Regulation of hepatic lipid metabolism in CB1$^{-/-}$ and Abcb4$^{-/-}$ -HBV surface protein transgenic mice

Inaugural-Dissertation

submitted to the

Faculty of Medicine

in partial fulfilment of the requirements

for the PhD-degree

of the Faculties of Veterinary Medicine and Medicine

of the Justus Liebig University Giessen

by

Irungbam Karuna Devi

of

Imphal, India

Giessen 2020

From the

Center for Internal Medicine, Gastroenterology

Head: Univ.-Prof. Dr. med. Elke Roeb, MHAC

Justus Liebig University, Giessen

First Supervisor and Committee Member: Univ.-Prof. Dr. med. **Elke Roeb**, MHAC

Second Supervisor and Committee Member: Univ.-Prof. Dr. **Reinhard Dammann**

Committee chairman: Univ.-Prof. Dr. **Nobert Weissmann**

Date of Doctoral Defense: 06-05-2020

TABLE OF CONTENTS

ABBREVIATIONS:

Abcb4	ATP Binding Cassette Subfamily B Member 4
ACC1	Acetyl CoA carboxylase
ACS	Acetyl Co synthase
ADRP	Adipocytes differentiation protein
AGPAT1	Acylglycerol-3-Phosphate O-Acyltransferase 1
AMPK	5' AMP-activated protein kinase
ATGL	Adipose triglyceride lipase
°C	Celsius degree
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CHOL	Cholesterol
CHOL-oleate	Cholesterol oleate
ChREBP	Carbohydrate-responsive element binding protein
CREB	cAMP response element-binding protein
DGAT1	Diacylglycerol O-Acyltransferase 1
DGAT2	Diacylglycerol O-Acyltransferase 2
DNL	*Denovo* lipogenesis
ECS	Endocannabinoid system
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FFAs	Free fatty acids
FXR	Farnesoid X receptor
g	gram
GPAT1	Glycerol-3-phosphate acyltransferase 1
h	hour
HBs	HBV surface protein transgenic mice
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HFD	High fat diet
HMG-CoA	HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme-A reductase
HSL	Hormone sensitive lipase
LAMP1	Lysosomal Associated Membrane Protein 1
LAMP2	Lysosomal Associated Membrane Protein 2
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
LDL	Low density lipoprotein
LDs	Lipid droplets
LPL	Lipoprotein lipase
MDR2	Multidrug resistant protein 2
MDR3	Multidrug resistant protein 3
mg	milligram
MGAT1	Monoacylglycerol O-Acyltransferase 1
MTTP	Microsomal triacylglycerides transfer protein

NAFLD	Non alcoholic fatty liver disease
NASH	Non alcoholic steatohepatitis
ng	nanogram
P62	Ubiquitin-binding protein p62 or Sequestosome 1(SQSTM1)
PFIC3	Progressive familial intrahepatic cholestasis type 3
PLIN1	Perilipin 1
PLIN2	Perilipin 2
PLIN3	Perilipin 3
PLIN4	Perilipin4
PLIN5	Perilipin 5
PPARα	Peroxisome Proliferator Activated Receptor alpha
PPARγ	Peroxisome Proliferator Activated Receptor gamma
PTCP	Phosphatidylcholine transfer protein
SCD1	Stearoyl-CoA Desaturase 1
SREBP1c	Sterol regulatory element-binding protein 1
TAG	Triacylglycerol
TGR5	Takeda-G-protein-receptor-5
VLDL	Very low density lipoprotein

SUMMARY

Nonalcoholic fatty liver disease (NAFLD) is currently one of the most prevalent liver diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) with fibrosis and cirrhosis. Other common liver diseases encompass HCV or HBV infections and cholestasis. The coexistence of HBV infection and NAFLD has become a common cause of liver damage due to the high prevalence of both entities. There are substantial studies citing the association of hepatic steatosis as a major risk factor for development of hepatocellular carcinoma (HCC) and mortality in chronic HBV infection. From the literature, it is known that the inhibition of CB1 receptor has hepatoprotective and anti-steatotic effects. Additionally, Abcb4 knockout mice showed an impairment of lipid homeostasis which is linked to liver injury and development of hepatic fibrosis. Keeping in view, the major role of hepatic lipid metabolism in the pathogenesis of chronic liver diseases, the present study was conducted to investigate the role of the endocannabinoid signaling system and Abcb4 knockout induced cholestasis in the hepatic lipid regulation in HBs transgenic mice.

The overall objective of this dissertation was to study the hepatic lipid regulation in HBs transgenic mice on two different genetic background mutations ($CB1^{-/-}$ and $Abcb4^{-/-}$) and its impact on disease development and progression. In the first part of the study, HBs transgenic mice were crossed with global CB1 receptor knockout mice on B/6 background to investigate the effect of $CB1^{-/-}$ on HBV induced hepatic steatosis and chronic liver disease progression. In the second part of the study, HBs transgenic mice were crossed with Abcb4 knockout mice on BALB/c background, to investigate the effect of cholestatic liver damage due to Abcb4 knockout and liver injury by transgenic HBs expression on the modulation of hepatic lipid metabolism.

The present study suggests a global CB1R KO mediated reduction of hepatic steatosis in HBs transgenic mice via enhancement of autophagy and in particularly autophagy of cytoplasmic LDs in hepatocytes. This mechanism might serve as a survival strategy to cope with metabolic stress induced by loss of body weight and decreased hepatic lipogenesis via CB1 receptor knockout. Our study has demonstrated for the first time an association between CB1 receptor signaling and PLIN2 protein expression. Loss of CB1 receptor was associated with reduction in PLIN2 both, *in vivo* as well as *in vitro* supporting the hypothesis of a cross talk between CB1 receptor signaling

pathways and PLIN2. The reduction of PLIN2 was linked with increased cytoplasmic lipases protein expression demonstrating enhanced association and accessibility with cytoplasmic LDs for further lipolysis and degradation. Thus, enhancement of autophagy more specifically lipophagy together with increased expression of cytoplasmic lipases might provide explanations for CB1 KO mediated increase in hepatic lipid catabolism and improvement of steatosis in HBs transgenic mice. Moreover, the CB1 receptor KO has shown to decrease hepatic fibrosis in HBs transgenic mice with slight improvement of liver injury as demonstrated by inclination to decrease expression of IL-1β and TNF-α and remarkable reduction of α SMA protein expression, suggesting a protective effect.

The second part of the project was planned with the hypothesis that deficiency of Abcb4 transporter mediates alteration in hepatic lipid metabolism in HBs transgenic mice which might be associated with acceleration of the cholestatic liver disease. To summarize the major findings, Abcb4$^{-/-}$ mediated enhancement of cholestasis in HBs transgenic mice was characterized by activation of AMPK-CREB signaling pathways, which concomitantly suppressed the hepatic *denovo* lipogenesis, lipid uptake, transfer, and biosynthesis of TAG resulting in decreased hepatic TAG esterification and storage. The decrease in TAG storage was further associated with the reduction of PLIN2. The activation of AMP kinase both *in vivo* and *in vitro* showed a reduction of PLIN2 suggesting a major role of AMPK in cholestasis mediated modulation of lipid metabolism. In parallel to the reduction of hepatic TAG, increased lipoprotein lipase expression further suggests the enhancement of TAG catabolism resulting in a more toxic accumulation of FFAs. The decreased TAG storage with increased FFAs might be a potentiating factor for accelerating liver damage in HBs/Abcb4$^{-/-}$ as supported by increased AST, ALT, and GGT in the serum in parallel with fibrosis and tumor induction as described in earlier studies.

Future research: HBs mice on Abcb4 knockout showed a reduction of hepatic steatosis. However, this reduction was not associated with improvement of liver injury and fibrosis but rather inflicted more severity of chronic liver disease development. From the literature, it is known that hepatic stellate cell (HSC) activation is important for liver fibrosis induction. Loss of cytoplasmic LDs is one of the reasons for HSC activation besides unconjugated bile acids. The results of the present study give a way forward for potential future research topics as listed below.

1. To investigate the role of bile acids in modulating PLIN2 expression in HSC and its association with fibrosis and inflammatory cytokine induction.
2. The studies with HBs-CB1$^{-/-}$ has some limitation as the global CB1 receptor knockout was not liver specific, which might be the reason for a weak protective response observed in HBs mice. Therefore, future studies should be focused on using a specific hepatic deletion of CB1 receptor. In addition, mice with fibrosis susceptible BALB/c strain should be considered. Secondly, future studies may be directed towards generating PLIN2 deleted HBs mice to investigate the effect on hepatic disease development in HBs transgenic mice.
3. It will be interesting to investigate the effect of dietary HFD supplementation to HBs/Abcb4$^{-/-}$ with regard to ameliorating liver injury and inflammation.
4. Further, the role of autophagy in hepatic lipid metabolism under cholestasis would be an interesting area for therapeutic intervention.
5. Hepatocytes express highest amounts of RXRa and therefore, studies may also be directed to investigate the role of RXRa and its cross talk with other transcriptional factors such as PXR, LXR, FXR, and PPARα in regulating hepatic and serum cholesterol, triglycerides and bile acids in the context of cholestatic liver injury.

ZUSAMMENFASSUNG:

Die nichtalkoholische Fettleberkrankheit (NAFLD) ist derzeit eine der häufigsten Lebererkrankungen weltweit, die in verschiedenen Ausprägungen von der einfachen Steatose über die nichtalkoholische Steatohepatitis (NASH) zur NASH-Fibrose und -Zirrhose bis hin zu sekundären Begleiterscheinungen bei anderen chronischen Lebererkrankungen wie HCV, HBV-Infektionen und Cholestase reicht. Die Koexistenz von HBV-Infektionen und NAFLD ist aufgrund der hohen Prävalenz beider Entitäten zudem eine häufige Ursache chronischer Lebererkrankungen. Es gibt umfangreiche Studien, die die Assoziation von Lebersteatose als Hauptrisikofaktor für die Entwicklung eines Leberzellkarzinoms (HCC) und die Mortalität bei chronischer HBV-Infektion nennen. Es ist bekannt, dass der Knockout des Cannabinoidrezeptors1 (CB1 KO) eine hepatoprotektive und antisteatotische Wirkung hat. Mäuse mit Abcb4 Knockout, ein bekanntes chronisches Cholestasemodell, zeigen eine Dysregulation der Lipid-Homöostase, die mit Leberschäden und der Entwicklung einer Fibrose einher geht. Unter besonderer Berücksichtigung des hepatischen Lipidstoffwechsels bei der Pathogenese chronischer Hepatitis B assoziierter Lebererkrankungen wurde die vorliegende Studie durchgeführt, um die Rolle des Endocannabinoid-Signalsystems und des ABCB4-Proteins bei der hepatischen Lipidregulation in transgenen HBs-Mäusen (mit Überexpression des Hepatitis B Oberflächenproteins) zu untersuchen.

Das übergeordnete Ziel dieser Dissertation war die Untersuchung der hepatischen Lipidregulation in HBs-transgenen Mäusen auf zwei verschiedenen genetischen Mutationshintergründen ($CB1^{-/-}$ und $Abcb4^{-/-}$) und deren Einfluss auf den Verlauf der chronischen hepatischen Schädigung. Im ersten Teil der Studie wurden transgene HBs-Mäuse mit CB1-Rezeptor-Knockout-Mäusen auf B/6-Hintergrund gekreuzt, um die Wirkung auf die hepatische Steatose und das Fortschreiten der HBs-induzierten chronischen Lebererkrankung zu untersuchen. Im zweiten Teil der Studie wurden transgene HBs-Mäuse mit Abcb4-Knockout-Mäusen auf BALB/c-Hintergrund gekreuzt, um die Wirkung einer Cholestase auf eine simultane HBs induzierte Leberschädigung unter besonderer Berücksichtigung des hepatischen Lipidstoffwechsels zu untersuchen.

Die vorliegende Studie deutet auf eine CB1 KO-vermittelte Reduktion der Lebersteatose in HBs transgenen Mäusen durch eine Verbesserung von Autophagie, insbesondere der Autophagie von

zytoplasmatischen Lipidtröpfchen in Hepatozyten hin. Dies könnte als Überlebensstrategie im Umgang mit metabolischem Stress, dienen der durch den Körpergewichtsverlust und die verminderte hepatische Lipogenese durch CB1-Rezeptor-Knockout induziert wird. Die Studie hat erstmalig einen Zusammenhang zwischen CB1-Rezeptor-Signaling und der Proteinexpression von PLIN2 gezeigt. Der Verlust des CB1-Rezeptors war mit einer Verminderung von PLIN2 sowohl *in vivo* als auch *in vitro* assoziiert. Dies stützt die Hypothese eines Crosstalks zwischen CB1-Rezeptor-Signalweg und PLIN2. Die Reduktion des mit zytoplasmatischen Lipid Droplets (cLDs) assoziierten Proteins PLIN2 war mit einer gleichzeitigen Erhöhung der zytoplasmatischen Lipasen assoziiert, was eine verbesserte Assoziation und Zugänglichkeit der cLDs für eine weitere Lipolyse und Degradation anzeigte. Somit könnte die gesteigerte Autophagie, genauer gesagt Lipophagie, zusammen mit einer erhöhten Expression zytoplasmatischer Lipasen eine Erklärung für die durch CB1-Knockout vermittelte Zunahme des hepatischen Lipidkatabolismus und die Verbesserung der Steatose in HBs transgenen Mäusen liefern. Darüber hinaus hat sich gezeigt, dass der CB1-Rezeptor-Knockout die hepatische Fibrose in HBs-transgenen Mäusen verringert und die Leberschädigung verbessert. Dies konnte durch eine Verringerung der inflammatorischen Zytokine IL-1β und TNF-α und eine Verringerung der Smooth muscle actin (αSMA)-Proteinexpression belegt werden, ein insgesamt protektiver Effekt.

Im zweiten Teil des Projekts wurden die Veränderungen des hepatischen Lipidstoffwechsels in HBs-transgenen Mäusen auf dem genetischen Abcb4 knockout Hintergrund untersucht. Unsere Hypothese lautete, dass ein Abcb4-Transporter Mangel eine Veränderung des hepatischen Lipidstoffwechsels in HBs-transgenen Mäusen vermittelt, der zu einer Beschleunigung der cholestasebedingten Lebererkrankung führt. Erstaunlicherweise detektierten wir eine durch $Abcb4^{-/-}$ vermittelte Verbesserung der hepatischen Steatose in HBs-transgenen Mäusen. Dieser antisteatotische Effekt wurde durch Aktivierung von AMPK-CREB-Signalwegen vermittelt, welche gleichzeitig die hepatische *denovo*-Lipogenese, die Lipidaufnahme und sowohl den TAG-Transfer als auch die TAG-Biosynthese unterdrückten. Daraus resultierte letztendlich eine verminderte Veresterung und Speicherung von TAG in der Leber. Die verringerte TAG-Speicherung war weiterhin mit der Reduktion von PLIN2 verbunden. Die Aktivierung der AMP-Kinase sowohl im vivo Cholestasemodell als auch *in vitro* zeigte eine Reduktion von PLIN2, was auf eine wichtige Rolle von AMPK bei der cholestasevermittelten Modulation des

Fettstoffwechsels hindeutet. Parallel zur Reduktion der hepatischen TAG deutet eine erhöhte Lipoprotein-Lipaseexpression auf einen gesteigerten TAG-Katabolismus hin, was zu einer stärkeren Akkumulation toxischer FFAs führte. Die verringerte TAG-Speicherung mit erhöhter FFA-Menge könnte der potenzierende Faktor für die Beschleunigung der Leberschädigung bei HBs/Abcb4$^{-/-}$ Tieren sein. Hierauf deuten erhöhte AST-, ALT- und GGT-Spiegel im Serum und z.B. Fibrose und Tumorinduktion, die bereits in früheren Studien beschrieben wurden, hin.

Zukünftige Forschung: HBs-Mäuse auf Abcb4-Knockout zeigten eine Reduktion der Lebersteatose, wobei diese Reduktion nicht mit einer Verbesserung des Leberschadens oder der Leberfibrose in Verbindung gebracht wurde, sondern eine tendenzielle Verstärkung der chronischen Leberkrankheit nach sich zog. Aus der Literatur ist bekannt, dass die Aktivierung der hepatischen Sternzellen (HSC) für die Induktion einer Leberfibrose wichtig ist. Der Verlust von zytoplasmatischen LDs ist neben unkonjugierten Gallensäuren einer der Gründe für die Aktivierung der HSC. Folgende Aspekte sind in zukünftigen Projekten zu klären.

1. Bedeutung der Gallensäuren bei der Modulation der PLIN2-Expression in HSC und bei der Induktion inflammatorischer Zytokine und Fibrose.

2. Die Studien mit HBs-CB1$^{-/-}$ Mäusen haben einige Einschränkungen, da der globale CB1-Rezeptor-Knockout nicht hepatospezifisch ist. Dies könnte u.a. ein Grund für die schwache protektive Wirkung des CB1 Knockout auf die HBs induzierte Leberschädigung sein. Zukünftige Studien sollten sich auf die Verwendung einer spezifischen Deletion des hepatischen CB1-Rezeptors konzentrieren und Mäuse auf einem Fibrose-suszeptiblem BALB/c-Hintegrund verwenden. Die Generierung von PLIN2-deletierten HBs-Mäusen kann einen weiteren Ansatz darstellen, die Wirkung einer Lipidmodulation auf die Entwicklung chronischer Leberschäden bei HBs-transgenen Mäusen zu untersuchen.

3. Die Wirkung einer diätetischen High Fat Diät-bei HBs/Abcb4-/- Mäusen auf Leberschädigung (Inflammation, Steatose) sollte untersucht werden.

4. Ferner wäre die Rolle der Autophagie im Leberlipidstoffwechsel bei Cholestase eine interessante Fragestellung.

5. Hepatozyten exprimieren die höchste Menge an RXRa. Daher könnten weitere Studien auch darauf ausgerichtet sein, die Rolle von RXRa und dessen Kreuzreaktion mit anderen Transkriptionsfaktoren wie PXR, LXR, FXR und PPARα bei der Regulierung des Leber- und Serumcholesterins und der Triglycerid- und Gallensäuren im Zusammenhang mit cholestatischen Leberschäden zu untersuchen.

1. INTRODUCTION

1. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) and its progressive and advanced form, nonalcoholic steatohepatitis (NASH) are major health problems and common causes for cirrhosis, and HCC, which often needs liver transplantation (Velkov et al. 2018). The global incidence of NAFLD is ~25% (Araújo et al. 2018), which together with NASH causes a great economic and health burden (Younossi et al. 2019). The NAFLD phenotype covers a wide range of diseases including chronic hepatitis C and B infection (Misra and Singh 2012). Hepatitis B virus (HBV) infection is a major health problem and globally 257 million people, or 3.5% of the world population, were living with chronic HBV infection as per estimates from World Health Organization (WHO) in 2015. So far, many aspects of the pathogenic mechanisms underlying the viral infection are clarified but little is known about the pathogenicity of the surface proteins of HBV with regard to hepatic steatosis, liver injury, cirrhosis, and malignant transformation. Since the 1980s, HBV transgenic mice models selectively expressing HBV proteins, have been used to study the role of these surface proteins (Chisari et al. 1987). However, the molecular pathways underlying the disease pathogenesis associated with surface protein have not been fully elucidated yet.

As per studies 28% prevalence rate of hepatic steatosis has been reported for chronic hepatitis B (CHB) patients (Fan and Chitturi 2008). However, an association of steatosis with CHB still remains controversial (Thomopoulos et al. 2006). It was also reported that steatosis per se in CHB is associated with metabolic disturbances rather than to fibrosis (Thomopoulos et al. 2006, Rastogi et al. 2011). Nevertheless, recent studies suggested that the presence of steatosis in CHB patients has a higher susceptibility to liver mortality and cancer development (Peleg et al. 2019). Additionally, recent *in vitro* studies showed increased lipid droplets accumulation concomitant with induction of HBVX protein (HBx) (Wang et al. 2019). Likewise, HBV surface protein transgenic mice (HBsAg) are also known to develop steatosis (Chisari et al. 1987, Chung and Wu 2018, Irungbam et al. 2019). HBsAg transgenic mice are able to induce liver injury, ER stress, and tumor formation (Churin et al. 2014). Induction of ER stress has also been associated with hepatic steatosis by HBsAg as reported earlier (Baiceanu et al. 2016). Therefore, the coexistence of HBV infection and NAFLD has become a common phenomenon in liver disease with association of metabolic disturbances such as obesity with HCC (Machado et al. 2011). Enhanced lipogenesis and lipid storage is the inflicting factor for the development and

progression of NAFLD (Pinzani, 2011). In general, hepatic lipid storage in NAFLD is the final result of imbalance in uptake process and secretion pathways (Ipsen et al. 2018) Substantial studies described and discussed the potential mechanisms associated with lipid storage in NAFLD, however the etiology still remains elusive (Hashimoto and Tokushige 2011). The occurrence of liver cirrhosis and hepatocellular carcinoma is linked with chronic HCV and HBV infection. Worldwide HCC holds the second position among all cancer related mortality (Mittal and El-Serag 2013). The molecular interplay between viral factors and host metabolic system in induction of viral pathogenesis in chronic HBV remains unclear.

The pathogenesis of liver disease and its associated factors is very complex and only partially understood. So far hepatic steatosis had gained considerable importance in the context of metabolic function within diseased liver. Since many years, endocannabinoids (EC) and their receptors which are G-protein-coupled receptors have emerged as major regulators of several pathophysiological aspects associated with chronic liver disease progression (Mallat et al. 2011). Basal hepatic expression of cannabinoid receptors is low, but higher expression was detected during liver injury in several pathological settings, depending upon the nature of insult (Mallat et al. 2011, Amini et al. 2020). Studies have revealed a significant relationship between expression of CB1 and CB2 with the degree of fibrosis in CHB patients (Dai et al. 2017). CB1 receptor signaling has been associated with enhancement of hepatic steatosis via production of 2-AG by hepatic stellate cells, increased lipogenesis with suppression of fatty acid oxidation, and blockade of VLDL production and secretion (Tam et al. 2011, Osei-Hyiaman et al. 2005). Metabolic disturbances due to obesity, insulin resistance and other phenotypes are known to be associated with ECs (Kunos et al. 2008). CB1 receptor signaling induced AMPK inhibition mediated enhancement of lipogenesis genes (Matias et al. 2006). Activated CB1 receptor mediates hepatic steatosis via activation of gene transcription of SREBP1 and its downstream targets such as ACC1, SCD1 and FAS expression. These effects are prevented by CB1 receptor antagonism (Kunos et al. 2008, Tam et al. 2011). Several studies have suggested beneficial and hepatoprotective effects of both genetic deletion and pharmacological antagonization of CB1 receptor in both rodents and human during high fat diet (HFD) or obesity condition via reduction of body weight and improvement of hepatic steatosis and inflammatory damage (Ravinet Trillou et al. 2003, Gary-Bobo et al. 2007).

CB1 receptor activation is known to mediate autophagic flux (Hiebel et al. 2014). Studies conducted in several cancer cell lines showed enhancement of autophagy due to ECs (Dando et al. 2013). Recently, the autophagic contributions to cytoplasmic lipid droplets (cLD) deterioration via fusion of autophagosomes with lysosomes has been identified (Singh et al. 2009). The association between autophagy and cLDs emerge from the observation that accumulation of cLDs occurs in various organs due to deficiency of lysosomal acid lipase (LAL) (Tuohetahuntila et al. 2017). Cytoplasmic lipid droplet protein, PLIN2 is reported to be degraded through chaperone-mediated autophagy (CMA) and macroautophagy. Autophagy helps in the clearance of hepatic LDs and also facilitates the recruitment of cytosolic lipases, adipocytes triglyceride lipase (ATGL) and other autophagy effector proteins to LDs (Kaushik and Cuervo 2015). Similarly, deficiency of PLIN2 reduces hepatic TAG storage via enhancement of autophagy (Tsai et al. 2017). The role of various autophagy and lipophagy genes involved in hepatic TAGs metabolism has been confirmed. (Yang *et al.*, 2010; Settembre *et al.*, 2015; Schroeder *et al.*, 2014). Hydrolysis of TAG stored in the LD compartment requires cytoplasmic lipase binding and activation that exerts LDs catabolism (Sathyanarayan et al. 2017). ATGL has a LC3 binding region, suggesting a crosstalk between ATGL and autophagy (Martinez-Lopez et al. 2016). Similarly, some studies have elucidated the complex interaction between perilipins 1, 2, and 5 with CGI-58, a cofactor for ATGL in controlling lipolysis (Granneman et al. 2009, Lord et al. 2016)

Chronic cholestatic liver diseases also fall within the spectrum of NAFLD (Shipovskaya and Dudanova 2018). Chronic cholestatic liver disorders are of multifactorial origin, damaging the bile ducts and causing accumulation of toxic bile, thus inducing liver tissue damage (Trauner et al. 1999, Reichert and Lammert 2018). Bile production is a complex process that involves a coordination of hepatocytes and cholangiocytes with numerous involvement of bile acid transporters (Trauner et al. 1999). Among the hepatocytes transporters, ATP-binding cassette, subfamily B, member 4 gene (*ABCB4*), present at the canalicular membrane of hepatocytes and translocating phosphatidylcholine into the bile canaliculi, an essential component of bile (Reichert and Lammert 2018), help to form mixed micellar aggregates along with cholesterol thereby reducing the detergent activity and cytotoxicity of bile and preventing cholesterol crystallization (Zollner and Trauner 2008, Cuperus et al. 2014). Mutations or deficiency of

Abcb4 gene are related with various forms of cholestatic diseases in human beings such as progressive familial intrahepatic cholestasis type 3, intrahepatic cholestasis of pregnancy, low-phospholipid-associated cholelithiasis, primary biliary cirrhosis, and cholangiocarcinoma. (Zollner and Trauner 2008, Trauner and Boyer 2003, Olsen et al. 2020). Mice with homozygous disruption of the Abcb4 gene were generated in 1990s. These mice suffer from liver disease characterized by severe necrotic damage of hepatocytes, strong portal inflammation, and proliferation and destruction of the canalicular and small bile ductular tracts (Smit et al. 1993) . Abcb4 knockout mice represent one of the best models to study human chronic cholestasis liver disease. The decreased expression of Abcb4 causes dysregulation in phospholipid metabolism and lipid homeostasis which are linked to aggravation of liver injury and fibrosis (Moustafa et al. 2012) also predisposing an aberrant pro-inflammatory lymphocyte response and an aggravated phenotype, which is observed in extrahepatic biliary atresia (EHBA) in neonatal mice (Carey et al. 2017). The deficiency of the Abcb4 transporter protein is associated with disruption in bile acid (BA) homeostasis with increased hepatic BA toxic levels leading to biliary fibrosis and higher incidence of hepatocellular carcinoma (HCC) (Sundaram and Sokol 2007). It was in early 1970s, that the TAG lowering effect of bile acids was recognized in patients suffering from gall-stones and treated with bile acids (Jiao et al. 2015, Angelin et al. 1987). Bile acids regulate nuclear receptors and G protein-coupled receptor (GPCR) signaling mechanism to modulate both, the hepatic lipid and the glucose metabolism (Chiang and Ferrell 2018). The nuclear receptor family member, farnesoid X receptor (FXR) is an endogenous ligand for bile acids, playing an important key role in hepatic TAG homeostasis. Its importance was proven in FXR-deficient mice exhibiting marked hepatosteatosis and hypertriglyceridemia with disturbances in bile acid homeostasis (Watanabe et al. 2004). Bile acids activated FXR controls TAG metabolism by suppressing hepatic *denovo* lipogenesis via downregulating the expression of SREBP-1c and FAS and thus promoting TAG oxidation and clearance (Thomas et al. 2008, Sinal et al. 2000). Chronic hepatitis B or C patients are also often associated with fibrosing cholestatic hepatitis (FCH), which is a rapidly progressive, and a fatal form of liver injury (Xiao et al. 2008). To further understand the cytopathic damage due to HBs transgenic mice together with cholestasis induced biliary liver damage, a murine $HBsAg^{+/-}/Abcb4^{-/-}$ model was generated by our working group for the first time. We were able to show that HBV surface protein (HBsAg) expression potentiated and expedited the cholestatic liver disease and the carcinogenic

progression in Abcb4$^{-/-}$-mice (Zahner et al. 2017). However, untill now, there is no information was available on the modulation of hepatic lipids in HBs/Abcb4$^{-/-}$ combined model of chronic liver diseases. Keeping in mind the public health burden due to chronic HBV, cholestasis, and its association with NAFLD, there is an urgent need to investigate the molecular interaction of the viral factors with host metabolism to unterstand the interplay of pathological mechanisms of viral steatosis and its association with chronic disease development. The deletion of CB1 receptor and Abcb4 transporter knockout are associated with a reduction of hepatic steatosis. To gain a deep understanding of the interplay between CB1 and ABCB4 in connection to hepatic steatosis, this thesis has been splitted into two projects as depicted in figure 1.

1.1. Aim of the study

PROJECT1:

AIM: The aim of the current study was to investigate the effect of CB1 knockout on regulation of hepatic lipid metabolism and its impact on the pathogenicity of HBs surface protein induced chronic liver disease. We hypothesized that CB1 knockout mediates suppression of lipogenesis and reduction of hepatic steatosis which might be associated with amelioration of HBs induced liver injury and fibrosis in HBs transgenic mice.

PROJECT2:

AIM: The aim of the current study was to investigate the effect of Abcb4 knockout on hepatic lipids in HBs transgenic mice. We hypothesized that the deficiency of Abcb4 transporter mediates alteration in hepatic lipid metabolism in HBs transgenic mice, which might be associated with the potentiation of cholestatic liver disease.

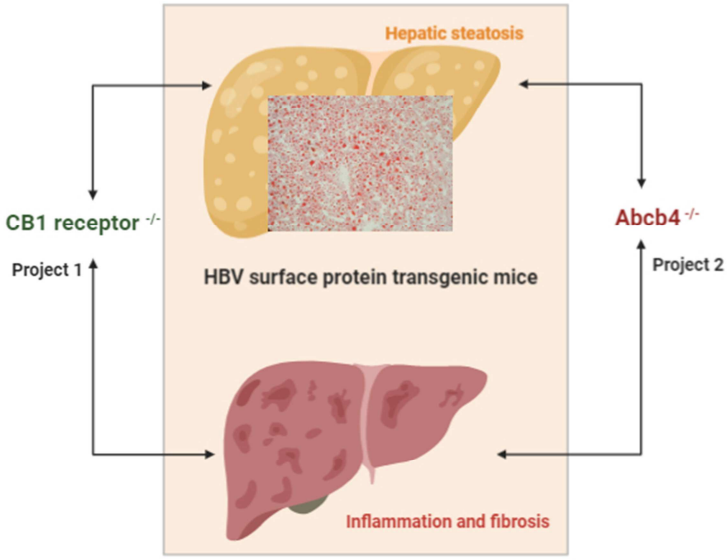


Figure 1: Schematic diagram illustrating the two different experimental projects using CB1 receptor knockout and Abcb4 knockout models, to analyse the effect on hepatic steatosis, inflammation, and fibrosis in HBV surfcae protein transgenic mice.

2. REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Liver and lipid metabolism

The liver is a highly specialized digestive organ that plays a major role in body metabolism including glycogen storage, plasma protein synthesis, lipid metabolism, and drug detoxification. Additionally, it produces bile, which is important for the digestion of lipids. Bile acts as surfactant and emulsifies lipids. The role of the liver in lipid metabolism is indispensable. It has multifaceted function such as cholesterol synthesis, lipogenesis, the production of triglycerides, and synthesis of bulk of the body lipoproteins. The liver is the central hub that performs all the functions concerning to lipid metabolism such as uptake, formation, utilization, and finally secretion into the circulation. Hepatic free fatty acids (FFAs) come from the diet (15–30% of the liver's FFA), *de novo* lipogenesis (up to 30% of the liver's FFA) and from the recycling of FFAs released from adipose tissue (Gluchowski *et al.* 2017). These hepatic FFAs are either utilized for formation of triglycerides, shuttle into β-oxidation for energy production, used in membrane synthesis or signaling pathways. Triglycerides formed in the liver are exported into the circulation as very low-density lipoproteins (VLDLs) that serve as source of lipids for extra hepatic tissues. Hepatic lipid metabolism is regulated by a complex interaction of transcription factors, nuclear receptors hormones, and intracellular signaling pathways. Under normal physiological conditions, liver tightly maintains normal hepatic lipid pools. However, deregulation in one of the other pathways leads to the accumulation of lipids and steatosis which subsequently develops into NAFLD (Baraona and Lieber 1979). Therefore, the formation of steatosis is the final physiological result from imbalances between uptake, utilization and the disposal of lipids. Lipid homoeostasis is regulated through four major pathways: uptake of circulating lipids, *de novo* lipogenesis (DNL), fatty acid oxidation (FAO), and export of lipids in very low-density lipoproteins (VLDL) as illustrated in Fig. 2.

