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*Abcb4<sup>−/−</sup>*-mouse model for cholestasis

- CB1 antagonism
- Rimonabant p.o.

- Bile acids
- c-JUN
- SREBP1

- Hepatocellular damage ↓
- Inflammation ↓
- Physiologic zonation ↑
Pharmacological antagonization of cannabinoid receptor 1 improves cholestasis in Abcb4−/− mice

Short title: Rimonabant improves cholestasis

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Abbreviations: Abcb4 ATP-binding cassette 4, ACEA arachidonyl-2’-chloroethylamide, AKT proteinkinase B, ALT alanine aminotransferase, AP-1 activator protein 1, AST aspartate aminotransferase, BA bile acid, CB1 cannabinoid receptor 1, Ccnd1 cyclin D1, CD45 cluster of differentiation antigen 45, CK19 cytokeratin 19, CPT1 carnitine palmitoyltransferase I, ECM extracellular matrix, Fasn fatty acid synthase, Fxr farnesoid X receptor, GAPDH glycerinaldehyde-3-phosphat-dehydrogenase, HCC hepatocellular carcinoma, HSC hepatic stellate cell, IkB inhibitor of κB, IL interleukin, Jnk c-Jun N-terminal kinase, LCN2 lipocalin 2, Mcp-1 monocyte chemoattractant protein-1, MMP matrix metalloproteinase, NF-κB nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells, PCK1 phosphoenolpyruvat carboxykinase, Ppar peroxisome proliferator activated receptor, qRT-PCR quantitative real-time polymerase chain reaction, Srebp-1 sterol regulatory element-binding protein 1, STAT3 signal transducer and activator of transcription 3, T-β-MCA, tauro-β-muricholic acid, TIMP tissue inhibitor of metalloproteinase, TNF-α tumor necrosis factor-α, WT wild type.

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Synopsis: Antagonization of endocannabinoid receptor 1 (CB1) by rimonabant ameliorated metabolic, inflammatory, and carcinogenesis-associated processes as well as serum bile acids in Abcb4-knockout mice. This leads to an improvement of cholestasis and reduced hepatic pathogenesis.
Abstract

Background & Aims: The endocannabinoid system is involved in the modulation of inflammatory, fibrotic, metabolic, and carcinogenesis-associated signaling pathways via the cannabinoid receptors 1 and 2 (CB1 and CB2). We hypothesized that the pharmacological antagonization of CB1 receptor improves cholestasis in Abcb4−/− mice.

Methods: After weaning, male Abcb4−/− mice were treated orally with rimonabant (an specific antagonist of CB1) or ACEA (an agonist of CB1) for up to 16 weeks of age. Liver tissue and serum were isolated and examined by means of serum analysis, qRT-PCR, western blot, immunohistochemistry, and enzyme function. Untreated Abcb4−/− and BALB/c wild type mice served as controls.

Results: Cholestasis induced symptoms such as liver damage, bile duct proliferation, and enhanced circulating bile acids were improved by CB1 antagonization. Rimonabant treatment also improved PEPCK expression, reduced inflammation and the acute phase response. The carcinogenesis-associated JNK/c-JUN and STAT3 signaling pathways activated in Abcb4−/− mice were reduced to wild type level by CB1 antagonization.

Conclusions: We demonstrated a protective effect of oral CB1 antagonization in chronic cholestasis using the established Abcb4−/− model. Our results suggest that a pharmacologic antagonization of the CB1 receptor could have a therapeutic benefit in cholestasis associated metabolic changes, liver damage, inflammation, and carcinogenesis.

Keywords: liver, rimonabant, bile acid, acute phase, fibrosis
Every year, more than 1.2 million people die from complications of cirrhosis which currently is the 11th most common cause of death worldwide. This number demonstrates the need to establish new and effective therapy options. After removing the cause, the damaged liver is usually able to restore its function, even in the presence of advanced cirrhosis. Up to now, however, there is still no effective pharmacological anti-fibrotic therapy in advanced liver injury resulting from chronic cholestasis such as sclerosing cholangitis.

A large number of studies have demonstrated that the endocannabinoid system is a significant mediator of acute and chronic liver disease. The function of the two cannabinoid receptors CB1 and CB2 has been a subject for scientific research for many years. In addition to their metabolic effects, new insights into their role in the development of liver inflammation and fibrosis in chronic liver damage are of great interest.

Teixeira-Clerc et al. were able to show that the CB2 had anti-inflammatory and antifibrotic effects in the early stages of liver damage. This protective influence was eliminated by the profibrotic effects of the activated CB1 if liver damage persists. In chronically damaged liver tissue, CB1 is strongly expressed and stimulated by increased release of endocannabinoids by hepatocytes, HSC, and Kupffer cells. It shows its strongest expression in non-parenchymal cells such as inflammatory cells, proliferating cholangiocytes, hepatic stellate cells and portal myofibroblasts.

Rimonabant, also called SR141716A, belongs to the active ingredient class of anorectics and is a selective CB1 antagonist. The CB1 blockade in the diseased liver showed positive effects on the development of steatohepatitis, fibrosis, and the metabolic syndrome in several animal models.

Recently we demonstrated that a global knockout of the Cb1 gene (Cb1−/−) reduced the expression of the lipid droplet binding protein PLIN2 in the livers of Cb1−/− and hepatitis
B surface protein (HBs)-transgenic mice, which spontaneously develop hepatic steatosis. In addition, the antagonization of CB1 in human cell culture also caused a reduction of PLIN2, a cytoplasmic lipid droplet binding protein involved in the storage of neutral lipids within the lipid droplets. The CB1 receptor is downregulated during cholestasis. Anandamide (AEA) a partial CB1 agonist suppresses cholangiocyte growth in BDL mice by induction of cholangiocyte apoptosis.

Abcb4−/− mice represent a well-characterized model for sclerosing cholangitis beginning with persistent cholestasis that progresses to cirrhosis and liver failure before late childhood. Although knowing the basic genetic defects and the pathology of the disease, being characterized by ductular proliferation in the liver and progressive intrahepatic cholestasis, there is still no successful therapeutic approach.

