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 murine/human NASH. Antiplatelet-therapy (APT - Aspirin/Clopidogrel; Ticagrelor) but not NSAID-therapy (Sulindac) prevented NASH and subsequent HCC development. Intravital microscopy showed that liver-colonization by platelets depends primarily on Kupffer cells at early and late stages of NASH, involving hyaluronan-CD44 binding. APT reduced intrahepatic platelet-accumulation and platelet-immune cell interaction frequency, thereby limiting hepatic immune-cell trafficking. Consequently, cytokine/chemokine release, macrovesicular steatosis and liver damage were attenuated. Analyses of distinct animal models identified platelet-cargo, platelet-adhesion/activation but not platelet-aggregation as pivotal for NASH-pathogenesis. In particular, platelet-derived GPIbα is critical for NASH and subsequent HCC, offering a potential interventional target against NASH.
INTRODUCTION

Nonalcoholic 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 nonalcoholic steatohepatitis (NASH) which may progress to cirrhosis and ultimately hepatocellular carcinoma (HCC)\(^4-6\). HCC is the third most common cause of cancer-related death worldwide and is one of the fastest rising cancer in the United States and Europe\(^7-9\). Major risk factors for NASH include metabolic syndrome, abdominal obesity, insulin resistance, glucose intolerance or type 2 diabetes mellitus and dyslipidaemia\(^3,10-16\).

We previously developed a pre-clinical model of chronic human metabolic syndrome, NASH and NASH-induced HCC\(^\text{15}\). 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\(^17\), but are also crucial for pathophysiological conditions like thrombosis\(^18\), obesity\(^19\), atherosclerosis\(^20,21\), metastasis\(^22\) and stroke\(^23\). In addition, a growing body of evidence highlights platelets as active players in liver disease and inflammation\(^24-28\). Notably, it has been reported that activated platelets contribute to cytotoxic T lymphocyte (CTL)-mediated liver damage in a model of viral hepatitis\(^29,30\). 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\(^30,31\). Additionally, in a recent
study of NAFL (but not NASH) patients, APT lowered serum markers of obesity and liver damage (REF).

There is an unmet need for efficacious, low-risk therapies against NASH and NASH-to-HCC transition. Although several drugs (e.g. decreasing blood-sugar level) are in phase 2 and 3 development\textsuperscript{32,33}, currently no approved pharmacological therapies are available that are capable of preventing NASH or related pathologies. Further, the role of platelets in NASH and HCC development is not well-characterized. Thus, we investigated whether antiplatelet therapy (APT) and molecules involved in platelet-function might interfere with NASH and NASH-induced HCC development.
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 were significantly increased in number compared to age-matched normal chow diet (ND)-fed controls (Fig. 1a). Moreover, platelet-aggregation (number, aggregate size) was strongly increased (Fig. 1b). 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. 1; Supplementary Fig. 1c,d), including high-fat, high-fructose, high-cholesterol, “Western-style” diets with or without trans-fat (WD-HTF; WD-NTF), a “Western-style” diet with fructose supplemented in drinking water (WD-FSDW)36, a methionine/choline-deficient diet (MCD) and an inducible knock-in mouse expressing the human unconventional prefoldin RPB5 interactor (URI) in hepatocytes (hURI-tetOFFhep)37. All models inducing NASH with varying degrees of fibrosis, which is a primary determinant of outcomes in NASH38,39, displayed a significant increase in intrahepatic platelet numbers compared to controls (Fig. 1c-e; Supplementary Fig. 1c-f). 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% high-fat diet (HFD), displaying only steatosis, or mice fed a 60% kcal HFD to induce both simple steatosis and insulin resistance40, lacked any
significant increase in intrahepatic platelet numbers (Fig. 1g,h; Supplementary Fig. 1g-i). Intrahepatic platelet activation in NASH was confirmed by electron microscopy (EM) (Supplementary Fig. 1j). Thus, abnormal intrahepatic platelet number, platelet aggregation and platelet activation could not be found in steatosis or obesity-linked insulin resistance but is present in NASH.

