos and images were acquired and processed using Volocity software (PerkinElmer).

1260 Quantification of platelet aggregation, Kupffer cell and neutrophil numbers and sinusoid

1261 diameters was also performed using Volocity software. Per mouse, ten fields of view (FOV)

1262 were randomly selected and assessed. Platelet aggregation was measured using the "find

1263 objects" function in Volocity software and the highest background was subtracted as

1264 described (Surewaard et al 2018). Kupffer cell and neutrophil numbers were manually

1265 counted in ten randomly selected FOV. For measurement of sinusoidal diameter, images

1266 were exported from Volocity software as .jpg files. ImageJ software (NIH) was used to

1267 measure sinusoid diameters. Per mouse we measured 10 randomly selected sinusoids in 5

1268 fields of view (FOV).

1269

1270 **Statistical Analyses**

1271 Mouse data are presented as the mean ± SEM. Pilot experiments and previous published

1272 results were used to estimate the sample size such that appropriate statistical tests could

1273 yield significant results. Statistical analysis was performed using GraphPad Prism software

1274 version 7.03 (GraphPad Software). Data of three or more groups were analyzed by analysis

1275 of variance with the post hoc Tukey or Bonferroni multiple comparison test. Data of three

1276 groups over time were analyzed by two way analysis of variance with the post hoc Tukey

1277 multiple comparison test. Analysis of two samples was performed with two-tailed Student's t-

1278 test, Mann-Whitney t test and statistics for HCC incidence were calculated using two-tailed

1279 Fisher's exact test. Statistical significance is indicated either as exact p-value or as follows:*p

1280 < 0.05, **p < 0.01, ***p < 0.001, , and ****p < 0.0001; "n.s." indicates not significant.

1281

1282 **Data availability Statement**

1283 Data that support the findings of this study have been deposited in uploaded to ArrayExpress
1284 (www.ebi.ac.uk/arrayexpress/) and the data set is available under the accession number E-
1285 MTAB-6073, entitled "Transcriptomic differences in livers of mice fed with normal diet and
1286 choline-deficient high-fat diet".

1287 Moreover, the data that support the findings of this study are available from the
1288 corresponding authors upon reasonable request. If not stated otherwise, the authors declare
1289 that all other data supporting the findings of this study are available within the paper and its
1290 supplementary information files.

1291 The Figures 6a and Supplementary Fig. 12 have associated source data that are uploaded to
1292 ArrayExpress (www.ebi.ac.uk/arrayexpress/) and the data set is available under the
1293 accession number E-MTAB-6073, entitled "Transcriptomic differences in livers of mice fed
1294 with normal diet and choline-deficient high-fat diet".

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AUTHOR CONTRIBUTIONS

1403 Design of the study: M.Malehmir, M.J.W., D.R., A.W., B.N., M.G. and M.

1404 Heikenwaelder. M.Malehmir, E.K., D.P., V.L., M.J.W., C.D., performed breeding and

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1411 T.S., M.P., L.S. D.H.A., C.N.-A., J.L. provided tissue samples or mouse strains

1412 and/or scientific input. K.U. and T.E. performed bio-statistical analyses. All authors

1413 analyzed data. M.Malehmir, M.E.H., D.P., S.G., M.Szydlowska, P.K., B.N., M.G.,

O.K., T.O., A.W., and M. Heikenwaelder wrote the manuscript, and all authors contributed to writing and provided feedback.

COMPETING FINANCIAL INTERESTS

FIGURE LEGEND

1431 **Figure 1: Increased platelet numbers and aggregates in liver sinusoids of murine and**
1432 **human NASH.**

1433 **(a)** CD42b staining and quantification of intrahepatic platelets (CD42b$^+$) in 6 months ND or
1434 CD-HFD fed mice, arrows indicate platelets, (n=7 mice/group), scale bar: 50 µm. **(b)** 3D
1435 confocal images of platelet (green)/liver endothelium (grey) interaction of 6 months ND or
1436 CD-HFD fed mice (n=4 mice/group), scale bar: 20 µm. See also Movies S1 and S2. **(c)**
1437 CD42b staining and quantification in 6 months ND, WD-HTF (n=6 mice/group) or **(d)** WD-
1438 NTF fed mice, arrows indicate platelets, (ND n=12 mice; WD-HTF n=4 mice), scale bar: 50
1439 µm. **(e)** CD42b staining and quantification of intrahepatic platelets (CD42b$^+$) in 2 months ND
1440 or MCD fed mice, arrows indicate platelets, (n=5 mice/group), scale bar: 50 µm. **(f)** CD61
1441 staining and quantification of platelets (CD61$^+$) in human livers, arrows indicate platelets,
1442 (non-diseased patients n=4; NASH patients n=21). **(g)** H/E, CD42b staining and
1443 quantification in 6 months ND or HFD-45% fed mice, (ND n=6 mice; HFD-45% n=7 mice),
1444 scale bar: 50 µm **(h)** H/E, CD42b staining and quantification in 6 months ND or HFD
1445 (60%kcal & low sucrose (LS)) fed mice, (ND n=8 mice; HFD n=6 mice), scale bar: 50 µm. All
1446 data are shown as mean ± SEM. All data were analyzed by two-tailed Student's t test.