In our present work, the effect of CB1 antagonization on cholestasis and its consequential damage was examined. For this purpose, Abcb4−/− mice were treated with the selective CB1 antagonist rimonabant. Our hypothesis was that the disease progression and consequences of cholestasis can be delayed by CB1 antagonization. Metabolic parameters and specific markers for inflammation, fibrogenesis, and carcinogenesis were examined. Untreated Abcb4−/− mice and mice fed with the CB1 agonist arachidonyl-2-chloroethylamide (ACEA) were included as controls.
Results

Cholestatic liver disease

The sterol regulatory element-binding protein-1 (SREBP1) plays a crucial role in the regulation of cholesterol metabolism and the fatty acid synthesis. SREBP1 is a downstream effector of CB1, thus contributing to the development of obesity and fatty liver via lipogenesis\textsuperscript{11}. It has been shown that cholestasis was associated with reduced mRNA expression of Srebp1 and diminished lipogenesis in Abcb4\textsuperscript{-/-}-mice\textsuperscript{12,13}. Here, we isolated nuclear proteins and analyzed the nuclear amount of the matured transcription factor nSREBP1. Interestingly, we found enhanced amounts of matured nuclear SREBP1 protein in Abcb4\textsuperscript{-/-}-mice compared to WT mice while neither rimonabant, nor ACEA altered nuclear SREBP1 significantly (Fig. 1A).

In the course of slowly progressing cholestasis an increase in connective tissue remodeling was observed in the liver (black arrows) of untreated Abcb4\textsuperscript{-/-} mice, most evident in the periportal fields (Fig. 1B, upper right). The liver structure and the remodelling of connective tissue in mice treated with rimonabant (Fig. 1B, lower right) was comparable to that of WT mice (Fig. 1B, upper left). Remarkably, pathological changes in the portal fields of the Abcb4\textsuperscript{-/-} mice were also reduced by the treatment with ACEA (Fig. 1B, lower left). Although histological grading suggested an improved scoring in rimonabant treated animals, statistical significance was not reached.

In the course of hepatocellular damage, accompanying cholestatic disease ALT (Fig. 1C) and AST (Fig. 1D) serum values increase. Serum levels of ALT and AST were reduced by the treatment with both, rimonabant and ACEA. There were no differences in the serum ALT and AST levels between rimonabant and ACEA treated mice (Fig. 1C and D).
CK19 immunostaining (black arrows) visualized a higher number of periportal bile ducts in Abcb4−/− mice and thus showed the highest level of bile duct proliferation in untreated Abcb4−/− mice (Fig. 1E upper right). Rimonabant treatment and - in a lesser degree - ACEA treatment resulted in reduced bile duct proliferation (Fig. 1E and Fig. 1F).

**Figure 1**

In order to determine the degree of cholestasis quantitatively, serum bile acids were analyzed. Cholestasis, particularly due to the Abcb4−/−, leads to a loss of the barrier function of bile ducts caused by changes in the tight junctions. This consequently leads to an increase of serum bile acids (Fig. 2A). ACEA and rimonabant tendentially reduced serum bile acid concentrations.

The bile acids transferred into the serum as part of the cholestatic disease showed a clear dominance of the taurine-conjugated bile acids T-ω-MCA and T-β-MCA in the serum of the Abcb4−/− mice. This predominance of taurine conjugates is typical in mice (Fig. 2B). Interestingly, hepatic bile acid content as well as the hepatic expression of Fxr was not significantly altered, neither in Abcb4−/− mice nor by treatment with ACEA or rimonabant (Fig. 2C and D).

**Figure 2**

**Hepatic metabolism**

Cytosolic phosphoenolpyruvate carboxykinase (PCK1) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, the rate-determining step of gluconeogenesis.
In the healthy liver, gluconeogenesis mainly takes place in periportal hepatocytes, where a higher expression of PCK1 positive cells can be displayed. A pathological disturbance of the metabolic zonation in Abcb4^{-/-} mice was detected by immunohistochemical PCK1 staining. The liver tissue of the untreated Abcb4^{-/-} mice showed an impaired structure (arrows) and zonation (arrowheads) (Fig. 3A). The treatment with rimonabant largely preserved a clear zonation, similar to the WT controls. Intriguingly, the treatment with ACEA also preserved zonation to a large extent, which might indicate additional physiologic relevance apart from CB1 agonization. The serum glucose levels remained unchanged in all groups (data not shown).

PPARα is involved in the regulation of lipid catabolism and glucose homeostasis. Gene expression of important regulators of the glucose metabolism, such as the peroxysome proliferator-activated receptors alpha (Ppara), Cpt1 and Pck1, were examined by qRT-PCR at mRNA level (Fig. 3B+C).

Ppara- but not Cpt1 gene expression showed a tendential downregulation in untreated Abcb4^{-/-} mice compared to the WT (p = 0.082). Rimonabant treatment normalized Ppara gene expression (p = 0.023) but had no effect on Cpt1. ACEA treatment had no effect on Ppara (Fig. 3B). Although Cpt1 was not altered in Abcb4^{-/-} mice we found reduced expression in ACEA-treated Abcb4^{-/-} mice in comparison to wt mice (Fig. 3B). In summary, zonation of PCK1 and gene expression of Ppara were normalized by rimonabant treatment. Serum glucose levels were largely unaffected by these changes. However, albeit zonal perturbation, the total amount of hepatic Pck1 mRNA did not change (Fig. 3C).

Figure 3
Since glucose and lipid metabolism are closely linked, the gene expression of fatty acid synthetase (Fasn) and the transcription factor peroxysome proliferator-activated receptor gamma (Pparγ) were examined, both having regulatory functions in lipid metabolism. The hepatic amount of Fasn mRNA was downregulated in untreated Abcb4−/− mice. Rimonabant treatment tendentially increased Fasn, while ACEA treatment normalized Fasn gene expression to WT levels (Fig. 3D). The gene expression of Pparγ was not altered in Abcb4−/− mice, compared to WT. Rimonabant treatment led to an upregulation, compared to untreated Abcb4−/− mice (Fig. 3D).