Asp-Clo treatment is an anti-platelet therapy (APT) currently used in several diseases (e.g. to prevent coronary stent thrombosis)41. We first addressed whether mice fed a CD-HFD would respond to APT. Compared to untreated mice on CD-HFD, Asp-Clo-treated mice displayed significantly lower intrahepatic platelet numbers (Supplementary Fig. 1k,l). Moreover, Asp-Clo-treated animals showed less platelet aggregation and activation when compared to CD-HFD-fed controls (Supplementary Fig. 1m,n). To investigate the effects of APT on human NAFLD, we started an initial prospective clinical trial (German Clinical Trials Register (DRKS) 587/2016BO2) with NAFLD patients (e.g. BMI>30; obesity, arterial hypertension, hyperlipidemia, diabetes mellitus type II, constantly increased LDL, vLDL and serum cholesterol levels) undergoing a heart catheter procedure and subsequent APT for 6 months (Supplementary Fig. 1o; Table S2). An initial cohort of n=24 patients was recruited for a small, preliminary clinical trial. Platelet function analyses revealed that patients generally responded well to APT (Supplementary Fig. 1p), although serum total LDL- and HDL- cholesterol levels remained unchanged (Supplementary Fig. 1p,q). For control, we investigated patients without APT (Table S3). NAFLD patients were monitored for 6 months after coronary angiography via serum analysis and sonography. APT-treated NAFLD patients showed significantly reduced liver volume and liver fat mass (Supplementary Fig. 1r-t). Liver fat was significantly reduced in statin-treated patients, whereas liver volume remained largely unchanged. Higher BMI did not affect liver fat or liver volume significantly. At its best, these underpowered data
supported the hypothesis that APT might have beneficial effects in human NAFLD/NASH.

Asp-Clo treatment attenuates NASH and NASH-associated conditions

We next investigated whether Asp-Clo (adjusted to the body weight) affects NASH or HCC development in mice. C57Bl/6 mice were given ND, CD-HFD or CD-HFD with Asp-Clo and analyzed after 12 months. Weight gain over time was significantly higher in CD-HFD and CD-HFD/Asp-Clo-fed mice compared to ND-fed controls (Fig. 2a). Similar to body weight, epididymal fat (eWAT) weight was not different to CD-HFD in CD-HFD/Asp-Clo-fed mice (Supplementary Fig. 2a). Low platelet numbers were found in eWAT and remained unaltered (Supplementary Fig. 2b,c). CD3+ T-cell infiltration was significantly reduced by Asp-Clo (Supplementary Fig. 2d). Liver damage, platelet numbers and aggregation state were significantly lower in livers of mice fed for 6 and 12 months CD-HFD/Asp-Clo (Fig. 2b; Supplementary Fig. 1k-m;2e,f). Asp-Clo significantly improved glucose tolerance (Fig. 2c), reduced liver triglycerides (Fig. 2d), and attenuated serum LDL- and HDL- cholesterol levels (Fig. 2e; Supplementary Fig. 2g, h). Several genes involved in fatty acid β-oxidation, lipolysis and cholesterol metabolism are dysregulated during NASH development15. Asp-Clo treatment prevented downregulation of several candidate genes from all three groups (Fig. 2f). CD-HFD/Asp-Clo-fed mice lacked statistically significant changes in oxygen consumption, respiratory exchange ratio (RER), activity or in food and water intake compared to CD-HFD-fed mice (Fig. 2g,h; Supplementary Fig. 2i). These data were corroborated in WD-HTF fed mice (Supplementary Fig2. j,k).

To analyze platelet activation, P-selectin, a marker of α-granule release, and integrin 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. Immune-cell infiltration was reduced in 6-month and 12-month CD-HFD/Asp-Clo-treated mice (Fig. 3a, data not shown). Flow cytometry demonstrated strong reductions in total number, effector differentiation (CD8+CD62L−CD44+CD69+), proportion of CD4+/CD8+ and NKT-cells (Fig. 3b,c).

Analyses of several inflammatory signaling pathways - potentially supporting carcinogenesis - activated under CD-HFD were dampened by Asp-Clo (Fig. 3d and Supplementary Fig. 3c). Asp-Clo significantly reduced CD11b+F4/80hi Kupffer cells (KCs) in CD-HFD livers (Fig. S3d-k). An unbiased t-distributed stochastic neighbor
embedding (t-SNE) based clustering approach identified 9 myeloid sub-clusters (Fig. S3h,i). Asp-Clo significantly reduced the abundance of Cluster 6, characterized by a high expression of CD11b, F4/80 and Gr1, closely resembling CD11b⁺F4/80⁺ monocyte-derived macrophages (MoMFs). Further, a multiplex gene expression analysis for FACS-isolated Ly6C⁺ MoMFs, LY6C⁻ MoMFs and KCs was performed. Principal component analysis of 561 genes revealed similar Ly6C⁺ MoMFs and Ly6C⁻ MoMFs in NASH-diet-fed mice with and without Asp/Clo treatment. However, KCs from Asp-Clo-CD-HFD livers clustered more closely to ND KCs than CD-HFD (Fig. S3h-k), indicating that Asp-Clo most effectively influences the KC compartment. Taken together, these data indicate that Asp-Clo treatment attenuated KC activation, alongside reduced inflammatory myeloid cell infiltration in the injured liver. Altogether, Asp-Clo prevented NASH, reduced intrahepatic immune cell influx and dampened pathways potentially supporting hepatocarcinogenesis⁴²,⁴³.