1447

1448 **Figure 2: Asp-Clo treatment results in reduction of steatosis, liver damage, NASH and**
1449 **NASH-associated conditions.**

1450 **(a)** Body weight development of 12 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (ND
1451 n=5 mice; CD-HFD n=5 mice; CD-HFD/Asp-Clo n=9). Statistic: ND vs. CD-HFD (black
1452 asterisks), ND vs. CD-HFD/Asp-Clo (green asterisks). **(b)** ALT of 12 months ND, CD-HFD or
1453 CD-HFD/Asp-Clo fed mice (6 months: ND n=14 mice; CD-HFD n=11 mice; CD-HFD/Asp-Clo
1454 n=4 mice; 12 months: ND n=7 mice; CD-HFD n=12 mice; CD-HFD/Asp-Clo n=18 mice). **(c)**
1455 IPGTT of 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n=5 mice/group). Statistic:
1456 ND vs. CD-HFD (black asterisks), ND vs. CD-HFD/Asp-Clo (green asterisks). **(d)** Liver
1457 triglyceride (6 months: ND n=11 mice; CD-HFD n=15 mice; CD-HFD/Asp-Clo n=7 mice; 12
1458 months: ND n=4 mice; CD-HFD n=9 mice; CD-HFD/Asp-Clo n=15 mice) and **(e)** serum
1459 cholesterol levels of 6 and 12 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (6 months:
1460 ND n=4 mice; CD-HFD n=7 mice; CD-HFD/Asp-Clo n=9 mice; 12 months: ND n=6 mice; CD-
1461 HFD n=17 mice; CD-HFD/Asp-Clo n=11 mice). **(f)** Real-time qPCR analysis for genes
1462 involved in lipid metabolism/β-oxidation of 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed
1463 mice (ND n=4 mice; CD-HFD n=5 mice; CD-HFD/Asp-Clo n=7 mice). Statistic: CD-HFD vs.
1464 CD-HFD/Asp-Clo (green asterisks). **(g)** Analysis of VO$_2$ and respiratory exchange ratio

1465 (RER) over time in 2 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (ND n=4 mice; CD-
1466 HFD n=8 mice; CD-HFD/Asp-Clo n=8 mice). **(h)** Analysis of food (g/mouse/day) and water
1467 intake (ml/mouse/day) (ND n=4 mice; CD-HFD n=8 mice; CD-HFD/Asp-Clo n=6 mice). **(i)**
1468 MRI analyses 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n=3 mice/group). T1
1469 (fast low-angle shot [FLASH]) OUT phase: dark color indicative of steatosis. T2 TurboRare:
1470 an increase in subcutaneous and abdominal fat and hepatic lipid accumulation (bright
1471 regions). **(j)** H/E staining and **(k)** NAS evaluation of 6 months ND, CD-HFD or CD-HFD/Asp-
1472 Clo fed mice (ND n=9 mice; CD-HFD n=9 mice; CD-HFD/Asp-Clo n=6 mice), scale bar:
1473 100µm in 10X, 50µm in 20X. All data are shown as mean ± SEM. Data in (a) and (c) were
1474 analyzed by two way analysis of variance with the post hoc Tukey's multiple comparison test;
1475 *: $P < 0.05$. **: $P < 0.01$. ***: $P < 0.001$. ****: $P < 0.0001$. Data in (b), (d), (e), (g), (h) and (k)
1476 were analyzed by one way analysis of variance with the post hoc Tukey's multiple
1477 comparison test. Data in (f) were analyzed by two-tailed Mann Whitney's test; *: $P < 0.05$. **:
1478 $P < 0.01$.