Immunohistochemical staining of FASN also demonstrated a disturbed zonation (arrowheads) and a preserved distribution of hepatocellular FASN protein expression after ACEA and rimonabant treatment (Fig. 3E).

In summary, a pathological remodeling of the liver was found in the untreated Abcb4−/− group with dissolved zonation and lobular structure. Analogous to the liver section of the WT, the rimonabant-treated (and also ACEA-treated) group showed a clear zonation of the liver. Normal liver structure and enzyme values at WT level were found in both treatment groups.

**Hepatic inflammation**

Former studies demonstrated that the alterations in lipid metabolism mediate inflammation, fibrosis, and proliferation in Abcb4−/−-mice. Immunostaining revealed an infiltration of a higher number of CD45 positive leukocytes in untreated-and ACEA-treated Abcb4−/− mice (Fig. 4A, B). Remarkably, the treatment with rimonabant reduced this cholestasis-associated infiltration of inflammatory cells. Histopathological
examination also suggested a lower score of the hepatic inflammation in the rimonabant-treated group, but statistical significance was not reached. Likewise, the H&E staining might indicate a moderate infiltration of inflammatory cells in this group (Fig. 1B).

Lipocalin-2 (LCN2 or NGAL), an important component of the acute phase reaction, is increasingly expressed in neutrophil granulocytes infiltrating the tissue, but also in hepatocytes activated by proinflammatory stimuli. Although neither, hepatic mRNA levels nor protein expression of Lcn2/LCN2 was significantly altered, an increased infiltration of LCN2-positive cells as a characteristic feature of the biliary inflammatory process was shown by immunohistochemistry in the untreated Abcb4−/− group (Fig. 4C-F). Interestingly Lcn2 was increased in the ACEA group in comparison to WT mice but normalized to wt levels in rimonabant treated mice (Fig. 4C). The same effects were observed for the infiltration of LCN2-positive cells (Fig. 4F). Moreover, rimonabant reduced the number of infiltrating LCN2-positive cells significantly (Fig. 4F).

Figure 4

Monocyte chemotactic protein 1 (Mcp-1), an inflammatory cytokine, recruits monocytes, T cells and dendritic cells to the site of inflammation. Untreated Abcb4−/− mice showed an upregulation of Mcp-1. Rimonabant reduced Mcp-1 to WT level (Fig. 5A). Tumor necrosis factor-α (TNF-α) has various functions in liver disease, including attraction and activation of inflammatory cells as well as mediation of hepatotoxicity and regeneration. Tnf-α was induced in untreated Abcb4−/− mice and reduced to WT level by rimonabant, while ACEA had no effect (Fig. 5B).
Collagen-1 is the main component of fibrotic tissue. Sirius Red staining indicated pronounced fibrosis in untreated $Abcb4^{-/-}$ mice with deposition of fibrillar collagen around the portal fields and around proliferating bile ducts (Fig. 5A). Histologically, rimonabant-treated mice showed a lower collagen deposition - approximately at WT level - compared to untreated $Abcb4^{-/-}$ mice. Also ACEA-treated mice showed an increased hepatic collagen deposition, however, below the level of collagen expression of untreated $Abcb4^{-/-}$ mice (Fig. 5C).

To analyze the locoregional correlation of collagen-1 and proliferating bile ducts, a collagen-1/CK19-co-staining was carried out (Fig. 5D). We observed a spatial coherence of proliferating bile ducts and collagen-1 deposits in the untreated $Abcb4^{-/-}$ group. $Abcb4^{-/-}$ mice showed a strong deposition of collagen-1 and increased proliferation of bile ducts (arrowheads). Moderate bile duct proliferation and collagen-1 deposition was observed in the rimonabant group, comparable to the WT. Bile duct proliferation and collagen-1 deposition in ACEA-treated mice were similar to untreated $Abcb4^{-/-}$ mice. Please note that CK19 positive cells, that do not form bile ducts, appear in the periphery of portal tracts in $Abcb4^{-/-}$ and ACEA mice (arrows). Nevertheless, it actually remains speculative if these cells indicate the initiation of newly forming bile ducts or other associated processes (Fig. 5D).

The cholestasis-dependent increase of hepatic hydroxyproline content in $Abcb4^{-/-}$ mice appeared slightly reduced by ACEA and rimonabant treatment but did not reach statistical significance (Fig. 5E).
Fibrosis-associated genes such as *Timp1* and *Mmp2* were subjected to qRT-PCR analysis.

**Figure 4**

In order to analyze the proteolytic potential of extracellular matrix (ECM) the gene expression of *Mmp2* was analyzed by qRT-PCR. Treatment with rimonabant induced *Mmp-2* gene expression in comparison to WT and untreated *Abcb4/-/-* mice while *Timp-1* mRNA levels were similar among all groups (Fig. 5F).

Taken together, fibrosis was clearly induced in *Abcb4/-/-* mice whereas fibrogenesis did not appear with significant lower extent in rimonabant-treated mice. Nevertheless histopathologic assessment of fibrosis was improved after rimonabant-treatment. Interestingly, we observed a pronounced proteolytic potential indicated by *Mmp-2* induction in rimonabant-treated mice.

**Malignancy-associated signaling and proliferation**

c-JUN and STAT3 are critical regulators of liver cancer development and progression\(^{14,15}\). Both pathways are also involved in cholestasis-associated carcinogenesis\(^{16}\). In order to analyze the activation of the JNK/c-JUN signaling pathway, the phosphorylation of c-JUN was examined by western blotting in comparison to the expression of its unphosphorylated form (Fig. 5A).
c-JUN was activated in liver tissue of Abcb4<sup>−/−</sup> mice while rimonabant treatment resulted in a reduced phosphorylation of c-JUN (Fig. 5A), and a reduced hepatocellular nuclear translocation (Fig. 5B). STAT3 phosphorylation was not significantly altered between the mouse groups (Fig. 5C). According to the regulatory role of c-JUN in cell cycle<sup>17,18</sup>, *Cyclin D1* expression was reduced to WT levels by treatment with rimonabant (Figure 5D).