Next, we studied the effects of Asp-Clo treatment on CD-HFD-induced HCCⁱ⁵. 13 out of 51 CD-HFD livers displayed macroscopically visible tumors (~25%) by 12 months (Fig. 3e-g). In contrast, CD-HFD/Asp-Clo-treated mice lacked macro- and microscopically visible liver tumors (Fig. 3e-g; Supplementary Fig. 3l). CD-HFD-fed mice treated with a lower dose of Asp-Clo (according to the initial body weight and not further adjusted to diet/age-related weight gain) developed significantly fewer HCC (3/53) compared to untreated CD-HFD-fed mice (13/51) (Supplementary Fig. 3m). Therefore, a sufficient dose of Asp-Clo is 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 (Supplementary Fig. 4a-i). Moreover, NASH-related enrichment of genes was associated with expression of TNF-superfamily members, cytokine/chemokine production and chemotaxis was found\textsuperscript{15,37} (Supplementary Fig. 4a-j). Asp-Clo treatment significantly attenuated the latter, some of which are also released from activated platelets (e.g. CCL5, TGFβ)\textsuperscript{44}.

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 (Supplementary Fig. 4k-r).

To exclude COX2-dependent effects of Asp-Clo on NASH pathology, we used another non-steroidal anti-inflammatory drug (NSAID; COX1/2 inhibitor): Sulindac. CD-HFD/Sulindac-treated mice exhibit obesity, no significant changes in liver/body weight ratio, hepatic triglycerides, glucose tolerance, severe steatosis and increased liver damage comparable to CD-HFD treated mice (Supplementary Fig. 5a-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 antagonist of the platelet P2Y12 receptor\textsuperscript{45,46} for coronary artery disease used in the clinic\textsuperscript{47}. Liver damage, liver triglycerides and serum cholesterol levels were significantly reduced in Ticagrelor-treated mice (Supplementary Fig. 6a-h). Downregulation of several genes involved in fatty acid oxidation and lipolysis that occurs during CD-HFD (Supplementary Fig. 6i,j) as well as histopathological features of NASH were
prevented by Ticagrelor (Supplementary Fig. 6k-m). Total numbers, activation and proportion of CD4+/CD8+, CD8+CD62LD44+CD69+ and CD3+NK1.1+ cells were also reduced (Supplementary Fig. 6n-o).

In contrast to liver tissues, an increase in mRNA expression of genes involved in inflammation and fibrosis was found in eWAT from CD-HFD or WD-HTF mice receiving Asp-Clop or Ticagrelor (Supplementary Fig. 6p).

In contrast to untreated CD-HFD mice, Ticagrelor treatment significantly reduced HCC development. Only one liver nodule (adenoma) was detectable in 29 CD-HFD/Ticagrelor-treated mice (Supplementary Fig. 6r).

The therapeutic potential of Ticagrelor was tested in CD-HFD mice with fully established NASH (4 months CD-HFD), followed by 8-weeks of Ticagrelor with CD-HFD. Intrahepatic platelet numbers, liver damage and liver fibrosis were reduced/reverted (Supplementary Fig. 6s,t,u). Similar results were obtained with therapeutic Ticagrelor treatment in the context of a WD-HTF (Supplementary Fig. 6v-y).

**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 and early 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. 7a-c). Platelets were the first non-resident cell-type to efficiently populate the liver at ≤4 weeks pdi in both CD-HFD and WD-HTF (Fig. 4a-d; Supplementary Fig. 7a-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. 7a-c, data not shown). Even at this early stage, mild steatosis, reduced sinusoidal diameter and hepatocyte swelling could be observed (Fig. 4e,f; Supplementary Fig. 7b). NAS was increased significantly in CD-HFD and WD-HTF (Fig. 4g,h; Supplemental Fig. 7c-g). Platelets interacted primarily with Kupffer cells, as determined by 3D high-resolution reconstruction (Figure 4i).

Although intrahepatic granulocyte-numbers remained unaltered in the first 8 weeks pdi, granulocytes might reflect a cell-type that supports intrahepatic platelet recruitment and NAFL/NASH induction. Administration of anti-Ly6G antibodies for 8 weeks via Alzet-pumps to CD-HFD-fed mice revealed no significant role of granulocytes in the early development of NAFL and NASH (Supplementary Figure 7h-j).