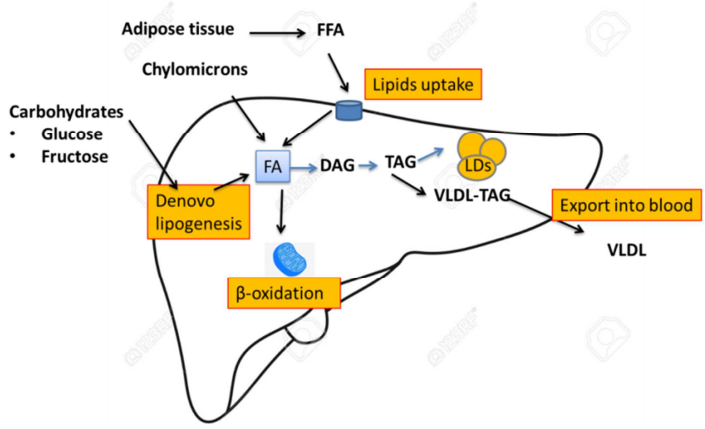


Fig. 2: Schematic diagram representing the major pathways of hepatic lipid regulation (modified and adapted from (Ipsen *et al.* 2018)).

2.1.1. Hepatic lipid uptake

One of the sources of hepatic FFAs is the plasma pool. During the fasting state, lipolysis occurs in adipocytes, which increases the plasma pool of FFAs. Adipocytes lipolysis is mainly suppressed by insulin while catecholamine, glucagon, and natriuretic peptides etc. promote it (Zhai 2010). On the contrary, 'insulin-resistant state' as observed in obesity and other metabolic syndromes, adipocyte lipolysis is rather enhanced, leading to sufficient increased in FFAs pool in the circulation independent of dietary source (Zhai 2010). FFAs enter cells via transporters or by diffusion mechanisms. The membrane bound proteins which are involved in FFAs transportation are fatty acid translocase (CD36/FAT), fatty acid transporter (FAT), fatty acid binding protein (FABP), caveolin-1, glutamate–oxaloacetate-transaminase-2(GOT2) (also known as mitochondrial aspartate aminotransferase), that facilitate the uptake of long-chain FFAs (Ipsen *et al.* 2018). CD36 is a membrane bound glycoprotein having multiple functions apart from facilitating FFA uptake. CD36 is mainly expressed in platelets, mononuclear phagocytes, adipocytes, and hepatocytes (Ipsen *et al.* 2018). CD36 is trafficked between endocytic vacuoles and the membrane depending on the stimulus such as PPARα activation or increased FFAs etc. (Ipsen *et al.* 2018). It has been reported that hepatic CD36 deficiency or insufficiency protects against systemic inflammation, steatosis, and insulin resistance in high fat diet (HFD) fed animals (Wilson *et al.* 2016). FATP2 and FATP5 are two dominant isoforms of FATPs found in the liver (Doege *et al.* 2006). FATP5 knockout mice are protected from fatty liver (Doege *et al.* 2006). All this studies testified the importance of hepatic lipid transporter in maintaining FFAs

flux and total lipid content. Fatty acid transporter protein (FATP) function is regulated in very complex way and the contribution of all these FATPs to FFA uptake is not well understood. Nevertheless, PPARα signaling is mainly responsible for coordinating the transcription of these transporter proteins, which are also regulated via hormones such as insulin and leptin (Ipsen *et al.* 2018; Nguyen *et al.* 2008).

2.1.2. *De novo* lipogenesis in the liver

De novo lipogenesis (DNL) is a cascade of enzyme catalyzed pathways that converts acetyl-coA derived mainly from carbohydrate catabolism into fatty acids. In healthy persons, the contribution of DNL to liver triglyceride production is minimal. Approximately ~5% of total triglyceride are incorporated into secreted VLDL (Fabbrini *et al.* 2010). On the other side, the contribution of DNL is ~25% in case of NAFLD patients (Fabbrini *et al.* 2010), as insulin triggers DNL in NAFLD patients via the SREBP pathway (Greco *et al.* 2008). Studies reported that mice fed with ethanol showed an increased hepatic DNL, seemingly through enhanced expression of SREBP1 (Gluchowski *et al.* 2017; Ji and Kaplowitz 2003).

As fatty acids are the structural elements required for cell membranes, DNL takes place in all cells, but more actively in metabolic tissues, such as liver, adipose tissues (ATs), and skeletal muscle (Hollands and Cawthorne 1981). Both, liver and adipose tissues can synthesize fatty acids via DNL in humans. *De novo* synthesis of fatty acids in adipose tissue, directly adds more fat accumulation and storage. In HFD, obesity, and NAFLD, the liver promotes DNL. In comparison to adipose tissues, liver is a significant contributor of lipogenesis in rodents. In contrast, carbohydrate-rich diets induced an increased DNL in ATs than the liver (Aarsland *et al.* 1997), suggesting that adipocytes are a second major site for fat synthesis in human beings.

Hormones and nutritional status control DNL in the liver. During fasting, activation of AMP-activated protein kinase (AMPK) occurs, which subsequently inhibits DNL (Kawaguchi *et al.* 2002) (Kawaguchi *et al.* 2002; Lu and Shyy 2006). However, after a carbohydrate-rich diet, a rise in blood glucose and insulin levels further stimulates DNL. Similarly, fructose- or sucrose-rich diets promote DNL in both, liver, and ATs in mice., a diet high in fat content rather inhibits DNL, thus suggesting the dramatic effect of food composition on DNL in both, liver and adipose tissues (Sanchez-Gurmaches *et al.* 2018).

The biosynthetic pathway of DNL starts with the substrate acetyl-CoA derived from non-lipid sources. The first committed step is catalyzed by acetyl-CoA carboxylase (ACC), which forms malonyl-CoA from acetyl-CoA. ACC has two isoforms (ACC1 and ACC2) (Brownsey *et al.* 2006) differing in their distribution and cellular localization pattern. ACC1 is present in the cytosol and in lipogenic tissues, while ACC2 is present in mitochondria of the skeletal and heart muscles (Abu-Elheiga *et al.* 2000). In insulin-stimulated conditions, unphosphorylated ACC is the active form. Unphosphorylated ACC formation results in increasing malonyl-CoA levels. By contrast, the phosphorylated form of ACC is the inactive form, which is induced by P-AMPK, inhibiting the malonyl-CoA production. Malonyl-CoA acts as an allosteric inhibitor of carnitine palmitoyl transferase 1 (CPT1) (McGarry and Foster 1977) and as long-chain fatty acids transporter in mitochondria, an important step in fatty acid oxidation. The fatty acids are formed by condensing malonyl-CoA onto an acyl-carrier protein group and are catalyzed by fatty acid synthase (FASN), the key regulator enzyme of DNL. The reaction is followed by a series of dehydration and reduction reactions that ultimately produce a 16-carbon long saturated fatty acid called palmitate. FASN is regulated by insulin and citrate, and inhibited by PKA, AMP and palmitoyl-CoA (Menendez *et al.* 2009). The fatty acids produced by DNL have multiple fates. They are either used for triglyceride (TAG) esterification, oxidized, or might function as intracellular signals including their action as PPAR ligands, LXR modulators, lipokines, and substrates for protein palmitoylation (Paglialunga and Dehn 2016; Solinas *et al.* 2015).

2.1.3. Fatty acid oxidation

The mitochondria, peroxisomes, and cytochromes are the major sites for fatty acid oxidation (FAO) in mammalian cells (Begriche *et al.* 2013). FAO is the most important source of energy production. This process is triggered by a surplus of FFA and is regulated by the PPARα (Ipsen *et al.* 2018; Kersten and Stienstra 2017). Several studies demonstrated that the loss of PPARα gene results in enhanced hepatic steatosis in ob/ob mice (Ipsen *et al.* 2018; Lee *et al.* 1995). In addition, PPARα and PPARα-target genes were enhanced in wild type and ob/ob mice when fasted for 24h (Gao *et al.* 2015). FAO is initiated with the uptake of FFA which is dependent on carnitine palmitoyl transferase 1 (CPT1), which is located in the outer mitochondrial membrane (Nassir and Ibdah 2014). Acetylcosynthase (ACS), an enzyme of the mitochondria cannot process long chain fatty acids, so the entry of long chain fatty acids are reulated by CPT1 (Reddy

and Rao 2006). Furthermore, CPTI activity can be inhibited by malonyl-CoA, the product of the first step of *de novo* lipogenesis (Alves-Bezerra and Cohen 2017). Negative energy balance causes malonyl CoA decrease with increase in fatty acid oxidation. The long chain fatty acids which cannot be oxidized via mitochondria are preferably metabolized via peroxisomal β-oxidation. During excessive fatty acids influx, cooperatively peroxisomal and microsomal CYP4A mediated ω-oxidation helps to cope with fatty acid overload on the mitochondrial β-oxidation system and thereby maintains fatty acid metabolism within the liver. In NAFLD, apart from mitochondrial β-oxidation, ω-oxidation maintained by the cytochromes also contributes to FAO, which generates considerable amounts of reactive oxygen species promoting oxidative stress and liver damage (Ipsen *et al.* 2018; Rao and Reddy 2001).

2.1.4. Triacylglycerol export

The formation of very low density lipoproteins (VLDLs) and their subsequent export into the circulation by the liver is a highly coordinated process maintaining the overall lipid homeostasis. The development of atherosclerosis is associated with excessive loads of VLDLs in the circulation (Ginsberg 2002). The size of VLDL particles ranges between 35 and 100 nm in diameter. VLDL mainly consist of neutral lipids (mostly triglycerides) core that are surrounded by cholesterol, cholesteryl esters, phospholipids, and specific apolipoproteins (Tiwari and Siddiqi 2012). Apolipoprotein B (apoB) is the most predominant apolipoprotein providing not only structural stability to the nascent VLDL particles (Tiwari and Siddiqi 2012), but it also maintains the structural framework for the assembly of triglyceride (TG)-rich lipoproteins in the liver and in the intestine. The turnover of apoB100 and apoB48 in a few species regulates the VLDL synthesis (Ipsen *et al.* 2018). The microsomal triacylglycerol transfer protein (MTTP) is a heterodimer protein complex having a lipid-binding and transfer domain (Jamil *et al.* 1996) as well as a 55-kDa protein disulfide isomerase (PDI) domain (Wetterau *et al.* 1990), that transfers the synthesized lipids from smooth endoplasmic reticulum to apoB (Hussain *et al.* 2003). Lipidation of apoB depends on the availability of triglycerides; without lipidation or with the limitation of TAGs, a significant amount of nascent apoB undergoes proteoasomal degradation mediated by cytosolic chaperons like hsp70 and hsp90 (Ipsen *et al.* 2018), through post translational degradation which can be stimulated by *n*-3 fatty acids and insulin (Ginsberg and Fisher 2009). The biogenesis of VLDL takes place in ER. After the assembly they move to *cis*-

Golgi in a transport vesicles, where the apoproteins are glycosylated and then bud off as secretory vesicles from the Golgi membrane. Subsequently, vesicles migrate to the sinusoidal membrane of the hepatocyte, from where the VLDL transported into the circulation (Alves-Bezerra and Cohen 2017).

The mechanism of VLDL synthesis or clearance has not been fully elucidated. Fatty acids chemical nature, chain length, and their degree of saturation can affect the rate of VLDL export (Sundaram and Yao 2010). For instance, earlier studies have shown that medium-chain length fatty acids compared to palmitate reduce VLDL secretion in chicken primary hepatocytes (Sato *et al.* 2005). Likewise, the presence of exogenous oleate supplementation remarkably increased VLDL secretion in McA-RH7777 cells (Sundaram *et al.* 2010). On the contrary, McA-RH7777 cells treated with poly-unsaturated fatty acids, showed a significant reduction in VLDL secretion (Tran *et al.* 2006). Besides, the incubation period of FFAs treatment also has a profound effect on VLDL synthesis and secretion. For instance, excessive TAG accumulation was observed during extended incubation time with higher concentrations of oleic acid, that promotes excessive degradation of apo-B100 in McA-RH7777 cells (Ota *et al.* 2008, 2008). Therefore, effective VLDL assembly and secretion is impaired during lipid overloading that exceeds the capacity of hepatocytes and causes hepatosteatosis. Lipid droplets associated proteins, PLIN2 and CideB play a role in VLDL export (Alves-Bezerra and Cohen 2017). McA-RH7777 cells with overexpression of PLIN2 promotes storage of cytosolic lipid droplets with subsequent impairment in VLDL secretion (Alves-Bezerra and Cohen 2017). Similarly, *Cideb*$^{-/-}$ mice are resistant and protected from HFD induced steatosis (Li *et al.* 2007, 2007). In mice, the inhibition of microsomal triacylglycerol transfer protein hampers the synthesis and secretion of VLDL and chylomicrons, thereby leading to steatosis (Liao *et al.* 2003). Other possible limitations may include a high rate of degradation of apoB100, or deficient synthesis of phosphatidylcholine and cholesterol, which affect the turnover of VLDL in liver (Sundaram and Yao 2010).

2.2. Hepatic lipid regulation

2.2.1. Role of AMPK in lipid metabolism

Basically, all the cells need constant supply of energy in the form of ATP, and therefore, a normal ATP homeostasis has to be maintained for cellular growth, differentiation and

physiological functions. Adenosine monophosphate-activated kinase (AMPK) acts as a master sensor and induces cellular cascades for maintaining energy homeostasis in the cell (Heidrich *et al.* 2010, 2010). The activation of AMPK occurs in response to various stimuli such as exercise, low glucose, hypoxia, ischaemia, heat shocks, increased reactive oxygen species (ROS), pharmacological agents etc. (Garcia and Shaw 2017; Villanueva-Paz *et al.* 2016). During various stress suitation, the relative increase in AMP/ADP ratio to ATP, thus promotes the activation of AMPK via phosphorylation (Garcia and Shaw 2017). Once AMPK is activated it induces enhancement of catabolic pathways to generate more ATP while inhibiting anabolic pathways as an adaptive response to the stressor.

AMPK is a heterotrimeric protein complex, which consist of three subunits namely α, β and γ. The alpha subunit is the catalytic subunit while β and γ are the regulatory domains. Binding to the γ regulating unit brings the conformational changes that promote and stabilize phosphorylation of AMPK (Xiao *et al.* 2011). Apart from AMP/ADP binding, phosphorylation of Thr 172 by other serine/threonine kinases such as LKB1 and CAMKK2 (CAMKKβ) kinase, the closest mammalian kinase to LKB1, can directly mediate the phosphorylation of AMPK (Mihaylova and Shaw 2011).

AMPK has a multifaceted role. Activated AMPK regulates the glucose homeostasis via inhibiting gluconeogenesis and promoting glucose breakdown, enhancement of translocation of GLUT4 and GLUT1 for more glucose uptake (Villanueva-Paz *et al.* 2016). It also regulates lipid metabolism by directly phosphorylating proteins or modulating gene transcription in specific tissues such as the liver, fat and muscle (Wang *et al.* 2018). SREBP1c, P-ACC1, FAS, SCD1, HMGCR, GPAT1, HSL, ATGL etc. are some of the genes involved either in synthesis, oxidation and lipolysis, which are modulated directly through phosphorylation or modulation at gene transcription level by activated AMPK (Wang *et al.* 2015). Activated AMP kinases regulate SREBP1 (sterol regulatory element binding protein 1), a master transcriptional regulator of lipid synthesis (Li *et al.* 2011), but also HNF4α (hepatocyte nuclear factor-4α) and ChREBP (carbohydrate-responsive element binding protein) (Kawaguchi *et al.* 2002). Therefore, activated AMPK mediate reprogramming of cells to limit glucose and lipid synthesis and favor oxidation of fatty acids as alternative source of energy.

2.3. Autophagy in lipid metabolism

2.3.1. Autophagy

Autophagy is a highly conserved and coordinated process of double walled autophagosome formation, sequestration of intracellular damaged proteins and their attachment to lysosomes for subsequent degradation (Mihaylova and Shaw 2011). Autophagy protects the cells from cytotoxic compounds and provides nutrients during starvation and maintains the cell homeostasis. During metabolic stress and low energy condition AMPK gets phosphorylated and activates autophagy by phosphorylating the ULK1 complex, which initiates the cascade of autophagy proteins (ATGs) (Egan *et al.* 2011; Kim *et al.* 2011; Mack *et al.* 2012). Autophagy is generally activated by starvation and nutrient deprivation to generate metabolic intermediates in order to maintain ATP production. In fact, the autophagy checkpoint is a major mechanism for the maintenance of intracellular homeostasis that can be up-regulated by nutrient deprivation and/or organelle damage (Green *et al.* 2014).

Autophagy might be non-selective or selective (Johansen and Lamark 2011). In non-selective autophagy the intracellular content is engulfed by autophagosomes without any discrimination whereas selective autophagy uses ATG8s proteins to selectively recruit the cytoplasmic components into autophagosomes. So far, numerous types of selective autophagy have been reported such as mitophagy (Lemasters 2005), pexophagy (peroxisomes) (Till *et al.* 2012), or even lipophagy (lipids) (Liu and Czaja 2013).

Autophagy is directly or indirectly associated to lipogenesis, lipolysis, fatty-acid oxidation, ketogenesis, and cholesterol efflux (Martinez-Lopez and Singh 2015). Lipophagy is a kind of selective autophagy of intracellular lipid droplets that provide energy and fatty acid for lipid biogenesis but also removal of excessive stored lipid droplets. Impairment in lipophagy is shown to aggravate pathophysiological consequences such as enhancement of liver lipid droplets and hepatic steatosis.

2.3.2. Lipophagy

2.3.2.1. Lipid droplets

Lipid droplets are dynamic organelle, representing an intracellualr compartments for neutral lipid storage (Welte 2015, 2015). They serve as storehouse for lipids which can be used at the time of

various cellular needs such as for energy production or use for membrane components and so on. Lipid droplets also protect the cells from toxic effects, which may be caused due to high concentration of toxics free fatty acids by safely sequestering them in the form of less harmful and inert triglycerides (Welte 2015). Because of the protective nature of LDs, its amount increases drastically in various metabolic disorders such as fatty liver and obesity (Greenberg *et al.* 2011). Lipid droplets are particularly important in tissues specialized for energy storage or lipid turnover, such as adipose tissue, the liver, and the intestine (Gross and Silver 2014). However, LD are also stored in other tissues such as skeletal muscle, the adrenal cortex, macrophages, and mammary glands (Walther and Farese 2012). Similarly, LDs are involved in lipid signaling of immune cells and often targeted by pathogens (Saka and Valdivia 2012, 2012).

2.3.2.2. Lipid droplets associated proteins

Lipid droplets associated proteins are mainly located on the surface of the lipid droplets conferring stability and protection from degradation (Gao *et al.* 2015; Gross and Silver 2014; Yang *et al.* 2015). Proteins belonging to perilipins and cell death-inducing DNA fragmentation factor-like effector (CIDE) families are the most proteins which are surface associated with LDs (Itabe *et al.* 2017). In case of eukaryotes, the family of perilipin consists of 5 proteins namely perilipin 1 to 5. CIDE family consists of 3 proteins namely A to C (Brasaemle 2007). The LDs associated proteins have differential tissue expression pattern. Among the perilipins, PLIN1 was first discovered. PLIN1 is mainly expressed in adipocyte tissues and steroidogenic cells (Greenberg *et al.* 1991).The interaction of PLIN1 with a regulatory protein called comparative gene identification-58 (CGI-58) on the surface of LDs prevents the access of cytoplasmic lipases (ATGL, HSL) to the LDs and thereby prevents the degradation of TAG (Greenberg *et al.* 2011; Zimmermann *et al.* 2004). However, in certain physiological conditions, the phosphorylated form of PLIN1 gets detached from the LDs surface and thereby fails to protect TAG degradation. The second member of perilipin family, PLIN2, also known as adipocytes differentiation related protein (ADRP) is ubiquitous in distribution pattern but its major site is the liver. PLIN2 functions as "**shielding effect"** and blocks the accessibility of cytoplasmic lipases to LDs surfaces (Kaushik and Cuervo 2015*;* Listenberger *et al.* 2007). PLIN2 expression is often associated with increased lipid loading into the cells such as during HFD and obesity, and other metabolic syndromes. PLIN2 deletion or -knockout mice are protected against steatosis

(Arisqueta *et al.* 2018; McManaman *et al.* 2013). Like PLIN2, PLIN3 is also associated with LDs and has a widespread expression in various tissues (Dalen *et al.* 2007; Greenberg *et al.* 2011) Another name of PLIN3 is TIP47. This protein is not only found on LDs surfaces also in the cytoplasm (Barbero *et al.* 2001). The main function of PLIN3 is to stabilize the LDs and compensate the function of other PLINs (Itabe *et al.* 2017). The fourth member of the perilipin family, PLIN4, is mainly expressed in adipocytes and skeletal muscles (Itabe *et al.* 2017). During exercise the expression of PLIN3 enhances in the skeletal muscle and also in hepatic stellate cells. Thus suggesting a role of PLIN3 in TAG storage and oxidation (Covington *et al.* 2014; Straub *et al.* 2008). PLIN5 is known as OXPAT protein with highest expression seen in myocardial tissues. It has been found in other tissues such as liver and skeletal muscle (Dalen *et al.* 2007; Itabe *et al.* 2017). PLIN5´s main role is not only providing the stability of LDs but is associated with the supply of FAs for mitochondrial oxidation (Dalen *et al.* 2007).

CIDE family proteins CIDE A to C are abundantly expressed in adipocyte tissues and liver. However, CIDEC is not detectable in normal liver. CIDE proteins also play an important role in TAG metabolism. Several studies reported beneficial effects of CIDE deletion on the protection from steatosis in HFD induced obesity and insulin resistance in rodents and humans (Gong *et al.* 2009; Itabe *et al.* 2017).

2.3.2.3. Lipophagy and NAFLD

The recent discovery, that LDs can be selectively degraded through a autophagy mechanism called lipophagy, advances the understanding the role of LDs in various diseases (Liu and Czaja 2013; Zhang *et al.* 2018). During starvation or low energy states, acute lipid stimulus increases autophagic sequestration of lipid droplets and their degradation in lysosomes (Martinez-Lopez and Singh 2015). Apart from autophagy, lipid droplets can also be degraded through cytoplasmic lipases such as hormone sensitive lipase and adipose triglyceride lipase (ATGL) (Zimmermann *et al.* 2004).

It was the remarkable study by Singh et al. which for the first time described the regulation of hepatic lipid metabolism through autophagy. Inhibition of autophagy led to enhancement of hepatic steatosis both, *in vivo* as well as *in vitro* (Singh *et al.* 2009). Several studies have shown that autophagy is involved in lipid metabolism, including lipogenesis, lipolysis, fatty-acid

oxidation, ketogenesis, and cholesterol efflux (Martinez-Lopez and Singh 2015). Depending upon the size of the LDs, autophagy can be either macroautophagy or microautophagy (Singh *et al.* 2009). Recently another form of autophagy of LDs has been reported, the so called chaperon mediated autophagy. This selective degradation of the LDs associated protein PLIN2 via P-AMPK suggests an interplay between AMPK and PLIN2 in maintaining intracellular TAG storage (Kaushik and Cuervo 2015).

Impairment in lipophagy has been associated with low body fat and with the enhancement of hepatic LDs during fasting as observed in superoxide dismutase 1(SOD1) knockout mice (Martina *et al.* 2014). Inhibition of SREBP-2 activity induced autophagic mediated reduction in hepatic steatosis in both, *in vitro* and in GNMT-KO mice, a NAFLD model (Zubiete-Franco *et al.* 2016). Therefore, all the above studies clearly show that lipophagy negate the progression of NAFLD. On the contrary, lipophagy promotes fibrosis in the liver and other tissues (Kounakis *et al.* 2019). Activated HSC associated with the reduction of extracellular lipid droplets apart from other features such as oxidative stress, endoplasmic reticulum stress, and overexpression of G proteins and the accumulation of p62 all induced fibrosis (Kim *et al.* 2018). Autophagy inhibition has induced anti-fibrogenetic effects in mouse and human HSC lines (Hernandez-Gea *et al.* 2013).

2.4. Nonalcoholic fatty liver disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) has emerged as most frequent form of chronic liver disease with a worldwide prevalence of 25% (Younossi *et al.* 2019). The history of fatty liver and its association with chronic liver injury is known for >50 years, however it was Jürgen Ludwig and colleagues in 1980, who described NASH for the first time (Ludwig *et al.* 1980). NAFLD can be defined as the presence of hepatic steatosis, as confirmed either by imaging or histology, with the absence of secondary causes that enhances hepatic fat storage such as excessive alcohol consumption, steatogenic medication, or monogenic hereditary disorders (Sanyal 2019; Younossi *et al.* 2019). NASH is the more advanced form of NAFLD, characterized with inflammation and hepatocyte damage (steatohepatitis), accompanied by pericellular fibrosis, that may progress to cirrhosis and eventually to HCC and death (Diehl and Day 2017). NAFLD patients tend to have a metabolic syndrome such as obesity, type 2 diabetes mellitus (T2DM), hyperlipidemia and hypertension (Younossi *et al.* 2019). The maximum of

NAFLD occurrence was reported in the Middle East and South America and lowest in Africa. While NAFLD is on rise in the Asian subcontinent and the far east countries (Sanyal 2019). It is estimated that by 2030, the burden of end-stage liver disease will be escalated to 2–3-fold in both, western nations as well as in several Asian countries (Sanyal 2019; Younossi *et al.* 2019). Younossi et al. published a detailed meta-analysis and presented the geographical occurrence of NAFLD among the T2DM patients as shown in Figure 2. Over the last few years, tremendous trends in research progress have been made to understand the spectrum of NAFLD and its pathomechanisms, to identify targets for therapeutic approaches and to start clinical trials. Despite the availability of enormous information on NAFLD and NASH, currently there is no medication available to cure NAFLD except for dietary and life style modification (Friedman *et al.* 2018; Roeb *et al.* 2015).

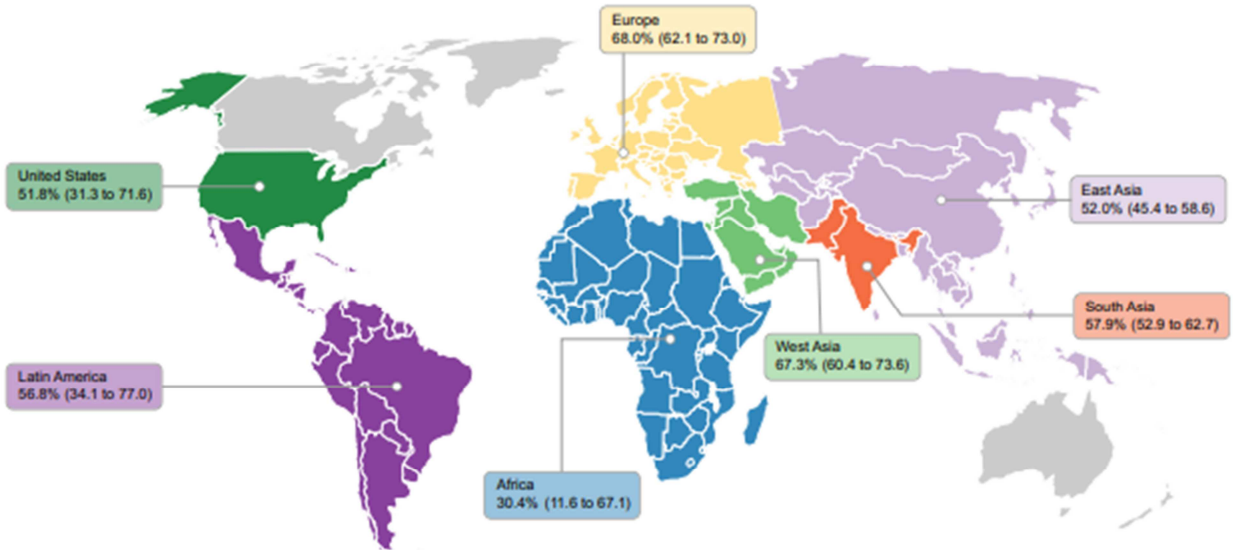


Fig 3: The geographical distribution pattern of NAFLD among patients with type 2 diabetes (modified from (Younossi et al. 2019, *Journal of Hepatology*)).

2.4.1. Pathogenesis of NAFLD:

2.4.1.1. Two Hit Model

The underlying mechanism for the development and progression of NAFLD is very complex and of multifactorial in origin**.** It was James and Day, who first explained the so called 'two hits hypothesis' in 1988 describing a two-step process leading to the development of NASH.

According to this hypothesis, hepatic lipid accumulation as results of a sluggish lifestyle, high fat diet, obesity, and insulin resistance act as the first hit thereupon sensitizing the liver to further insults as a 'second hit'. The 'second hit' triggers inflammatory cascades and fibrotic processes. Oxidative stress and subsequent lipid peroxidation, proinflammatory cytokines, adipokines and mitochondrial function were included among these factors (James and Day 1998; Peverill *et al.* 2014).

2.4.1.2. Multiple hit hypotheses

NAFLD together with NASH are dynamic and evolving diseases which often associated with several factors acting in parallel and synergistically for the disease development and progression. The two hit hypothesis cannot simulate and explain the complexity of human NAFLD by its own. Thereupon, a multiple-hit hypothesis has been emerged to explain the multifactorial causes for NAFLD and NASH.