In summary, our results show that c-JUN was activated by chronic cholestatic liver damage in Abcb4 knockout mice. The treatment with rimonabant reduced this activation to WT levels, while ACEA had no significant influence. Furthermore, c-JUN-associated proliferation, here shown by *Ccnd1* expression, followed the same trend.


Discussion

The common final outcome of chronic liver diseases is the development of liver inflammation, fibrosis, and cirrhosis\textsuperscript{19}. Every year an estimated 1,200,000 people worldwide die from its complications, including portal hypertension and hepatocellular carcinoma, thus demonstrating the need to establish new and effective treatment options for chronic liver diseases\textsuperscript{20}. The removal of the cause of liver injury can lead to regeneration of the damaged liver even with advanced cirrhosis\textsuperscript{21}. If this is not possible, there is still no effective antifibrotic or anticirrhotic therapy available to date.

Our present work has shown that the administration of the CB1 antagonist rimonabant in \textit{Abcb4\textsuperscript{-/-}} mice

1. reduced damage, inflammation, and histopathological fibrosis of the liver,

2. maintained liver integrity and zonation, and

3. reduced the activation of carcinogenesis-associated signaling pathways.

Thus, modulating the liver endocannabinoid system might be a potential therapeutic option to treat liver injury associated with cholestasis.

In different mouse models like \textit{CCl4\textsuperscript{-}}, thioacetamide\textsuperscript{-}, and bile duct ligation\textsuperscript{-} induced fibrosis, in resected human cirrhotic livers, and in cell cultures of human hepatic stellate cells, and hepatic myofibroblasts, the stimulation of CB1 induces profibrotic effects, while the stimulation of CB2 results in the opposite outcome\textsuperscript{2,22,23}. In diet\textsuperscript{-} induced obese mice, rimonabant had positive effects on liver metabolism and induced a reduction of liver fibrosis\textsuperscript{2,6,24,25}.

Here, the influence of a pharmacological modulation of CB1 by the CB1 antagonist rimonabant and ACEA (a CB1 agonist) in \textit{Abcb4\textsuperscript{-/-}} mice on BALB/c genetic background\textsuperscript{26} was examined for the first time. Upregulation of SREBP-1c and fatty acid synthetase (\textit{Fasn}) in the signal cascade of activated CB1 contributes to the
development of obesity and fatty liver via increased lipogenesis\textsuperscript{11}. In bile duct ligated mice the CB1 receptor is downregulated during cholestasis\textsuperscript{8}. Accordingly, in \textit{Abcb4}\textsuperscript{-/-} mice lipogenesis is reduced during cholestasis\textsuperscript{13} and these alterations in lipid metabolism mediate inflammation, fibrosis, and proliferation\textsuperscript{12}. In our study, we demonstrated an increase of nuclear matured SREBP-1 in untreated \textit{Abcb4}\textsuperscript{-/-} mice. Neither ACEA- nor rimonabant-treated \textit{Abcb4}\textsuperscript{-/-}-mice displayed significant alterations of nuclear SREPB-1.

**Damage caused by cholestasis**

In our study, increased ALT and AST values in untreated \textit{Abcb4}\textsuperscript{-/-} mice demonstrated increased liver cell damage, which did not occur in rimonabant-treated (and ACEA-treated) mice. The ALT values were significantly reduced under rimonabant, but still above the reference values of healthy WT mice (50 U/l)\textsuperscript{27}. An incomplete ALT normalization could be attributed to the blood concentration of rimonabant being too low due to the oral dosage form, an incomplete absorption in the intestine or any further metabolization\textsuperscript{23,28}. Nevertheless, an optimized pharmacological antagonization of CB1 might be a promising target to handle liver damage in cholestasis.

ALT reduction in the ACEA group was interesting, since in analogy to other models of chronic liver diseases, the values were expected to worsen\textsuperscript{29}. Repeated administration of CB1 agonists, however, could induce CB1 internalization or a reduction of CB1 protein synthesis\textsuperscript{30}. Coupling of ACEA to other receptors with protective effects on liver damage was unlikely for a long time due to its previously assumed high specificity for CB1. However, it could be shown that ACEA acts as an agonist of the transient receptor potential vanilloid 1 (TRPV1)\textsuperscript{31}. Activation of TRPV1 by capsaicin e.g. led to a lower lipid droplet formation in the liver of highfat diet (HD)-fed mice\textsuperscript{32}. Taking this into account, we speculate that ACEA administration may have a protective effect on
some cholestasis-associated liver changes via the agonism at TRPV1, which could explain some of the coherent effects by rimonabant and ACEA that we describe with the current study.

Due to an increased permeability of bile duct epithelium, increased bile acid concentrations can be measured in the blood circulation of Abcb4\(^{-/-}\) mice\(^{33}\). The conjugation of bile acids with amino acids such as taurine or glycine increases their detergent properties and prevents their precipitation in an acidic environment. It is known that the bile acids in rodents are 80-90% taurine-conjugated\(^{34,35}\). Lower concentration levels of total bile acids in the serum were measured after ACEA and rimonabant treatment compared to the untreated group. Missing statistical significance might be due to the limited group size and high number of different bile acids measured. Interestingly, ACEA-treated mice reached lower levels than rimonabant-treated mice with regard to inflammation and bile acids concentration. In the analysis of bile acids in the serum, the dominance of taurine-conjugated, in particular, the tauro-\(\beta\)-muricholic acid, tauro-\(\alpha\)-muricholic acid and tauro-\(\omega\)-muricholic acid, was confirmed in all samples\(^9\).

The reduced parenchymal damage (Fig. 1B and C) indicates that the agonization of the CB1 receptor might also have hepatoprotective effects in Abcb4\(^{-/-}\) mice. As described above, this may be due to further interactions of ACEA on hepatic receptors. However, the protective effect of rimonabant in Abcb4\(^{-/-}\) mice is congruent with other models of chronic liver damage such as CCl\(_4\)-induced, thioacetamide-induced, and bile duct ligation-induced liver fibrosis\(^2\).