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

**Kupffer cell-dependent platelet recruitment supports early- and late-stage of NAFL and NASH, involving hyaluronan-CD44 binding**

To investigate the functional role of Kupffer cells and molecules involved in platelet-LSEC/immune-cell interaction (e.g. hyaluronan, CD44) in early NASH, CD-HFD or WD-HTF mice were treated with Clodronate liposomes (CLL), control liposomes (CL), hyaluronidase (HYAL), HYAL/CLL (double treatment), CD44-binding/HA-blocking blocking AB (clone KM81) or CD44-binding/HA-non-blocking AB (clone IM7), for control. Treatment with CLL or HYAL significantly reduced intrahepatic platelet
recruitment in CD-HFD (Fig. 5a,c). CLL and CLL/HYAL but not CL treatment reduced Kupffer cell numbers in CD-HFD- and WD-HTF-fed mice (Fig. 5b, Supplementary Fig. 8c,d). CLL and HYAL reduced NAS significantly in CD-HFD and WD-HTF-fed mice (Fig. 5b). A significant reduction in liver damage was found following HYAL but not CLL (Fig. 5d). Similar data were obtained using WD-HTF mice (Supplementary Fig. 8d-e). In addition, treatment with a CD44-binding/HA-blocking but not with a CD44-binding/HA non-blocking antibody led to a reduction in NAS and liver damage (Fig. 5e,f).

Notably, significantly reduced NAS, platelet accumulation, triglycerides, and liver damage by 2.5 weeks of CLL-treatment in mice fed CD-HFD for 6 months demonstrate the therapeutic potential of CLL even in a short treatment scheme (Fig. 5g,h; Supplementary Fig. 8i,j). Thus, Kupffer cells, hyaluronan and platelet CD44 are important players in the early and late stages of NAFL and NASH.

**Platelet cargo is indispensable for NASH development**

Platelets release bioactive factors from intracellular granules in response to cellular activation. During thrombo-inflammatory reactions, the mostly proteinous components of α-granules are essential for immune cell recruitment and tissue damage. Nbeal2 knockout mice (Nbeal2/−), which lack α-granules in platelets and are thus protected from thrombosis and thrombo-inflammatory tissue damage were fed CD-HFD for 6 months. Importantly, CD-HFD-fed Nbeal2/− displayed no significant difference in intrahepatic platelet number, platelet aggregation and gained weight similarly to CD-HFD-fed controls (Fig. 5i; Supplementary Fig. 8l). Still, significantly lower serum ALT and AST levels were found in CD-HFD-fed Nbeal2/− mice compared to CD-HFD-fed (Fig. 5j). This was paralleled by a significant decrease in liver triglycerides, lower serum cholesterol (Fig. 5k,l), and improved glucose tolerance in CD-HFD-fed
Nbeal2−/− mice (Supplementary Fig. 8m). Deregulation of lipid metabolism genes in CD-HFD C57BI/6 livers was partially prevented in CD-HFD/Nbeal2−/− (Supplementary Fig. 8n). CD-HFD Nbeal2−/− livers lacked histological features of steatosis or NASH, corroborated by reduced NAS (Figure 5m; Supplementary Fig. 8o). Suppression of NASH in Nbeal2−/− mice on CD-HFD was corroborated by significant diminution of lipid content and macro-vesicular steatosis (Figure 5n). In line, decreased T-cell infiltration, neutrophil accumulation and macrophage activation was found in Nbeal2−/− mice on CD-HFD (Supplementary Fig. 8p). Together, these results indicated that platelet α-granule components contribute to NASH and NASH-associated conditions.

CD-HFD-induced NASH could not be rescued in mice lacking the GPIIib subunit of the platelet fibrinogen receptor, GPIIib/IIIa (integrin α2β3; Itga2b−/− mice) which harbors activatable platelets unable to aggregate (Supplementary Fig 10a-h). This is in agreement with a recent study indicating that deletion of the platelet integrin α2β3 binding motif of fibrinogen not altering NASH52.

Moreover, mice lacking the major platelet adhesion receptors P-selectin53 (Selp−/−), von-Willebrand-factor (vWF) 54,55, Mac1 -cognate GPIibα ligands – the activating platelet collagen receptor glycoprotein VI (GPVI; Gp6−/−)56, platelet-derived C-type lectin-like receptor 2 (Clec-2−/−) and hematopoietic-specific podoplanin (PDPN−/−) all developed NASH and NASH-associated conditions upon CD-HFD feeding (Supplementary Figs. 9-14).

Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) is known for its interaction with leukocyte L-selectin and LPAM-1 (alpha4/beta7), driving leukocyte homing to secondary lymphatic organs, mucosa and inflamed tissues. MAdCAM-1 was recently discovered as an important player in NASH57. Animals with genetic inactivation of MAdCAM-1 presented lower NAS and less liver damage after 6-month
of WD-HTF. We analyzed livers of WD-HTF-fed mice lacking MAdCAM-1 (MAdCAM-1<sup>-/-</sup>), L-selectin (L-sel<sup>-/-</sup>), integrin beta7 (β7<sup>-/-</sup>) and both L-selectin and integrin beta7 (L-sel/Beta 7<sup>-/-</sup>). MAdCAM-1<sup>-/-</sup> mice displayed significantly reduced intrahepatic platelet numbers correlating with a partial protection from NASH (Supplementary Fig. 15a-b). In contrast, genetic inactivation of MAdCAM-1 ligands integrin beta7, L-selectin and L-selectin/integrin beta7 did not affect intrahepatic platelet numbers or platelet aggregation and did not or only partially prevent NASH (Supplementary Fig. 15a-b).