The hypothesis behind the pathogenesis of NAFLD is highly debatable. So far two hypotheses, the "two hit hypothesis" (Dowman *et al.* 2010), and the recently emerged "multiple hit hypothesis" are very popular, however the multiple hit hypothesis prevails among the scientific community (Ratziu *et al.* 2010). The earlier hypothesis suggests that accumulation of lipid in liver cells due to insulin resistance acts as first hit which might induce subsequent hepatic inflammation, fibrosis, and liver injury together acting as second hit. Contrary to this the "multiple hit hypotheses" suggests that there are multiple factors rather than sole lipid accumulation which are significantly responsible for subsequent liver damages. Others than lipid accumulation and insulin resistance, these multiple factors are genetic, epigenetic, environmental, dietary, gut associated, and altered inflammatory pathways significantly responsible for the onset of NAFLD (Alisi *et al.* 2012; Ayonrinde *et al.* 2015; Fang *et al.* 2018).
The age, sex and ethnicity also have an impact on the progress of NAFLD with cases appearing more in boys than girls (Marzuillo *et al.* 2014; Schwimmer *et al.* 2005). Gene polymorphisms of certain proteins (such as TM6SF2, PNPLA3) are closely associated to the genetic basis of hepatic steatosis, fibrosis and cancer (Giudice *et al.* 2011; Romeo *et al.* 2008; Speliotes *et al.* 2010). DNA methylation of the peroxisome proliferator-activated receptor γ coactivator 1α (PPARGC1A), mitochondrial transcription factor A (TFAM) promoters and alteration in mitochondrial DNA content are closely associated to insulin resistance and NAFLD (Sookoian *et*

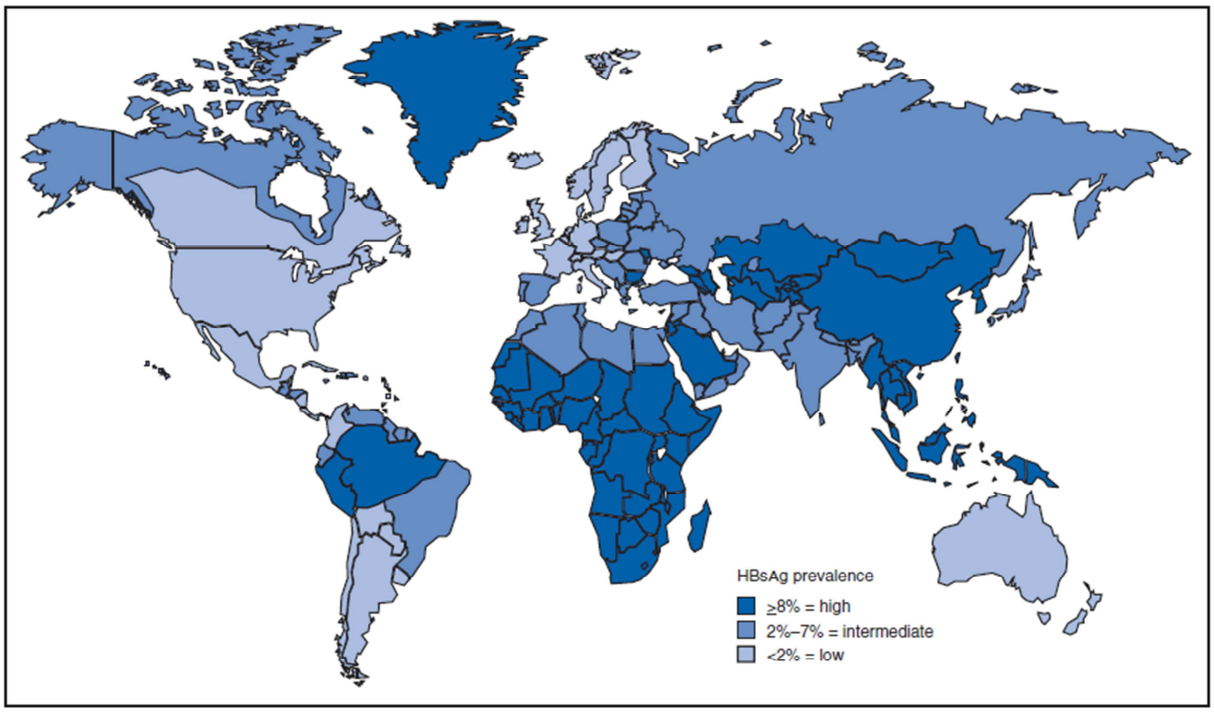
al. 2010). Environmental factors such as abnormal intestinal microbiome with hepatic inflammatory signaling are reported as well as causatives for NAFLD (Clemente *et al.* 2016; Rotman and Sanyal 2017). Alterations in circadian rhythm and its impact on nuclear receptors (FXR and CAR) which maintain the bile acid production and also influence steatohepatitis (Shi *et al.* 2019; Yang *et al.* 2017). Many inflammatory pathways (such as JNK-AP-1 and IKK-NF-κBD, TLRs, ILs) are also found to be deregulated during NAFLD and aggravate the disease (Cai *et al.* 2005; Hotamisligil 2006). The gut-liver axis plays a pertinent role in the regulation of bacterial load to the liver. Intestinal barrier and gut microbiota (GM) are critically associated to the bacterial burden. A compromised intestinal barrier and upraised intestinal microbiota allow a higher bacterial load in the liver, which causes necrosis, inflammation and liver damage (Clemente *et al.* 2016) . High carbohydrate (fructose) and fat rich diet are also etiologies behind the onset of NAFLD and NASH (Ventura *et al.* 2011). The higher sugar intake imparts lipid accumulation in the liver by converting excess acetyl CoA into fatty acid. Fructose seems to enhance the transcriptional and translational activity of lipogenic genes (such as SREBP-1c and carbohydrate-responsive element-binding protein, ChREBP) associated to fatty acid accumulation in liver (Cicerchi *et al.* 2014; Lanaspa *et al.* 2012).

2.5. Hepatitis B infection

HBV infection leads to a wide spectrum of liver disease ranging from acute to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Glebe and Bremer 2013; Velkov *et al.* 2018; WHO). It is estimated that 300 million humans are chronically infected with hepatitis B virus (HBV) (Liang 2009). Among individuals with chronic HBV infection who are untreated, 15% to 40% progress to cirrhosis, which may lead to liver failure and liver cancer (Tang *et al.* 2018). About 887,000 annual deaths are attributed to HBV, mostly due to the long-term sequelae liver cirrhosis and hepatocellular carcinoma (HCC) (Velkov *et al.* 2018).

Most infections are acquired through perinatal transmission at birth, through horizontal transmission by sexual contact, infected body fluids, and through injecting drugs in adolescents and adults (Lok and McMahon 2009). The epidemiology of hepatitis B can be described in terms of the prevalence of hepatitis B surface antigen (HBsAg) in a population, broadly classified into high- (8% HBsAg prevalence), intermediate (2%–7%) and low prevalence (<2%) areas (MacLachlan and Cowie 2015; Previsani N 2002). Prevalence of Hepatitis B is found to be

highest in Sub-Saharan Africa and East Asia, where 5–7% of the adult population is chronically infected (MacLachlan and Cowie 2015). The lowest prevalence was discovered in North America, Western and Northern Europe, and Australia etc. (Hou *et al.* 2005).



* For multiple countries, estimates of prevalence of hepatitis B surface antigen (HBsAg), a marker of chronic HBV infection, are based on limited data and might not reflect current prevalence in countries that have implemented childhood hepatitis B vaccination. In addition, HBsAg prevalence might vary within countries by subpopulation and locality.
Source: CDC. Travelers' health; yellow book. Atlanta, GA: US Department of Health and Human Services, CDC; 2008. Available at http://wwwn.cdc.gov/travel/yellowbookch4-HepB.aspx.

Fig 4: Worldwide distribution of chronic HBV prevalence (Source: Centre of disease control, 2008)

2.5.1. Hepatitis B virus epidemiology

Hepatitis B virus (HBV) belongs to the Hepadnaviridae (hepatotropic DNA virus) family having a strong predilection for infecting liver cells. Related viruses are also found in other species such woodchucks, ground squirrels, tree squirrels, Peking ducks, and herons (Liang 2009). So far, 9 different genotypes of HBV namely A to I with specific geographical distribution pattern has been reported (Velkov *et al.* 2018). The figure below shows the geographical distribution pattern of different genotypes of HBV across the globe (Velkov *et al.* 2018). 96% of chronic HBV

infections worldwide are caused by five of these nine genotypes: C, D, E, A and B. Whereas 2% of global chronic HBV infections are caused by genotypes F to I.

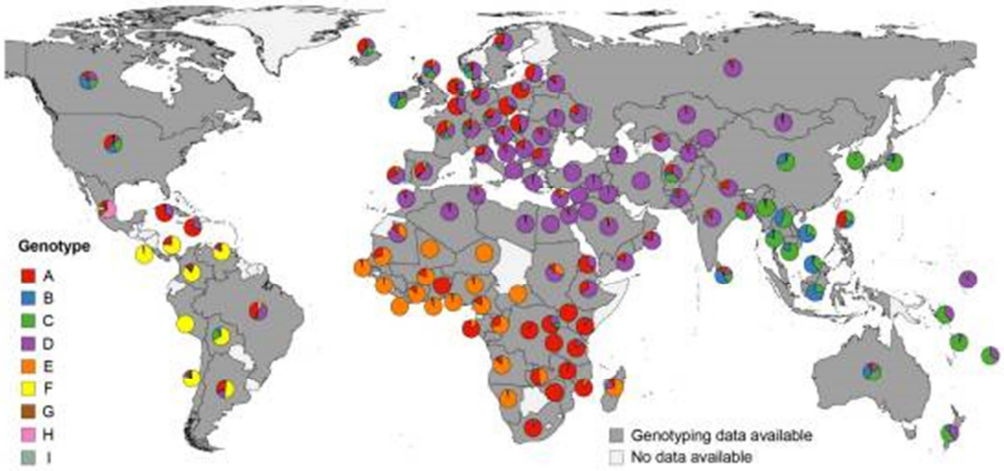


Fig 5: Country wise distribution of HBV genotypes (modified from (Velkov *et al.* 2018)).

2.5.2. Virus structure

HBV virus particle (virion) are double enveloped spherical particles that are about 40–42 nm in diameter (Klingmuller and Schaller 1993). The outer envelope consists of lipoprotein, containing three envelope glycoproteins (LHB, MHB, and SHB) also named as surface antigens (HBsAg) (Klingmuller and Schaller 1993). The inner envelope encloses the viral nucleocapsid composed of HBcAg proteins together with the viral genome and a polymerase (Breiner *et al.* 1998). The viral genome is partially double stranded, relaxed-circular DNA of 3.2 kb (HBV rcDNA), and polymerase that is responsible for the synthesis of viral DNA (Churin *et al.* 2015; Kang *et al.* 2015). The host cellular enzymes transform the HBV rcDNA into a covalently closed circular DNA (cccDNA) inside the nuclei of infected cells in the host (Churin *et al.* 2015). This episomal HBV cccDNA remains as stable minichromosome organized by histone and non-histone proteins in the infected hepatocytes (Churin *et al.* 2015). The viral genome encodes four overlapping open reading frames (ORFs) namely, S, C, P, and X (Liang 2009). The S ORF encodes for the viral surface envelope protein (HBsAg). This region is structurally and functionally divided into the pre-S1, pre-S2, and S regions. The C ORF encodes for the structural protein of nucleocapsids and the 'e' antigen (HBeAg) (Churin *et al.* 2015; Datta *et al.* 2012; Liang 2009*a*). The precore ORF encodes for a signal peptide that directs the translation product to the endoplasmic

reticulum, where the protein is further processed to form the HBeAg. The X ORF encodes for a 16.5 kDa multifunctional protein called HBxAg (Bouchard and Schneider 2004; Datta *et al.* 2012). The *P* ORF encodes for the DNA polymerase (DNA Pol), the reverse transcriptase (RT), and the RNase H, which also functions as the terminal protein (TP) (Bartenschlager and Schaller 1988; Datta *et al.* 2012). Apart from the virion produced by the virus, HBV infected cells also produce two distinct subviral lipoprotein particles: 20-nm spheres and filamentous forms (Breiner *et al.* 1998). These viral particles are the "incomplete forms" as they either contain only the envelope glycoproteins and host-derived lipids or the envelope glycoproteins enclosing a nucleocaspid without genome (Hu and Liu 2017).

2.5.3. Animal model for HBV infection

2.5.3.1. Animal model

Animal models are an important source for understanding and solving the issues associated to HBV infection. However, good animal models are limited due to the very small host range. Chimpanzees are the sole non-human primate fully at risk of having HBV infection; however their use in HBV research is strongly restricted (Wieland 2015). Several non-primate species that are susceptible to HBV includes the tree shrew (Tupaia belangeri), showing a close phylogenetic relationship with primates (Xiao *et al.* 2017). Additionally, primary Tupaia hepatocytes are suitable for HBV studies, due to easy availability compared with primary human hepatocytes, and also due to the presence of sodium taurocholate co-transporting polypeptide (NTCP) (Yan *et al.* 2015).

The first human liver chimeric mouse model was developed in immunodeficient ($Rag2^{-/-}$, SCID, SCID/beige) mice with the urokinase-type plasminogen activator (uPA) transgene. The expression of the uPA gene can induce necrosis of hepatocytes, resulting in subacute liver failure in young mice, and making it possible to transplant human hepatocytes into mouse livers. Transplantation of human hepatocytes into uPA-SCID mice leads to a liver-humanized model with a high human hepatocyte reconstitution rate and suitable for hepatitis B and C infection (Tsuge *et al.* 2005). With further refinement in art of generating chimeric mouse models with better human liver transplant efficiency, another chimeric mouse model was constructed using FRG ($Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$) mice (Azuma *et al.* 2007), with almost 95% repopulation of mouse liver with human liver cells. Human liver cells repopulated in FRG mice support HBV and

hepatitis C virus (Bissig *et al.* 2010). Researchers have further developed a variety of strategies to develop chimeric mice dually reconstituted with both, immune cells and hepatocytes of human origin, including AFC8 (Washburn *et al.* 2011) and A2/NSG (Bility *et al.* 2014) to enable adaptive immune and immunotherapy strategies studies.

2.5.3.2. MOUSE MODEL

2.5.3.2.1. HBV transgenic mouse model

From the start of 1980s, HBV transgenic mice selectively expressing HBV proteins, including HBsAg, e antigen, or x protein, have been generated to check the role of those proteins (Chisari *et al.* 1987). Guidotti et al. developed transgenic mouse models with full HBV genome, which produced infectious HBV virions morphologically indistinguishable from human-derived virions (Guidotti *et al.* 1995). HBV is immune-tolerant and cannot be spontaneously cleared as its genome is integrated in the host/mouse genome; this model has made important contributions in deciphering the knowledge on the pathogenesis of HBV infection and is also used for testing many antiviral drugs, such as lamivudine, adefovir dipivoxil, and entecavir (Ebert *et al.* 2011).

2.5.3.2.2. HBV transfected mouse model

Viral vectors supported adenoviruses or adeno-associated viruses containing HBV genomes are efficiently transduced into the hepatocytes of immunocompetent mice. After shot of high doses of HBV containing adenoviral vectors (Ad-HBV), HBV replication lasts less than three months because transduction of ad-HBV leads to a robust immune reaction against the adenovirus itself. This problem can be minimized by the injection of low doses of Ad-HBV (Huang *et al.* 2012, 2012). Delivery of naked HBV DNA via injection into tail vein referred to as “hydrodynamic injection” was also used successfully to deliver replication-competent HBV genomes into the livers of immunocompetent mice, with a viremia peaking after 6 d and rapidly declining thereafter (Yang *et al.* 2002). The HBV replication and duration in mice relies on the plasmid backbone, mouse strain, and also the sex. To further closely mimic chronic HBV infection with the persistence of HBV cccDNA in humans. Further, a surrogate mouse model with HBV cccDNA persistence was established using a replication-defective recombinant adenoviral vector, recombinant HBV cccDNA was successfully delivered into the mouse liver (Li *et al.* 2018) .

2.5.4. HBV associated hepatic steatosis

Concurrent steatosis is a common histological feature of hepatitis C infection, while the prevalence and link of hepatic steatosis to HBV is quite limited and also controversial. According to recent epidemiological studies, the risk of nonalcoholic fatty liver disease development is reduced by HBV infection (Hu *et al.* 2018; Machado *et al.* 2011). In contrast, there are some studies which reveal the presence of hepatic steatosis in the range 25% and 51% in patients with HBV (Baclig *et al.* 2018; Machado *et al.* 2011). Furthermore, recent studies showed that HBV may be an inflicting source for the development of NAFLD *in vitro* via increased production of mitochondrial ROS (Wang *et al.* 2019). Regardless of the baseline HBV viral load, CHB patients with liver steatosis are at higher risk of mortality and HCC development than CHB patient without liver steatosis (Peleg *et al.* 2019). Indeed, there are some studies which present an opposite view. Studies conducted by Machado et al. (Machado *et al.* 2011) showed no positive association of HBV infection and hepatic steatosis. Likewise, HBV replication is adversely affected with hepatic steatosis inducing higher chance of HBV seroclearance in an immunocompetent HBV replicating mouse model. Similarly, a recent study reported hypolipidemia in HBV patients (Quaye *et al.* 2019).

The mechanism of steatosis in HBV infection is still elusive. HBV X protein (HBx) is known to promote lipid storage *in vitro* (Kim *et al.* 2007). Furthermore, a recent study has shown that HBx induces steatosis through inhibition of apoB secretion and induction of peroxisome proliferator activated receptor γ (PPARγ), and sterol regulatory element binding protein 1c (SREBP-1c) (Kim *et al.* 2007). Steatosis is also induced through a direct interaction with liver X receptor a (LXRa) or tumor necrosis factor (TNF) receptor 1 leading to NFkB activation and TNF production (Kim et al. 2010). Chronic hepatitis B infection with cytokines surges could alter plasma lipid distributions (Quaye *et al.* 2019**).** Several studies have demonstrated that proinflammatory cytokines can induce lipogenesis (Fabris *et al.* 1997). Recent studies also supported that HBx-induced hepatic lipid accumulation via regulation of FABP1, HNF3β, C/EBPα, and PPARα (Wu *et al.* 2015). To further understand the HBV-steatosis conundrum humanized mouse models of HBV may provide a more detailed insight into the disease development (Bility *et al.* 2014).

2.6. Endocannabinoid system (ECS)

2.6.1. Introduction and ECS receptors

Since time immemorial psychoactive and medicinal properties of Marijuana (cannabis) is known. With the discovery of the endocannabinoid system, the pharmacological activity and the mechanistic pathways of cannabinoids have been developed. The endocannabinoid system consist of two specific G-protein coupled receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), their endogenous lipid ligands (endocannabinoids), and the enzymes involved in their synthesis and degradation (Mallat *et al.* 2013).

The endocannabinoid system (ECS) plays an important role in many functions both, in central and peripheral tissues. ECS influences food intake, cardiovascular, gastrointestinal, immune, behavioral, anti-proliferative, and reproductive functions. The ECS is a lipid signaling system, consisting of the endogenous cannabis-like ligands (endocannabinoids) anandamide (AEA) and 2-arachidonoylglycerol (2-AG), derived from arachidonic acid (Mouslech and Valla 2009).

Both CB1 and CB2 receptors are G-protein-coupled protein receptors (GPCR) characterized by seven trans-membrane domains (Schatz *et al.* 1997, 1997). CB1 receptor is mainly expressed in the brain areas which are connected with motor control, emotional responses, behavioral, and energy homeostasis. Similarly, CB1 receptor is also expressed in peripheral tissues such as adipose tissue, pancreas, liver, gastrointestinal tract, skeletal muscles, heart, and the reproduction system (Cota *et al.* 2003). The activation of CB1R is associated mainly with regulation and maintenance of energy homeostasis. The CB2R is only expressed in cells of the immune system, such as B cells, monocytes, and T-cells. The expression level varies among different immune cells and their activation state (Howlett 2002), indicating their role in inflammatory processes. For example, CB2R expression was enhanced with degree of immune cells differentiation (Munro *et al.* 1993). In a nutshell, CB2R were mainly linked with a variety of immune cell functions, such as migration, proliferation, and cell death (Kloet and Woods 2009). Apart from the endogenous ligand, exogenous ligands namely Δ9- Tetrahydrocannabinol (THC) has been discovered from cannabis, having similar binding affinity to both, CB1 and CB2 receptor (Tam *et al.* 2011). Endocannabinoids are synthesized and transported into cells via receptors mediating uptake and degradation by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) enzymes (Mallat *et al.* 2013).

2.6.2. Role of ECS in hepatic steatosis and chronic diseases

During the normal physiological condition, the expression level of CB1R in the liver is very low. However, during any liver injury such as HFD induced steatosis, chronic HCV or HBV infection etc., the expression of CB1R is elevated (Osei-Hyiaman *et al.* 2008). On the other side, the endogenous hepatic ligands of CB1R, both AEA and 2-AG expression in the liver are comparable to that of the brain (Osei-Hyiaman *et al.* 2005). The CB1 and CB2 expression pattern in the liver has been linked with metabolic disorders, such as obesity, NAFLD, etc. (Tam *et al.* 2011). Excess energy intake in HFD or obesity triggered the hepatic CB1R signaling, which orients hepatic metabolism toward energy storage, but may be a reason for body fat accumulation (Osei-Hyiaman *et al.* 2008). Hepatic CB1R activation is linked with elevated *de-novo* synthesis of FA (Osei-Hyiaman *et al.* 2005) via activation of lipogenic transcription factors, steroid regulatory element-binding protein-1c (SREBP-1c), fatty acid synthase (FAS), and acetyl-coA carboxylase-1 (ACC-1) as demonstrated in wild type mouse mice compared to CB1-knockout mice fed with chow diet (Tam *et al.* 2011). Therefore, all these studies supported a connection between excess energy storage and the activation of the ECS resulting in hepatic steatosis, insulin resistance, hyperglycaemia, and dyslipidaemia (Auguet *et al.* 2014).

Liver fibrosis is a typical hallmark response to chronic liver injury resulting in cirrhosis, liver failure, and hepatocellular carcinoma (Teixeira-Clerc *et al.* 2006). The endocannabinoid system exerts either profibrotic effects possibly mediated by CB1 receptors and antifibrogenic effects via CB2 receptors (Tam *et al.* 2011). In a normal physiological state, CB1 and CB2 receptor expression remains undetectable in the liver, but they are prominently expressed either in injured and fibrotic mouse liver or in human CHB patients (Dai *et al.* 2017; Julien *et al.* 2005). However, during development of fibrosis the pro-fibrogenic CB1 receptor may outweigh the anti-fibrogenic CB2 receptor (Dai *et al.* 2017). CB2 mediates its anti-fibrotic and hepatoprotective effects by inducing anti-proliferative effects and apoptosis of myofibroblasts and stellate cells (Julien *et al.* 2005). Similarly $CB2^{-/-}$ mice showed enhanced response to fibrogenic stimuli (Julien *et al.* 2005). Substantial studies have elucidated the use of non-psychoactive CB2 agonists in the treatment of liver fibrosis proving a great therapeutic potential (Julien *et al.* 2005). In contrast, human HCV patients with daily use of cannabis showed increased fibrosis rather than reduction (Hézode *et al.* 2008). Different mouse models of liver

fibrosis showed enhanced CB1 receptor expression in stellate cells and hepatic myofibroblast. Genetic deletion or pharmacological ablation of CB1 receptors showed protection against liver fibrosis (Teixeira-Clerc *et al.* 2006).

2.7. ATP Binding Cassette Subfamily B Member 4 (ABCB4)

2.7.1. Introduction

A hallmark of cholestasis is hepatic and biliary damage due to retention of toxic biliary components. As a result of damage, inflammation, fibrosis, cirrhosis, and eventually carcinogenesis were observed (Cuperus *et al.* 2014). Various hepatocellular transporter proteins present at the basolateral and canalicular membrane, which are responsible for the transport of bile, salts and lipids (Reichert and Lammert 2018). Various ATP-binding cassette (ABC) transporter proteins which are present in the canalicular membrane are associated to bile formation and secretions of bile acids (ABCB11) (Gerloff *et al.* 1998), bilirubin (ABCC2) (Paulusma *et al.* 1997), phosphatidylcholine (ABCB4) (Smit *et al.* 1993), cholesterol (ABCG5/G8) (Berge *et al.* 2000), and drugs (ABCB1, ABCC2, ABCG2) (Cuperus *et al.* 2014).

Among them, ATP-binding cassette subfamily B member 4 (ABCB4) is a phospholipid floppase which “flops” phosphatidylcholine into bile, a critical function that confers protection to cholangiocyte membranes from being exposed to high concentrations of detergent effect of bile acids (Reichert and Lammert 2018). ABCB4 is 170kDa protein with two nucleotide-binding and two 6-helical transmembrane domains. The ABCB4 gene is localized on 7q21.1 chromosome (Lincke *et al.* 1991). ABCB4 is mainly expressed in the canalicular membrane of the hepatocytes. Low levels of Abcb4 transcripts have been detected in other organs such as adrenal gland, tonsil, brain, spleen and also in muscle (Cui *et al.* 2009).

The protein has several names in different species. It is named as MDR3 P-glycoprotein in humans and called Mdr2 P-glycoprotein in rodents (Oude Elferink, Ronald P. J. and Paulusma 2007). With different homologues found in rats and mice, which make the nomenclature more confusing. Therefore, the new nomenclature of ABC transporters is now uniformly called as ABCB4 in humans and Abcb4 in non-human species (Oude Elferink, Ronald P. J. and Paulusma 2007).

ABCB4 expression is regulated via transcriptional and posttranscriptional mechanisms. Studies have demonstrated enhancement of Abcb4/ABCB4 expression after treatment with fenofibrate, a PPARα agonist in cultured rat and human hepatocyte cell lines (Ghonem *et al.* 2014) . Bile salts and especially cholate feeding may induce Abcb4 expression in rodents (Gupta *et al.* 2000). Similarly, the artificial FXR agonist GW4064 also induced *Abcb4*/*ABCB4* expression in both, rat and human hepatocytes (Liu *et al.* 2003).

2.7.2. ABCB4 and chronic cholestatic liver disease

ABCB4 protein defects or deletion is associated with damage of bile canaliculi and small bile ducts, that subsequently leads to chronic and progressive liver disease (Stapelbroek *et al.* 2010). Mutation of the Abcb4 gene has been associated with several disease conditions such as progressive familial intrahepatic cholestasis type 3 (PFIC3), intrahepatic cholestasis of pregnancy (ICP), and low phospholipid-associated cholelithiasis (LPAC) (Reichert and Lammert 2018), chronic cholangiopathy, drug-induced liver injury (DILI), adult biliary fibrosis or cirrhosis, and transient neonatal cholestasis (TNC) (Sticova and Jirsa 2019). PFIC3 is a inheritable liver disease with chronic and progressive cholestasis that may progress to the end stage liver disease (Reichert and Lammert 2018). Other fibrotic biliary diseases in human such as primary sclerosing cholangitis has been associated with Abcb4 transporter deficiency (Thoeni *et al.* 2019). The coordinate cooperation between bile salt export pump (Bsep) (ABCB11) and ABCB4 transporter helps in the detoxification of bile compounds from the hepatocytes (Baghdasaryan *et al.* 2016). PFIC2 and 3 is caused by mutation of *ABCB11* and *ABCB4* genes, responsible for severe cholestatic liver disease in children (Baghdasaryan *et al.* 2016). Alteration of lipid homeostasis due to the lack of Abcb4 provokes aberrant inflammatory response and exacerbates EHBA phenotype in neonatal mice (Carey *et al.* 2017). Apart from maintaining phospholipid homeostasis, ABCB4 also plays a critical role in glucose homeostasis in mice and humans mainly mediated via the LRH-1-dependent PC pathway (Hochrath *et al.* 2012).

2.7.3. Bile acids metabolism and chronic cholestasis

Bile acids are synthesized from cholesterol in the liver. Bile synthesis occurs through a cascade of enzymatic reactions. Herein 7α-hydroxylase enzyme catalyzes the rate limiting step which converts cholesterol into 7α-hydroxycholesterol (Cuperus *et al.* 2014). Subsequently, the bile acids are than conjugated with either taurine or glycine and called as “bile salts”. These bile salts

are excreted via ABCB11 and ABCC2 into the canalicular lumen. 95 % of secreted bile is reabsorbed in the terminal intestine through a sodium dependent transport mechanism. They return back to the liver via the enterohepatic circulation and the basolateral bile transporters mediate their uptake. The sodium/taurocholate cotransporting polypeptide (NTCP) transporter mediates the majority uptake (~90%) of these bile acids. The remaining 10% are transported through sodium independent organic anion transporters such as OATP1A2, OATP1B1, and OATP1B3 (Hagenbuch and Meier 1994).

The nuclear receptor, farnesoid X receptor (FXR) maintains bile acid homeostasis (Makishima *et al.* 1999). In rodent and human hepatocytes, bile acid activated FXR mediates reduction of hepatic bile acid uptake (NTCP) and synthesis (CYP7A1/CYP8B1), while promoting canalicular (ABCB11, ABCC2) and basolateral bile acid excretion (Cuperus *et al.* 2014; Gerloff *et al.* 1998). FXR mediates the activation of the short heterodimer partner (SHP), further represses the transcription of NTCP, CYP7A1, and CYP8B1 (Gupta *et al.* 2000). Bile acid detoxification via CYP3A4, SULT2A1, and UGT2B4, is also mediated by FXR therefore conferring hepatic protection (Zollner *et al.* 2006). There are other nuclear receptors such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) that are also involved in bile acid metabolism (Huang *et al.* 2003). The three main solute components of bile are bile acids, phospholipids and cholesterol. These major solutes together form mixed micelles that enhance cholesterol solubility and also reduce the toxicity of bile acids. Therefore, any impairment in bile acid or phospholipid secretion or toxic bile acid accumulation serves as potential source for hepatobiliary system damages with bile duct epithelial cells injury and inflammation (Zollner *et al.* 2006). Intrahepatic cholestasis is potentially induced either by congenital or acquired defects in canalicular membrane transporter proteins (Zollner and Trauner 2008).

Promising therapeutic approaches for the treatment of Abcb4-associated cholestasis have been published. For instance, hepatocarcinogenesis in Abcb4$^{-/-}$ mice is aborted with long-term administration of nuclear bile acid receptor, FXR agonist (Cariello *et al.* 2017). Enhanced IL-1β tone is correlated with the degree of liver fibrosis and HSC proliferation in Abcb4$^{-/-}$ mice and pharmacological antagonism of IL-1 showed anti-fibrotic effects *in vitro* but not in Abcb4$^{-/-}$ mice (Reiter *et al.* 2016). A recent study reported the use of a unique synthetic variant of FGF19,

called FGF19-M52, that suppressed hepatic bile acid synthesis and protected from fibrosis and hepatocarcinogenesis (Gadaleta *et al.* 2018). Dietary supplementation of lecithin could also protect from cholestatic liver disease in cholic acid fed Abcb4 deficient mice (Lamireau *et al.* 2007). The production of fibrotic scars in livers of *Mdr2*$^{-/-}$ mice was mainly contributed to activated portal fibroblasts and hepatic stellate cells, and these cells might be therapeutic targets for cholestatic injury and fibrosis (Nishio *et al.* 2019). Recent studies showed the therapeutic potential of the inhibition of NADPH oxidase (NOX) in reversing cholestatic fibrosis in *Mdr2*$^{-/-}$ mice (Nishio *et al.* 2019) .

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Animals

3.1.1. CB1$^{-/-}$-HBs– (Project 1)

Global CB1 receptor knockout mice were purchased from the European Mouse Mutant Archive. Generation and characteristics of the transgenic lineage C.B6J-Tg (Alb1HBV) 44Bri (HBVTg/c) have been described previously (Churin *et al.* 2014). Hybrids of CB1$^{-/-}$ crossbred with HBVTg/6 mice on the C57BL/6 genetic background were generated. At the age of 12, 26, and 52 weeks mice were anaesthetized by isofluran inhalation and subsequently killed by cervical dislocation. Body and liver weight analyses were performed. Liver and serum samples were collected and preserved at -80°C until further analysis. To determine routine laboratory analysis, serum samples were used for analysis of liver function enzymes, aspartate and alanine aminotransferases (AST, ALT) using Reflotron plus analyzer (Roche, Mannheim, Germany).

Transgenic mice were maintained in the central animal laboratory of the Justus Liebig University Giessen under specified pathogen-free conditions. This study was carried out in strict accordance as per the recommendations laid in the guideline for the care and use of laboratory animals of the German law of animal welfare. The mice received humane care and all experiments were approved by the committee on the ethics of animal experiments of the Regierungspraesidium Giessen, Germany (approved no, GI 20/10 A5/2012 and 128/2014).

3.1.2 Abcb4$^{-/-}$ -HBs –(Project 2)

BALB/c-Abcb4$^{-/-}$ mice (C.FVB (129P2)-Abcb4tm1Bor named as Abcb4$^{-/-}$ mice) were bred and housed as described previously (Roderfeld *et al.* 2010). Confirmation of the Abcb4$^{-/-}$ genotype mice was performed by polymerase chain reaction (PCR) as described earlier (Roderfeld *et al.* 2010). Generation and characteristics of the transgenic lineages Tg (Alb-1HBV) (C57BL/6J-Tg (Alb1HBV) 44Bri/J) have been described (Churin et al. 2014). These mice were crossed back to the genetic background BALB/cJ (C.B6J-Tg (Alb1HBV) 44Bri N10 herein called HBs mice) for 9 generations. HBs mice were crossed with Abcb4$^{-/-}$ mice, resulting in the F2 generation BALB/c-Abcb4/Alb1-HBV hybrid mice (C.Cg-Tg (Alb1HBV) 44Bri-Abcb4tm1Bor herein called HBs/Abcb4$^{-/-}$mice). At the age of 12-19 weeks, mice were killed by CO_2-inhalation (n = 5 per age and both sex). The body and liver weight analyses were done. Liver and serum samples

were collected and preserved at −80°C until further analysis. For routine laboratory analyses, serum samples were used for analysis of liver function enzymes such as aspartate and alanine aminotransferases (AST, ALT), alkaline phosphatase (AP) along with triacylglycerides (TAG) and cholesterol (CHOL) by using Reflotron Plus Analyzer (Roche, Mannheim, Germany). This study was carried out in strict accordance as per the recommendations in the guideline for the care and use of laboratory animals of the German law of animal welfare. All experiments were approved by the Committee on the ethics of Animal Experiments of the Regierungspraesidium Giessen, Giessen, Germany (permit number: V54-19c 2015c GI20/10 Nr. A36/2011, Nr. A5/2012 andNr.52_2011).