**CB1 in view of hepatic glucose and lipid metabolism**
In the context of cholestatic diseases, the liver parenchyma and thus the liver architecture and the sophisticated zonation is disrupted. In the present work, an abolished zonation in untreated *Abcb4*<sup>−/−</sup> mice as well as in ACEA-treated *Abcb4*<sup>−/−</sup> mice could be shown by immunohistochemical staining for PCK1 and FASN (Fig. 3 A and E). Physiologic zonation was regained by treatment with rimonabant.

The endocannabinoid system contributes to the development of steatosis, dyslipidemia, and insulin resistance<sup>36</sup>. PCK1, the rate-determining enzyme of gluconeogenesis, did not show a clear staining in the periportal zone, but a diffuse distribution of PCK1-positive hepatocytes<sup>37</sup>. Ghafroory et al. showed that the CCl<sub>4</sub>-induced liver damage led to a strong change in the gene expression for enzymes of glucose metabolism in the periportal and perivenous zones<sup>38</sup>. The typical zonal expression of PCK1 was largely preserved through rimonabant treatment (Fig. 3A), which also reflects serious protective effects of rimonabant on metabolism. *Pck1*’s gene expression levels did not show any differences between the groups (Fig. 2C) and serum glucose concentrations were within the normal range in all groups. Thus, euglycaemia might probably be attributed to a compensated metabolism in the stage of liver fibrosis, but not yet cirrhosis or decompensation.

Various models have shown that hepatic CB1 activation increases de novo lipogenesis by activating *Srebp1c* and *Fasn* and at the same time reduces fatty acid oxidation<sup>11,39</sup>. Analogous to a previous study<sup>11</sup>, untreated *Abcb4*<sup>−/−</sup> mice showed a downregulation of *Fasn* expression in our study. The normalization of *Fasn* expression by rimonabant was associated with a normalization of metabolic processes. IHC for FASN indicated a diffuse distribution of FASN positive hepatocytes in untreated *Abcb4*<sup>−/−</sup> mice and a normalization of the typical zonation in rimonabant-treated mice.

Recently, we demonstrated that the CB1 knockout *in vivo* and pharmacologic antagonization of CB1 in cell culture decreased PLIN2 expression, which might be an
essential step in lipid breakdown\textsuperscript{7}. In this study, we show that the pharmacologic modulation of CB1 represents a novel therapeutic approach for the treatment of cholestatic liver injury.

**Regulation by peroxysome proliferator-activated receptors**

In the liver, a protective effect of PPAR\(\alpha\) on the development of NASH and inflammation was demonstrated by the downregulation of NF\(\kappa\)B, AP-1, STATs and IL-6\textsuperscript{40}. In the present work, \(Ppara\) was tendentially reduced in untreated \(Abcb4^{-/-}\) mice compared to WT. In the rimonabant group, normalization of \(Ppara\) to WT level was observed, while \(Ppara\) expression remained unchanged with ACEA treatment (Fig. 3B). Since there were no differences in the serum glucose concentrations, the cholestatically reduced \(Ppara\) expression appears to have no effect on stable hepatic glucose metabolism. Among others, PPAR\(\gamma\) regulates the differentiation of adipocytes and contributes to lipid accumulation in the liver. These effects are moderated by induction of SREBP-1c, ACC and FASN\textsuperscript{41}. Interestingly, \(Ppar\gamma\) expression was significantly induced in the rimonabant group. The ACEA group however, showed no differences compared to the untreated \(Abcb4^{-/-}\) mice.

**Hepatic inflammation**

The enhanced number of infiltrated CD45\(^+\) leukocytes in \(Abcb4^{-/-}\) mice was abolished by rimonabant, which indicates an anti-inflammatory effect of pharmacologic CB1 antagonization during cholestasis. Upregulation of lipocalin 2 during ER stress-induced inflammatory responses protects hepatocytes from being overwhelmed by unfolded protein response upon liver injury\textsuperscript{42}. On the other side LCN2 is secreted into the serum from liver cancer tissue in humans and mice\textsuperscript{43}. \(Lcn2\) was increasingly expressed in the ACEA group, whereas the
rimonabant group showed normalized Lcn2 expression on WT level. A clinical study of 716 patients with cirrhosis revealed that LCN2 might be a biomarker of acute-on-chronic liver failure and prognosis in cirrhosis\textsuperscript{44,45}. Since LCN-2 is a good candidate for HCC diagnosis and screening, the reduction of Lcn-2 might indicate a beneficial development. Interestingly, the untreated group and the ACEA group showed neither upregulation of IL-6, NF-κB, I-κB, AKT nor other mediators inducing the aforementioned LCN2 (data not shown). It must be assumed that LCN2 is activated by the damaged hepatocytes via alternative routes. The measurement of pro-inflammatory markers such as LCN2, TNF-α, and MCP-1 demonstrates the anti-inflammatory effect of rimonabant, which correlated with reduced liver damage in our murine model.

**Fibrosis**

As expected, untreated Abcb4\textsuperscript{-/-} mice exhibited an increased overall hepatic collagen deposition, which was not altered by treatment with ACEA or rimonabant. Nevertheless histopathological assessment of fibrosis was improved after rimonabant treatment. The net deposition of scar tissue depends on the balance between synthesis and degradation\textsuperscript{3,46}. The latter reflecting the relative activity of MMPs and their inhibitors TIMPs, which are primarily produced by HSC. The activation of MMPs leads to the dissolution of the deposition of extracellular matrix (ECM). The activity of the MMPs thus leads to fibrosis regression\textsuperscript{46,47}. On gene expression levels, the treatment with rimonabant led to an upregulation of the proteolytic potential, enabling enhanced degradation of ECM in the fibrotic liver. This fact may be reflected by the moderate level of fibrosis in rimonabant treated animals.