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 drive NASH. Platelet-derived GPIbα has been described to influence platelet attachment and activation (REF).

We thus hypothesized that GPIbα might mediate platelet-trafficking/activation in inflamed livers during NASH, contributing to efficient immune cell recruitment to the liver. We first analyzed the interaction of GPIbα with parenchymal and non-parenchymal liver cells (LSECs; Kupffer cells etc.) in NASH (Fig. 6a,b). 3D reconstruction revealed most frequent interactions between GPIbα<sup>+</sup> platelets and Kupffer cells (Fig. 6a,b).

Thus, we blocked the major ligand binding domain of GPIbα in 6-months CD-HFD-fed mice using Fab fragments of the anti-GPIbα antibody, pop/B<sup>58</sup> for 5 weeks. Notably, already this relatively short treatment significantly reduced intrahepatic platelet accumulation in the presence of CD-HFD (Fig. 6c,d). Consequently, steatosis, NAS,
liver damage and immune cell infiltration were reduced, fibrosis was dampened (Figure 6c-h; Supplementary Fig. 16a-c). In addition, anti-GPIbα antibody treatment reduced protein expression of several pro-inflammatory and homeostatic cytokines/chemokines – linking intrahepatic platelet activation with mediators of inflammation (Supplementary Fig. 16d).

We next tested whether therapeutic anti-GPIbα antibody treatment would prevent fatty liver to NASH transition in early disease progression (e.g. after a 6 week CD-HFD; see also Figure 4). However, these treatments did not ameliorate NAFL/NASH - most likely due to lack of or low expression of unidentified GPIbα ligands at early disease stages (Supplementary Fig. 16e,f). These data highlight distinct mechanism of platelet recruitment in early versus established NASH – still involving Kupffer cells at both disease stages.

To corroborate the above data in a genetic model, we fed transgenic mice expressing an IL-4α/GPIbα fusion-protein in a GPIbα/− background59 in which the ligand-binding ectodomain of GPIbα is replaced by the α-subunit of the human IL-4 receptor (hIL4α/GP1ba-Tg)59 a CD-HFD for 6 months. Remarkably, platelet aggregate size, platelet area and platelet-liver endothelium coverage were significantly lower in CDHFD-fed hIL4Ra/GPIbα-Tg mice compared to CD-HFD-fed C57Bl/6 controls (Supplementary Figure 16g-i). Both hIL4ra/GPibα-Tg and C57Bl/6 mice gained weight similarly when fed CD-HFD (Supplementary Fig. 16j). Serum cholesterol, liver triglycerides, serum ALT and AST levels were significantly lower in CD-HFD/hIL4ra/GPibα-Tg mice (Fig. 6i,j; Supplementary Fig. 16k), accompanied by less LDL- and HDL-cholesterol (Supplementary Fig. 16k). Similarly, dysregulated mRNA expression of lipid metabolism-related genes in CD-HFD-fed C57Bl/6 livers was prevented in livers of CD-HFD/hIL4ra/GPibα-Tg mice (Supplementary Fig. 16l).
Concomitant with the attenuation of liver damage, we observed a strong and significant reduction in intra-hepatic CD8+ T- and NKT-cells by flow cytometry analysis (Fig. 6k), mechanistically linking platelet-attachment/activation to efficient intrahepatic immune cell attraction. In addition, reduced neutrophil accumulation and macrophage influx/activation were observed by immunohistochemistry (Supplementary Fig. 16m-n). In line, CD-HFD/hIL4rα/GP1bα-Tg mice showed lower protein expression of several pro-inflammatory and homeostatic cytokines/chemokines (Supplementary Fig. 16o). CD-HFD/hIL4rα/GP1bα-Tg mice lacked histological features of NASH, paralleled by a reduction in lipid accumulation and absence of macro-vesicular steatosis analyzed by H/E and Sudan red staining (Fig. 6l-o). Interestingly, mice lacking the major platelet adhesion receptors P-selectin, von-Willebrand-factor (vWF) or Mac-1, the cognat ligands of GPIbα, displayed full blown NASH in the context of a CD-HFD (6 months).