1479

1480 **Figure 3: Anti-platelet treatment with Asp-Clo abrogates immune cell infiltration into**
1481 **the liver and prevents NASH-induced HCC development.**

1482 **(a)** CD3, F4/80, MHCII and Ly-6G staining and quantification of 6 months ND, CD-HFD or
1483 CD-HFD/Asp-Clo fed mice (CD3: ND n=5 mice; CD-HFD n =11 mice; CD-HFD/Asp-Clo n=5
1484 mice; F4/80: ND n=6 mice; CD-HFD n=12 mice; CD-HFD/Asp-Clo n=5 mice; MHCII: ND n=9
1485 mice; CD-HFD n=12 mice; CD-HFD/Asp-Clo n=5 mice), scale bar: 50µm. **(b) (left)**
1486 Representative FACS plots and quantification of hepatic CD4/CD8 ratio, **(right)** NKT cells
1487 and **(c)** activated CD8+ cells of 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (CD8$^+$:
1488 ND n=6 mice; CD-HFD n=6 mice; CD-HFD/Asp-Clo n=4 mice; CD3$^+$NK1.1$^+$: ND n=4 mice;
1489 CD-HFD n=4 mice; CD-HFD/Asp-Clo n=3 mice; CD8$^+$CD62L$^-$CD44$^+$CD69$^+$: ND n=6 mice;
1490 CD-HFD n=6 mice; CD-HFD/Asp-Clo n=4 mice). **(d)** Western blot images of 6 months ND,
1491 CD-HFD or CD-HFD/Asp-Clo fed mice (n=2 mice/group). kDa: kilo Dalton. **(e)** Representative
1492 macroscopical images of livers from 12 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice.
1493 (ND n=0 tumors in 27 mice; CD-HFD n=13 tumors in 52 mice; CD-HFD/Asp-Clo n=0 tumors
1494 in 20 mice). White arrow head indicate HCC, scale bar: 7.5 mm. **(f)** HCC incidence of 12
1495 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice. (T=tumor; NT=non-tumor), (ND n=0
1496 tumors in 27 mice; CD-HFD n=13 tumors in 52 mice; CD-HFD/Asp-Clo n=0 tumors in 20
1497 mice). **(g)** HCC characterization by H/E and collagen IV (Col IV) of 12 months ND, CD-HFD
1498 or CD-HFD/Asp-Clo fed mice, dashed line indicates tumor (T) border, scale bar: 2 mm (upper
1499 row H/E) and 200 µm (lower H/E; Col IV). All data are shown as mean ± SEM. Data in (a), (b)

and (c) were analyzed by one way analysis of variance with the post hoc Tukey's multiple comparison test. Data in (f) were analyzed by two-sided Fisher`s exact test.

Figure 4: Platelets efficiently populate the liver early during fatty liver pathogenesis.

(a) Intravital microscopy of livers of 4, 5, 6 and 8 weeks ND or CD-HFD fed mice. Analysis of Kupffer cells (violet), platelets (blue) and granulocytes (red), (4 weeks: ND n=2 mice; CD-HFD n=2 mice; 5 weeks: ND n=2 mice; CD-HFD n=2 mice; 6 weeks: ND n=4 mice; CD-HFD n=4 mice; 8 weeks: ND n=3 mice; CD-HFD n=3 mice), scale bar: 40 µm. **(b)** CD3 staining and quantification of 6 (ND n=4 mice; CD-HFD n=8 mice) or **(c)** 8 weeks ND or CD-HFD fed mice (ND n=5 mice; CD-HFD n=4 mice), scale bar: 50 µm. **(d)** Quantification of platelet area by intravital microscopy of mice shown in (a) (ND: 4 weeks n=2 mice and 40 FOV; 5 weeks n=2 mice and 40 FOV; 6 weeks n=4 mice and 40 FOV; 8 weeks n=2 mice and 40 FOV; CD-HFD: 4 weeks n=2 mice and 20 FOV; 5 weeks n=2 mice and 20 FOV; 6 weeks n=4 mice and 30 FOV; 8 weeks n=2 mice and 19 FOV). **(e)** Analysis of liver sinusoid diameter by intravital microscopy of mice shown in (a) (ND: 4 weeks n=2 mice and 101 sinusoids; 5 weeks n=2 mice and 150 sinusoids; 6 weeks n=4 mice and 100 sinusoids; 8 weeks n=2 mice and 150 sinusoids; CD-HFD: 4 weeks n=2 mice and 100 sinusoids; 5 weeks n=2 mice and 150 sinusoids; 6 weeks n=4 mice and 100 sinusoids; 8 weeks n=2 mice and 100 sinusoids). **(f)** Hepatocyte swelling measurement by H/E of mice shown in (b) and (c) (6 weeks: ND n=4 mice; CD-HFD n=3 mice; 8 weeks: ND n=3 mice; CD-HFD n=4 mice). **(g)** NAS evaluation of 6 or 8 weeks ND or CD-HFD fed mice (6 weeks: ND n=19 mice; CD-HFD n=18 mice; 8 weeks: ND n=3 mice; CD-HFD n=6 mice). **(h)** Liver triglycerides of 6 weeks ND or CD-HFD fed mice (n=3 mice/group). **(i)** 3D confocal images and quantification of platelet (green)/Kupffer cells (red) interaction of 6 months ND or CD-HFD fed mice (n=4 mice/group). Liver endothelium (grey), scale bar: 20 µm. **(j) (left)** Representative images of intravital microscopy of 6 weeks ND or CD-HFD fed mice. Analysis of Kupffer cells (violet, violet arrowhead), HABP (red, red arrowhead) and LSECs (blue), scale bar: 43 µm. **(right)** Representative high magnification images of intravital microscopy of mice shown in (i), Analysis of Kupffer cells (violet, violet arrowhead), HABP (red, red arrowhead) and LSECs (blue), (ND n=4 mice; CD-HFD n=4 mice), scale bar: 43 µm. All data are shown as mean ± SEM. All data were analyzed by two-tailed Student's t test.