**Signal transduction**
In acute and chronic liver damage, JNK1 and its downstream signals are activated and contribute to disease progression\textsuperscript{48}. In the present work, western blot analysis of untreated \textit{Abcb4}\textsuperscript{-/-} mice showed an increased activation of c-JUN. The hepatoprotective effect of rimonabant treatment was reflected in a lower activation c-JUN, which was normalized to WT level. In 2001 Gupta et al. could already show a direct activation of JNK1 by taurine-conjugated bile acids in a model of rat hepatocytes\textsuperscript{49}. p-c-JUN, \textit{Cyclin D1} were activated in untreated \textit{Abcb4}\textsuperscript{-/-} mice (Fig. 6). Rimonabant reduced this activation to WT level. Treatment with ACEA had no effect.

**Limitations**

A limitation of the study is the way how medication was administered via the drinking water, which means that different concentrations of active substances cannot be ruled out on an individual basis. Both, the amount of substances absorbed via the intestine and the further metabolization remain unknown factors. Alternatives such as intravenous or intra-abdominal injection did not appear physiological enough. In addition, these procedures, however, might be technically difficult, associated with animal stress, and time-consuming.

In summary, the CB1 blockade in \textit{Abcb4}\textsuperscript{-/-} mice by rimonabant enabled a reduction of liver damage as well as inflammatory and acute phase markers such as LCN2, \textit{Mcp1}, and \textit{Tnf-\alpha}. With regard to liver metabolism, treatment with rimonabant resulted in the preservation of the typical liver zonation. Proliferation and carcinogenesis-associated signaling pathways were normalized to WT levels by treatment with rimonabant. We thus conclude that the regulation of JNK signaling pathway by CB1 modulation might play an important role in cholestatic liver diseases and if applicable in hepatic carcinogenesis.
Acknowledgement: The authors thank Annette Tschuschner and Heike Müller for excellent technical assistance.
**Material & Methods**

**Animal treatment**

The present study was performed with permission of the State of Hesse, Regierungspraesidium Giessen, according to section 8 of the German Law for the protection of animals and conforms to the NIH guide for the care and use of laboratory animals. All experiments were approved by the committee on the ethics of animal experiments of the Regierungspraesidium Giessen, Germany (permit number: V54-19c2015 (1) G II 20 / 10 No. 52/2011). BALB/c-Abcb4^{-/-} mice (C.FVB(129P2)-Abcb4^{-/-} mice) were bred and housed as described previously. Characterization of Abcb4^{-/-} genotype, sample collection, and routine analysis have been described elsewhere. In this project, the population of male Abcb4^{-/-} knockout mice was divided into three groups. One group (n = 4) remained untreated and received standard chow. The second group (n = 4) was treated with 1 mg/kg body weight/day ACEA after weaning from the mother in the third week of life. A third group (n = 5) was fed with 1 mg/kg body weight/day rimonabant. Standard chow (R/M-H) supplemented with ACEA and rimonabant was obtained from Sniff (Spezialdiäten GmbH, 59494 Soest, Germany). 20 kg chow was charged with 133 mg rimonabant or 125 mg ACEA, respectively.

Mice were sacrificed at the age of 16 weeks. Livers and blood sera were isolated and stored at -80°C. 16 weeks old wild-type mice (WT) were used as healthy supercontrols.

**Histology and Immunohistochemistry**

Preparation of 3 µm paraffin sections, H&E staining, Sirius red staining, immunohistochemistry, microphotography, and scoring was performed as described before. The following specific primary antibodies were used for immunohistochemistry: CK19 Abcam #ab15463-1, PCK1 home-made (BC) antibody.
was raised in rabbits using a synthetic peptide comprising amino acids 385-399 of the cytosolic form of phosphoenolpyruvate carboxykinase, FASN CST #3189, CD45 CST 70257, LCN2 Santa Cruz #sc-80234, type I collagen SantaCruz #sc-33111, pc-Jun Cell Signaling #3270. Unspecific isotype IgGs were used for control.

Bile Acid Analysis
Bile acids were quantified by Ultra performance liquid chromatography-Tandem mass spectrometry (UPLC-MS/MS) as has been described in literature51.

Hydroxyproline Assay
Total hepatic hydroxyproline content was quantified as described previously with minor modifications52,53. Briefly, 50mg mouse liver tissue was hydrolyzed in 1ml 6 N HCl at 110°C for 14 h. Hydrolysates were filtered through 45 µm pore filters (Sartorius, Göttingen, Germany). 15 µl of the hydrolysate was dried under nitrogen flow and subsequently redissolved in 50 µl 50% 2-propanol. 100 µl of the 0.6 % chloramine-T (Merck, Germany) solution was added to the samples and hydroxyproline standard probes (4-hydroxy-L-proline, Sigma-Aldrich, Taufkirchen, Germany) and incubated for 10 min at room temperature. 100 µl of Ehrlich’s solution (3 g dimethylamino-benzaldehyde (Sigma-Aldrich, Taufkirchen, Germany) in 26 ml 2-propanol + 8 ml 70% perchloric acid) was added and the samples were again incubated for 45 min at 50°C. Absorbance was measured at 570 nm using a microplate reader (Packard BioScience, Meriden, CT, USA). Hydroxyproline levels were calculated against standard curves of and expressed as mg hydroxyproline per gram liver tissue.

Quantitative Real-Time-PCR
RNA extraction and cDNA synthesis as well as qRT-PCR were performed as described before\textsuperscript{50}. Briefly, hepatic RNA was extracted using the RNeasy Mini- (QIAGEN, Hilden, Germany) and elimination of genomic DNA was performed with TURBO DNasefree-Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. RNA integrity and purity were analyzed by gel electrophoresis and spectrophotometry and equal amounts of RNA were transcribed into cDNA, using the iScript cDNA Synthesis-Kit (Bio-Rad, Hercules, CA, USA). qPCR was carried out using a StepOnePlus real-time PCR system and SYBR-Green/ROX dye. Primers were purchased by Microsynth (Göttingen, Germany). Individual gene expression was calculated according to the $\Delta\Delta\text{Ct}$-method\textsuperscript{54}.