3.2. Cells culture

3.2.1. Chemicals consumables and instruments used in cell culture experiments

Table 1a: Chemicals and consumables for cell culture

Chemicals	Manufacturer	Product number
Dimethyl sulfoxide (DMSO)	Carl Roth	A994.1
Dulbecco's Modified Eagle Medium (DMEM) F12	Gibco, Grand Island, USA	11320033
Fetal calf serum (FCS)-Good	PAN BiotechGmbH, Aidenbach, Germany	P40-37500
Pen-Strep-Amphotericin B mix	PAN BiotechGmbH, Aidenbach, Germany	P06-07350
BSA conjugated Oleic acids	Sigma-Aldrich (Merck), Darmstadt, Germany	O3008-5ML
Rimonabant	Sigma-Aldrich (Merck), Darmstadt, Germany	SML0800-5MG
Methanandamide	Sigma-Aldrich (Merck), Darmstadt, Germany	M186-5MG
IST (100x) mixture	Gibco, Thermo scientific, U.S.	41400045
Dexamethasone	Sigma-Aldrich, St. Louis, MO	D8893
Bile salts	Sigma-Aldrich (Merck), Darmstadt, Germany	48305-50G-F
Dorsomorphin	Merck, Darmstadt, Germany	P5499-5MG
Sterile DPBS	Lonza, Basel, Switzerland	17-512F
Collagen I, Rat tail	Thermo scientific	A10483-01
Trypsin EDTA	PAN BiotechGmbH, Aidenbach, Germany	P10-0231SP

Accutase	Invitrogen, Thermo scientific, U.S.	00-4555-56
Consumables		
CELLSTAR® cell culture dish	Greiner bio-one, Austria	664160
CELLSTAR® 6 Well Cell Culture Plate	Greiner bio-one, Austria	657160
CELLSTAR® 12 Well Cell Culture Plate	Greiner bio-one, Austria	66510
CELLSTAR® 24 Well Cell Culture Plate	Greiner bio-one, Austria	662102
CELLSTAR® serological pipette 2ml	Greiner bio-one, Austria	P7990-1000EA
CELLSTAR® serological pipette 5ml	Greiner bio-one, Austria	P7615-200EA
CELLSTAR® serological pipette 10ml	Greiner bio-one, Austria	P7740-200EA
Biosphere Filter tips	Sarstedt, Nümbrecht, Germany	70.760.211, 70.762.211,70.1130.210,
Cover slips	R Lagenbrinck GmbH, Emmendingen, Germany	01-0012/1

Table 1b: Equipments used in cell culture

Instruments	Manufacturer	Version and Product number
CRYO SAFE -1C Freeze control	SP Scienceware,Pennsylvania	F18844-0000
Heraeus safty cabinet LaminAir	Heraeus, Hanau, Germany	HBB 2448
Incubator	Thermo Scientific™, U.S.	HeraCell 150i
Microscopes	Leica Microsystems, Wetzlar, Germany	Leitz DMR3 DM/LH6
Electronic analytical balances	Sartorius Göttigen, Germany Faust,Schaffhausen, Switzerland	Analytical scale, A200S Table scale, FA-1500-2
Thermomixer	Eppendorf, Hamburg Germany	5436
UltraRocker™ Rocking Plattform	Bio-Rad	1660719EDU

Vortex	Janke & Kunkel IKA Labortechnik, Staufen, Germany	AL.16.P.0.10942
Water Bath	GFL, Hanover, Germany	Model no: 1003
-80°C freezer	Thermo Scientific™, U.S.	HFU500TV
Camera	Nikon, Minato, Japan	CoolHx540
Centrifuges	Beckmann, California, U.S.	GC-6KR

3.2.2. AML12 cell culture protocol

AML12 (alpha mouse liver 12) cell line derived from mouse hepatocytes (CD1 strain, line MT42) and transgenic for human TGF alpha was received as a kind gift from Prof. Ralf Weißkirchen, Institut für Molekulare Pathobiochemie, Experimentelle Gentherapie und Klinische Chemie, Aachen, Germany. The cells were cultured in 10 ml of DMEM-F12 medium (Gibco, Grand Island, USA) supplemented with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium (ITS mixture, Gibco), 40 ng/ml dexamethasone (Sigma-Aldrich, St. Louis, MO) in a culture dish with 10% fetal calf serum, 1% penicillin, 1% streptomycin, 1% fungizone at 37 °C in a humidified atmosphere with 5% CO2 air. Cells were plated at a split ratio of 1:4. In all the experiments, the treatment was done in a pre-confluent stage (80-90%). The pre-confluent cells were pre-treated with oleic acid (BSA conjugated oleic acid, Sigma, #O3008-5ML) at a concentration of 200µM for 12h. Then medium was changed, washed with sterile PBS and re-incubated with fresh medium. The CB1 receptor antagonist and agonist, rimonabant and methanadamide were used for cell stimulation at conc. of 1µM for 24h. At the end of the experiments, the cells were fixed using 4% PFA at -20 °C for 15 min followed by washing in sterile cold PBS for three times before performing immunofluorescence staining. For western blot analysis, the cells were lysed directly using 1x Laemmli buffer, boiled at 99 °C for 10 min and followed by short centrifugation. The lysates were stored at -20 °C till used.

3.2.3. HepG2 cell culture protocol:

HepG2 cell lines were obtained from CLS services Germany. The cells were cultured in cell culture dishes using 12 ml of DMEM-F12 medium supplemented with 10% fetal calf serum, 1% penicillin, 1% streptomycin, 1% fungizone and grown at 37°C humidified air with 5% CO2 air. Cells were plated at a split ratio of 1:4 into 12 well cultured plates. The cells were grown up to pre-confluent cells (80-90%) and were left either untreated (control cells) or pretreated with oleic

acid (BSA conjugated oleic acid, Sigma # O3008-5ML), at 250µM concentration for 12h. Subsequently, treatment with bile salts (cholate and deoxy cholate) (Sigma-Aldrich, cat no. 48305-50G-F) at150µM concentration was done in fresh serum free medium after washing the cells once in PBS. Similarly, for inhibitor assays, the cells preloaded with oleic acid were pre-treated with dorsomorphin (AMPK inhibitor, Merck, P5499-5MG) at 10µM concentration for 1h prior to treatment with the bile salts. After the treatment for 1h, the cells are washed in sterile PBS once and fresh serum free DMEM-F12 was added. Bile acid was added and cells were further incubated for the next 24h. At the end of the experiments, the cells were lysed directly using 1x Laemmeli buffer. The cell lysates were boiled at 99 °C for 10 min followed by short centrifugation and the samples were stored at -20 °C till used for western blot analysis.

3.3. Western blot

Western blotting, also called immunoblotting is a technique to detect a specific antigen using a specific antibody. It was introduced by Towbin, et al. in 1979 (Towbin *et al.* 1979), since then became a routine technique for protein analysis. It is a rapid and sensitive assay for detection and characterization of proteins, which is based on the principle of immunochromatography, where proteins are separated into polyacrylamide gel according to their molecular weight. After the electrophoresis, the gel is placed onto a nitrocellulose membrane and assembles between the layers of filter papers; then electrotransferred either by wet methods or semi dry methods. The transferred proteins on the membrane are detected by specific primary and secondary enzyme labeled antibody and substrate.

3.3.1. Chemicals

Table 2: A list of chemicals and reagents used in western blotting

Chemicals and reagents	Manufacturer	Product number
Ammonium persulfate (APS)	Carl Roth, Karlsruhe, Germany	9592.2
p-Coumaric acid	Sigma Aldrich, Germany	C9008-10G
Luminol	Sigma Aldrich, Germany	A8511-5G
Disodium phosphate ($Na2HPO_4$)	Carl Roth, Karlsruhe, Germany	
Potassium dihydrogen phosphate (KH2PO4)	Carl Roth, Karlsruhe, Germany	P030.2
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany	3904.1

Potassium chloride (KCl)	Carl Roth, Karlsruhe, Germany	3957.1
Magnesium chloride	Carl Roth, Karlsruhe, Germany	KK36.2
Rotiphorese ® Gel 30	Carl Roth, Karlsruhe, Germany	3029.01
Tris-base	Carl Roth, Karlsruhe, Germany	4855.2
Glycine	Carl Roth, Karlsruhe, Germany	3908.2
Sodium dodecyl sulfate (SDS)	Serva Electrophoresis	0765.03
Tetramethylethylenediamine (TEMED)	Serva Electrophoresis	35925.01
Hydrochloride (HCL)	Carl Roth, Karlsruhe, Germany	K025.1
Methanol	Carl Roth, Karlsruhe, Germany	4627.1
Milk powder	Carl Roth, Karlsruhe, Germany	T145.3
Nitro blue tetrazolium chloride (NBT)	Carl Roth, Karlsruhe, Germany	4421.3
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	Carl Roth, Karlsruhe, Germany	6368
PageRuler^{TM} Prestained Protein Ladder	Thermo Fisher Scientific	26617
Tween 20	Sigma Aldrich, Germany	P1379-500ML
Bromophenol Blue	Carl Roth, Karlsruhe, Germany	A512.1
DDT	Carl Roth, Karlsruhe, Germany	6908.3

3.3.2. Buffers and solutions

a. 10x PBS recipe:

- 80 g sodium chloride (NaCl) (=1.37 M)
- 2 g potassium chloride (KCl) (=27 mM)
- 2.45 g potassium dihydrogen phosphate (KH2PO4) (=18 mM)
- 11.5 g disodium phosphate ($Na2HPO_4$) (81 mM)
- Addition of distilled H_2O (final volume: 1 L), pH 7.4

b. 10x TBS recipe:

- 24.23 g Tris base
- 80 g NaCl
- Addition of distilled H_2O (final volume: 1 L), pH 7.5

For 1x TBS-T buffer: 10x TBS 1:10 + 1mL TWEEN® 20 (for 1 L)

c. 10x electrophoresis running buffer

- 10 g SDS (=1% w/v resp. 35 mM)
- 30.3 g Tris-base (=0.25 M)
- 144 g Glycin (=1.92 M)

- Addition of distilled H_2O (final volume: 1 L), pH 8.3

d. 2M Tris/HCL buffer (pH 8.8)

- Tris base: 242.28 grams
- Addition of HCl for adjusting the pH to 8.8
- Addition of distilled H_2O (final volume: 1000 mL)

e. 4x Tris-Cl buffer (SDS-PAGE stacking gel)

- 6.05 g Tris-base (=0.5 M) in 40 mL H_2O_{dd}
- adjust pH to 6.8 with 1 M HCl
- addition of 100 mL distilled H_2O
- 0.4 g SDS (0.4%)

f. Alkaline phosphatase (AP) incubation buffer

- 2.92 g NaCl
- 0.23 g $MgCl_2$
- 6.06 g Tris base

g. Western blot transfer buffer

- 5.32 g Tris base
- 3.93 g glycine
- Addition of 500 mL H_2O_{dd}
- 200 mL methanol
- Addition of distilled H_2O (final volume: 1 L), pH 9.2

h. Acrymalide: Bisacrylamide (30:5) (ready made from Carl Roth, cat no. 3029.1)

Table 3: 4x sample buffer (loading buffer)

Reagents/chemical	Amount
40% v/v Glycerol	20ml
0.25 M Tris (pH6.8) buffer	6.25ml
0.02% w/v Bromophenol blue	10mg
8% w/v SDS	4g
400mM Dithiothreitol	3.08g
Adjust the final volume with Distilled water (H_2O_{dd})	23ml
Total	50ml

Table 4: Various gel percentage composition for making separating gel

Reagents	6%	8%	10%	12%	15%
Distilled water (H_2O_{dd})	5.85	5.25	4.45	3.85	2.85
Acrylamide/bisacrylamide (30% 0.8w/v)	2ml	2.6ml	3.4ml	4ml	5ml
2 M Tris (pH8.8)	1.95ml	1.95ml	1.95ml	1.95ml	1.95 ml
10% (w/v) SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate(APS)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl
Total volume	10ml	10ml	10ml	10ml	10ml

Table 5: Stacking gel (3.3%) compositions

Reagents	volume
Distilled water (H_2O_{dd})	2130 µL
Acrylamide/bisacrylamide(30%0.8w/v)	330 µL
4x Tris- buffer pH 6.8	830 µL
10%(w/v) ammonium persulfate(APS)	50µl
TEMED	5µl

Table 6: List of primary antibodies used for western blotting

Primary antibody	Species	Manufacturer	Dilution used
P-AMPK	Rabbit	Cell signaling,2535S	1:1000
AMPK	Rabbit	Genetex, GTX50863-100	1:1000
PLIN2	Rabbit	Proteintech, 15294-1-AP	1:1000
PLIN3	Rabbit	Progen, 70011	1:1000
PLIN4	Rabbit	Merck,ABS526	1:1000
PLIN5	Rabbit	Progen, 70011	1:1000
GAPDH	Mouse	Cloud clone, CAB932Hu01	1:5000
β-actin	Mouse	Santa cruz,	1:5000
LC3B	Rabbit	Novus, NB100-2220SS	1:1000
p62/SQSTM1	Rabbit	Novus, NBP1-48320SS	1:1000
P-CREB	Rabbit	Cell signaling, 9198S	1:1000
PPARγ	Rabbit	Cell signaling, 2435S	1:1000
FASN	Rabbit	Cell signaling, 3180S	1:1000
ACC1	Rabbit	Cell signaling ,3676S	1:1000
CIDEC	Rabbit	Novus,NB100-430SS	1:1000

Table 7: List of secondary antibodies

Secondary antibody	Species	Manufacturer	Product number
Mouse anti-goat IgG HRP-linked	Goat	Invitrogen	A16072
Rabbit anti-goat IgG HRP-linked	Goat	Invitrogen	A16104
Mouse anti-goat IgG AP-linked	Goat	Invitrogen	A16069
Rabbit anti-goat IgG AP-linked	Goat	Invitrogen	A16107

3.3.3. Consumables and equipment:

a. Mini protean electrophoresis system (BIORAD™)
b. Western blot transfer machine (Biometra, An analytik Jena company)
c. Nitrocellulose (Merck, Darmstadt, Germany)
d. PVD membrane (Merck, Darmstadt, Germany)
e. Filter paper (Whatmann, Merck, Darmstadt, Germany)
f. Amersham Hyperfilm™ ECL (GE Healthcare, limited, UK)
g. X ray machine (AGFA, CP1000, USA)
h. Intas chemostar (Intas science imaging instrument, Göttingen, Germany)

3.3.4. Procedure:

Liver tissues from each group of animals were weighed and mixed with 1x Laemmli buffer at a ratio of 1:40, subjected to a short homogenization followed by boiling at 95°C for 10 min and then briefly centrifuged for 5 min. Alternatively, liver samples were weighed and homogenized in 1% Tritonx-100 buffer (1% Tritonx-100,150mM NaCl, 50mM Tris pH 8.0 with protease inhibitor) briefly for few seconds and the protein concentration of the triton liver lysates was determined using the BCA kit assay method. The liver lysates were aliquoted and stored at -80°C till used. Just before use, the triton liver lysate was mixed with 4x Laemmli and boiled for 5min. After SDS-PAGE, the gel was transferred to nitrocellulose membrane and immunoblotting procedures were followed by the standard protocol. Proteins recognition was done by using specific antibodies against the primary antibodies. The proteins were visualized using peroxidase-conjugated secondary antibodies and chemiluminescent reagent, developed on X-ray developer machine (AGFA, CP1000), using high sensitivity film (Hypersensitive film, Amersham) or Intas chemostar (Intas imaging instrument, Göttingen). Alternatively, proteins

were visualized using alkaline phosphatase conjugated secondary antibodies and developed with soluble 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

3.4. Immunohistochemistry

The principle of immunohistochemistry is the specific binding of an antibody to its antigen on either a frozen or paraffin-embedded tissue. This method detects antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The antibody-antigen binding reaction can envisage in different manners using secondary antibody conjugated with Horseradish Peroxidase (HRP) or alkaline phosphatase (AP) enzymes and substrate are used to produce a color- reaction.

3.4.1. Reagents (for Paraffin):

a. **Xylol**, (Merck, Darmstadt, Germany)

b. **0.1M Citrate-Buffer, pH 6.0**

- Stocksolution A: 0,1M Citricacid ($C_6H_8O_7$) = 21.01g,
  1L of aqua dest. water was added (buffer pH 2.1)

- Stocksolution B: 0.1M tri-Na-Citratedihydrat ($C_6H_5Na_3O_7$ x $2H_2O$) = 29.41g
  1L of aqua dest. water was added
- Working solution: 9ml solution A + 41ml solution B added to 450ml aqua dest. water, pH= 6, each buffer stored at 4°C.

c. Phosphor-Buffered-Saline (Carl Roth, Karlsruhe, Germany cat# Art. No. 1112.2)

d. 30% Hydrogen-Peroxide (H_2O_2), 250ml (Merck, Darmstadt, Germany)

e. 10% BSA (Carl Roth,Karlsruhe, Germany, cat# Art. No. 8076.1)

f. Mayer's Haematoxylin (Carl Roth, Karlsruhe, Germany)

g. MicroSlides (Thermo Scientific™ SuperFrost Ultra Plus™ Adhesion Slides).

h. Aquatex, 50ml (Merck, Darmstadt, Germany)

i. Vector ImmPRESS ™ HRP REAGENT KIT, Vector ImmPRESS ™ AP REAGENT KIT (Vector laboratories, Inc, Burlingame, U.S.; cat# CA 4010)

j. Vector@ Red, alkaline phosphatase substrate kit, Vector@ Blue alkaline phosphatase substrate kit, Vector@ VIP Peroxidase substrate Kit, Vector DAB peroxidase substrate kit (Vector laboratories, Inc, Burlingame, U.S.; CA 4010).

3.4.2. Reagents (for Cryo section):

a. Phosphor-Buffered-Saline (Carl Roth, Karlsruhe, Germany cat# Art. No. 1112.2)
b. 10% BSA (Carl Roth, cat# Art. No. 8076.1)
c. Vector ImmPRESS ™ HRP REAGENT KIT, Vector ImmPRESS ™ AP REAGENT KIT (Vector laboratories, Inc, Burlingame,U.S.; CA 4010)
d. Microscop Slides: Micro Slides, x-traTM Adhesive (Surgipath-Leica, Wetzlar, Gemany)
e. Aquatex (Merck, Darmstadt, Germany)
f. 4% neutral buffered formaldehyde (Merck, Darmstadt, Germany) solution prepared in 1x PBS with slight NaOH addition to increase the solubility and final pH adjustment to 7.0 using conc. HCL.
g. Methanol (Merck, Darmstadt, Germany)
h. Acetone (Merck, Darmstadt, Germany)

3.4.3. List of antibodies used for IHC

Table 8: List of primary antibodies used for IHC

Primary antibody	Species	Manufacturer	Product number	Dilution used
PLIN2	rabbit	Proteintech	15294-1-AP	1:200
ATGL	mouse	Cell signaling	CST 61038	1:100
LPL	Rabbit	Proteintech;	21133-1-AP	1:100
GLUT1	Rabbit	Abcam	ab115730	1:100
LAMP1	Mouse	Genetex	GT25212#3302	1:100
LC3B	Rabbit	Novus	NB100-2220SS	1:200
p62/SQSTM1	Rabbit	Novus	NBP1-48320SS	1:100
MGAT1	Mouse	Santa cruz	sc-376079	1:50/100
Lipase	Rabbit	Novus,	NBPI-54155SS	1:100

3.4.4. Procedure:

Immunohistochemistry (IHC) was performed with paraffin sections of mice samples as per standard protocol described in **table 9**. Details of the antibodies used for IHC experiments are listed in table 8. Double immunostaining was performed as described previously (Graumann *et*

al. 2016). The color reaction was developed with VECTOR VIP Peroxidase Substrate Kit, (Vector Laboratories) or HighDef® red IHC AP chromogen Enzo.

Table 9: Protocol for immunohistochemistry using paraffin sections

Incubation (min)	Reagent	Note
10	Xylol	
10	Xylol	
5	Xylol	
5	Ethanol abs. 99,6%	
5	Ethanol abs. 99,6%	
5	Ethanol abs. 96%	
2	Ethanol abs. 70%	
5	Tapwater	Running tap water
5 x 2	Microwave with Citric buffer	After antigen retrieval, cooled down at RT for 20-30 min
5	Tapwater	Under running tap water
3 x 2	PBS	
15	H_2O_2-PBS-Mix 15%	For blocking endogenous peroxidase, take equal vol. of H_2O_2 (30%) and equal vol. of PBS
5	Water	Aqua dest water
3 x 2	PBS	
60	30ul 10% BSA + 30µl 2.5% horse-Serum from Impresskit (For MOM block: 20µl of MOM + 480µl of 10% BSA)	For anti mouse primary antibodies, mouse tissue has to be blocked using MOM kit, vector.
3x2	PBS, pH=7.4	
over night	1st-antibody	Required dilution prepared in 10% BSA
5x4	PBS, pH=7.4	
30	2rd-antibody	Secondary
5x4	PBS	
3-5	Developed with VECTOR VIP Peroxidase	

	Substrate Kit or HighDef® red IHC AP chromogen Enzo
5	Water
6	Counterstaining: Haematoxylin
5	water
10s	Ethanol 96%
10s	Isopropanol
10s	Isopropanol
10s	Xylol
10s	Xylol
10s	Xylol
	embedding with Pertex and air drying

3.5. Immunofluorescence Microscopy:

The widely used immunohistochemistry is based on the use of fluorophores to visualize the location of bound antibodies in a tissue section. Immunofluorescence is a particularly robust and broadly applicable method used to assess both the localization and endogenous expression levels of proteins of interest. This technique is applicable for tissue sections (paraffin and cryo), isolated cells or cultured cells that are fixed by a variety of methods. There are two classes of IF techniques: primary (or direct) and secondary (or indirect). In the direct immunoflourence method, the primary antibody is directly conjugated to a fluorescent dye when bound to its target protein; the complex can be visualized directly under the microscope. This method is faster, decreases background signal and helps to avoid issues with antibody cross-reactivity or non-specificity. The indirect immunofluorescence uses two antibodies: a primary antibody, which recognizes the target antigen and binds to it, and a secondary antibody labeled with a fluorescent dye, which recognizes and binds to the primary antibody and indirectly localizes the target, and helps in visualization by the microscope.

3.5.1. Reagents and Equipment (Paraffin):

a. Phosphor-Buffered-Saline
b. Xylol (Merck, Darmstadt,Germany)
c. Ethanol (Carl Roth, Kalsruhe, Germany)
d. 2.5 % normal horse Serum, Vector Lab, #S-2012
e. Goat serum (Vector Laboratories, California, U.S., cat# s-1000)
f. BSA (Carl Roth, Karlsruhe, Germany, cat# Art. No. 8076.1)

g. Fluoromount^{Tm} aqueous mounting medium (Sigma-Aldrich,Germany, Cat# F4680)
h. Microscope DMIL (Leica, Wetzlar, Germany)
i. Microwave, Alaska, Germany

3.5.2. List of fluorochrome dyes conjugated antibodies and nuclear stained used in IF

Table 10: List of Fluorochrome conjugated secondary antibodies.

Fluorochrome	λex (nm)	λem (nm)	Color
Alexa 488 (anti rabbit)	488	497 to 643	Green
Alexa 546 (anti mouse)	530/545	610/675	Red
FITC (anti Rabbit)	495	519	Green
DAPI (nuclear staining)	358	461	Blue
Hoechst 33342(nuclear staining)	352	461	Blue

3.5.3. Procedure:

Paraffin embedded liver sections were used for performing the immunofluorescence staining. The sections were deparaffinized as per the standard protocol as described in table 11. Unmasking was done by boiling the sample in 1x citrate buffer in a microwave oven for 10 min. Blocking was done using 10% BSA, 5% goat serum and 5% mouse IgG (MKB-2213, Vector Labs) in phosphate-buffered saline (137 mM sodium chloride, 3 mM potassium chloride, 7 mM disodium hydrogen phosphate, 11 mM dipotassium hydrogen phosphate, pH 7.4). Primary antibodies were used for double staining; anti-mouse ATGL, 1:100 (Novus, NBP1-25852), and anti-rabbit PLIN2, 1:200 (Proteintech, 15294-1-AP), anti-mouse LAMP1, 1:100 (Genetex, GT25212), p62, 1:100 (Proteintech, 18420-1-AP). Fluorochrome-conjugated secondary antibodies, Alexa-488 or 546 (Invitrogen,) were used. Nucleus was stained with DAPI. Cover slips were mounted on using Fluoromount^{Tm} aqueous mounting medium (Sigma-Aldrich, Germany, Cat# F4680). All the images were acquired with a fluorescent microscope (Leica, Leitz, and DMR3) mounted with a Nikon camera (Coolpix 5400) and prepared with Adobe Photoshop 7.0 and Image (NIH, Bethesda, MD, USA).

Table 11: Protocol for Immunoflourence staining using paraffin sections

Incubation (min)	Reagent	Note
10	Xylol	
10	Xylol	
5	Xylol	
5	Ethanol abs. 99,6%	
5	Ethanol abs. 99,6%	
5	Ethanol abs. 96%	
2	Ethanol abs. 70%	
5	Tapwater	running tap water
5 x 2	Microwave with Citric buffer	after antigen retrieval, cooled down at RT for 20-30 min
5	Tapwater	under running tap water
3 x 2	PBS	
60	480µl 10% BSA +20 µl Goat-Serum (additionally, for MOM block: 20µl of MOM + 460µl of 10%BSA + 20µl of goat serum)	for anti-mouse primary antibody, mouse tissue blocked using MOM kit, vector.
3x2	PBS, pH=7.4	
over night	primary antibody	required dilution prepared in 10% BSA + 5% goat serum.
5x4	PBS, pH=7.4	
30	2rd-antibody	AlexaFlour 488, 546(anti-mouse), AlexaFlour 488, 546(anti- rabbit),required dilution prepared in 10% BSA + 5% goat serum (1:1000 dilution)
5min	DAPI/ Hoechst	nuclear staining
5x4	PBS	
	Embedding with Fluoromount^{Tm} aqueous mounting medium (Sigma-Aldrich, Germany, Cat# F4680, F4680).	air dried protecting from light

3.6. Oil red O staining

The main mechanism of lipid staining is derived from the polyazo dyes with increased solubility in the lipids than in the hydroalcholic solvents. The polyazo group of dyes includes the oil red

series, the Sudan red series, and the Sudan blacks, which are used invariably to dye the lipids. The methods and details of the protocol are adapted from IHC world website.

3.6.1. Reagent Formula

I. Oil red O stock stain

Oil red O (Sigma, Cat#CI 26125) 0.5 g

Isopropanol (Carl Roth) 100.0 ml

The dye was dissolved in isopropanol with gentle stirring on a hot plat magnetic stirrer till dissolved completely. This is the stock stain.

II. Oil Red O working solution

30 ml of the stock stain was diluted with 20 ml of distilled water, kept for standing for 10 min, filtered into couplin jar and covered immediately.

The working stain was filtered and made fresh each time.

III. Glycerine Jelly Mounting Medium

Gelatin (Kitchen grade)	10 g
Distilled Water	60 ml
Glycerol	70 ml
Phenol	0.25 g

The gelatine was dissolved in the distilled water using sufficient heat to melt it, the glycerol and phenol was added. The solution was mixed well, transferred to a small capped bottle and refrigerated. Alternatively, aqueous mounting medium which were available commercial were used.

3.6.2 Procedure:

Frozen cryo tissue sections of 10 mm thickness were cut and air dried before proceeding to subsequent staining steps. The section were fixed in 4% formaldehyde for 10 min and washed in running tap water for 5 min. Followed by a quick dip in 60% isopropanol. Then the slides were stained with freshly prepared ORO working stain for 15 min at RT. The slides were then rinsed in 60% isopropanol. The nucleus was lightly stained by dipping in hematoxylin for 1 min. The slides were washed under the running tap water for 5 min and mounted in aqueous mountant (Sigma-Aldrich, Germany, Cat# F4680).

3.7. Thin layer chromatography:

Chromatography is a technique for separation of mixtures of two or more compounds. The separation is accomplished by the partitioning of the mixture between two phases: stationary and mobile. The principle behind this technique is based on differential solubility and adsorption affinity of different substances to mobile and stationary phases between which they are partitioned. Among the different types of chromatography methods, thin Layer Chromatography (TLC) is a solid-liquid technique, in which the stationary phase is solid and the mobile phase is liquid, which is moving. Most commonly used solid adsorbents are silica gel alumina (Al_2O_3 x H_2O) and (SiO_2 x H_2O). TLC is a sensitive, fast, simple and inexpensive analytical technique for separation and detection. The method is easy to perform. The samples are spotted at one end of a sheet of glass or Al-sheet coated with a thin layer of an adsorbent. After spotting, the TLC plate is placed inside the covered jar containing a shallow layer of solvent. Because of the capillary action the solvent rises up through the adsorbent and differential partitioning occurs between the components of the mixture dissolved in the solvent and the stationary adsorbent phase. The substance having higher adsorption affinity to the stationary phase will be separated earlier than the substance with lower affinity (Timothy 2002) .

3.7.1. Reagents and equipment:

a. TLC chamber (Carl Roth, Karlsruhe, Germany)
b. TLC silica gel 60G F254 plates (Merck, Darmstadt, Germany cat # 1.00390.0001)
c. Mobile phase (n-Hexane, Diethyl ether, chloroform, n-heptane, acetic acid, HPLC-grade methanol; Carl Roth, Karlsruhe, Germany)
d. Hair dryer (Moulinex hair dryer)
e. Filter paper (Whatman, Merck, Germany)
f. Hamilton syringe (Hamilton company, USA)
g. Polar lipids standard (Avanti, U.S., cat#181108P)
h. TLC Non polar lipid standards (Matreya LLC,U.S., cat # 1129)
i. Sulphuric acid (Carl Roth, Karlsruhe, Germany)

3.7.2. Procedure:

The lipid extractions from liver tissues was followed as per the protocol described earleir (Laggai *et al.*, 2013) with slight modifications. 20 mg of frozen liver tissue were weighed and then

homogenized with hexane/2-propanol 3:2(v/v) to a final volume of 20 times the volume of the tissue sample (1g in 20 ml of solvent mixture) for 1h. After dispersion, the whole mixture was centrifuged at 4°C at 10000 *g* for 10 min. The supernatant was transferred to a new vial and dried under a nitrogen gas, re-suspended in an appropriate volume of chloroform/methanol 2:1 (v/v). The TLC plate was prewashed in chloroform: methanol 2:1 (v/v) and then heated to 110℃. The samples and standard lipids were spotted on the plate at equidistance with the help of Hamilton syringe. Then, the plate was air dried with a hair dryer for ~1 min. The plate was then immersed into the TLC tank pre-conditioned with mobile phase (Hexane/diethylether/ acetic acid; 90:3:1) run up to 2/3 of the plate. The plate was removed from the tank and air dried for a few min and sprayed with conc. sulphuric acids and charred by incubating in the oven at 120℃ for another few mins till brown bands appear.

3.8. High performance thin layer chromatography

HPTLC (high-performance thin layer chromatography) is higher and refined form of TLC, which provides superior separation efficiency. HPTLC is more precise, accurate, sensitive and with higher repeatability and reproducibility of the results compared to TLC techniques. HPTLC is most commonly used in food industries, pharmaceutical industries for detection of adulterants and identification and quantification of impurities, bioactive substances etc. The background principle of HPTLC is based on adsorption chromatography, where the chemical constituent in the mobile phase and their subsequent interaction with the stationary phase. The chemical substances gets separated on the chromatographic plate depending upon different adsorption affinities towards stationary phase, so less affinity substance moves faster along with the mobile and vice versa. HPTLC method coupled to mass spectrometric analysis enables for very accurate identification of substances even from very small amount (Krüger *et al.* 2015; Taha *et al.* 2015).