Figure 5: Intrahepatic platelet accumulation depends on Kupffer cells, hyaluronan and

1535 **cargo function**.

1536 **(a)** Representative images of intravital microscopy after treatment (clodronate liposomes

1537 (CLL) or hyaluronidase (HYAL)) in 6 weeks ND, CD-HFD, CD-HFD +CLL or CD-HFD +HYAL

1538 fed mice. Analysis of Kupffer cells (violet), platelets (blue, blue arrowhead), and granulocytes

1539 (red), (n=4 mice/group), scale bar: 40 µm. **(b)** H/E and F4/80 staining with quantification and

1540 NAS evaluation after treatment in 6 weeks ND, CD-HFD, CD-HFD +CLL or CD-HFD +HYAL

1541 fed mice (H/E: ND n=10 mice; CD-HFD n=14 mice; CD-HFD +CLL n=9 mice; CD-HFD

1542 +HYAL n=8 mice; F4/80: ND n=7 mice; CD-HFD n=10 mice; CD-HFD +CLL n=4 mice; CD-

1543 HFD +HYAL n=5 mice), scale bar: 50 µm. **(c)** Quantification of platelet area by intravital

1544 microscopy of mice shown in (a) (ND n=4 mice and 40 FOV; CD-HFD n=4 mice and 30 FOV;

1545 CD-HFD +CLL n=4 mice and 40 FOV; CD-HFD +HYAL n=4 mice and 30 FOV). **(d)** ALT

1546 levels of mice shown in (b) (ND n=17 mice; CD-HFD n=13; CD-HFD +CLL n=7 mice; CD-

1547 HFD +HYAL n=8 mice). **(e-g)** H/E histology, ALT levels and NAS evaluation after anti-CD44

1548 antibody treatment (anti-CD44 antibody blocking- (KM81) or non-blocking (IM7) HA-binding

1549 site) in 6 weeks ND, CD-HFD, CD-HFD +IM7 (non-HA blocking) or CD-HFD +KM81 (HA-

1550 blocking) fed mice (ALT: ND n=16 mice; CD-HFD n=12 mice; CD-HFD +IM7 n=4 mice; CD-

1551 HFD +KM81 n=4 mice; H/E and NAS: ND n=19 mice; CD-HFD n=15 mice; CD-HFD +IM7

1552 n=4 mice; CD-HFD +KM81 n=4 mice), scale bar: 50 µm. **(h)** Representative H/E, CD42b

1553 staining and **(i)** NAS evaluation and platelet quantification after CLL treatment in 6 months

1554 CD-HFD or CD-HFD +CLL fed mice (H/E and NAS: CD42b: CD-HFD n=5 mice; CD-HFD

1555 +CLL n=3 mice; CD42b: CD-HFD n=5 mice; CD-HFD +CLL n=3). **(j)** Body weight

1556 development of 6 months ND, CD-HFD or CD-HFD/Nbeal2$^{-/-}$ fed mice (ND n=6 mice; CD-