Western Blotting

Western blot experiments were performed as described before\textsuperscript{55} using antibodies against SREBP1 (#bs-1402R, BIOSS, Woburn, USA), LCN2 (#AF1857 R&D Systems, Abingdon, UK), as well as P-c-JUN (#3270), c-JUN (#9165), P-STAT3 (#9145), and STAT3 (#4904P) all purchased from Cell Signaling Technology, Inc., Danvers, MA, USA) while mouse anti β-Actin monoclonal antibodies (sc-47778, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or Ponceau C stained blots were used for loading controls. Semiquantitative analysis of obtained signals was performed utilizing ImageJ Software\textsuperscript{56}.

Triglyceride measurement

Triglyceride quantification was performed according to the manufacturer’s instructions (#ab65336 Abcam, Cambridge, USA).

Statistics
The data were processed and analyzed with IBM SPSS Statistics version 26.0. The
distribution of the residuals was checked with graphic methods (QQ plot) and no
significant deviation from the normal distribution was found. All parameters were
analyzed with one-way-ANOVA test and post-hoc Bonferroni test. Bonferroni corrected
significance levels are presented.

All authors had access to the study data and had reviewed and approved the final
manuscript.
References


**Figure Legends**

**Figure 1** Rimonabant and ACEA reduced cholestatic liver injury in *Abcb4*<sup>−/−</sup> mice. (A) Nuclear SREBP (nSREBP)<sub>1</sub> was induced in *Abcb4*<sup>−/−</sup> mice. Treatment with rimonabant or ACEA tendentially reduced hepatic nSREBP1. Densitometric analysis was performed with ImageJ Software. N = 3-4 mice per group. These are representative immunoblotting data of three independent experiments. (B) H&E-staining suggested that the cholestatic changes in the portal fields of the *Abcb4*<sup>−/−</sup> mice were improved by the treatment with ACEA and rimonabant. 200x, bars 100µm. Arrows indicate cholestasis induced periportal alterations of hepatic architecture. (C and D) ALT and AST serum levels were induced in *Abcb4*<sup>−/−</sup>-mice. CB1 antagonization by rimonabant but also treatment with ACEA caused a considerable reduction in serum levels of alanine amino transferases (ALT and AST) indicating reduced hepatocellular injury. Serum aminotransferases were quantified in two independent experiments. N = 3-5 mice per group. (E and F) CK19-immunostaining (arrows) visualized enhanced ductular reaction in *Abcb4*<sup>−/−</sup>-mice. CK19 immunostaining demonstrated reduced ductular proliferation in ACEA and rimonabant treated mice. The relative area that was stained for CK19 by immunohistochemistry was quantified using ImageJ. 200x, bars 100µm. One-way ANOVA with post-hoc Bonferroni-test were used for statistical analysis in panels (A), (C), (D), and (F).

**Figure 2** Serum- and hepatic bile acid level. (A) Total serum bile acid concentration was increased in *Abcb4*<sup>−/−</sup>-mice. ACEA and rimonabant treatment tendentially reduced serum bile acid concentrations. (B) Individual serum bile acid concentrations showed considerable interindivudual differences within the groups. Serum bile acid quantification was performed once. (C) Interestingly, hepatic bile acid...
concentrations were not significantly different between the groups. (D) Quantitative real-time PCR demonstrated equal amounts of hepatic mRNA levels of Fxr1 and Fxr2 in all groups.

**Figure 3** Treatment with ACEA and rimonabant ameliorated cholestatic metabolic changes in *Abcb4*–/–-mice. (A) Immunostaining revealed periportal localization of PCK1 in healthy liver (upper left panel). Zonation of PCK1 expression was disrupted in *Abcb4*–/– mice (upper right panel). Treatment with ACEA and rimonabant largely preserved the healthy zonation pattern of PCK1 (lower panels). 100x, bars 100µm. Arrowheads indicate the disturbed zonal expression of PCK1 in *Abcb4*–/–-mice. Arrows indicate periportal fibroinflammatory infiltrates without PCK1 expression. # central vein, * portal tract. (B) *Ppara* was tendentially reduced in *Abcb4*–/–-mice. The treatment with rimonabant normalized hepatic *Ppara* mRNA to healthy control level. Interestingly, PPARα-regulated gene expression of *Cpt1* was not altered in *Abcb4*–/–-mice but reduced by treatment with ACEA. (C) *Pck1* mRNA levels were constant among all groups. (D) Hepatic *Pparγ* transcription was tendentially reduced in *Abcb4*–/– mice. Rimonabant treatment increased *Pparγ*. *Fasn* mRNA was significantly decreased in cholestatic *Abcb4*–/– liver. The treatment with ACEA normalized *Fasn* while the induction of *Fasn* by rimonabant was not statistically significant. (E) Immunostaining of FASN depicted the zonated expression of FASN. Similar to the disturbed distribution of PCK1 (shown in (A)), also the zonation of FASN was disrupted in cholestatic liver of *Abcb4*–/– mice and preserved by the treatment with ACEA and rimonabant. 100x, bars 100µm. # central vein, * portal tract. One-way ANOVA and post-hoc Bonferroni-test were used for statistical analysis in panels (B), (C), and (D). N = 3-5 mice per group. Depicted are representative data of one independent experiment out of three.
**Figure 4** Rimonabant reduced hepatic inflammation in *Abcb4<sup><s>-</s></sup>-mice. (A and B) Immunostaining of CD45 and counting of positive cells per portal field indicated enhanced numbers of CD45<sup>+</sup> cells (arrowheads) infiltrated in the portal tracts of *Abcb4<sup><s>-</s></sup>* and ACEA treated mice while treatment with rimonabant reduced the numbers of these cells. 200x, bar 100µm, * portal vein. N = 4-5 mice per group. CD45<sup>+</sup> cells in at least five randomly chosen portal tracts were counted. (C) Hepatic mRNA of *Lcn2* was enhanced in ACEA treated mice and normalized to wildtype control levels by rimonabant treatment. (D) Western blot analysis suggested comparable protein regulation of LCN2 protein as observed on mRNA level (C). Nevertheless, densitometric and statistic analysis did not show significant differences between the groups. These are representative immunoblotting data of two independent experiments. (E-F) Immunohistochemistry demonstrated enhanced numbers of LCN2 positive cells in *Abcb4<sup><s>-</s></sup>* mice and reduced hepatic infiltration of LCN2 positive cells (arrowheads) in rimonabant treated mice. 100x, bars 100µm, * portal vein. One-way ANOVA and post-hoc Bonferroni-test were used for statistical analysis in panels (B), (C), (D), and (F). N = 3-5 mice per group. One independent experiment out of three mRNA analyses is shown.