Finally, we investigated whether hIL4rα/GP1bα-Tg mice would develop HCC upon a long term CD-HFD. hIL4rα/GP1bα-Tg mice receiving CD-HFD for 12 months displayed significantly lower fibrosis, serum ALT levels, and lacked any macro- or microscopical evidence of HCC (Fig. 6p-u).
DISCUSSION

It is becoming increasingly clear that beyond their central role in hemostasis and wound repair after vascular injury\textsuperscript{60}, platelets are key players in multiple pathophysiological conditions\textsuperscript{24,29,31,61,62,27} including cytotoxic T lymphocyte (CTL)-mediated liver damage and associated pathologies\textsuperscript{29}. Here, we identified Kupffer cells as key players in the recruitment of platelets to the liver in early and late stages of NASH. In the early stages of NASH this involved hyaluronan and platelet CD44. Notably, we found that GPIb\(\alpha\) - expressed by platelets - appeared to be primarily involved in the interaction of platelets with Kupffer cells at late stage but not at early stages of NASH. This underlines distinct mechanisms of intrahepatic platelet recruitment according to the stage of fatty liver disease – involving Kupffer cells.

Moreover, we found no evidence for a role of platelet-derived GPIIb/IIIa (\(\text{Itga2b}^{\pm}\) mice) in NASH, suggesting platelet activation and adhesion to be important and platelet aggregation to be dispensable.

What is the function of platelets recruited to the liver? Our results indicate a major contribution of platelet \(\alpha\)-granule components to NASH, as shown by the marked protection of \(\text{Nbeal2}^{\pm}\) mice. The exact nature of these \(\alpha\)-granule constituents is currently still unclear. However, we found that several chemokines/cytokines and platelet-secreted factors are reduced upon anti-GPIb\(\alpha\) antibody treatment suggesting that platelet cargo function directly or indirectly correlates with an increase of immune-cell attracting chemokines/cytokines.

The key role of GPIb\(\alpha\) in NASH identified in this study parallels a similarly vital function of this receptor in the development of experimental autoimmune encephalomyelitis (EAE), in which it orchestrates the recruitment of leukocytes to the inflamed CNS\textsuperscript{63}. So far, it is unknown which ligands of GPIb\(\alpha\) are relevant for EAE or NASH. Our results -
similar to the EAE study - argue against a key role of the three known cognate interaction partners of GPIbα: vWF, Mac-1 and P-selectin and rather point to the pro-inflammatory function of α-granules in intrahepatic immune cell attraction. In line, selectins have been shown to be dispensable for leukocyte recruitment into the inflamed liver microvasculature. Other interaction partners might be involved (e.g. coagulation factors XI, XII). It is also conceivable that GPIbα exerts its function in disease development independent of a ligand. Moreover, due to the complex pathogenesis underlying NASH, it is conceivable that GPIbα is not the only molecule involved.

From a clinical standpoint, we demonstrate that platelets and platelet-derived glycoprotein GPIbα are potential interventional therapeutic targets of NASH and subsequent HCC development, thus providing the rationale for a new treatment modality against a metabolic disease of major public health relevance. So far, no firm recommendations for NASH treatments can be made. The use of pioglitazone (most efficacy data, but off-label outside T2DM because of side effects) or vitamin E (better safety and tolerability in the short-term) or their combination could be used for NASH treatment. Other agents like obeticholic acid have been shown to improve histological features of NASH as well, data with respect to their long-term benefits are still being awaited.

We demonstrate that APT (e.g. Asp-Clo) used for acute and long-term treatment of patients with myocardial infarction, as well as Ticagrelor attenuate NASH and NASH-induced HCC in a preventive fashion. In contrast, the NSAID Sulindac did not prevent NASH in mice. Thus, rather than the use of NSAIDs in general, therapies that specifically block intrahepatic platelet accumulation/platelet function seem to be required to prevent NASH and NASH-associated conditions.
For NASH we could also observe a therapeutic effect of APT – stopping the sequel to NASH triggered HCC, although potential therapeutic effects of APT in the context of pre-existing HCC was not tested. Similarly, we have observed a therapeutic anti-NASH effect in the context of anti-GPIbα antibody treatment. Remarkably, in both cases therapeutic Ticagrelor or anti-GPIbα antibody treatment fibrosis was partially reverted. Moreover, we show that treatment with a P2Y12 antagonist, depletion of functional GPIbα or lack of α-granules not only abolished activation, accumulation and adhesion of platelets to the liver endothelium but also reduced intrahepatic immune-cell trafficking, consequently reducing liver damage and attenuating disease development. It has been reported that the mean platelet volume (MPV) is increased in NAFLD patients\(^1\), and the MPV is correlated with histological severity of steatosis and fibrosis in NASH\(^2\). In a prospective human cohort study, we demonstrated that APT reduces liver volume and liver fat accumulation in NAFLD patients, supporting the results of our \textit{in vivo} experiments. However, results of this study - that can be at best taken as a starting point for further studies - have to be taken with caution as it is currently underpowered and as effects on liver steatosis correlated with statin treatment. Interestingly, a cross-sectional analysis suggests that regular aspirin use may be associated with a lower prevalence of NAFLD\(^3\).