1557 HFD n=6 mice; CD-HFD/Nbeal2$^{-/-}$ n=4 mice). Statistic: ND vs. CD-HFD (black asterisks), CD-

1558 HFD vs. CD-HFD/Nbeal2$^{-/-}$ (blue asterisks). **(k)** ALT, AST levels, **(l)** liver triglycerides and **(m)**

1559 serum cholesterol levels of mice shown in (i) (ALT: ND n=7 mice; CD-HFD n=18 mice; CD-

1560 HFD/Nbeal2$^{-/-}$ n=4 mice; AST: ND n=4 mice; CD-HFD n=8 mice; CD-HFD/Nbeal2$^{-/-}$ n=3 mice;

1561 liver triglycerides: ND n=5 mice; CD-HFD n=7 mice; CD-HFD/Nbeal2$^{-/-}$ n=4 mice; serum

1562 cholesterol: ND n=4 mice; CD-HFD n=11 mice; CD-HFD/Nbeal2$^{-/-}$ n=3 mice;). **(n)**

1563 Representative H/E of mice shown in (i) (CD-HFD n=9 mice; CD-HFD/Nbeal2$^{-/-}$ n=10 mice),

1564 damaged hepatocytes (asterisks) are indicated, scale bar: 50µm. **(o)** Fat quantification by

1565 Sudan red staining of mice shown in (i) (ND n=4 mice and 17 fields; CD-HFD n=4 mice and

1566 14 fields; CD-HFD/Nbeal2$^{-/-}$ n=4 mice and 35 fields;), scale bar: 100 µm. All data are shown

1567 as mean ± SEM. Data in (b), (c), (d), (f), (g), (k), (l), (m) and (o) were analyzed by one way

1568 analysis of variance with the post hoc Tukey's multiple comparison test. Data in (i) were

1569 analyzed by two-tailed Student`s t test. Data in (j) were analyzed by two way analysis of

variance with the post hoc Tukey's multiple comparison test; *: P < 0.05. **: P < 0.01. ***: P <

0.001. ****: P < 0.0001.

Figure 6: Anti-GPIbα antibody treatment as well as genetic dysfunction of GPIbα

reduces NASH, fibrosis and HCC development.

(a) Representative 3D confocal images of GPIbα (green, green arrowheads)/Kupffer cells

(red, red arrowheads) interaction of 6 months ND or CD-HFD fed mice (ND n=4 mice; CD-

HFD n=4 mice). Liver endothelium (grey), scale bar: 30 µm. **(b)** High magnification 3D

confocal images and quantification of GPIbα (green)/Kupffer cells (red) and GPIbα

(green)/LSECs (grey) interaction in 6 months ND or CD-HFD fed mice (ND n=4 mice and 2

FOV/mouse; CD-HFD n=4 mice and 2 FOV/mouse), scale bar: 3 µm. For visualization of

intravascular events, the transparency of the sinusoidal rendering was set to 50%. **(c)**

Representative H/E and CD42b staining after 5 weeks of GPIbα blocking or control Fab in 6

months CD-HFD fed mice, scale bar: 50 µm. Platelets are indicated by arrows. **(d)** Platelet

quantification, **(e)** NAS evaluation, **(f)** ALT levels, **(g)** liver triglycerides and **(h)** Sirius red-

positive areas quantification of mice shown in (c) (CD42b staining and platelet quantification:

CD-HFD +control Fab n=8 mice; CD-HFD +GPIbα blocking Fab n=8 mice; H/E, NAS and

ALT: CD-HFD +control Fab n=5 mice; CD-HFD +GPIbα blocking Fab n=4 mice; liver

triglycerides: CD-HFD +control Fab n=4 mice; CD-HFD +GPIbα blocking Fab n=4 mice;

Fibrosis: CD-HFD +control Fab n=5 mice; CD-HFD +GPIbα blocking Fab n=4 mice). **(i)**

Serum cholesterol, liver triglycerides and **(j)** ALT levels of 6 months ND, CD-HFD or CD-

HFD/*hIL4rα/GPIbα*-Tg fed mice (serum cholesterol: ND n=4 mice; CD-HFD n=5 mice; CD-

HFD/*hIL4rα/GPIbα*-Tg n=4 mice; liver triglycerides: ND n=6 mice; CD-HFD n=5 mice; CD-

HFD/*hIL4rα/GPIbα*-Tg n=4 mice; ALT: ND n=4 mice; CD-HFD n=3 mice; CD-

HFD/*hIL4rα/GPIbα*-Tg n=4 mice). **(k)** Quantification by flow cytometry of intrahepatic immune

cells (**(left)** CD8+ T-cells, **(middle)** activated CD8+ T-cells, **(right)** NKT-cells) of mice shown

in (i) (CD8$^+$, activated CD8$^+$ and NKT cells: ND n=3 mice; CD-HFD n=3 mice; CD-