**Figure 5** ACEA and Rimonabant did not alter hepatic fibrosis. (A and B) Hepatic mRNA levels of *Mcp-1* (A) and *Tnf-α* (B) were enhanced in *Abcb4<sup><s>-</s></sup>*-mice. The treatments with rimonabant significantly reduced Tnf- α whereas Mcp-1 was normalized to wt-levels in tendency. (C and D) Sirius Red staining (C) and immunohistochemical costaining (D) of type I collagen (red) and CK19 (grey) indicated reduced periportal fibrosis and ductular reaction by the treatment with ACEA and rimonabant. Magnification 100x (C) and 200x (D), arrowheads depict
collagen accumulation, arrows bile ducts, * portal vessels. (B), bars 100µm. (E)

Hydroxyproline quantification demonstrated enhanced hepatic fibrosis in Abcb4^{-/-} mice as well as in ACEA-treated mice and moderate induction of fibrosis in rimonabant treated mice. N = 4-5 mice per group. The experiment was carried out two times. (F) Remarkably, transcriptional levels of the fibrosis marker Timp-1 was not significantly altered among the groups while the hepatic expression of Mmp-2 was induced in rimonabant treated mice. One-way ANOVA and post-hoc Bonferroni-test were used for statistical analysis in panels (A), (B), (E), and (F). N = 3-5 mice per group. One independent experiment out of three mRNA analyses is shown.

Figure 6 Rimonabant reduced the activation of c-JUN signaling as well as Ccnd1 in Abcb4^{-/-}-mice. (A) Western blot analysis demonstrated the activation of c-Jun in Abcb4^{-/-}-mice and the reduction of hepatic c-JUN activation by rimonabant. (B) Immunohistochemistry showed more c-JUN-positive nuclei in hepatocytes of Abcb4^{-/-}-mice and the decline of hepatocellular c-JUN activation (arrowheads) by the treatment with rimonabant. * portal vessels, # central veins. (C) The level of activation was not significantly altered among the groups. (D) Cholestasis induced induction of hepatic Ccnd1 transcription in Abcb4^{-/-}-mice was normalized to WT level by rimonabant. One-way ANOVA and post-hoc Bonferroni-test were used for statistical analysis in panels (A), (C), and (D). N = 3-5 mice per group. One independent experiment out of three mRNA analyses is shown. For protein expression, representative data of three independent experiments are depicted.
**Figure 1**

**A**

![Box plot](image)

P = .039

WT  Abcb4⁻/⁻  ACEA  Rimo

nSREBP1/Control

WT  Abcb4⁻/⁻  ACEA  Rimo

Ponceau S

50 kDa  25 kDa

**B**

![Images of tissue sections](image)

WT  Abcb4⁻/⁻  ACEA  Rimo

**C**

![Box plot](image)

P < .001  P < .001  P < .001

ALT (U/L)

WT  Abcb4⁻/⁻  ACEA  Rimo

**D**

![Box plot](image)

P = .002  P < .001

AST (U/L)

WT  Abcb4⁻/⁻  ACEA  Rimo

**E**

![Images of tissue sections](image)

WT  Abcb4⁻/⁻  ACEA  Rimo

**F**

![Box plot](image)

P < .001  P < .001  P = .001

%CK19-stained area

WT  Abcb4⁻/⁻  ACEA  Rimo

P = .003  P = .007
Figure 2

A

P = .026  P = .079

serum BA (µM)

WT  Abcb4⁻/⁻  ACEA  Rimo

B

serum BA (µM)

WT  Abcb4⁻/⁻  ACEA  Rimo

TLCA  TDCA  THCA  T-b-MCA  TCDCA  THDCA  T-a-MCA  TUDCA  T-ω-MCA

C

hepatic BA pmol/mg

WT  Abcb4⁻/⁻  ACEA  Rimo

D

Fxr1  Fxr2

Fxr1/2 (x-fold)

WT  Abcb4⁻/⁻  ACEA  Rimo
Figure 3

A

WT

Abcb4^+/−

ACEA

Rimo

B

Ppara and Cpt1 (x-fold)

WT Abcb4^+/− ACEA Rimo

P = .044

P = .023

C

Pck1 (x-fold)

WT Abcb4^+/− ACEA Rimo

D

Ppara and Fasn (x-fold)

WT Abcb4^+/− ACEA Rimo

P = .034

P = .078

P = .005

P = .001
Figure 4

A

WT  |  Abcb4⁻/⁻  |  ACEA  |  Rimo

B

CD45⁺ cells/portal field

WT  |  Abcb4⁻/⁻  |  ACEA  |  Rimo

C

Lcn2 (x-fold)

WT  |  Abcb4⁻/⁻  |  ACEA  |  Rimo

D

LCN2/β-Actin

WT  |  Abcb4⁻/⁻  |  ACEA  |  Rimo

E

WT  |  Abcb4⁻/⁻  |  ACEA  |  Rimo

F

LCN2⁺ cells/portal field

WT  |  Abcb4⁻/⁻  |  ACEA  |  Rimo
Figure 5

A

Mcp-1 (x-fold)

WT  Abcb4<sup>−/−</sup>  ACEA  Rimo

P = .034  P = .110

B

Tnf-α (x-fold)

WT  Abcb4<sup>−/−</sup>  ACEA  Rimo

P = .002  P = .043

C

WT  Abcb4<sup>−/−</sup>  ACEA  Rimo

D

WT  Abcb4<sup>−/−</sup>  ACEA  Rimo

E

hydroxyproline [μg/g]

WT  Abcb4<sup>−/−</sup>  ACEA  Rimo

P = .021  P = .006  P = .002

F

Mmp-2 and Timp-1 (x-fold)

WT  Abcb4<sup>−/−</sup>  ACEA  Rimo

P < .001  P = .002  P = .038