The prevention of NASH ultimately suppressed subsequent HCC formation, mostly because the pro-carcinogenic NASH-related environment (e.g. intrahepatic inflammation, hepatocyte damage) was lacking. Here, we have demonstrated that two distinct APT (Ticagrelor and Asp/Clo) were able to prevent HCC occurrence in experimental models. These novel approaches might have an important impact for chemo-preventive strategies, since no such treatments are currently available for NASH patients to prevent HCC development. 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 hamper NASH to HCC transition.
METHODS
Methods, including statements of data availability and any associated accession codes and references are available in the online version of the paper.

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FIGURE LEGEND

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

(a) CD42b staining and quantification of intrahepatic platelets (CD42b*) in 6 months ND or CD-HFD fed mice, arrows indicate platelets, (n= 10/group), scale bar: 50 µm. (b) 3D confocal images of platelet (green)/liver endothelium (grey) interaction of 6 months ND or CD-HFD fed mice (n= 4/group), scale bar: 20 µm. See also Movies S1 and S2. (c) CD42b staining and quantification in 6 months ND, WD-HTF or (d) WD-NTF fed mice, arrows indicate platelets, (n= 10/group), scale bar: 50 µm. (e) CD42b staining and quantification of intrahepatic platelets (CD42b*) in 2 months ND or MCD fed mice, arrows indicate platelets, (n= 10/group), scale bar: 50 µm. (f) CD61 staining and quantification of platelets (CD61*) in human livers with or without NASH, arrows indicate platelets, (non-diseased n=4; NASH patients n=18). (g) H/E, CD42b staining and quantification in 6 months ND or HFD (60%kcal & low sucrose (LS)) fed mice, (n= 8/group), scale bar: 50 µm. (h) H/E, CD42b staining and quantification in 6 months ND or HFD -45% fed mice, (n≥ 7/group), scale bar: 50 µm. All data are shown as mean ± SEM.*: P < 0.05. **: P < 0.01. ***: P < 0.001. ****: P < 0.0001. N.s.: Not significant.

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

(a) Body weight development of 12 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n= 10/group). Statistic: ND vs. CD-HFD (black asterisks), ND vs. CD-HFD/Asp-Clo (green asterisks). (b) ALT (n≥ 7/group) of mice shown in (a). (c) IPGTT of 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n= 5/group). Statistic: ND vs. CD-HFD (black asterisks), ND vs. CD-HFD/Asp-Clo (green asterisks). (d) Liver triglyceride and (e) serum cholesterol levels of 6 and 12 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n≥ 7/group). (f) Real-time qPCR analysis for genes involved in lipid metabolism/β-oxidation of mice shown in (b) (n≥ 4/group). Statistic: CD-HFD vs. CD-HFD/Asp-Clo (green asterisks). (g) Analysis of VO2 and respiratory exchange ratio (RER) over time in 2 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n≥ 4/group). (h) Analysis of food (g/mouse/day) and water intake (ml/mouse/day) of mice shown in (g). (i) MRI analyses 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice. T1 (fast low-angle shot [FLASH]) OUT phase: dark colour indicative of steatosis. T2 TurboRARE: an increase in subcutaneous and abdominal fat and hepatic lipid accumulation (bright regions).
(j) H/E staining and (k) NAS evaluation of mice shown in (f) (n= 10/group), scale bar: 100µm in 10X, 50µm in 20X. All data are shown as mean ± SEM.*: P < 0.05. **: P < 0.01. ***: P < 0.001. ****: P < 0.0001. N.s.: Not significant.

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

(a) CD3, F4/80, MHCII and Ly-6G staining and quantification of 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n= 5/group), scale bar: 50µm. (b) (left) Representative FACS plots and quantification of hepatic CD4/CD8 ratio, (right) NKT cells and (c) activated CD8+ cells of 6 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice (n≥ 4/group). (d) Representative Western blot images of mice shown in (b). kDa: kilo Dalton. (e) Representative macroscopical images of livers from 12 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice. White arrowhead indicate HCC, scale bar: 7.5 mm. (f) HCC incidence of 12 months ND, CD-HFD or CD-HFD/Asp-Clo fed mice. (T=HCC; NT=non-tumor), (ND n= 17; CD-HFD n= 13/51; CD-HFD/Asp-Clo n= 0/20). (g) HCC characterization by H/E and Collagen IV (Col IV) of mice shown in (f), dashed line indicates tumor (T) border, scale bar: 2 mm (upper row H/E) and 200 µm (lower H/E; Col IV). All data are shown as mean ± SEM.*: P < 0.05. **: P < 0.01. ***: P < 0.001. ****: P < 0.0001. N.s.: Not significant.