HFD/*hIL4rα/GPIbα*-Tg n=4 mice). **(l)** Representative H/E staining of mice shown in (i),

indications of damaged hepatocytes (asterisks) and satellitosis (arrows), scale bars: 100 µm

in 10X and 25 µm in 40X. **(m)** Sudan red staining and **(n)** quantification of Sudan red-positive

areas, **(o)** NAS evaluation of 6 months ND, CD-HFD or CD-HFD/*hIL4rα/GPIbα*-Tg fed mice

(H/E and NAS: ND n=7 mice; CD-HFD n=13 mice; CD-HFD/*hIL4rα/GPIbα*-Tg n=8 mice;

Sudan red staining and quantification: n=5 mice/group). **(p)** Fibrosis quantification and **(q)**

Sirius red staining of 12 months ND, CD-HFD or CD-HFD/*hIL4rα/GPIbα*-Tg fed mice (H/E

and NAS: ND n=7 mice; CD-HFD n=13 mice; CD-HFD/*hIL4rα/GPIbα*-Tg n=8 mice; Sudan

red: n=5 mice/group; fibrosis and Sirius red: NKT cells: ND n=4 mice; CD-HFD n=9 mice;

1606   CD-HFD/*hIL4rα/GPIbα*-Tg n=10 mice). **(r)** ALT levels of 12 months ND, CD-HFD or CD-

1607   HFD/*hIL4rα/GPIbα*-Tg fed mice (ND n=12 mice; CD-HFD n=16 mice; CD-HFD/*hIL4rα/GPIbα*-

1608   Tg n=9 mice). **(s)** Macroscopical images of tumors of mice shown in (r), tumor nodules are

1609   indicated by arrowhead) (CD-HFD: n=13 tumors in 52 mice; CD-HFD/*hIL4rα/GPIbα*-Tg: n=0

1610   tumors in 24 mice), scale bar: 750 µm. **(t)** HCC characterization by CD44v6, Collagen IV

1611   (Coll IV) and Ki67 staining form mice shown in (r). Arrowheads indicate positive hepatocytes,

1612   dashed line indicates tumor (T) border, scale bar: 200 µm (CD44v6 and Coll IV), 50 µm

1613   (Ki67). **(u)** HCC incidence (T=HCC; NT=non-tumor) from 12 months CD-HFD or CD-

1614   HFD/*hIL4rα/GPIbα*-Tg fed mice, CD-HFD: n=13 tumors in 52 mice; CD-HFD/*hIL4rα/GPIbα*-

1615   Tg: n=0 tumors in 24 mice). All data are shown as mean ± SEM. Data in (b), (d), (e), (f), (g)

1616   and (h) were analyzed by two-tailed Student`s t test. Data in (i), (j), (k), (n), (o), (p) and (r)

1617   were analyzed by one way analysis of variance with the post hoc Tukey's multiple

1618   comparison test. Data in (u) were analyzed by two-sided Fisher`s exact test.

Malehmir et al., Figure 1

a

Body weight [g] vs Age [months]
- ND
- CD-HFD
- CD-HFD/Asp-Clo

b

ALT [U/l]
- ND
- CD-HFD
- CD-HFD/Asp-Clo

6 months: P < 0.0001, P = 0.0340
12 months: P < 0.0001, P = 0.0002

c

Blood glucose [mmol/l] vs time [min]

d

Liver triglycerides [μg/mg tissue]

6 months: P = 0.0017, P = 0.0345
12 months: P = 0.0046, P = 0.0125

e

Cholesterol [mg/dl]

6 months: P = 0.0005, P = 0.0124
12 months: P = 0.0016, P = 0.0005

f

FAs oxidation: Acsm2, Acad10, Acat3, Acat2, Acot2, Acsbg1, Acox3, Acsl1, Cpt1, Pparδ
Lipolysis: El, HL, Lipe, Lpl
CHOL metabolism: Gpat, Sqle, Mvd, Abca1