3.8.1. Reagents and equipment

a. CAMAG TLC chamber (CAMAG, Muttenz, Switzerland)
b. CAMAG TLC automatic sampler (CAMAG, Muttenz, Switzerland)
c. CAMAG TLC developing chamber (CAMAG, Muttenz, Switzerland)
d. CAMAG TLC scanner (CAMAG, Muttenz, Switzerland)
e. HPTLC plates (Merck, Darmstadt, Germany)

f. Mobile phase (Hexane, Acetic acids, diethylether –HPLC grade, Carl Roth, Karlsruhe, Germany).

3.8.2. Procedure:

The lipid extractions from liver tissues was followed as per the protocol of Laggai (Laggai *et al.*, 2013) with slight modifications. 20 mg of frozen liver tissue were weighed and then lipids were extracted with of hexane/2-propanol 3:2 (v/v) to a final volume 50 times the volume of the tissue sample (1g in 50ml of solvent mixture) and kept in shaking platform for 1h. After dispersion, the whole mixture was centrifuged at 4°C at 10000 *g* for 10min. The supernatant was transferred to a new vial and dried under a nitrogen gas, further re-suspended in 100µl volume of chloroform/methanol 2:1 (v/v). The stock lipid samples were further diluted to 1:4 in chloroform: methanol before application onto the HPTLC plate (50ul of stock + 150ul of solvent) was made. Similarly, the standard (25mg/ml) was also diluted in chloroform: methanol (1:1 v/v) to have 300 ng/µl working conc.

For lipid quantification, protocol were followed as described earlier with slight modification in the solvent system (Krüger *et al.* 2015). The samples and standard solutions were sprayed with the Automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) as 8 mm bands allowing up to 21 tracks on HPTLC plate of 20 × 10 cm (distance from lower edge 8 mm, distance from left edge 14.5 mm, automatic distance between bands). For calibration, the standard solution (300ng/µl) of 1, 20, 40, 70µL resp. was sprayed on the HPTLC plate to have range of different conc. of standard (300–2100 ng/band). And the sample volumes used was 2.5 µL. Chromatography development was performed on HPTLC-MS plates (preheated to 110°C for 15 min) with a mixture of n Hexane/ Diethyl ether/Acetic acid (8:2:0.4, v/v/v) in the TLC developing chamber (CAMAG). Before development, the chamber was saturated for 20 min with filter paper lining. The migration distance was 65 mm (from the lower edge of the plate). After application of the samples and also after development, the plate was dried for 0.5 and 2 min, respectively with the help of hair dryer. **Derivatization:** For visualization of the non-polar lipids, the developed plates were dipped into a primuline solution (100 mg of primuline in 200 mL of acetone/water 4:1, v/v) at a speed of 3 cm/s and an immersion time of 1 s using a TLC Chromatogram Immersion Device (CAMAG). The plate was dried in a stream of warm air (hair dryer at 30 cm distance from the plate) for 2 min. **Densitometry and Documentation**. The

densitometric evaluation via fluorescence measurement at UV 366/>400 nm (mercury lamp, measurement slit size 6.0 mm × 0.2 mm, scanning speed 20 mm/s, K400 optical filter) was performed using a TLC Scanner 4 (CAMAG). Plate images were documented at 366 nm by a TLC Visualizer (CAMAG). All CAMAG instrumentation used so far as well as data obtained was processed with winCATS, version 1.4.6.2002 (CAMAG).

3.9. Real time PCR

Real time PCR is a method used for amplifying and quantifying the nucleic acid present in the sample in real time as the cycle progresses based on the fluorescence signal detected by the detector. The principle of real time PCR is solely based on fluorescent dye chemistry. There are two types: 1. Intercalating fluorescent dye based method, 2. Sequence specific fluorescent labelled oligos methods. Unlike conventional PCR method, this method is more sensitive, specific and cost effective too. Like the conventional PCR, it includes also three steps, denaturation, annealing, and extension. The real time PCR methods can be either used for absolute quantification of unknown samples based on the known template or for relative quantification.

3.9.1. Reagents and equipment:

a. Applied Biosystem ™ PCR thermo cycler (BIORAD, California, U.S.)
b. SYBR green master mix (Thermo Fisher Scientific, Massachusetts, United States Cat# 4309155)
c. Primers (Microsynth, Switzerland)
d. Sterilized tips and pipette (Sarstedt AG, Nümbrecht, Germany)
e. Direct-zol ™ mini RNA prep plus (Zymo Research, USA)
f. High capacity c DNA synthesis Kit (Thermo Fisher Scientific, Massachusetts, United States, cat# 4374966)
g. Nanodrop machine (Thermo Fisher Scientific, Massachusetts, United States, Cat# ND-ONE-W)

3.9.2. Details of real time PCR composition, conditions and primers.

Table 12: Real time PCR reaction composition

Reagent	Volume*
SYBR Green PCR master mix	6.3 µL
H_2O_{dd}	4.7 µL
Primer	0.5µL(0.25µLeach (forward/reverse)
cDNA	1 µL

*The volumes are calculated for one reaction

Table 13: Real time-PCR conditions

Step	Temperature [°C]	Time	Number of cylces
Initialization	95	10 min	1
Denaturation	95	10 sec	40
Annealing	60	1 min	
Elongation	72	10 sec	

Table 14: List of Primers

Gene	Forward	Reverse	Reference
MTT	5'-CTC TTG GCA GTG CTT TTT CTC T-3'	5'-GAG CTT GTA TAG CCG CTC ATT-3'	PRIMER BANK ID: 6678960A1
PLIN2	5'-GAC CTT GTG TCC TCC GCT TAT-3'	5'-CAA CCG CAA TTT GTG GCT C-3'	PRIMER BANK ID:31982516a1
RLP32	5'-TTA AGC GAA ACT GGC GGA AAC-3'	5'-TTG TTG CTC CCA TAA CCG ATG-3'	PRIMER BANK ID:25742730A1
FASN	5'-GGA GGT GGT GAT AGC CGG TAT-3'	5'-TGG GTA ATC CAT AGA GCC CAG-3'	PRIMER BANK ID:30911099a1
SCD-1	5'-TTC TTG CGA TAC ACT CTG GTG C-3'	5'-CGG GAT TGA ATG TTC TTG TCG T-3'	PRIMER BANK ID:31543675a1
SREBP-1c	5'-GATGTGCGAACTGGACACAG-3'	5'-CAT AGG GGG CGT CAA ACA G-3'	PRIMER BANK ID:27753981a1
LPL	5'-GGGAGTTTGGCTCCAGAGTTT-3'	5'-TGT GTC TTC AGG GGT CCT TAG-3'	PRIMER BANK ID:6678710a1

DGAT1	5’-TCC GTC CAG GGT GGT AGT G-3’	5'-TGA ACA AAG AAT CTT GCA GAC GA-3’	PRIMER BANK ID:6753632a1
DGAT2	5’-GCG CTA CTT CCG AGA CTA CTT-3’	5'-GGG CCT TAT GCC AGG AAA CT-3’	PRIMER BANK ID:16975490a1
ACOT1	5’-ATA CCC CCT GTG ACT AC CTG-3’	5'-CAA ACA CTC ACT ACC CAA CTG-3’	PRIMER BANK ID:6753550a1
CD36	5’-ATG GGC GT GAT CGG AAC TG-3’	5’-GTC TTC CCA ATA AGC ATG TCT-3’	PRIMER BANK ID:31982474a1
PPARγ	5’-AGA GCC CCA TCT GTC CTC TC-3’	5'-ACT GGT AGT CTG CAA AAC CAA-3’	PRIMER BANK ID:18875426a1
PCTP	5'-TTC TCG GAC GAG CAG TCC C-3'	5'-CCG GTA GAT GGT TAT GCC TGA G-3'	PRIMER BANK ID:6679235a1
APOE	5'-CTG ACA GGA TGC CTA GCC G-3'	5'-CGC AGG TAA TCC CAG AAG C-3'	PRIMER BANK ID:6753102a1
HMGCoA	5'-AGCTTGCCCGAATTGTATGTG-3'	5'-TCTGTTGTGAACCATGTGACTTC-3'	PRIMER BANK ID:26347907a1
GPAT1	5'-CAGCCAGGTTCTACGCCAAG-3'	5'-TGA TGC TCA TGT TAT CCA CGG T-3'	PRIMER BANK ID:23956162a1
AGPAT1	5'-TAAGATGGCCTTCTACAACGGC-3'	5'-CCA TAC AGG TAT TTG ACG TGG AG-3'	PRIMER BANK ID:26352009a1
β-actin	5'-AAGACGAAACTGTGAGTGACTG-3'	5'-TGCAACTGCTTAAACTTGTGGTT-3'	PRIMER BANK ID:500747a1
MGAT1	5'-TTGTGCTTTGGGGTGCTATCA-3'	5'-CCACAGTGGGAACTCTCCA-3'	PRIMER BANK ID:31981620a1
ACC1	5'-GATGAACCATCTCCGTTGGC-3'	5'-GACCCAATTATGAATCGGGAGTG-3'	PRIMER BANK ID:14211280a1
IL-1β	5’-GCAACTGTTCCTGAACTCAACT-3’	5’ ATCTTTTGGGGTCCGTCAACT-3’	PRIMER BANK ID: 6680415a1
TNFα	5’-GGAACACGTCGTGGGATAATG-3’	5’-GGCAGACTTTGGATGCTTCTT-3’	PRIMER BANK ID:14097828a1
PPAR-α	5’-AGAGCCCCATCTGTCCTCTC-3’	5’-ACTGGTAGTCTGCAAAACCAAA-3’	PRIMER BANK ID: 31543500a1

3.9.3. Procedure:

Mouse liver tissue of 20-30 µg was weighed and homogenized with 600 µl of Trizol™ reagents (Thermo Fisher scientific, cat# 15596026), followed by brief incubation at room temp. Further RNA isolation steps were followed as described in the Direct-zol™ RNA extraction kit (Zymoresearch). The isolated total RNA quantification was performed in Nanodrop. cDNA synthesis was done as per the protocol illustrated in High capacity cDNA synthesis kit (Thermo, 4374966). Real time PCR experiments were performed in Step one, Applied biosystem (BIORAD). Primers were ordered from Microsynth (Switzerland). The expression of all genes was normalized to β-actin to determine the relative mRNA expression (ΔCT). The fold change ($2^{\wedge- \Delta \Delta CT}$) was calculated as described earlier (Livak and Schmittgen 2001).

3.10. Electrophoretic mobility shift assay (EMSA)

EMSA was established as a method for detection of DNA binding proteins (Fried and Crothers 1981; Hellman and Fried 2007). The assay principle is based on the observation that the electrophoretic mobility of a protein-nucleic acid complex is typically less than that of the free nucleic acid. The basic technique is simple to perform, robust enough to accommodate a wide range of binding conditions using radioisotope-labeled nucleic acids, the assay is highly sensitive. While there are other variants such as fluorescence, chemiluminescence and immunohistochemical based detection method.

3.10.1. Reagents/ Equipments:

a. Intas ECL chemostar (Intas Science Imaging Instruments GmbH, Göttingen, Germany)
b. Centrifuge machine (VWR, Darmstadt, Germany, Cat#Micro Star17R)
c. Mini Protean electrophoresis system (BIO-RAD ™, U.S.)
d. Western blotting fast blot B43 (Biometra, Analytika, Jena AG Göttingen, Germany)
e. Filter paper (Whatman, Merck, Darmstadt, Germany)
f. NC membrane/PVD membrane (Merck, Darmstadt, Germany)
g. 1x TBE (Carl Roth, Karlsruhe, Germany)
h. NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo scientific, U.S., cat #. 78833)
i. Protein quantification kit (Pierce ™ BCA protein assay kit, Thermo scientific, U.S.)
j. LightShift™ Chemiluminescent EMSA Kit (Thermo Fisher Scientific, U.S. Cat#20148)

3.10.2. Procedure:

The following protocol describes a chemiluminescence based detection protocol for EMSA. The target oligo's probe sequence for CREB consensus binding site was from Santa Cruz (sc-2504). The probe sequences were as follows: CREB consensus sequences (F) 5' – AGA GAT TGC CTG ACG TCA GAG AGC TAG – 3'(R) 5'-CTA GCT CTC TGA CGT CAG GCA ATC TCT-3'. PPAR consensus binding sequences: (F) 5'-CAA AAC TAG GTC AAA GGT CA-3',(R) 5'-TGA CCT TTG ACC TAG TTT TG-3'. The 5′ ends of all single-stranded probes were biotin labeled. Non-biotin-labeled probes were also synthesized (a competitor probe) from Microsynth, Germany.

Nuclear proteins extraction from liver samples was done using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher scientific, cat# 78833). For the EMSA reaction, 2 µg of the nuclear protein was incubated with reaction reagents as per the protocol given in Light shift Chemiluminescent EMSA Kit (Thermo Scientific, cat# 20148). After a 10-min equilibration to 25°C, biotin-labeled (Hot probe) and excess of non-biotin probes (cold probe) were added and further incubated for 30 min at room temp. After hybridization, the complexes were resolved by electrophoresis on 5% non-denaturing polyacrylamide gels in 0.5× TBE (Tris-borate-EDTA) buffer at 10 V/cm for 1 h. The gel was then transferred to a positively charged nylon membrane using a semidry transfer cell. DNA was detected using Light shift Chemiluminescent EMSA Kit (cat no 20148, Thermo Scientific). Image processed by Intas Imager (Intas ECL chemostar, Göttingen, Germany).

3.11. Triglyceride Colorimetric Assay

Triglyceride Assay Kit (Abcam, Cambridge, U.K., Cat# ab65336) is a sensitive and easy assay to measure the triglyceride concentration in mammalian samples. As per the assay protocol triglycerides are converted to free fatty acids and glycerol. Glycerol is then oxidized to generate a product, which reacts with a probe to generate color (spectrophotometry at λ= 570 nm) and fluorescence (Ex/Em = 535/587 nm).

3.11.1. Reagents and equipment:

a. Microplate reader (Infinite M Plex, Tecan Männedorf, Switzerland)
b. Enzymes and buffers as supplied with the kit (Cayman,)

c. Microplate (Nunc™ maxisorp™ ELISA plates, Thermo scientific, U.S.)
d. Multichannel pipette (Eppendorf, Hamburg, Germany)

3.11.2. Procedure:

Approx. 20mg of liver samples were weighed from each group and the lysates was prepared using the buffer provided with the kit. The assay was initiated by adding samples and standards to wells as per the protocol given in the kit. Then assay buffer and lipase enzyme were added to each well, and kept for incubation at room temp. for 20 mins. Then triglycerides reaction mix was added into each well and incubated for next 60 mins. The glycerol released was subsequently measured by a coupled enzymatic reaction system with a colorimetric readout at 540 nm.

3.12. Free Fatty acid quantification:

Fatty Acids play a very important role in normal metabolism and many disease developments. They are precursors to a number of bioactive classes of compounds such as prostaglandins, leucotrienes and others, and have been implicated in diverse functions such as autism, immune system and inflammation response. PromoCell's Free Fatty Acid Quantification Kit provides a convenient, sensitive enzyme-based method for detecting the long-chain free fatty acids in various biological samples, such as serum, plasma and other body fluids, food, growth media, etc., and for accurate measurement of adipolysis in cell culture. In the assay, fatty acids are converted to their CoA derivatives, which are subsequently oxidized with the concomitant generation of color or fluorescence. C-8 (octanoate) and longer fatty acids can then be easily quantified by either colorimetric (spectrophotometry at λ = 570 nm) or fluorometric (at Ex/Em = 535/587 nm) methods with a detection limit 2 µM free fatty acids in a variety samples.

3.12.1. Reagents and equipment

a. Tecan Microplate reader (Infinite M Plex, Tecan Männedorf, Switzerland)
b. Enzymes and buffers as supplied with the free fatty acid quantification kit(Promokine, Cat# PK-CA577-K612)
c. Microplate (Nunc™ maxisorp™ ELISA plates, Thermo scientific, U.S.)
d. Multichannel pipette (Eppendorf, Hamburg, Germany)
e. Tips (Sarstedt, Nümbrecht, Germany)

3.12.2. Procedure:

10 mg liver tissue samples were weighed from each group and homogenized with 200 µl of chloroform-Triton X-100 (1% Triton X-100 in pure chloroform). The extract was spinned at high speed in a microcentrifuge for 5-10 minutes. Organic phases (lower phase) were collected, air dried at 50°C to remove the chloroform. Vacuum dried was performed for 30 min to remove trace chloroform. The dried lipids (in Triton X-100) were dissolved in 200 µl of fatty acid assay buffer by vortexing extensively for 5 min (the solution was slightly turbid). 20 µl of extracted sample were used per assay. Acyl-CoA Synthesis, 2 µl was added into all the standard and sample wells. Wells were mixed and the reaction incubated at 37°C for 30 min. A master reaction mix was prepared, enough for the number of assays and standard with the following composition of reaction mix as given below.

- 44 µl assay buffer
- 2 µl fatty acid probe
- 2 µl enzyme mix
- 2 µl enhancer

50 µl of the Reaction Mix was added to each well containing the standard or the test samples. The reaction was incubated for 30 min at 37°C, protected from light. O.D. was measured at 570 nm for colorimetric assay or fluorescence at Ex/Em =535/590 nm in a micro-plate reader. For the calculation background value (the 0 Control) was subtracted from all standard and sample readings. Standard curve nmol/well vs. OD570nm or fluorescence readings was plotted. Then the sample readings were applied to the standard curve to obtain fatty acid amount in the sample wells.

Fatty Acid Concentration = Fa/Sv (nmol/µl or mM)

(Fa is the Fatty Acid amount (nmol) in the well obtained from standard curve.

Sv is the sample volume (µl) added to the sample well).

3.13. Lipase activity assay:

Lipases perform essential roles in the digestion, transportation, and processing of dietary lipids by controlling the clearance of triglyceride-rich lipoproteins from the circulation. Cayman's lipase activity assay provides a fluorescence-based method for detecting lipase activity in

plasma, serum, tissue homogenates, and cell culture samples. In the assay, lipase hydrolyzes arachidonoyl-1-thioglycerol to arachidonic acid and thioglycerol. Thioglycerol reacts with the thiol fluorometric detector to yield a highly fluorescent product which can be analyzed with an excitation wavelength of 380-390 nm and an emission wavelength of 510-520 nm. The limit of detection is 0.1U/ml (±0.05 U/ml) lipase from animal/ bovine.

3.13.1. Reagents and equipment

a. Tecan Microplate reader (Infinite M Plex, Tecan Männedorf, Switzerland)
b. Enzymes and buffers as supplied with the Lipase activity assay kit (Cayman, U.S. cat#700640)
c. Microplate (Nunc™ maxisorp™ ELISA plates, Thermo scientific, U.S.)
d. Multichannel pipette (Eppendorf, Hamburg, Germany)
e. Tips (Sarstedt, Nümbrecht, Germany)

3.13.2. Procedures:

Liver samples were weighed from each group and lysates prepared in PBS containing protease inhibitor in cold condition as per protocol described in the kit (Cayman cat no. 700640). The assay was initiated by measuring the standards wells first. 170µl of 1x assay buffer, 10µl of thiol detector, 10 µl of lipase substrate and standards were added to their respective wells. The plate was covered and incubated for 15 min at 37℃. Fluorescence reading was taken at an excitation wavelength of 380-390 nm and emission wavelength of 510-520 nm by setting appropriate *gain* value.

For the samples wells 170µl of 1x assay buffer, 10µl of thiol detector, 10µl of lipase substrate, and 10µl of samples were added. Similarly, for lipase positive control wells, 170 µl of 1x assay buffer , 10µl of thiol detector, 10µl of lipase substrate, 10µl of lipase positive sample were added. At last for sample background wells, 180µl of 1x assay buffer, 10µl of thiol detector, 10 µl of samples were added. The plate was covered and incubated for 15 min at 37C. Fluorescence reading was taken at an excitation wavelength of 380-390 nm and emission wavelength of 510-520 nm at the same *gain* value set for standard reading. The data obtained were analysed as per protocol given below.

Determination of lipase activity

1. The average fluorescence of each sample and sample background at each time point was determined. The fluorescence was ploted as a function of time.
2. The change in fluorescence RFU per minute by was determined by selecting two pints on the curve. The change in fluorescence during that time was calculated using the following equation:

$$\text{RFU/min} = \frac{\text{RFU (Time 2)} - \text{RFU (Time 1)}}{\text{Time 2 (min)} - \text{Time 1 (min)}}$$

3. The sample background rate (RFU/min) was subtracted from all of the samples. Than, the lipase activity was calculated using the following equation given below. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of thioglycerol per minute at 37°C.

$$\textbf{Lipase Activity} = \left[\frac{\text{RFU/min}}{\text{Slope from standard curve (RFU/µM)}}\right] \times \text{Sample dilution}$$

3.14. Statistics

All the measurements were performed in technical triplicates repeated minimum of 3 times. Similarly, real time PCR analysis was performed in technical duplicate with repetition of 2 times. All results are expressed as means ± standard error of the mean (SEM). Significant differences between the four different groups were determined by one-way ANOVA (parametric) with Tukey's multiple comparisons test was used to compare the test groups, while two groups comparison was determined by Mann-Whitney's U test. All statistical analyses were performed with GraphPad Prism software (v6.0; GraphPad Software Inc., San Diego, CA) and p-values $p^{*}<0.05$, $p^{**}<0.01$, $p^{***}<0.001$, were considered statistically significant.

4. RESULTS AND DISCUSSION

4.1. Modulation of hepatic lipid metabolism by CB1 receptor (CB1R) knockout in HBs (HBV surface protein transgenic) mice

4.1.1. Results:

4.1.1.1. Body and liver weight reduced by CB1R KO in HBs mice

Genetic and pharmacological manipulation of CB1 receptor is associated with weight reduction in human and rodents (Ravinet Trillou *et al.* 2003). Endocannabinoids are known regulators of body weight and energy homeostasis via modulating the appetite behavior and peripheral energy metabolism (Cota *et al.* 2006). To this end, we explored the effect of $CB1^{-/-}$ on body and liver weight in HBs mice. $CB1^{-/-}$ and $HBs/CB1^{-/-}$ showed significant decreases in body weight in comparison to wild type mice (WT) and HBs mice resp. as shown in the **Fig.6A**. Similarly, the liver weight analysis showed a significant reduction of liver weight in $CB1^{-/-}$ compared to WT mice while there is slight decrease in liver weight in $HBs/CB1^{-/-}$ compared to HBs as shown in **Fig.6B**. Liver to body weight ratio analysis showed slight but no significant reduction in $CB1^{-/-}$ and $HBs/CB1^{-/-}$ as compared to WT (**Fig.6C**).

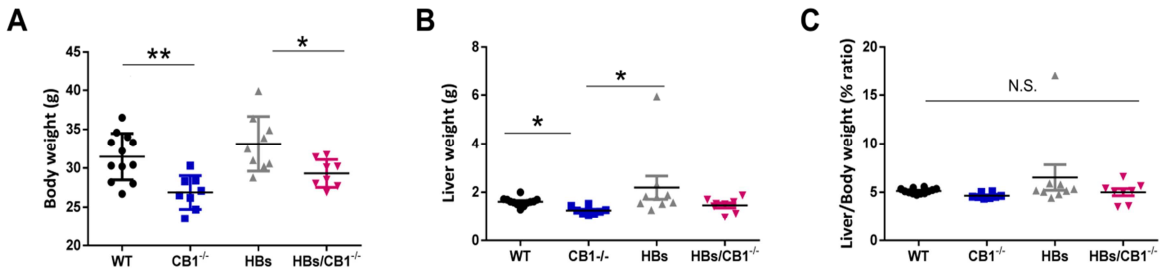
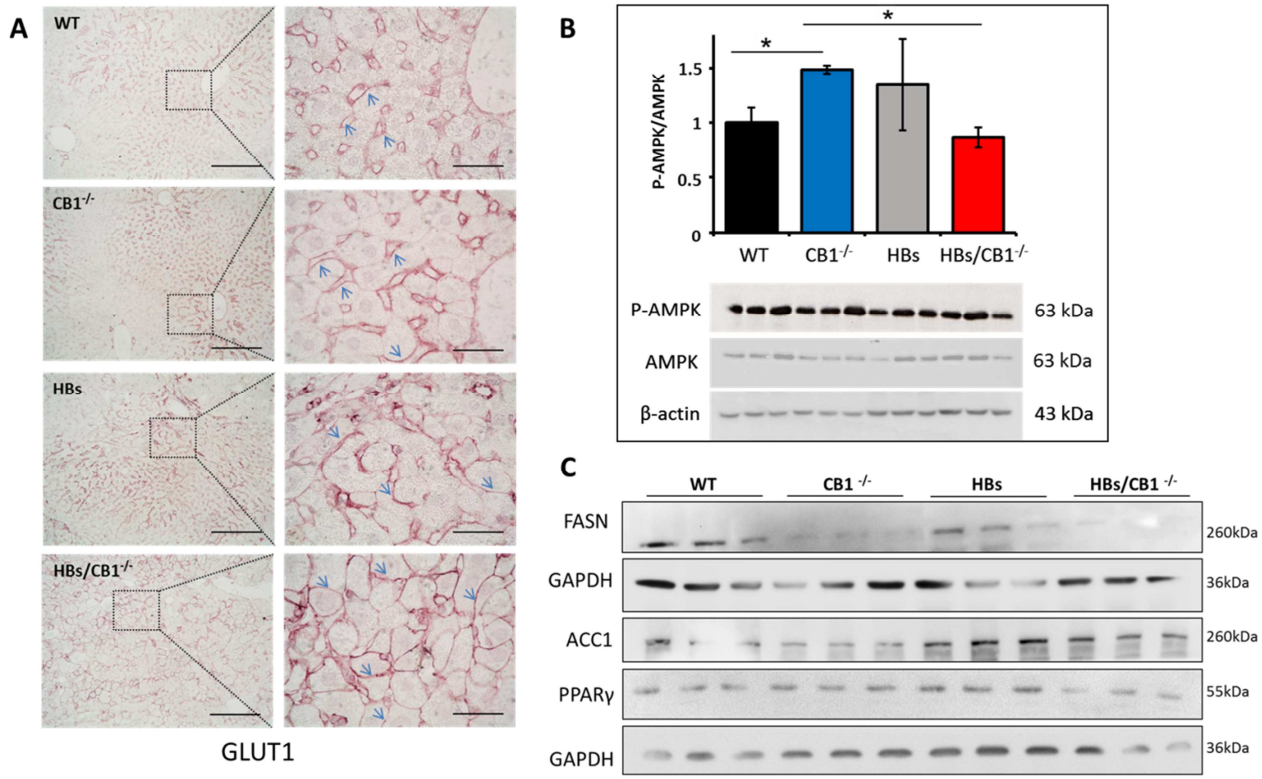


Figure 6: CB1receptor KO reduced body and liver weight in HBs mice. **(A)** Graph demonstrates a remarkable decrease of body weight in $CB1^{-/-}$ and HBs/ $CB1^{-/-}$ mice in comparison to WT and HBs. (**B**) Grpah showing liver weight analysis among the groups. **(C)** Graph depicting percentage liver/bodyweight analysis. Depicted are means ± SEM, n = 8-12, 52 weeks old mice. Statistical analysis, One way ANOVA with Tukey's multiple comparison test was performed, P value *<0.05, **< 0.01, N.S.; not significant was indicated (modified from (Irungbam *et al.* 2019)).

4.1.1.2. Induction of metabolic stress and suppression of lipogenesis by CB1R KO in HBs mice

The decrease in body and liver weight further prompted us to investigate the effect of $CB1^{-/-}$ on glucose and energy homeostasis. Disturbed glucose uptake could be an indicator of metabolic stress (Hayashi *et al.*, 2000). We therefore, explored the effect of $CB1^{-/-}$ on glucose transporter1 (GLUT1). GLUT1 staining analysis revealed an increased plasma membrane localization of GLUT1 in hepatocytes of HBs/$CB1^{-/-}$ compared to HBs (**Fig.7A**) suggesting increased uptake and demand of glucose within the hepatocytes. We further investigated GLUT1 expression at mRNA level, which remained unchanged (data not shown). It was reported that CB1KO increased hepatic AMPK activation (Jeong *et al.*, 2008; Kunos *et al.*, 2008). To this end, our Western blot analysis showed no significant activation of AMPK in HBs/$CB1^{-/-}$ compared to WT and HBs (**Fig.7B**). We further investigated the downstream targets of AMPK involved in *de novo* lipogenesis. Western blot analysis of major target genes, FASN, ACC1, and PPARγ revealed decreased protein levels in $CB1^{-/-}$ and HBs/$CB1^{-/-}$ in comparison to WT and HBs (**Fig. 7C**) suggesting a reduction of lipogenesis by CB1 knockout in HBs mice (Irungbam *et al.* 2019).



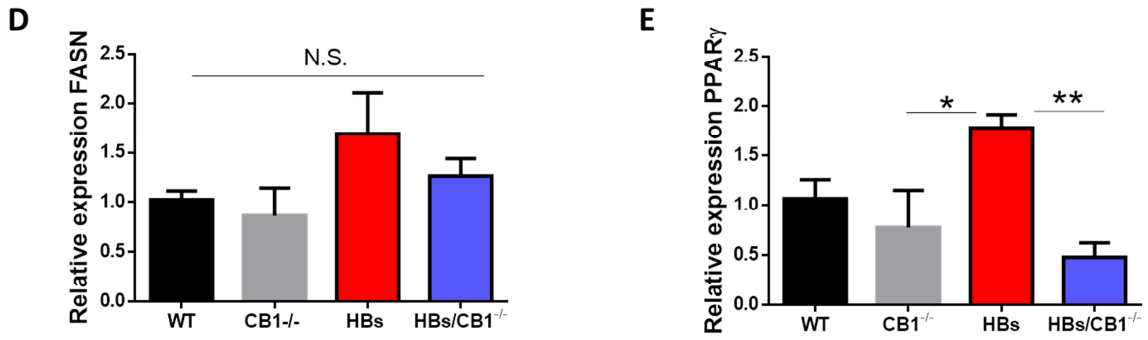


Figure 7: Increased hepatic membrane GLUT1 localization and supression of lipogenesis by CB1 receptor KO in HBs mice. (A) GLUT1 immunohistochemistry staining using paraffin-embedded liver sections from 52 weeks old mice was performed. Images on the right side are the enlarged areas from the boxed areas in the images on the left. Original magnification 200x (left) and 1000x (right), bars 200µm (left), 40µm (right). **(B)** Activation of AMPK was analysed using specific anti-phospho-AMPK in the liver lysates from 52 weeks old mice. Similar levels of AMPK expression were confirmed using anti-AMPK antibody. Equal protein loading was confirmed using anti-β-actin antibody. **(C)** Major genes of lipogenesis were analyzed using liver lysates from 52 weeks old mice with specific anti-FAS, Anti-ACC1 and anti-PPARγ antibodies. GAPDH antibody was used as loading control. **(D-E):** Relative expression of FASN and PPARγ normalized against β-actin (modified from (Irungbam *et al.* 2019)).

4.1.1.3. Reduced hepatic TAG storage by CB1R knockout in HBs mice

The reduction of body weight has been associated with loss of hepatic fat (Hayashi *et al.* 2000). The observed loss of body weight prompted us to investigate the effect of $CB1^{-/-}$ on hepatic lipid storage. Interestingly, the liver TAGs quantification analysis using liver lysate of 52w old mice samples revealed a significant increased in hepatic TAGs in HBs mice, which got reduced in $HBs/CB1^{-/-}$ **(Fig.8A).** Additionally, thin layer chromatogram (TLC) demonstrated decreased TAGs in $CB1^{-/-}$ and $HBs/CB1^{-/-}$ in comparison to WT and HBs mice resp. **(Fig.8B).** Furthermore, oil red staining showed clearly reduced lipid droplets amount in $CB1^{-/-}$ and $HBs/CB1^{-/-}$ compared to WT and HBs mice **(Fig.8C).** Taken together, our findings clearly suggest that the global CB1 receptor KO leads to reduction in hepatic LDs and TAGs storage in HBs mice.