Figure 4: Platelets efficiently invade 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), scale bar: 40 µm. (b) CD3 staining and quantification of 6 or (c) 8 weeks ND or CD-HFD fed mice (n= 4/group), scale bar: 50 µm. (d) Quantification of platelet area by intravital microscopy of mice shown in (a) (n≥ 2/group). (e) Analysis of liver sinusoid diameter by intravital microscopy of mice shown in (a) (n≥ 2/group). (f) Hepatocyte swelling measurement by H/E of mice shown in (b) and (c) (n= 4/group). (g) NAS evaluation of 6 or 8 weeks ND or CD-HFD fed mice (n≥ 4/group). (h) Liver triglycerides of 6 weeks ND or CD-HFD fed mice (n≥ 4/group). (i) 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. (j) (left) 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), scale bar: 43 µm. (right) 3D confocal images and quantification of platelet (green)/Kupffer cells (red) interaction of 6 months ND or CD-HFD fed mice (n= 4/group). Liver
endothelium (grey), scale bar: 20 µm. All data are shown as mean ± SEM.*: P < 0.05. **: P < 0.01. ***: P < 0.001. ****: P < 0.0001. N.s.: Not significant.

**Figure 5: Intrahepatic platelet accumulation depends on Kupffer cells, hyaluronan and cargo function.**

(a) Representative images of intravital microscopy after 2.5 weeks treatment (Clodronate liposomes (CLL) or hyaluronidase (HYAL)) in 6 weeks ND, CD-HFD, CD-HFD +CLL or CD-HFD +HYAL fed mice. Analysis of Kupffer cells (violet), platelets (blue, blue arrowhead), and granulocytes (red), scale bar: 40 µm. (b) H/E and F4/80 staining with quantification and NAS evaluation after 2.5 weeks treatment in 6 weeks ND, CD-HFD, CD-HFD +CLL or CD-HFD +HYAL fed mice (n≥ 7/group), scale bar: 50 µm. (c) Quantification of platelet area by intravital microscopy of mice shown in (a) (n= 4/group). (d) ALT levels of mice shown in (b) (n≥ 7/group). (e) NAS evaluation by H/E staining after 2.5 weeks anti-CD44 antibody treatment (anti-CD44 antibody blocking- (KM81) or non-blocking (IM7) HA-binding site) in 6 weeks ND, CD-HFD, CD-HFD +IM7 (non-HA blocking) or CD-HFD +KM81 (HA-blocking) fed mice (n≥ 4/group), scale bar: 50 µm. (f) ALT levels of mice shown in (e) (n≥ 4/group). (g) Representative H/E, CD42b staining and (h) NAS evaluation after 2.5 weeks CLL treatment in 6 months CD-HFD or CD-HFD +CLL fed mice (n≥ 3/group). (i) Body weight development of 6 months ND, CD-HFD or CD-HFD/Nbeal2−/− fed mice (n≥ 4/group). Statistic: ND vs. CD-HFD (black asterisks), CD-HFD vs. CD-HFD/Nbeal2−/− (blue asterisks). (j) ALT, AST levels, (k) Liver triglycerides and. (l) serum cholesterol levels of mice shown in (i) (n≥ 4/group). (m) Representative H/E of mice shown in (i), damaged hepatocytes (asterisks) are indicated, scale bar: 50µm. (n) Fat quantification by Sudan red staining of mice shown in (i) (n≥ 4/group), scale bar: 100 µm. All data are shown as mean ± SEM.*: P < 0.05. **: P < 0.01. ***: P < 0.001. ****: P < 0.0001. N.s.: Not significant.

**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. 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 (n= 4/group), 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) (n≥ 5/group). (i) Serum cholesterol, liver triglycerides and (j) ALT levels of 6 months ND, CD-HFD or CD-HFD/hIL4ra/GP1ba-Tg fed mice (n≥ 4/group). (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) (n≥ 4/group). (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, (p) fibrosis quantification and (q) Sirius red staining of 6 months ND, CD-HFD or CD-HFD/hIL4ra/GP1ba-Tg fed mice (n≥ 4/group). (r) ALT levels of 12 months ND, CD-HFD or CD-HFD/hIL4ra/GP1ba-Tg fed mice (n≥ 10/group). (s) Macroscopical images of tumors of mice shown in (r), tumor nodules are indicated by arrowhead), scale bar: 750 mm. (t) HCC characterization by CD44v6, Collagen IV (Coll IV) and Ki67 staining form mice shown in (r). Arrowheads indicate positive hepatocytes, dashed line indicates tumor (T) border, scale bar: 200 µm (CD44v6 and Coll IV), 50 µm (Ki67). (u) HCC incidence (T=HCC; NT= non-tumor) from 12 months CD-HFD or CD-HFD/hIL4ra/GP1ba-Tg fed mice, (CD-HFD: n=13/51; CD-HFD/hIL4ra/GP1ba-Tg: n=0/24). All data are shown as mean ± SEM.*: P < 0.05. **: P < 0.01. ***: P < 0.001. ****: P < 0.0001. N.s.: Not significant.
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