ND, CD-HFD, CD-HFD/Asp-Clo

log2: 10 — 0 — -10

g

ND, CD-HFD, CD-HFD/Asp-Clo

VO_2 (ml/h) — DARK DARK DARK — 19:00 07:00
CD-HFD vs CD-HFD/Asp-Clo - n.s

RER (VCO_2/VO_2) — DARK DARK DARK — 19:00 07:00
CD-HFD vs CD-HFD/Asp-Clo - n.s

h

ND, CD-HFD, CD-HFD/Asp-Clo

Food intake (g/mouse/day): P < 0.0001
Water intake (ml/mouse/day): P < 0.0001

i

T1 (FLASH) IN Phase — T1 (FLASH) OUT Phase — T2 TurboRare
ND, CD-HFD, CD-HFD/Asp-Clo

j

H/E (10 X) — H/E (20 X)
ND, CD-HFD, CD-HFD/Asp-Clo

k

ND, CD-HFD, CD-HFD/Asp-Clo

NAS — P < 0.0001, P = 0.0071 — 6 months

Malehmir et al., Figure 2

a

	CD3	F4/80	MHC II	Ly6G
ND				
CD-HFD				
CD-HFD/Asp-Clo				

ND ☐   CD-HFD ■   CD-HFD/Asp-Clo ■

CD3$^+$ cells/mm^2
P = 0.0257 P = 0.0197

F4/80$^+$ aggregates/mm^2
P = 0.0005 P = 0.0178

MHC II$^+$ aggregates/mm^2
P = 0.0163

Ly6G$^+$ cells/mm^2

b

ND   CD-HFD   CD-HFD/Asp-Clo

CD8 vs CD4
ND: 7%, 14.7%
CD-HFD: 15.7%, 13.7%
CD-HFD/Asp-Clo: 7.2%, 11.8%

CD8$^+$
P = 0.0008 P = 0.0016

CD3$^+$ Nk1.1$^+$
P = 0.0296 P = 0.0110

Total # of cells/liver (×10^4)

c

CD8$^+$CD62L$^-$ gated

ND   CD-HFD   CD-HFD/Asp-Clo

CD44 vs CD69
ND: 49.8%, 12.2%, 37.4%, 0.6%
CD-HFD: 39%, 43%, 16%, 2%
CD-HFD/Asp-Clo: 46%, 19%, 34%, 1%

CD8$^+$ CD62L$^-$ CD44$^+$ CD69$^+$
P = 0.0002 P = 0.0047
Total # of cells/liver (×10^4)

d

kDa	ND	CD-HFD	CD-HFD/Asp-Clo	
65				p-p65
65				p65
43				p-p38
43				p38
74				COX-2
37				GAPDH

e

ND   CD-HFD   CD-HFD/Asp-Clo

f

	ND	CD-HFD	CD-HFD/Asp-Clo
		P = 0.0032	P = 0.0143

T
NT

g

ND   CD-HFD   CD-HFD/Asp-Clo

H/E — T, NT, T, T
H/E — NT, T
Col IV — NT, T

Malehmir et al., Figure 3

a

Hepatocytes Kupffer Cells Platelets Neutrophils

4 weeks   5 weeks   6 weeks   8 weeks

ND

CD-HFD

b

ND 6 weeks CD-HFD

CD3

6 weeks

CD3+ cells/mm²

c

ND 8 weeks CD-HFD

8 weeks

CD3+ cells/mm²

d

Platelets area (µm²)/FOV

ND
CD-HFD

P < 0.0001   P < 0.0001   P < 0.0001   P < 0.0001

4 weeks   5 weeks   6 weeks   8 weeks

e

Ø Sinusoids (µm)

P < 0.0001   P < 0.0001   P < 0.0001   P < 0.0001

4 weeks   5 weeks   6 weeks   8 weeks

f

ND
CD-HFD

6 weeks

Hepatocyte area (µm²)

P = 0.0007

8 weeks

Hepatocyte area (µm²)

P = 0.0001

6 weeks

H/E   ND   CD-HFD

g

ND
CD-HFD

6 weeks

NAS

P < 0.0001

8 weeks

NAS

P = 0.0080

h

ND
CD-HFD

Liver triglycerides [µg/mg tissue]

P = 0.0621

i

ND

Coll IV
CD41
F4/80

CD-HFD

Coll IV
CD41
F4/80

ND
CD-HFD

PLT aggregate average size attached to KC (µm³)