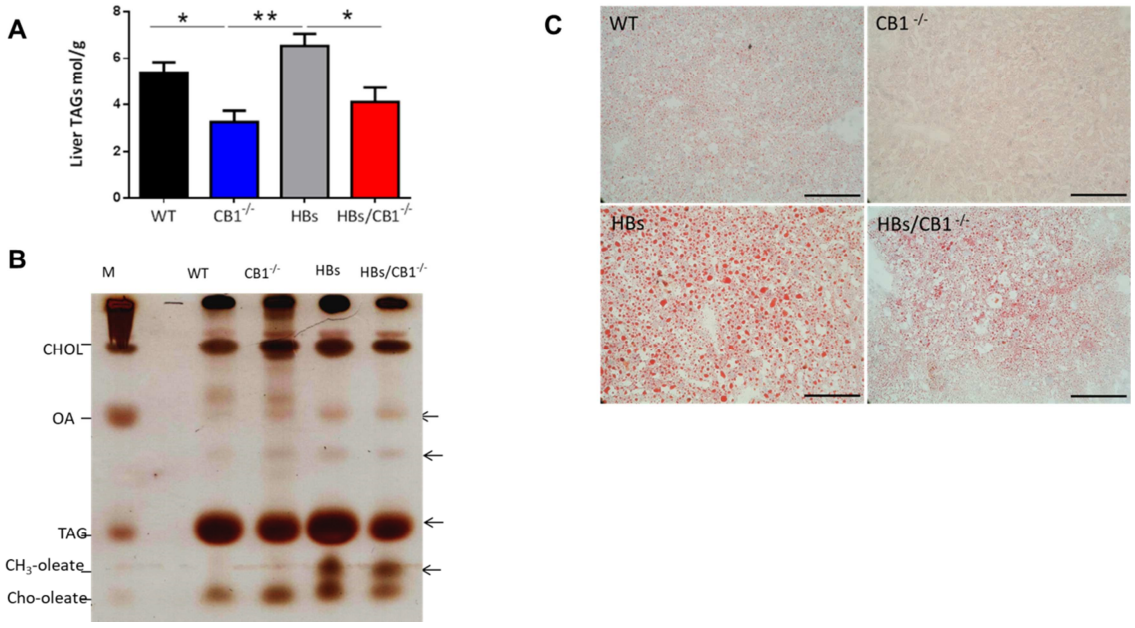


Figure 8: CB1 receptor KO reduced hepatic LDs storage in HBs mice. (**A)** Liver triglyceride quantification using liver samples from 52 weeks old $CB1^{-/-}$ and $HBS/CB1^{-/-}$ mice. n = 8-12, 52w old age, statistical test: ANOVA, Tukey's multiple comparison test, means ± SEM, P value *<0.05, **< 0.01. **(B)** Lipid separation using thin layer chromatography methods showed different total lipid profiles from 52 weeks old mouse liver tissues. CHOL: cholesterol, OA: oleic acid, TAGs: triglycerides. (**C)** Accumulation of neutral lipids depots as demonstrated by Oil red O staining using cryosections of 52 weeks old mouse liver was performed. Image magnification, 400x, bar 100µm (modified from (Irungbam *et al.* 2019)).

4.1.1.4. Reduced LDs associated protein, perilipin 2 by CB1R knockout in HBs mice

The reduction in TAG storage and lipid droplets (LDs) further prompted us to investigate the effect of CB1 receptor knockout on LDs associated proteins. Perilipins are the major cytoplasmic lipid droplet-associated proteins which are involved in intracellular lipid droplet formation and stability (Brasaemle 2007). The protein family consists of five members (Sztalryd and Brasaemle 2017)**.** Among them, PLIN2 represent the constitutive and ubiquitously expressed protein used as a protein marker of LDs (Tsai *et al.* 2017).

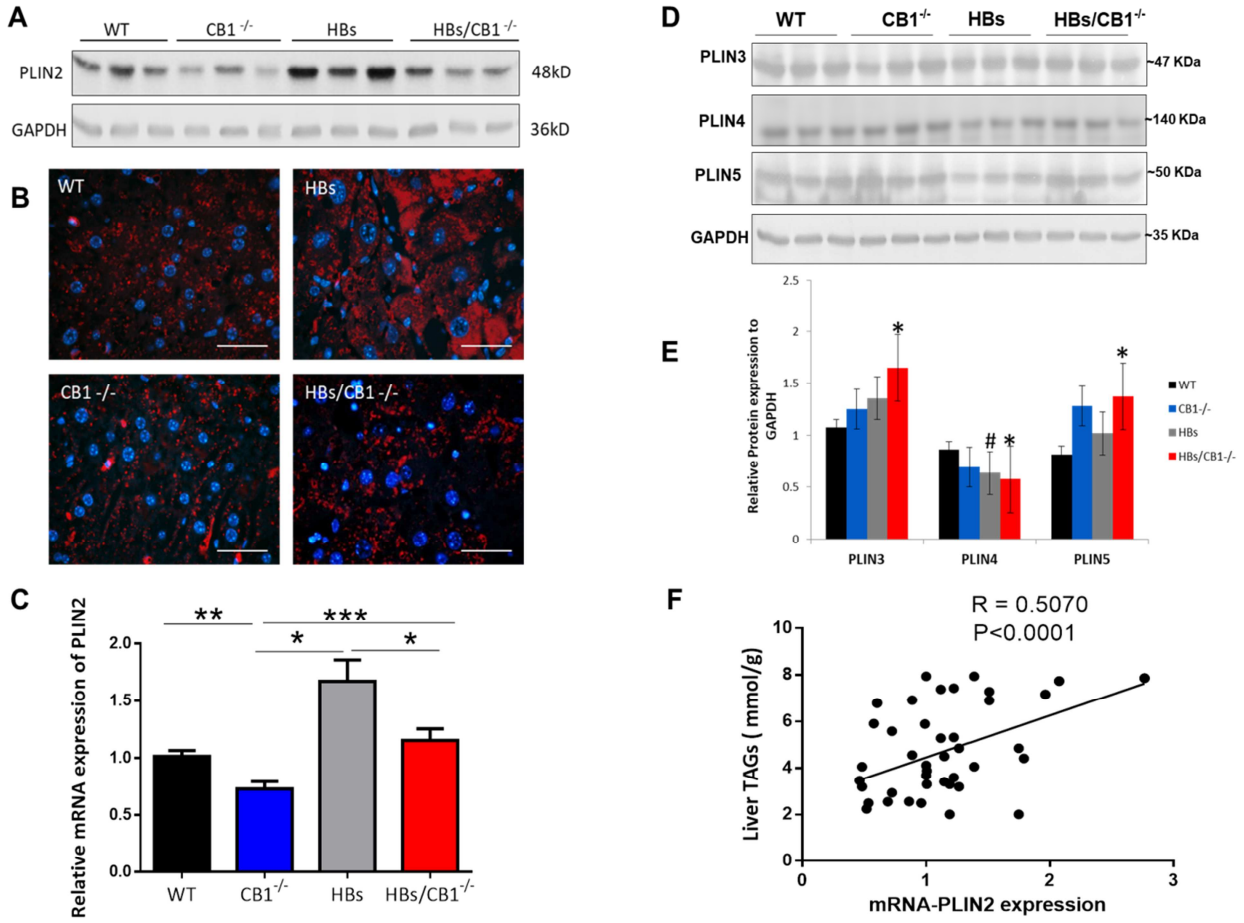


Figure 9: Reduction of LDs associated protein, perilipin 2 by CB1R KO in HBs mice. (A) PLIN2 protein expression as demonstrated by Western blot analysis of total protein lysates from the liver of 52 weeks old mice was performed using specific anti-PLIN2 antibody. Equal protein loading was confirmed using anti-β-actin**. (B)** Immunofluorescence analysis of paraffin-embedded liver sections from 52 weeks old mice was performed using specific anti-PLIN2 antibody (red-Alexa 546). Nuclei were stained with DAPI (blue). Original magnification1000x, bar 20µm. **(C)** Relative PLIN2 transcripts level as determined by real time PCR analysis. The PLIN2 expression was normalized to β-actin as reference gene and shown as relative expression (ΔΔCt). Statistical analysis was performed in Graph Pad prism 6.01, one way ANOVA, Tukey´s multiple comparison test was performed. Means ± SEM, P value *<0.05, **<0.01, ***<0.001. **(D)** PLIN3, 4, and 5 protein expressions as demonstrated by Western blot analyses of total protein lysates from the liver of 52 weeks old mice using specific anti-PLIN3, PLIN4 and PLIN5 antibodies. Equal protein loading was confirmed using anti-GAPDH**. (E)** Graph showing

normalized protein expression of PLIN3, 4, and 5 to GAPDH (note * indicated significance drawn between WT and HBs/CB1$^{-/-}$, # significance drawn between WT and HBs^{-}) (modified from (Irungbam *et al.* 2019)) (**F**) Linear regression graph showing positive correlation between mRNA PLIN2 expression levels and liver TAGs in the liver..

Here, we observed the reduction of PLIN2 protein expression in the liver of CB1$^{-/-}$ and HBs/CB1$^{-/-}$ compared to WT and HBs mice (**Fig.9A**). Similarly, PLIN2 mRNA levels were decreased in CB1$^{-/-}$ and HBs/CB1$^{-/-}$ compared to WT and HBs mice, respectively (**Fig.9C**). Immunofluoresence staining visualized the decreased PLIN2 expression in the hepatocytes of HBs/CB1$^{-/-}$ compared to HBs mice (**Fig.9B**). The reduction of PLIN2 mRNA was correlated with the reduction of TAG (**Fig. 9F**). Because, murine livers also express PLIN3 and PLIN5 (Langhi *et al.* 2014), in addition the expression of other PLIN family members were analyzed by Western blotting (**Fig.9D-E**). PLIN3 protein expression was upregulated in HBs/CB1$^{-/-}$ while PLIN4 was down regulated in HBs and HBs/CB1$^{-/-}$. Likewise, PLIN5 protein expression was decreased in the liver of HBs mice in comparison to HBs/CB1$^{-/-}$ (**Fig.9E**). The reduction of hepatic PLIN2 was accompanied with minor increased PLIN 3 and PLIN5 levels in CB1$^{-/-}$ and HBs/CB1$^{-/-}$ mice (**Fig. 9E**).

4.1.1.5. *In vitro* reduction of LDs associated protein, perilipin2 by CB1R antagonist

Furthermore, *in vitro* cell culture based experiments were performed to further strengthen the hypothesis that CB1 receptor signaling mediates the reduction of PLIN2 expression. In order to simulate the HBs induced hepatic steatosis, AML12 cells were pre-treated with oleic acid to increase the intracellular LDs. The cells were than subsequently treated with rimonabant at different concentrations which caused a dose dependent reduction in PLIN2 protein expression (**Fig.10A**). Similarly, AML12 cells pre-treated with oleic acid followed with CB1 receptor antagonist, rimonabant showed a reduction in PLIN2 expression in comparison to control vehicle and CB1 receptor agonist, methanadamide (**Fig.10B-C**). As a whole, our findings from *in vitro* experiments suggested reduction of hepatic PLIN2 expression by pharmacological manipulation of CB1 receptor (Irungbam *et al.* 2019).

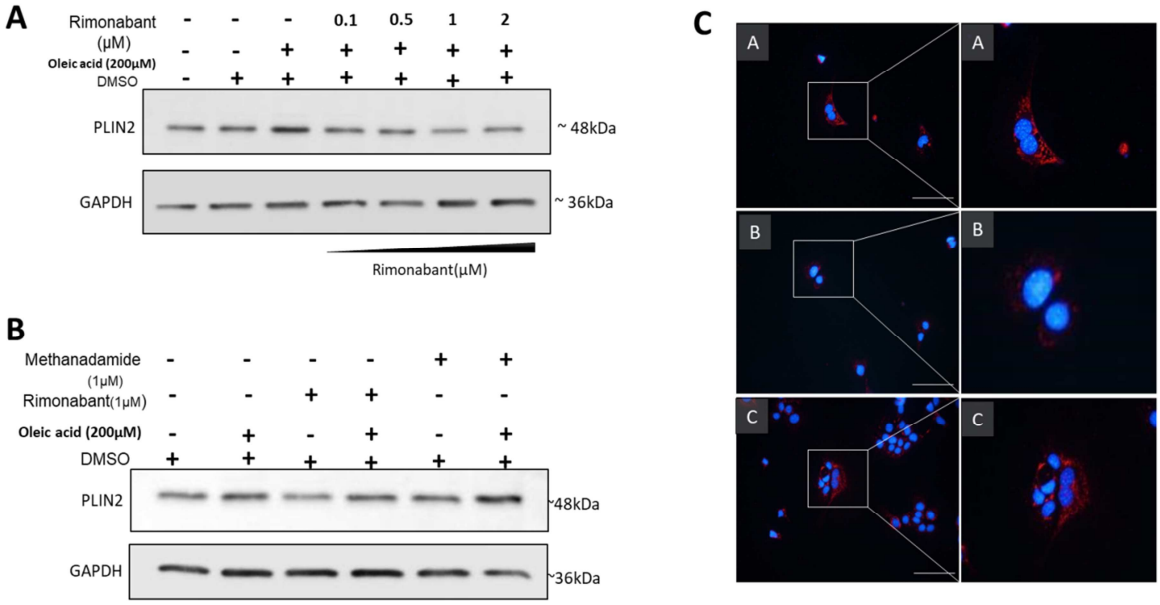


Figure 10: AML12 cells stimulation with CB1R antagonist reduced the expression of PLIN2. (A) Dose dependent decreased in PLIN2 expression after rimonabant treatment. AML12-Cells were treated with rimonabant at different doses (0.1, 0.5, 1, 2 µM) resp. in presence of oleic acid (200µM) for 48h. Western blot analysis of the cells lysates was performed using specific anti-PLIN2 antibody. Equal protein loading was confirmed using anti-GAPDH antibody. **(B)** AML12 cells were treated with rimonabant and methanandamide (1µM final conc.) for 48h in absence and presence of oleic acid (200µM). Western blot analysis was performed with cell lysates using specific anti-PLIN2 antibodies. Equal protein loading was confirmed using anti-GAPDH antibody. **(C)** Immunofluorescence analysis of AML12 cells treated with rimonabant and methanandamide (1µM) for 48h was performed using specific anti-PLIN2 antibody (red). Nuclei were stained with DAPI (blue). Original magnification 400x, bar 200µm (Irungbam *et al.* 2019)).

4.1.1.6. Elevation of autophagy by CB1R knockout in HBs mice

Autophagy is a cellular response to low energy state, an alternative strategy for survival under shortage of energy supplies (Kim *et al.* 2011). Here, we investigated whether there is an enhancement of autophagy due to metabolic stress induced by CB1KO. Expression levels of LC3B and p62 proteins, classical markers for autophagy, were tested (Klionsky *et al.* 2016). The immunostainings using anti LC3B antibody revealed increased autophagosomes puncta in

HBs/CB1$^{-/-}$ in comparison to WT and HBs mice (**Fig.11A-B**). Surprisingly, immunoblotting analysis using specific antibody against LC3B showed decreased LC3BII expression in HBs/CB1$^{-/-}$ compared to HBs (**Fig.11C-D**) suggesting either enhancement or inhibition of autophagy. To further understand the issue of autophagic induction or inhibition, the development of the autophagy process over a time period, a comparative Western blot analysis using total liver protein from HBs and HBs/CB1$^{-/-}$ mice groups of different ages (12, 26 and 52w) using anti-LC3B antibody was performed. The immunoblotting revealed increased LC3B-II formation in HBs/CB1$^{-/-}$ compared to HBs at 12 and 26 weeks. Interesting, LC3B-II protein level showed a reduction at 52 weeks (**Fig.11E**) in a similar way as observed in **Fig.11D.** The decrease in both LCB3I and LCB-II at an older age might be due to stronger nutrient stress mediated by the combined effects of CB1KO and HBs expression in the hepatocytes (Hiebel *et al.* 2014), leading to enhancement of autophagic degradation, supporting autophagic induction rather than inhibition. To further strengthen our hypothesis that CB1KO enhanced autophagy in HBs mice, we investigated p62 protein expression by Western blot. Our results demonstrated a slight decreased p62 expression in HBs/CB1$^{-/-}$ compared to HBs mice (**Fig.11C**). Additionally, immunofluorescence staining using anti-lysosomal associated membrane protein 1 (LAMP1) as another indicator for autophagy revealed an enhanced expression of LAMP1 in CB1$^{-/-}$ and HBs/CB1$^{-/-}$ in comparison to WT and HBs mice (**Fig.11F**). Further, double immunofluorence (IF) stainings using anti LC3B and anti-LAMP1 demonstrated increased colocalization in HBs/CB1$^{-/-}$ compared to HBs mice (**Fig.11G**) suggesting for enhanced autolysosome formation. Further, double IF for p62 and LAMP1 suggested a partial colocalization in HBs/CB1$^{-/-}$ compared to HBs (**Fig.11H**) suggesting autophagy of p62 marked proteins. To further support the *in vivo* findings, cell culture based experiments were performed with AML12 to assess the autophagy induction with CB1 receptor antagonization. Rimonabant treatment induced increased LC3BII protein expression in a time dependent manner (**Fig.11I**). Moreover, treatment of AML12 cells with rimonabant in absence or presence of oleic acid supplementation showed an increased LC3BII expression compared to control vehicle (**Fig.11J**). The prominent reduction of p62 after rimonabant treatment in presence of oleic acid compared to control (**Fig.11J**), suggests autophagy induction due to CB1 receptor antagonization.

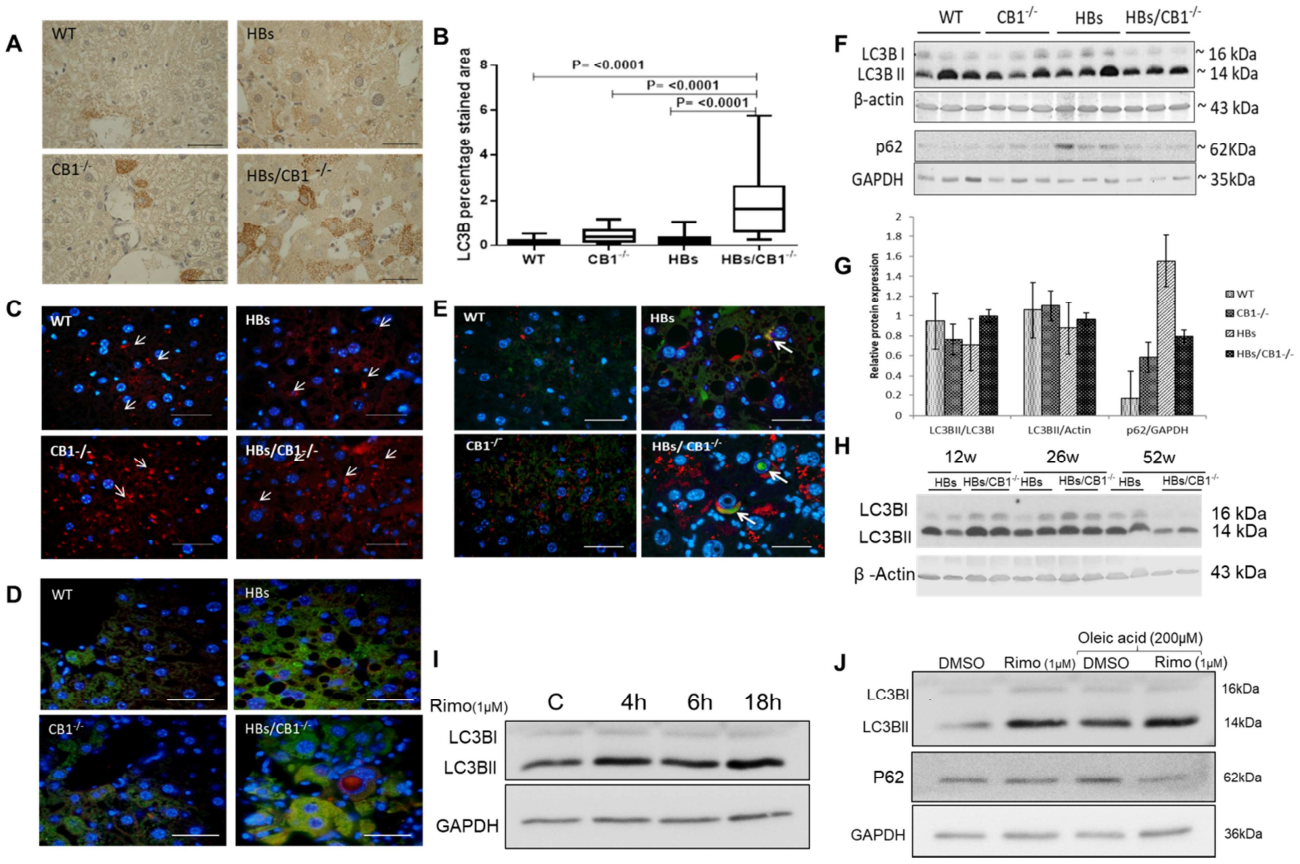


Figure 11: Elevation of autophagy mediated by CB1 receptor KO in HBs mice. **(A)** LC3B staining was performed using paraffin-embedded liver sections from 52 weeks old mice. Original image magnification 1000x, bar 40µm (**B**) The quantification of LC3B puncta was performed using ImageJ software and expressed as percentage of LC3B staining/field. Statistical analysis, ANOVA with tukey's multiple comparison tests were performed using GraphPad Prism 6.01 **(C)** LAMP1 expression as demonstrated by immunofluorescence staining using paraffin-embedded liver sections of 52 weeks old mice and specific anti-LAMP1 (red). DAPI (blue) was used to stain the nuclei. Magnification 1000x, bar 40 µm. **(D)** Double immunofluorescence staining of paraffin-embedded liver sections of 52 weeks old mice was performed using specific anti-LC3B (green) and anti-LAMP1 (red). DAPI (blue) was used to stain the nuclei. Yellow color showed the co-localization of these two proteins. Magnification 1000x, bar 40 µm. **(E)** Double immunostaining staining of p62 and LAMP1 was performed using paraffin-embedded liver sections of 52-week old mice with specific anti-p62 (green) and LAMP1 (red) antibodies. DAPI (blue) was used to stain the nuclei. Yellow color showed the co-localization of these two

proteins. Magnification 1000x Scale bar 40 µm. **(F)** LC3B and p62 proteins expression in the liver lysates from 52 weeks old mice was performed using specific anti-LC3B, anti-p62 antibodies. Equal protein loading was confirmed using anti-β-actin and GAPDH antibodies. **(G)** Densitometric analysis of relative protein expression normalized to GAPDH. **(H)** Comparative Western blot analysis of LC3B protein expression was performed using two representative liver lysates from 12-, 26- and 52 weeks old HBs and HBs/CB1$^{-/-}$ mice. Equal protein loading was confirmed using anti-β-actin antibody. **(I)** AML12 cell lysates treated with rimonabant (1µM) for different time point (0, 4, 6,18h) was tested for LC3B protein expression using specific anti-LC3B antibody. Equal protein loading was confirmed using anti- GAPDH antibody. **(J)** AML12 cell lysates treated with rimonabant (1µM) for 48h in absence and presence of oleic acid supplementation (200µM) was tested for LC3B protein expression using specific anti-LC3B antibody. Equal protein loading was confirmed using anti-GAPDH antibody (modified from (Irungbam *et al.* 2019)).

4.1.1.7. Induced autophagy of lipid droplets by CB1R knockout in HBs mice

Reduced LDs storage together with enhancement of autophagy in hepatocytes of HBs/CB1$^{-/-}$ mice further prompted us to explore the role of autophagy in lipid metabolism within hepatocytes. Lysosomal acid lipase (LAL) is an acidic hydrolytic enzyme, which is responsible for lipid digestion. Its expression is assessed as an indicator of autophagy mediated digestion of lipid droplets (Tuohetahuntila *et al.* 2017). Immunostaining of LAL revealed an increased expression of LAL protein in the hepatocytes of HBs/CB1$^{-/-}$ mice compared to WT and HBs mice (**Fig.12A**). Further, double staining of PLIN2 (blue), a LDs associated protein and LAMP1 (red) showed an enhanced co-localization in hepatocytes of HBs/CB1$^{-/-}$ (**Fig.12B**, arrows), that pointed out the induction of autophagy of LDs (known as lipophagy). Our data confirmed increased LAL expression and enhanced co-localization LAMP1 with PLIN2, suggesting an induction of lipophagy in the liver of HBs/CB1$^{-/-}$ mice.

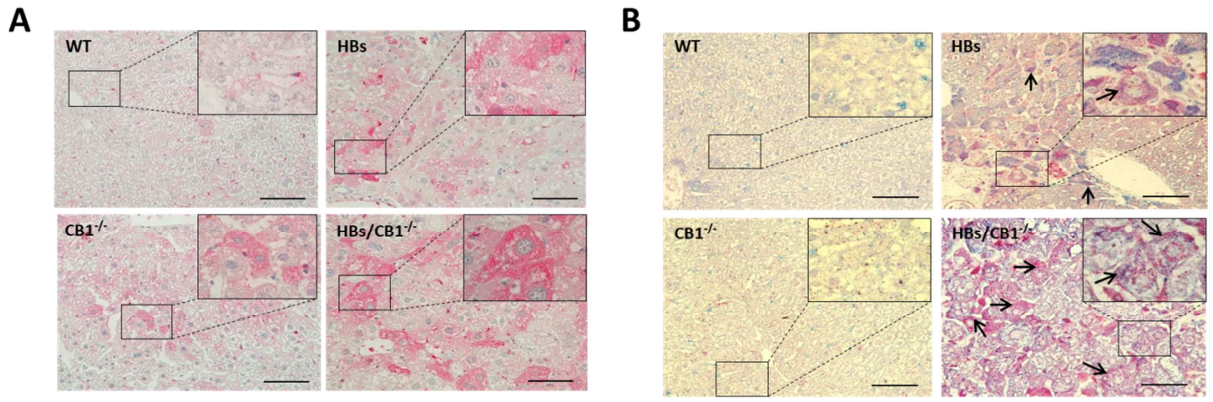


Figure 12: CB1 receptor KO induced autophagy of LDs in HBs mice. (**A**) Immunohistochemical analyses of paraffin-embedded liver sections from 52 weeks old mice were performed using anti-LAL antibody. Original image magnification 200x, bar 100 µm. **(B**) Double immunostaining analyses of paraffin-embedded liver sections of 52 weeks old mice was performed using specific anti-PLIN2 (blue) and LAMP1 (red) antibodies. Original image magnification 200x, bar 100 µm.

4.1.1.8. Increased lipase expression by CB1receptor KO in HBs mice

Apart from the selective autophagic mediated degradation of LDs, cytoplasmic lipolysis is the classical pathway that promotes LDs degradation (Lord *et al.* 2016; Sathyanarayan *et al.* 2017; Zhang *et al.* 2018). To this end, we therefore investigated whether the $CB1^{-/-}$ affected cytosolic lipoprotein lipase (LPL) and ATGL expression in hepatocytes of HBs mice. Immunohistochemical analysis showed an increased expression of LPL in $CB1^{-/-}$ and $HBs/CB1^{-/-}$ compared to WT and HBs mice (**Fig.13A**). Furthermore, double immunofluorescence staining of paraffin embedded liver sections of 52 weeks old mice using specific anti-PLIN2 and anti-ATGL antibodies showed a partial co-localization of PLIN2 and ATGL in $HBs/CB1^{-/-}$, while increased ATGL expression levels exhibited no colocalization with PLIN2 as observed in HBs mice (**Fig.13B**). These results suggest an increased ATGL accessibility to LDs in $HBs/CB1^{-/-}$ with the reduction of PLIN2 protein. Thus, CB1 knockout increased the cytoplasmic lipases expression in hepatocytes of HBs mice.

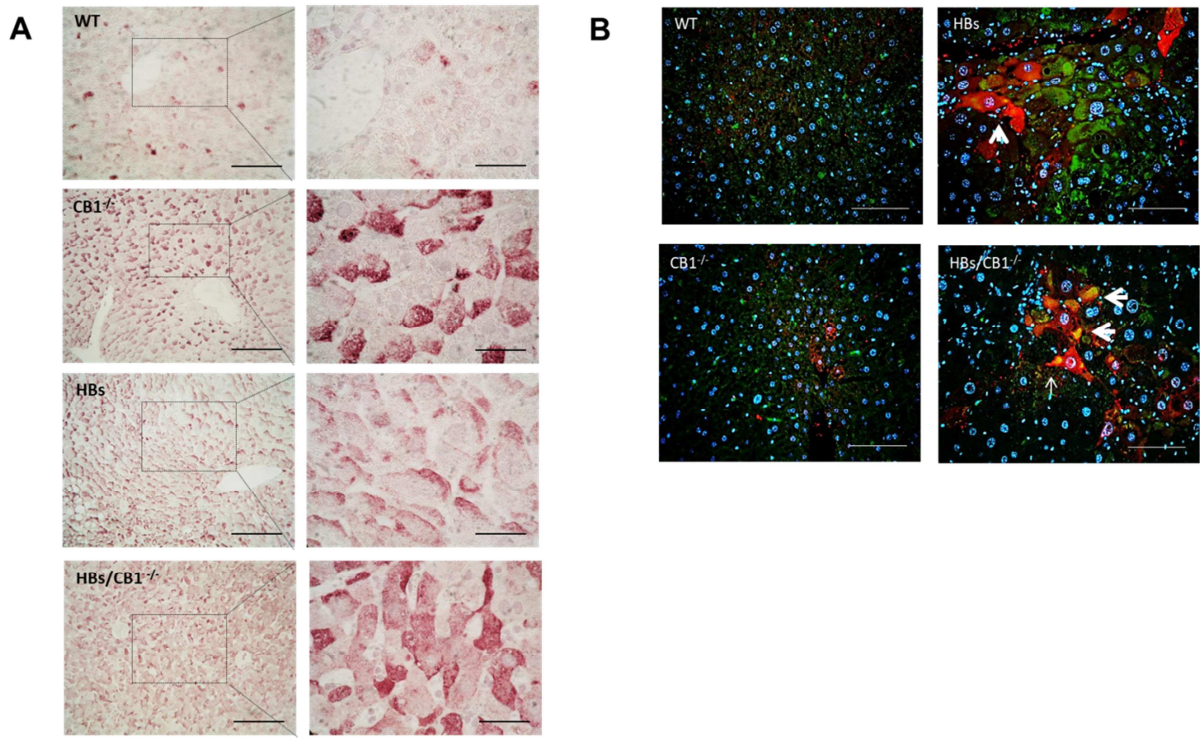


Figure 13: CB1 receptor KO enhanced lipases expression in HBs mice. (A) Lipoprotein lipase immunostaining using paraffin section of 52 weeks old mice stained using specific anti hepatic lipase (LPL). Image magnification (Left panel, 200x, right panel, 1000x; bar 200, 40µm). **(B)** Representative double immunostaining using PLIN2 (green) and ATGL (Red). Arrow depicting partial localization in HBs/CB1$^{-/-}$ mice. Image magnification 1000x, bar 40µm.

4.1.1.9. CB1 receptor KO reduced liver injury and fibrosis in HBs mice

Next, we explored the influence of CB1 receptor knockout on HBs induced liver injury and fibrosis. ALT and AST showed a prominent elevation in HBs and HBs/CB1$^{-/-}$ compared to WT and CB1$^{-/-}$ (**Fig.14A-B**). Real time PCR analysis of inflammatory gene expression of IL-1β and TNF-α showed a slight (but not significant) reduction in HBs/CB1$^{-/-}$ compared to HBs (**Fig. 14C-D**). Further, Western blot analysis using anti-αSMA revealed a reduction in CB1$^{-/-}$ and HBs/CB1$^{-/-}$ compared to WT and HBs (**Fig.14E**). These data indicate that CB1 receptor knockout displays a protection against fibrosis although a significant reduction of inflammation could not be shown in our experiments.

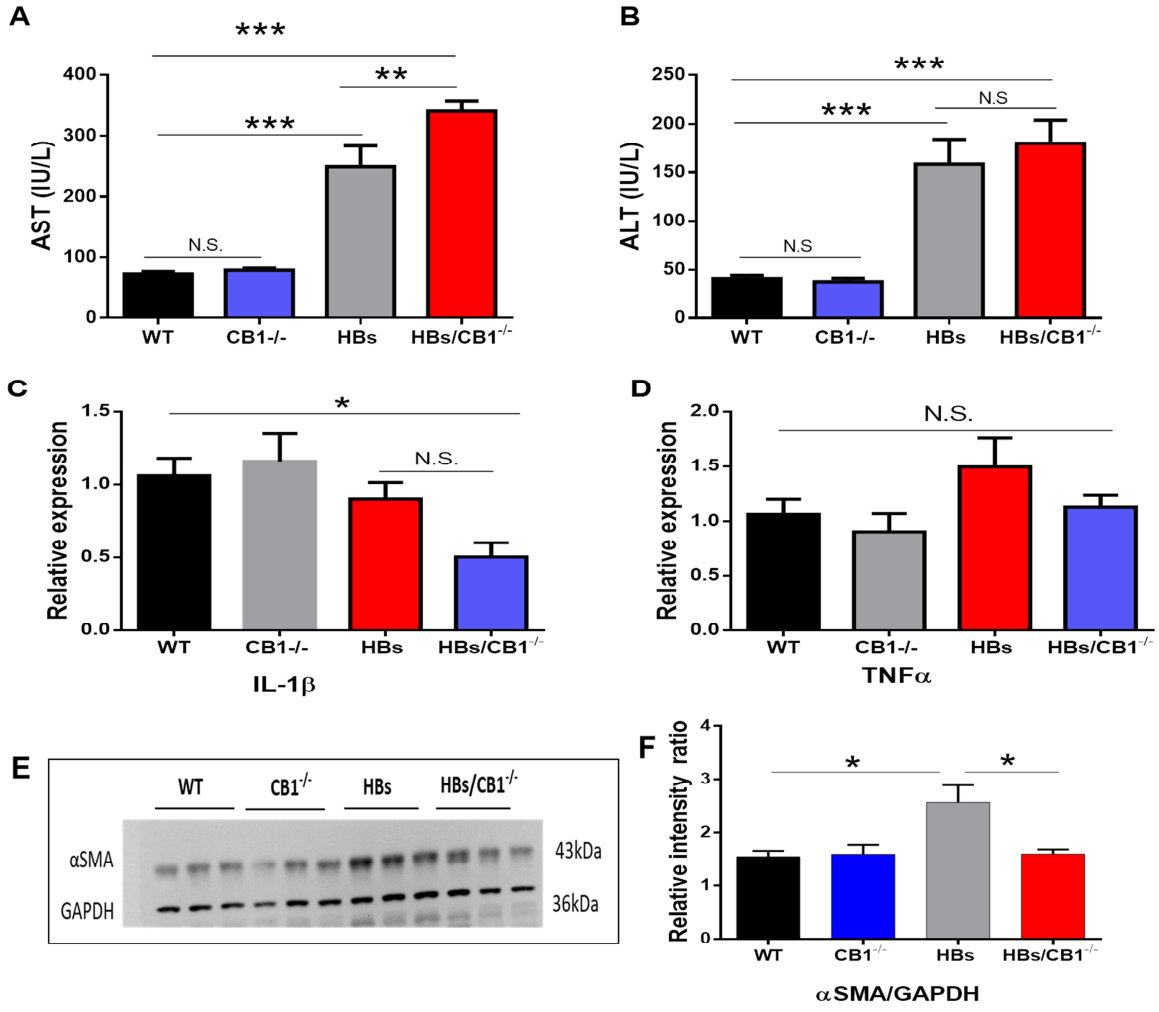


Figure 14: CB1 receptor KO effect on liver injury and fibrosis in HBs mice. (**A-B**) Bar graph representing ALT and AST analysis in 52w old mice of four different genotypes (n=8-10). Statistical analysis was performed in Graph Pad prism 6.01, one way ANOVA, Tukey´s multiple comparison test was performed. Means ± SEM, P value *<0.05, **< 0.01, ***< 0.001, N.S; not significant are indicated. **(C-D)** Quantitative real time PCR (qRT-PCR) analysis of IL-1β and TNFα transcript was performed. The amount of RNA in the different samples was normalized to β-actin as reference gene and shown as relative expression (2^-ΔΔCt). Statistical analysis was performed in Graph Pad prism 6.01, one way ANOVA, Tukey´s multiple comparison test was performed. Means ± SEM, P value *<0.05, **< 0.01, ***< 0.001, N.S; not significant are indicated. **(E)** Western blot analysis of αSMA protein expression was performed using liver lysates of 52-weeks old mice. Equal protein loading was confirmed using anti-GAPDH antibody.

(F) Densitometric analysis of relative protein expression of αSMA normalized to GAPDH. (modified from (Irungbam *et al.* 2019)).

4.1.2. Discussion:

For many years, endocannabinoids (EC) and their receptors have been emerged as major regulators of chronic liver disease development and progression (Mallat *et al.* 2011). In the current study, we have explored the effect of global CB1 receptor knockout on hepatic lipid storage and chronic liver disease development in HBs mice. Several studies involving genetic deletion or pharmacological antagonism of CB1 receptor have demonstrated anorectic, body weight reduction, anti-obesity effects and also shown improvements of hepatic steatosis and metabolic disturbances (Ravinet Trillou *et al.* 2003; Shi *et al.* 2014). In our current study, we also observed global CB1 KO mediated reduction in body and liver weight in HBs/CB1$^{-/-}$ as compared to HBs (Fig. 6A-B). Our results are in agreement with previous findings indicating that lack of CB1 signaling leads to reduction of body weight (Ravinet Trillou *et al.* 2003). The data on food intake was limited in our studies. Nevertheless, the reduction in body weight hinted towards decreased appetite and less food intake due to global CB1 receptor knockout. The weight loss associated with CB1 receptor blockade might induce metabolic stress. In response to metabolic stress, the organism tries to mobilize body's energy stores to cope with the stress situation. AMP-activated protein kinase is a metabolic sensor that responds to low cellular ATP concentration to maintain cellular energy homeostasis (Hardie 2011). However, our data showed no increased activation of AMPK in HBs/CB1$^{-/-}$ compared to HBs (Fig. 7B). Lack of AMPK activation might be due to an age dependent or hormone mediated affect such as glucagon, which coincides with elevated fat oxidation (Berglund *et al.* 2009). The effect of hormonal changes and effect of age need further investigation. Despite, lack of activation of AMP kinase in HBs/CB1$^{-/-}$, the downstream targets of AMPK, FAS, ACC1, PPARγ expression was slightly reduced in CB1$^{-/-}$ and HBs/CB1$^{-/-}$ (Fig. 7C), which is in agreement with earlier reports indicating CB1KO mediated inhibition of lipogenesis (Cota 2007). Wafa Abbud *et al.*, have reported that stimulation of AMPK is associated with enhancement of GLUT1-mediated glucose transport. Our study demonstrated increased hepatic membrane translocalization of GLUT1 in CB1$^{-/-}$ and HBs/CB1$^{-/-}$. The production of endogenous HBV surface proteins in the hepatocytes of mice might induce additional cellular stress, which might explain for higher energy surge as indicated

by more enhanced GLUT1 membrane localization in HBs/CB1$^{-/-}$ compared to CB1 KO (Fig. 7A). Unfortunately, information on fasting and re-feeding serum glucose parameter was limited in this study. The reduction of body weight with increased hepatocytes membrane glucose transporter protein localization suggested for higher energy surge and hints towards metabolic stress. At the transcriptional level GLUT1 was not regulated (data not shown) that excluded the effect of hypoxia in our model. Taken together, our findings support that CB1 knockout induce metabolic stress and reduce lipogenesis in the hepatocytes of HBs mice.

Studies have shown that CB1KO can modulate autophagy induction (Hiebel *et al.* 2014). Here, we also observed enhancement of autophagy by CB1 knockout in HBs mice. Autophagic flux refers to autophagic degradation activity (Klionsky *et al.* 2016). So far, the reliable biological marker used for identifying autophagic flux is LC3-II turnover or SQSTM1/p62 degradation, although it imposes challenges in the experimental set up to access the magnitude of autophagic flux especially in a steady state condition (Ben Loos *et al.* 2014). In similar line, our study demonstrated decreased LC3B-II and p62 in HBs/CB1$^{-/-}$ (Fig. 11F-G) along with an increased amount of autophagosomes puncta (Fig. 11A-B), suggesting an autophagic flux progression. Similarly, *in vitro* results also showed increased LC3BII and reduced p62 protein expression following rimonabant treatment (Fig. 11I-J), further supporting that CB1 receptor antagonization enhances autophagy.

HBV surface protein transgenic mice (Chisari *et al.* 1987), exhibit nuclear inclusions in murine hepatocytes (Thakur *et al.* 2014). The formation of insoluble protein inclusion in the hepatocytes displays a characteristic of a damaged liver in both, mice and humans (Zatloukal *et al.* 2002). It is known that p62 is a common component of insoluble protein inclusions or aggregates (Zatloukal *et al.* 2002). Therefore, the increased p62 expression in HBs and HBs/ CB1$^{-/-}$ might be associated with HBV transgenic proteins which are marked for degradation either through the ubiquitin- proteasomal or autophagic pathway (Liu *et al.* 2016). Increased p62 colocalization with LAMP1 in HBs/CB1$^{-/-}$ supports autophagic degradation of protein inclusion bodies (Fig. 11E).

Autophagy is a multistep process and doesn't provide a complete picture especially in steady state condition such as in animal models only with LC3B and p62. Some studies have reported

the measurement of autolysosome formation as another process to understand autophagy flux (Klionsky *et al.* 2016; Singh *et al.* 2009). Similarly, LAMP1 expression measurement is an important evaluator for autophagic flux (Pugsley 2017). In this context, we demonstrated increased LAMP1 protein expression in $CB1^{-/-}$ and $HBs/CB1^{-/-}$ compared to WT and HBs (Fig. 11C). Enhanced colocalization of LAMP1 with LC3B further supports the autophagic flux mediated through CB1KO in HBs mice (Fig. 11D).

It is known that $CB1^{-/-}$ promotes increased peripheral energy expenditure, increases lipid catabolism in the liver, and adipose tissues etc. (Cota 2007; Lee *et al.* 2019). We demonstrated decrease in lipid droplets size, numbers, and triglycerides content in the liver of $HBs/CB1^{-/-}$ (Fig. 7A-C) suggesting an enhancement of lipid catabolism. However, the mechanism of CB1 KO induced increased lipid catabolism is still elusive. The role of lipophagy and lysosomal acid lipases in reducing hepatic steatosis was described earlier (Singh *et al.* 2009). Here, we propose lipophagy as demonstrated by increased expression of LAL in $HBs/CB1^{-/-}$ mice (Fig. 12A) and partial co-localization of perilipin2 (PLIN2) and LAMP1 (Fig. 12B) which altogether supports lipophagy in murine hepatocytes. Removal of the LD surface proteins, PLIN2 and PLIN3, by macroautophagy or chaperon mediated autophagy (CMA) represents the prerequisite step that helps to unlock the lipid components of the LD core for degradation by lipophagy or cytosolic lipases (Kaushik and Cuervo 2015). Our results also demonstrated a decreased PLIN2 expression both, at transcriptional as well translational level (Fig. 9A-C). The reduction of PLIN2 was correlated with reduction of liver TAG content (Fig.9F). Further, the elevation of PLIN3 as observed in $HBs/CB1^{-/-}$ mice possibly be a compensatory mechanism regulating lipid droplet accumulation in the absence of PLIN2 (Sztalryd *et al.* 2006). Oxidative tissues such as skeletal muscle mostly expresseses PLIN5 and considered to promote fat oxidation (Dalen *et al.* 2006). The slight increase in PLIN5 in $CB1^{-/-}$ and $HBs/CB1^{-/-}$ mice suggests an increased oxidative degradation of TAGs (Fig. 9D, E).

The increased colocalization of PLIN2 with LAMP1 in $HBs/CB1^{-/-}$ mice (Fig. 11J) indicates the induction of either microautophagy or macroautophagy of LDs thereby promoting PLIN2 degradation. Cell culture experiments further support the reduction of PLIN2 expression following rimonabant treatment compared to vehicle or methanadamide (Fig. 10A-C) suggesting involvement of CB1signaling in modulation of PLIN2 expression. The major LDs associated

protein, PLIN2 protects and stabilizes, providing a "concealing effect'' and regulates the lipase's accessibility to LDs (Kaushik and Cuervo 2015; Listenberger *et al.* 2007). Decreased PLIN2 expression therefore reciprocally enhances the expression of hepatic lipases (Fig. 13A). Interestingly, the absence of colocalization of PLIN2 and ATGL in HBs might suggest the lack of association and accessibility with LDs. However, the partial colocalization observed in HBs/CB1$^{-/-}$ might indicate an increased accessibility on the LDs surface with reduction of PLIN2 (Fig. 13B). These data are in coherence with earlier reports (Sathyanarayan *et al.* 2017). Elevated lipophagy along with increased neutral lipolysis might reduce hepatic steatosis by promoting lipid turnover in HBs mice.

Studies have revealed a significant correlation with CB1 and CB2 expression pattern to the degree of fibrosis in CHB patients (Dai *et al.* 2017). Carbon tetrachloride (CCl_4) induced fibrosis showed improvement due to CB1 receptor depletion (Mallat and Lotersztajn 2008). Our findings also demonstrated a reduction of αSMA protein expression due to CB1KO in HBs mice along with a slight reduction in inflammatory cytokines which might indicate an improvement of fibrosis and liver damage in HBs mice (Fig. 14C-F). However, the improvement in hepatic steatosis was not associated with improvement of liver injury as indicated by elevated AST and ALT levels in HBs/CB1$^{-/-}$ compared to HBs mice (Fig. 14A-B). This might be an effect of over expressing the HBV transgenic proteins, thus conferring liver injury rather than hepatic steatosis *per se*.

Conclusion: Conclusively, our data suggest that CB1 knock out promotes a reduction in body and liver weight as well as reduction of hepatic fat in HBs mice. A schematic diagram (Fig.15) illustrates the major findings. The reduction of hepatic fat is associated to increased autophagy of lipid droplets in the hepatocytes of HBs/ CB1$^{-/-}$ mice, which might serve as a survival strategy to cope with with the metabolic stress induced by CB1 receptor deficiency. Lipophagy mediates not only the degradation of stored TAGs in LDs but also LDs associated protein, PLIN2, which ultimately enhances the association and accessibility of cytoplasmic lipases to LDs. Thus, enhanced lipophagy together with elevated lipase expression pave the mechanistic basis for CB1 knockout improvement in steatosis in HBs mice. Further, genetic deletion of CB1 receptor slightly improves fibrosis in HBs mice suggesting a beneficial role of CB1 receptor modulation on NAFLD and associated chronic liver disease.

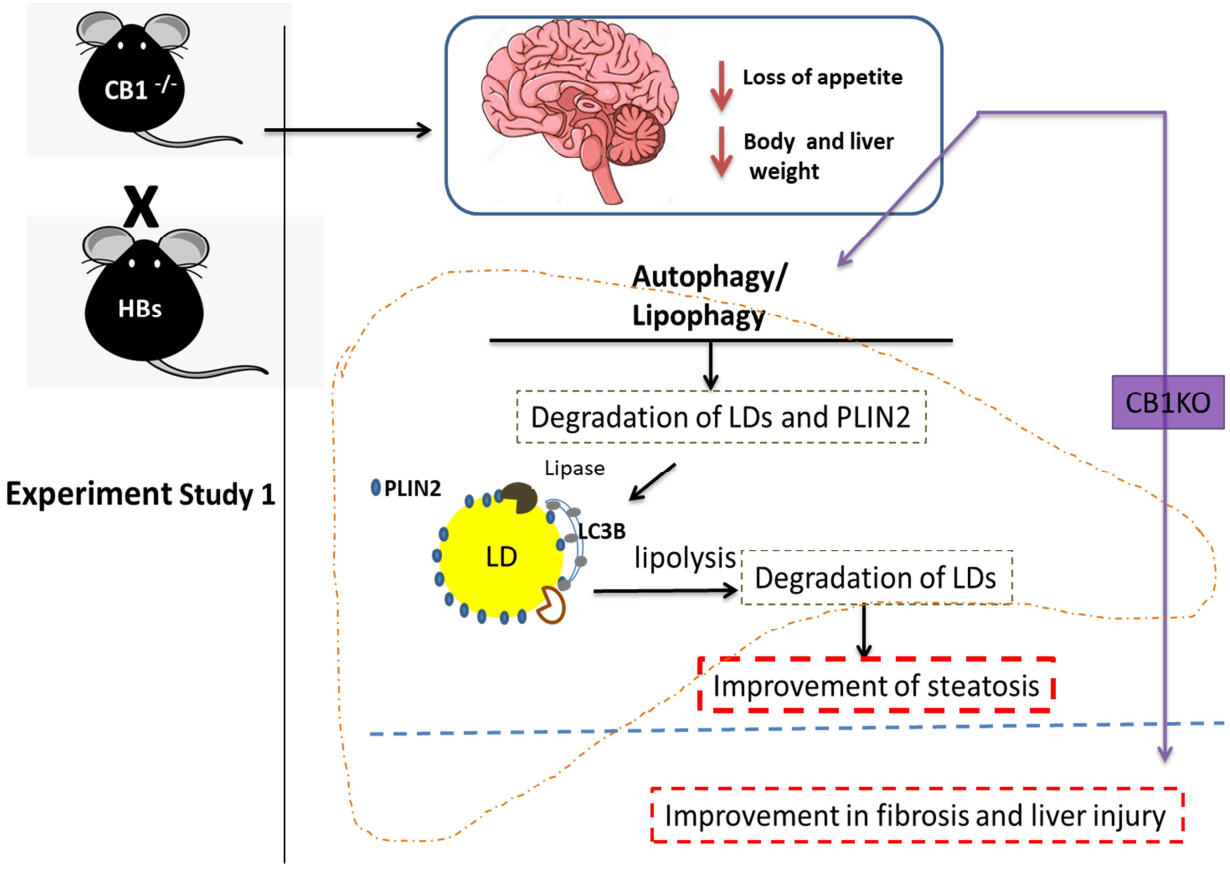


Figure 15: Loss of CB1 receptor signaling alleviates hepatic steatosis in HBs mice. Global CB1 receptor knockout mediates anorexia and subsequent reduction in body weight and hepatic steatosis in HBs mice via enhancement of autophagy.

4.2. Modulation of hepatic lipid metabolism by Abcb4 receptor knockout in HBs (HBV surface protein transgenic) mice.

4.2.1. Results:

4.2.1.1. Loss of hepatic lipid droplets and TAG storage mediated by Abcb4$^{-/-}$ in HBs mice.

In this study we investigated the combined effect of Abcb4$^{-/-}$ induced cholestasis on steatosis induced in HBs mice. Previously, it was reported that Abcb4$^{-/-}$ mice showed a dysregulation in lipid metabolism which is associated with the disease development and progression (Moustafa *et al.* 2012). To understand the alterations in the hepatic lipid profile in HBs/Abcb4$^{-/-}$, HPTLC-FLD (high performance thin layer chromatography-fluorescence detection) method was performed. Using this method, multiple lipids can be separated on a single HPTLC plate. Our results showed separation of 5 different lipids (cholesterol, free fatty acids, triacylglycerol, methyl oleate, and cholesterol oleate) from the total hepatic lipid extract. HPTLC analysis revealed a reduction of hepatic triacylglycerol in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ mice in comparison to WT and HBs resp. (**Fig.16A**). The identity of the lipids separated on the HPTLC plates was confirmed by mass spectrometry and the respective spectra are illustrated in **Fig.17A-H** showing identity of each lipid separated based on the standard input. Further, densitometric quantification demonstrated a significant reduction in the amount of triacylglycerol (TAG) in the liver of HBs/Abcb4$^{-/-}$ compared to HBs (**Fig.16B**). Interestingly, free fatty acid amount was significantly increased in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ as compared to WT and HBs resp. (**Fig.16C**). The levels of cholesterol and cholesteryl-oleate did not alter among the groups (**Fig. 16D-E**). Oil red O staining showed remarkable reduction in neutral lipid depots in the hepatocytes of both, Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ mice, compared to WT and HBs mice resp. (**Fig.16F**). These results are in line with the HPTLC findings. Additionally, liver TAG and FFAs quantification assays also demonstrated a reduction in hepatic TAG levels with an increase in the amounts of FFA in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ mice (**Fig.16G-H**). To investigate the changes in serum lipids parameter, cholesterol (CHOL) and triacylglycerol (TAG) analysis were performed. Our results demonstrate a significant reduction of cholesterol and triacylglycerol in Abcb4$^{-/-}$and HBs/Abcb4$^{-/-}$ compared to WT (**Fig.16 I-J**). Very interestingly, the hepatic Abcb4 receptor knockout

pertinently reduced hepatic TAG levels along with reduction of serum TAG and CHOL in HBs/Abcb4$^{-/-}$ mice.

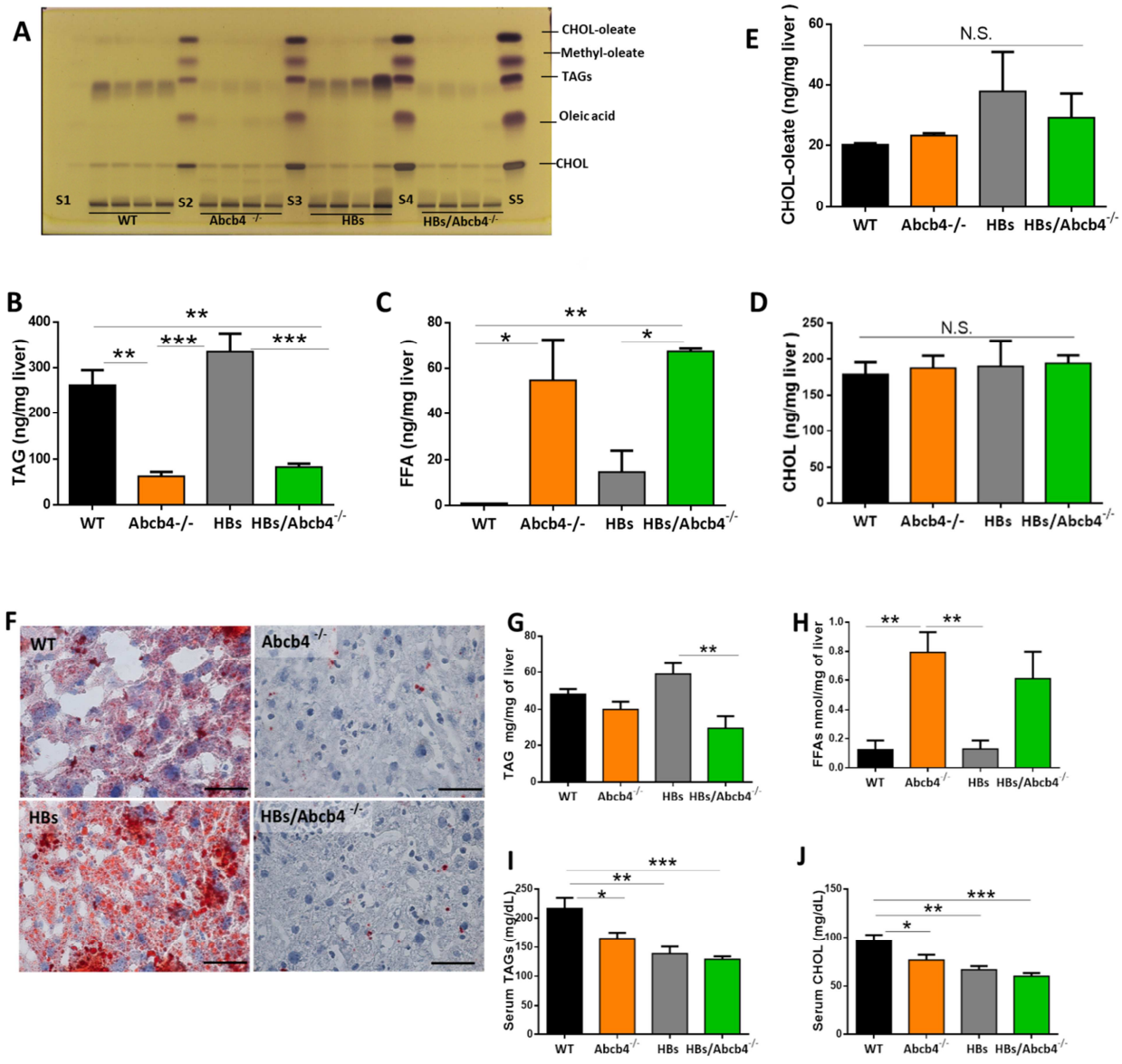


Figure 16: Reduction of hepatic LDs and TAG mediated by Abcb4$^{-/-}$ in HBs mice. (A) High performance thin-layer chromatography (HPTLC) chromatogram of non-polar lipids visualized by sulphuric acid derivation method. **(B-E)** Densitometric analysis of different lipids via fluorescence measurements of HPTLC-MS plate using TLC scanner 4 (CAMAG), plate images were documented at 366 nm by a TLC Visualizer (CAMAG). Bar graphs for triacylglycerol, cholesterol, free fatty acids, and cholesterol-oleate quantification are shown. Results represent

the mean ± SE from at least two independent HPTLC-MS plates with n = 4 in each group. (**F**) Oil red O (ORO) staining demonstrated decreased accumulation of neutral lipids in Abcb4$^{-/-}$ and HBs/ Abcb4$^{-/-}$ compared to WT and HBs mice (1000x, bar 50). (**G-H**) Graph depicting TAG and FFAs analysis from liver lysates using kit based methods. N=4 samples in each group. **(I-J)** Serum triacylglycerol and cholesterol estimation using Reflotron kit, n= 10 (5 female + 5 male) in each group. Statistical analysis: ANOVA with Tukey's multiple tests were performed. P value *<0.05, **<0.01, ***<0.001 and N.S. not significant are indicated (Paper in revision).

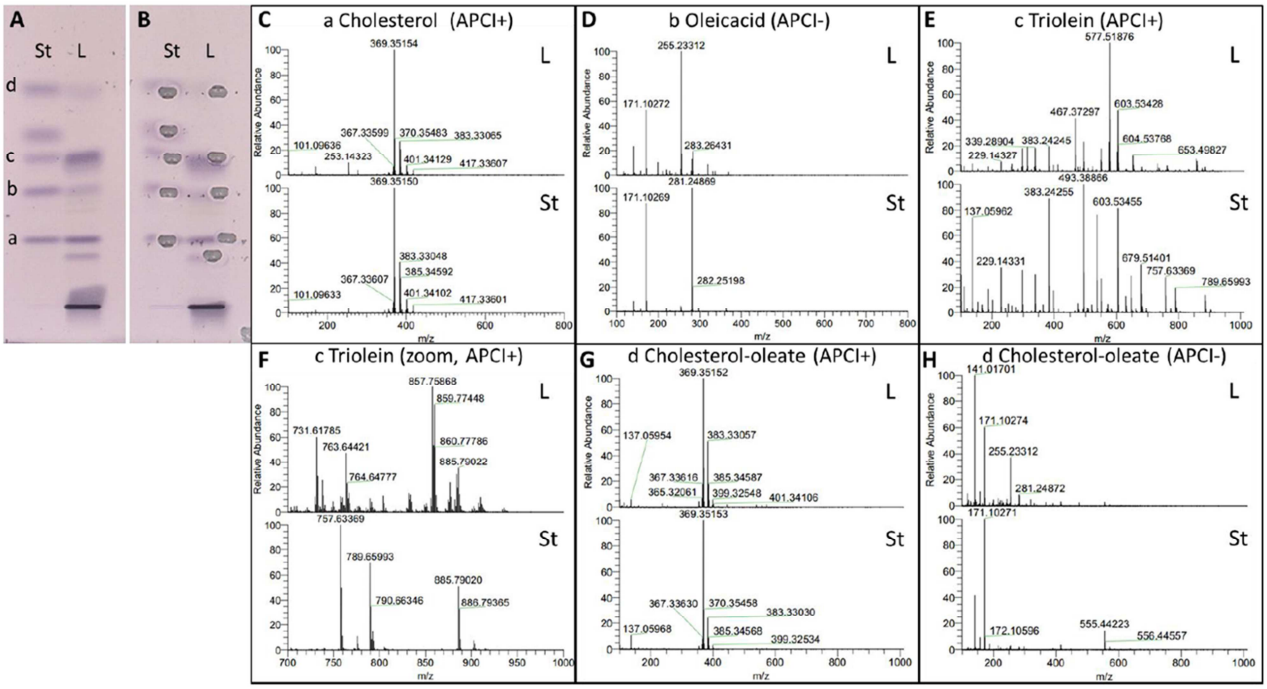


Figure 17: **HPTLC-MS (APCI) mass spectra of four different lipids**. HPTLC plates with lipids (**A**) and plate after elution of the selected zones **(B)** via TLC-MS Interface 2 into the HRMS, HPTLC-HRMS mass spectra **(C-H)** mass spectra of four different lipids (band a-d) from the liver samples (**L**) and lipid standards (**St**).

4.2.1.2. Effect of Abcb4$^{-/-}$ on body, liver weight and liver enzymes in HBs mice

The reduction in liver fat prompted to investigate the impact of Abcb4 knockout on the liver and bodyweight in HBs mice. The body weight analysis showed significant increases in HBs and HBs/Abcb4$^{-/-}$ compared to WT (**Fig.18A**). Interestingly, liver weight analysis revealed significant increases in Abcb4$^{-/-}$, HBs, and HBs/Abcb4$^{-/-}$ compared to WT (**Fig.18B**). Similarly,

liver to bodyweight ratio was significantly increased in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs resp. (**Fig.18C**). Additionally, the serum analysis of ALT, AST, and GGT showed remarkable increases in HBs/Abcb4$^{-/-}$ compared to HBs and also in Abcb4$^{-/-}$ compared to WT. (**Fig.18D-F**). Taken together, our results showed increased in body and liver weight along with enhancement of serum amino transaminases in HBs/Abcb4$^{-/-}$ mice.

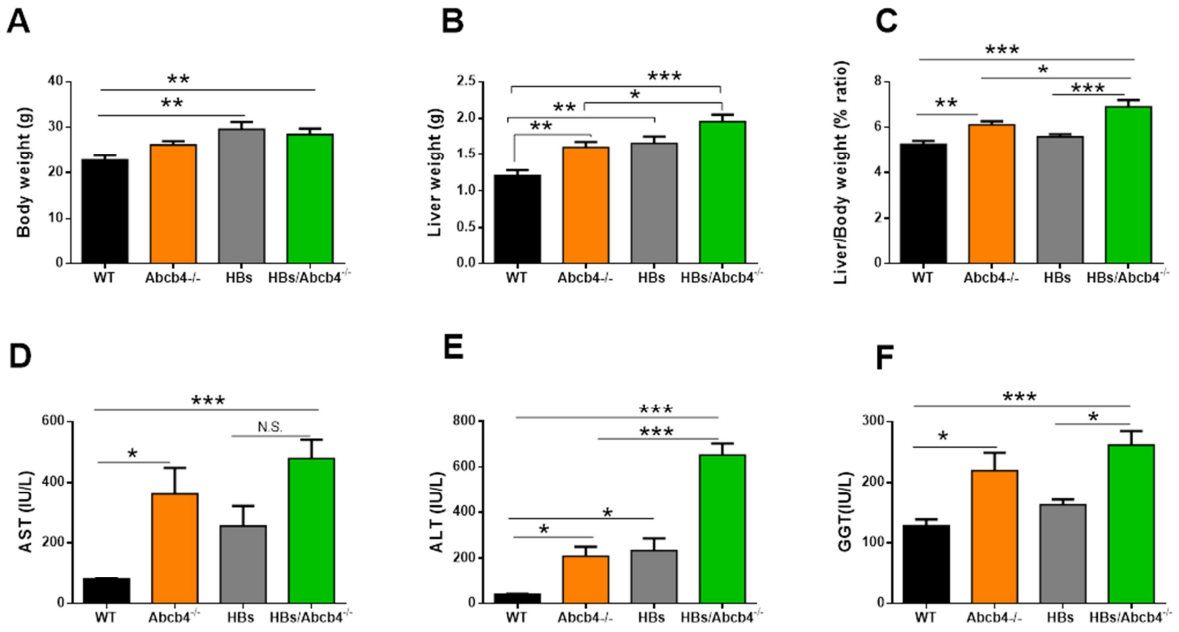


Figure 18: Body, liver weight and serum enzyme analysis. **(A-C)** Bar graph representing body weight, liver weight, and liver/body weight percentage ratio analysis of 12-19 weeks old mice, n=8-10. Statistical analysis: One way ANOVA (parametric) with Tukey's multiple tests were performed to compare the groups. P value *<0.05, **<0.01, ***<0.001 indicated in the figures. **(D-F)** Liver injury assessed by serum AST, ALT, and GGT analysis, n=8-10. Statistical analysis: One way ANOVA (parametric) with Tukey's multiple tests were performed to compare among the group. P value *<0.05, **<0.01, ***<0.001 indicated in the figures. All the analysis was performed using GraphPad, 6.0 versions.