P = 0.0053

j

Hepatocytes Kupffer Cells HABP LSECs

ND   CD-HFD   CD-HFD

Malehmir et al., Figure 4

a

Hepatocytes Kupffer Cells Platelets Granulocytes 6 weeks

ND CD-HFD CD-HFD + CLL CD-HFD + HYAL

b

ND CD-HFD CD-HFD + CLL CD-HFD + HYAL

H/E

F4/80

NAS
P < 0.0001
P < 0.0001
P < 0.0001

F4/80 aggregates /mm²
P = 0.0084

ND
CD-HFD
CD-HFD +CLL
CD-HFD +HYAL

c

Platelets area (µm²)/FOV
P < 0.0001
P < 0.0001
P < 0.0001

d

ALT [U/l]
P = 0.0471
P = 0.0015

ND
CD-HFD
CD-HFD +CLL
CD-HFD +HYAL

f

ALT [U/l]
P = 0.0368
P = 0.0393

ND
CD- HFD
CD- HFD +IM7
CD- HFD +KM81

e

N D CD-HFD +IM7 (non-HA blocking) +KM81 (HA-blocking)

H/E

CD-HFD CD-HFD

g

NAS
P = 0.0419
P = 0.0041
P < 0.0001
P < 0.0001

N D
C D-HFD
C D-HFD +IM7
C D-HFD +KM81

h

CD-HFD CD-HFD + CLL CD-HFD CD-HFD + CLL

H/E CD42b

i

NAS
P = 0.0338

Platelet count/mm²
P = 0.0417

CD-HFD
CD-HFD +CLL

j

ND
CD-HFD
CD-HFD/ Nbeal2 -/-

Body weight [g]
*** **** **** **** **** **

age [months]

k

ALT [U/l]
P = 0.0005 *P* = 0.0323

AST [U/l]
P = 0.0130 *P* = 0.0290

l

Liver triglycerides [µg/mg tissue]
P = 0.0004 *P* = 0.0259

m

Cholesterol [mg/dl]
P = 0.0167

ND
CD-HFD
CD-HFD/ Nbeal2 -/-

n

CD-HFD CD-HFD/Nbeal2-/-

H/E (20X)

o

ND CD-HFD CD-HFD/Nbeal2-/-

6 months

Sudan Red area (%)
P <0.0001 *P* <0.0001

Malehmir et al., Figure 5

a

ND   CD-HFD

Coll IV   GPIb α   F4/80

b

ND   CD-HFD

Coll IV   GPIbα   F4/80

ND   CD-HFD

Platelet adherence to Kupffer cells/LSECs (%)

$P = 0.0051$
$P = 0.0051$

PLT-Kupffer   PLT-LSEC

c

H/E   CD42b

Control

GPIb blocking ab

d   CD-HFD (ctrl ab)   CD-HFD/GPIbα blocking ab

Platelet count/mm^2

$P = 0.0210$

e

NAS

$P = 0.0331$

f

ALT [U/l]

$P = 0.0131$

g

Liver triglycerides [µg/mg tissue]

$P = 0.0437$

h

% Fibrosis

$P = 0.1193$

i   ND   CD-HFD   CD-HFD/GPIbα-IL4r-Tg

Cholesterol [mg/dl]

$P = 0.0162$
$P = 0.0001$ $P = 0.0177$

Liver triglycerides [µg/mg tissue]

$P = 0.0034$ $P = 0.0130$

j

ALT [U/l]

$P = 0.0026$ $P = 0.0075$

k

CD8$^+$

Total # of cells/liver ($\times 10^4$)

$P = 0.0321$
$P = 0.0012$ $P < 0.0001$

CD8$^+$ CD62L$^-$ CD44$^+$ CD69$^+$

Total # of cells/liver ($\times 10^4$)

$P = 0.0040$ $P = 0.0013$

CD3$^+$ Nk1.1$^+$

Total # of cells/liver ($\times 10^4$)

$P = 0.0466$ $P = 0.0076$

l

H/E (10 X)   H/E (40 X)

CD-HFD

CD-HFD/GPIbα-IL4r-Tg

m

ND   CD-HFD   CD-HFD/GP1bα-IL4r-Tg

6 months

q

Sirius Red

ND

CD-HFD

CD-HFD/GP1bα-IL4r-Tg

n

Sudan Red area (%)

$P = 0.0032$
$P < 0.0001$ $P < 0.0001$

o

NAS

$P < 0.0001$ $P = 0.0027$

p

% Fibrosis

$P = 0.0101$ $P = 0.0358$

r

12 months

ALT [U/l]

$P = 0.0038$
$P < 0.0001$ $P = 0.0287$

s

CD-HFD

CD-HFD/GPIbα-IL4r-Tg

t

CD44v6   Coll IV   Ki-67

CD-HFD

NT   T

CD-HFD/GPIbα-IL4r-Tg

u

$P = 0.0067$

T

NT

CD-HFD   CD-HFD/GPIbα-IL4r-Tg

Malehmir et al., Figure 6