4.2.1.3. AMPK-CREB activation mediated by Abcb4$^{-/-}$ in HBs mice

Next, we explored the effect of Abcb4$^{-/-}$ induced reduction in hepatic TAGs on the metabolic and energy sensor, AMPK. Immunoblotting demonstrated increased phosphorylation of AMPK in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ as compared to WT and HBs resp. (**Fig.19A**). The activation of

AMPK suppresses lipids synthesis and activates catabolic pathways (Garcia and Shaw 2017). The transcription factor CREB has been widely investigated as a metabolic sensor and regulator of glucose and fat homeostasis in liver and fat tissue (Altarejos and Montminy 2011). Immunoblotting results demonstrated an increased activation of CREB in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs mice resp. (**Fig.19A**). Similarly, immunohistochemical studies using specific P-CREB antibody also confirmed enhanced nuclear localization in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs mice resp. (**Fig.19B**). Additionally, EMSA demonstrated a more efficient binding of CREB oligonucleotides to nuclear proteins of Abcb4$^{-/-}$ and HBs/ Abcb4$^{-/-}$ mice in comparison to WT and HBs, with more enhanced binding noted in HBs/Abcb4$^{-/-}$ compared to Abcb4$^{-/-}$ and HBs mice resp. as shown in the **Fig.19C**.

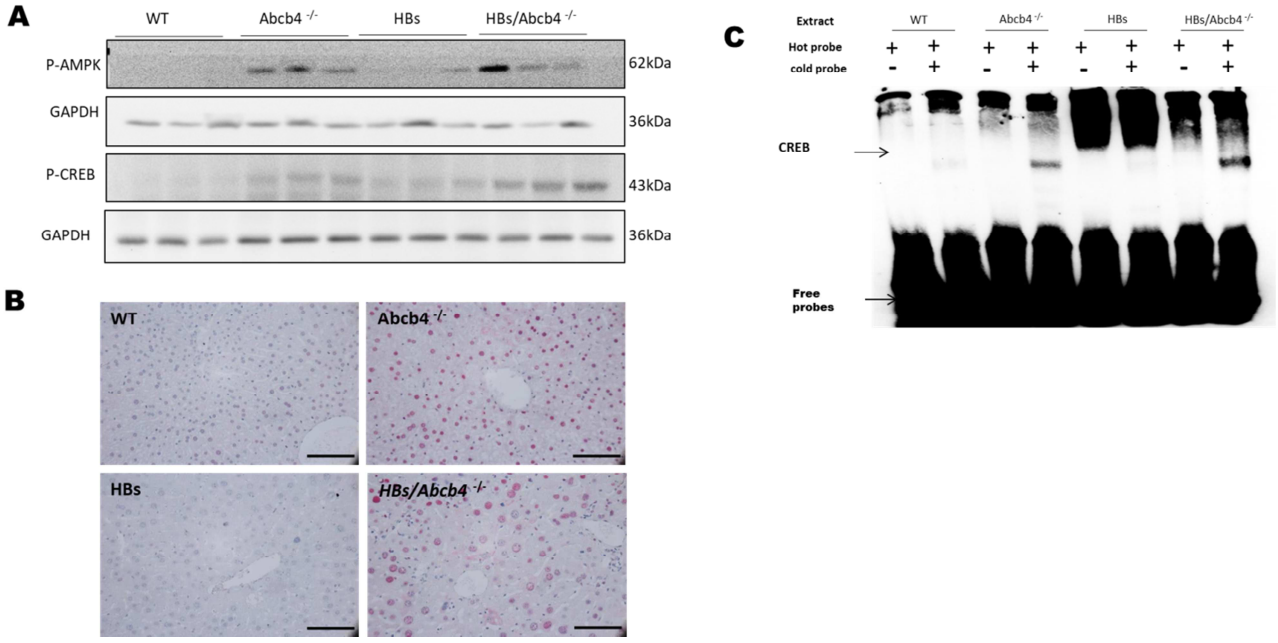


Figure 19: Abcb4$^{-/-}$ activated AMPK-CREB signaling in HBs mice. (A) Western blot analysis using specific antibodies against P-AMPK, AMPK, and P-CREB were performed. Equal loading was confirmed by GAPDH analysis. **(B)** Increased P-CREB nuclear localization was observed in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ as demonstrated by IHC staining (x400, bar 200µm). **(C)** EMSA demonstrated a more efficient binding of CREB oligonucleotides to hepatic nuclear proteins of Abcb4$^{-/-}$ and HBs/ Abcb4$^{-/-}$ mice in comparison to WT and HBs.

4.2.1.4. Suppression of hepatic lipogenesis mediated by Abcb4$^{-/-}$ in HBs mice

Next, we elucidated the effect of AMP-activated protein kinase (AMPK) activation on hepatic lipogenesis pathways. The target genes and transcriptional factors regulated by AMPK involved in lipogenesis, SREBP1-c, ACC1, FASN, Scd1, HMG-CoA, and PPARγ, in addition to genes involved in lipid uptake and transport (CD36, MTTP, apoE, PCTP) in the liver tissues were analyzed by real time PCR.

Sterol regulatory element-binding protein 1 (SREBP-1) is the transcriptional factor which is directly regulated via AMPK (Garcia and Shaw 2017). SREBP1c further modulates transcription of genes encoding enzymes of fatty acid synthesis and uptake, including acetyl CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl CoA desaturase-1(Scd-1), HMG-CoA and lipoprotein lipase (Cheng *et al.* 2018). qPCR analysis showed tendential downregulation of SREBP-1c expression in Abcb4$^{-/-}$, HBs and HBs/Abcb4$^{-/-}$ as compared to WT (**Fig.20A**). Two important genes of *de novo* fatty acid synthesis, acetyl-CoA carboxylase1 (ACC1) and fatty acid synthase (FASN) showed a significant downregulation in HBs/Abcb4$^{-/-}$ compared to HBs and WT resp. (**Fig.20B-C**). Similarly, Scd1, HMGCoA, and peroxisome proliferator-activated receptor gamma (PPARγ) showed significant downregulation in HBs/Abcb4$^{-/-}$ compared to HBs resp. (**Fig.20D-F**). Genes involved in lipid uptake and transportations namely, CD36 (fatty acid translocase), ApoE (apolipoprotein E), MTTP (microsomal triglyceride transfer protein), PCTP (phosphatidylcholine transfer protein), were suppressed in HBs/Abcb4$^{-/-}$ as compared to HBs (**Fig. 20G-J**). Our results suggested that Abcb4 knockout mediated activation of AMPK is associated with suppression of *de novo* lipogenesis, uptake, and transports of lipids in HBs mice.

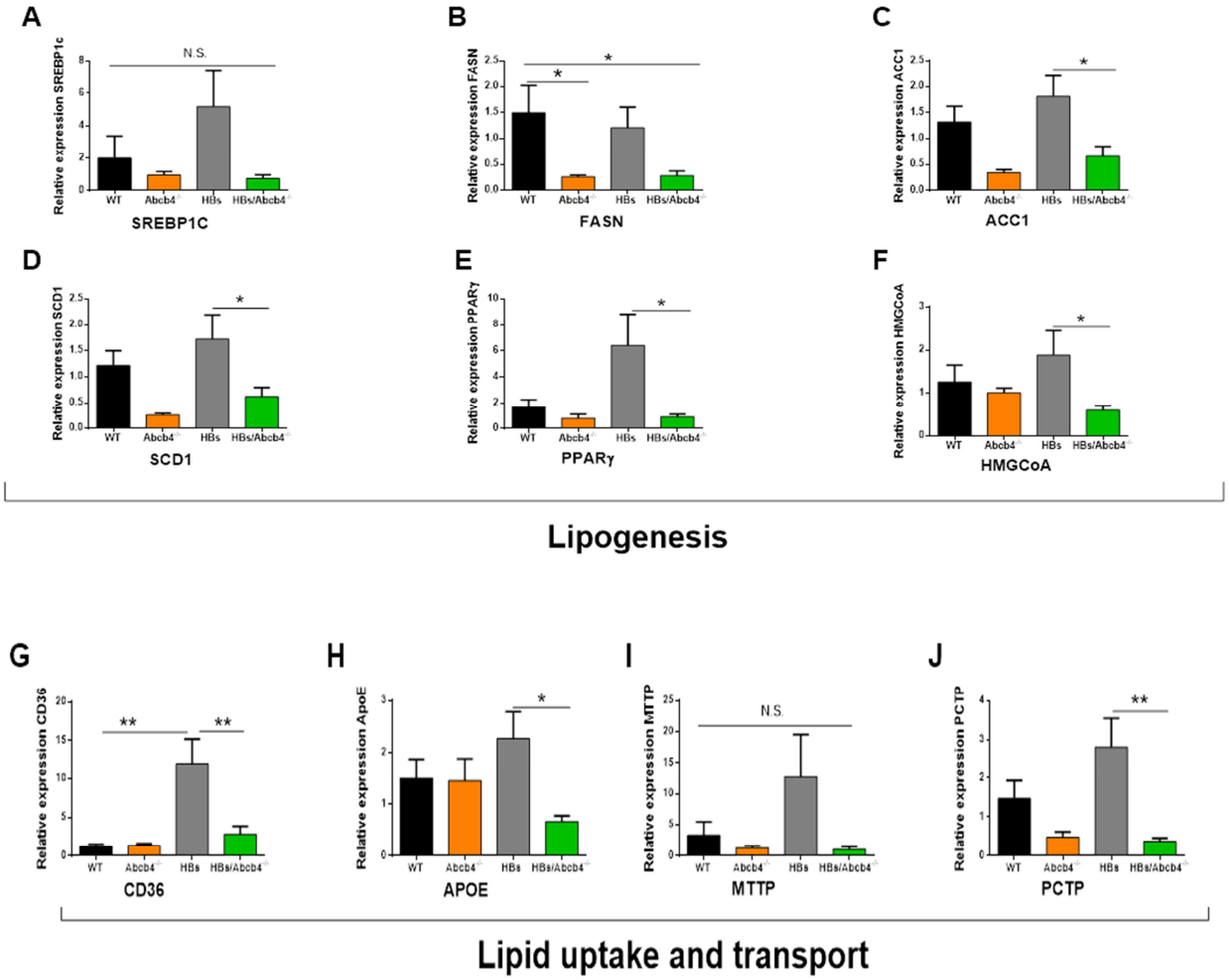


Figure 20: Abcb4$^{-/-}$ altered hepatic lipid metabolism gene expression in HBs mice. (A-K) Relative mRNA expression of major genes involved in *de novo* lipogenesis, SREBP-1c, FASN, ACC1, Scd-1, HMGCoA, PPARγ, CD36, ApoE, MTTP, PCTP in liver of four different groups were analysed. The primers that were used for RT-PCR are listed in table 14. Results are presented as mean ± S.E., n=10 in each group. Analyses were performed in duplicates. Statistical tests and computation of S.E were performed on ΔCT-values. ANOVA with Tukey`s multiple comparison tests was performed, P*<0.05 P**<0.01, P***<0.001, N.S.-Not significant.

4.2.1.5. Suppression of TAG synthesis mediated by Abcb4$^{-/-}$ in HBs mice

With the reduction of hepatic TAG storage, next we investigated the effect of Abcb4$^{-/-}$ induced cholestasis on TAG synthesis pathways. Therefore, we investigated the genes involved in TAGs synthesis, namely 1-acyl-glycerol-3-phosphate acyltransferase1 (AGPAT1), glycerol-3-

phosphate acyltransferase1 (GPAT1), monoacylglyceroltransferase1 (MGAT1), diacylglycerol acyltransferase 1 and 2 (DGAT1 and DGAT2). GPAT1 and AGPAT1 are involved the *de novo* synthesis of triacylglycerol (TAG) and glycerophospholipids (Yamashita *et al.* 2014). Our results demonstrated a down regulation of GPAT1 and AGPAT1 at transcriptional level in both Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ as compared to WT and HBs resp. (**Fig.21A-B**). MGAT1 synthesizes diacylglycerol by catalyzing the acylation of monoacylglycerol. We observed a reduction of MGAT1 at transcriptional level in HBs/Abcb4$^{-/-}$ as compared to HBs resp. (**Fig.21C**). Both, DGAT1 and DGAT2 catalyze the final committed step of TAG synthesis (the acylation of diacylglycerol with a fatty acyl-CoA) (Yen *et al.* 2008). At transcriptional level, we observed a significant downregulation DGAT2 in HBs/Abcb4$^{-/-}$ as compared to WT, while, DGAT1 expression did not significantly differ among the groups (**Fig.21D-E**). The protein expression level of MGAT1 and DGAT1 showed a decrease in HBs/Abcb4$^{-/-}$ as compared to HBs mice (**Fig.21F-G**). Taken together, our findings suggest that the Abcb4$^{-/-}$ mediated suppression of TAGs synthesis went along with reduction of LDs in mice on HBs background.

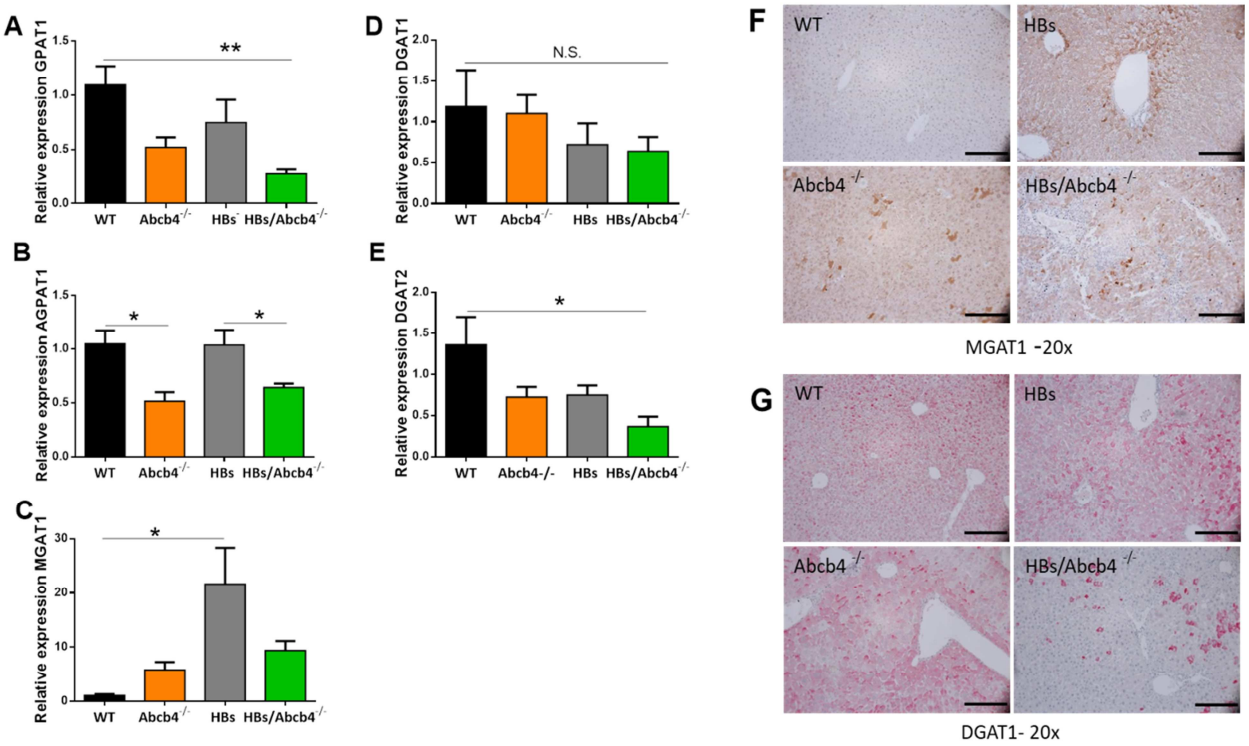


Figure 21: Abcb4$^{-/-}$ reduced TAGs synthesis and storage in HBs mice. (A-E) Bar graph representing relative mRNA expression of major genes involved in triacylglycerol synthesis; MGAT1, DGAT1, DGAT2, GPAT1, and AGPAT1 in the liver of four different groups, normalized against β-actin gene. The primers used are listed in table 14. Results are presented as mean ± S.E., n = 10 in each group, experiments were performed in duplicates. Statistical analysis: ANOVA with Tukey`s multiple comparison test, P*<0.05 P**<0.01, P***<0.001, N.S.-Not significant indicated. **(F)** Representative Immunohistochemical staining using specific antibodies against MGAT1 (x200, bar 200µm). **(G)** DGAT1 immunohistochemical staining using specific antibody against DGAT1 (x200, bar 200µm).

4.2.1.6. Loss of LDs associated proteins mediated by Abcb4$^{-/-}$ in HBs mice

The reduction in amount of LDs in hepatocytes as demonstrated by oil red staining further prompted us to investigate the effect on LDs associated proteins. PLIN2 is a most constitutively and ubiquitously expressed protein associated with LDs and a marker of LDs which directly correlated with the amount of LDs or TAGs storage (Tsai *et al.* 2017). Next, we investigated the effect of Abcb4$^{-/-}$ on PLIN2 expression by using western blotting, real time PCR and immunohistochemistry. PLIN2 expression was reduced in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ as compared to WT and HBs mice resp. both, at transcriptional and translational level (**Fig.22A-C**). Furthermore, the expression of other PLINs was not altered (data not shown). Additionally, other LDs associated proteins, CIDEC protein showed remarkable reduction in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ as compared to WT and HBs mice resp. (**Fig.22A**).

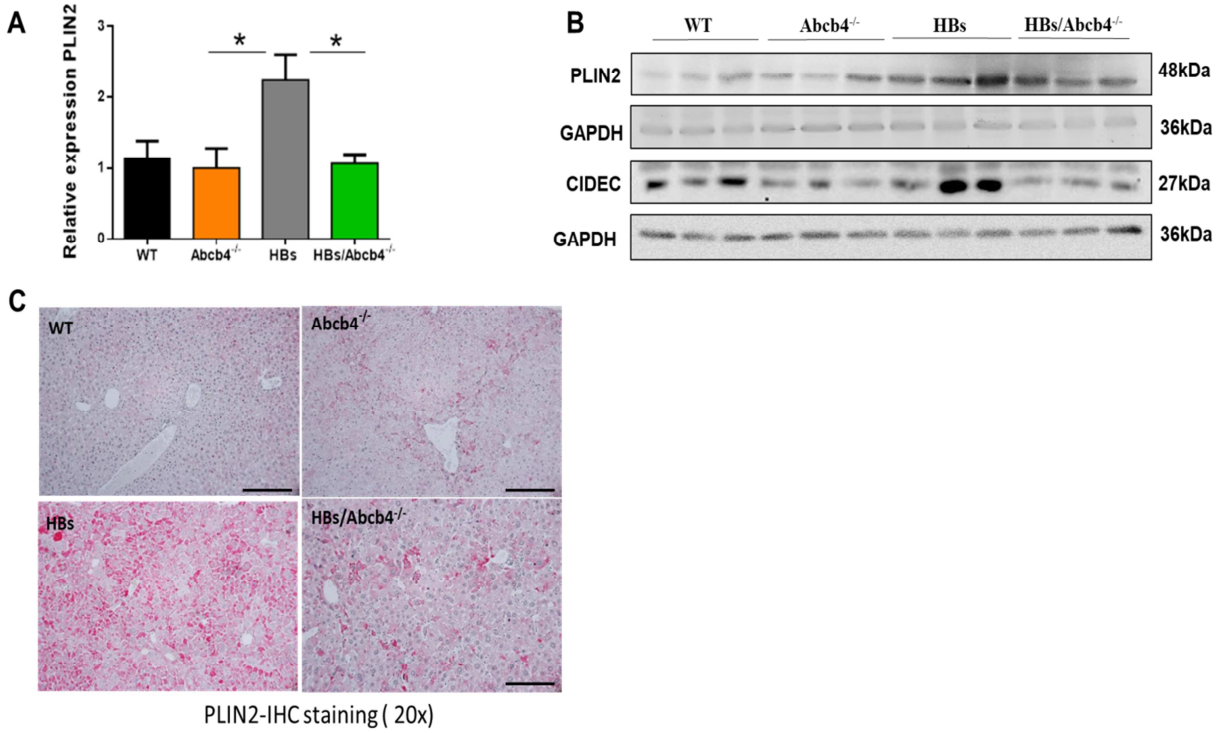


Figure 22: Reduced LDs associated proteins induced by Abcb4$^{-/-}$ in HBs mice. **(A)** Graph representing relative mRNA expression of PLIN2 gene in liver of four different groups, normalized against β-actin gene. Statistical analysis: ANOVA with Tukey`s multiple comparison tests, P value *<0.05 indicated. (**B**) Reduction of PLIN2 and CIDEC in the liver lysates of Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs mice. Equal loading was confirmed by GAPDH analysis. **(C)** Representative Immunohistochemical staining depicts decreased PLIN2 expression in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs mice. Original image magnification 200x, bar 200µm.

4.2.1.7. Enhanced lipoprotein lipase expression mediated by Abcb4$^{-/-}$ in HBs mice

We have observed a reduction of hepatic TAG storage and increased hepatic FFAs in HBs/Abcb4$^{-/-}$ with a reduction of lipogenesis and TAG synthesis. The increased hepatic FFAs may have been results of enhanced TAG hydrolysis and reduced esterification of FFAs to TAG for further storage. Abcb4$^{-/-}$ demonstrated enhanced FFAs due to increased TAG hydrolytic activity, that activate peroxisome proliferator activated receptor alpha (PPARα) expression (Moustafa *et al.* 2012). In this line, we further investigated the effect on total lipase activity and PPARα activation in HBs/Abcb4$^{-/-}$ mice. The total lipase activity assay was performed using

liver lysates (**Fig.23A**). We observed increased lipase activity in Abcb4$^{-/-}$ mice but no differences between HBs and HBs/Abcb4$^{-/-}$ mice (**Fig.23A**). Next, we analyzed whether PPARa activation went along with increased amounts of free fatty acids. The transcriptional analysis demonstrated significant up-regulation of PPARα in Abcb4$^{-/-}$ compared to WT mice with no differences between HBs and HBs/Abcb4$^{-/-}$ (**Fig.23B**). Therefore, the increased in FFA in HBs/Abcb4$^{-/-}$ neither correlates with PPARα activation nor the total lipase activity. However, activated PPARα can activate its downstream targets such as lipoprotein lipase (LPL), adipocytes triglycerides lipase (ATGL), lysosomal acid lipase (LAL) (Deng *et al.* 2006). Here, our data showed a significant up regulation of LPL expression level in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs mice resp. (**Fig.23C**). Additionally, the protein expression of LPL was remarkably increased in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ as compared to WT and HBs resp. (**Fig.23D**). Apart from LPL, other lipases such as ATGL and LAL showed no significant differences in expression among the group (data not shown). Taken together, our results demonstrate that the enhanced lipoprotein lipase expression in HBs/Abcb4$^{-/-}$ mice.

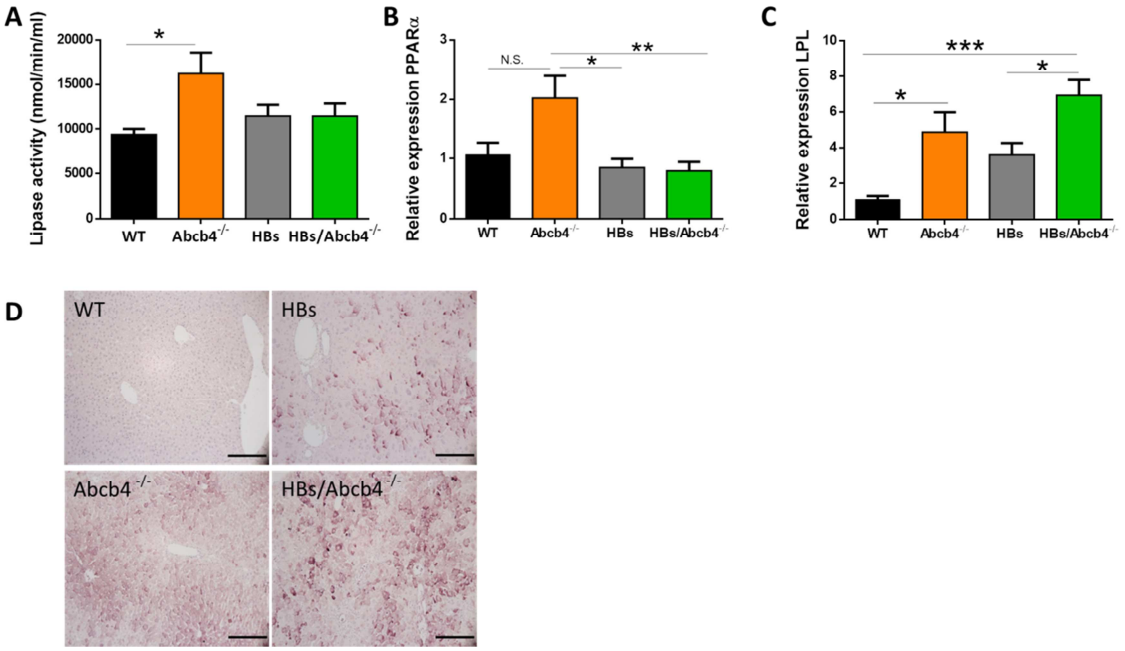


Figure 23: Enhanced LPL expression mediated by Abcb4$^{-/-}$ in HBs mice. **(A)** Total lipase activity assay was performed using liver lysate prepared as per the protocol given in Cayman lipase assay kit (Cat. No.700640), n = 6 were used. **(B-C)** Relative mRNA expression of major

genes involved in beta oxidation and lipolysis, PPARα, lipoprotein lipase (LPL), in the liver of four different groups were analyzed. Details of the primers used are listed in table 14. Results are presented as mean ± S.E., n = 10 in each group; experiments were performed in duplicates. Statistical analysis: ANOVA with Tukey`s multiple comparison tests, P*<0.05 P**<0.01, P***<0.001, indicated in the graph. (**D**) Enhanced LPL protein expression in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs mice. Image magnification x200, bar 200µm.

4.2.1.8. *In vitro* activation of AMPK-CREB signaling mediated by bile acid treatment

To further prove the mechanistic aspect of cholestasis induced disturbances in lipid metabolism in HBs mice, the human cell line HepG2 was used for the *in vitro* studies. In order to simulate the HBs induced hepatic steatosis, HepG2 cells were pre-treated with oleic acid to increase the intracellular LDs. The subsequent treatment of HepG2 with bile acid at the indicated concentration for 24h increased the phosphorylation of AMPK and CREB (**Fig.24A**). Similarly, the protein expression of FAS and PLIN2 showed remarkable reduction in the treated groups, which was concomitant with the activation of AMPK signaling by bile acid treatment (**Fig.24A**). To further confirm that the effect of bile acids on decreased TAG synthesis and storage was dependent on AMPK-CREB signaling, HepG2 cells were pretreated with a specific AMPK inhibitor (dorsomorphin), in presence and absence of bile acids at the indicated conc. for 24h. As a result, the activation of P-AMPK and P-CREB was blocked with treatment of dorsomorphin (**Fig.24B**). Importantly, the downstream targets of AMPK, FASN showed no increase, but PLIN2 protein expression was increased after treatment with the inhibitor (**Fig.24B**). Our data suggest that the increase of bile acid concentrations may decrease TAG synthesis and storage. The regulation of PLIN2 expression by BA may be associated to AMPK-CREB signaling.

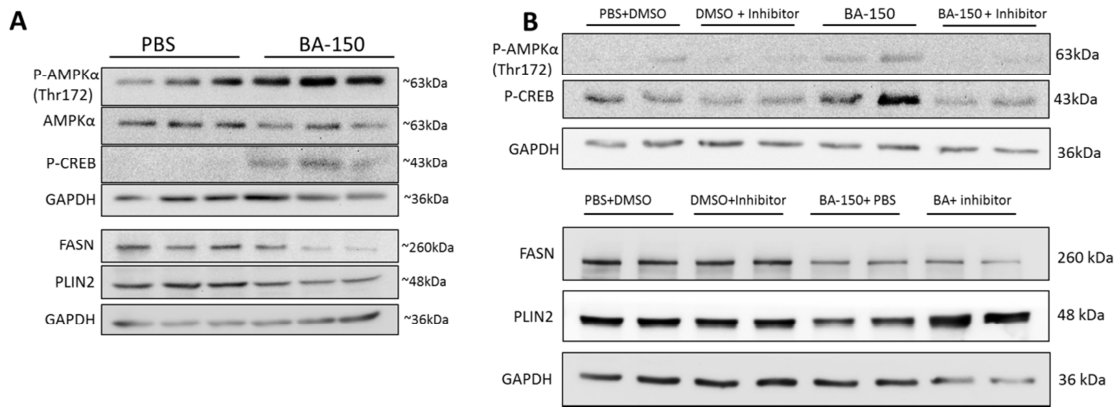


Figure 24: **Activation of AMPK-CREB signaling mediated by bile acids treatment**. **(A)** Western blot analysis of P-AMPK (α172), AMPKα, P-CREB, FAS, and PLIN2 protein expression levels in HepG2 cells pretreated with 250µM of oleic acid for 12h followed by treatment with bile salt (cholic-deoxycholate salt) (150µM conc.) for next 24h. GAPDH was used as a loading control, n = 3 **(B)** Western blot analysis of P-AMPK (α172), AMPKα, P-CREB, FAS, PLIN2 protein expression levels in HepG2 cells pretreated with 250µM of oleic acid for 12h followed by treatment with bile salt (cholic-deoxycholate salt) (150µM) in presence and absence of dorsomorphin (10µM), an AMPK inhibitor for next 24h. GAPDH was used as a loading control. n = 3.

4.2.2. Discussion:

The coexistence of HCV and HBV infection with cholestasis and NAFLD (Misra and Singh 2012; Shipovskaya and Dudanova 2018) has become a common phenomenon in chronic liver disease. Owing to increasing economic burden from chronic HBV infection and cholestasis (Xiao *et al.* 2008), a combined mice model of HBV surface protein transgenic mice and Abcb4$^{-/-}$ was generated for the first time (Zahner et al 2017) to comprehend the combined pathogenicity on chronic liver disease progression. Zahner et al. reported that HBsAg expression potentiated and accelerated cholestatic liver disease and carcinogenesis development on Abcb4$^{-/-}$ mice background (Zahner *et al.* 2017). Keeping in view, the important role of hepatic lipids in mediating cholestatic liver injury and fibrosis in Abcb4$^{-/-}$ (Moustafa *et al.* 2012), the present study was aimed to characterize the alteration of hepatic lipid metabolism in a model in the context of accelerating cholestatic liver disease development.

HBV transgenic mice with overexpression of surface proteins (HBs), develop steatosis apart from other pathological features (Chung and Wu 2018; Churin *et al.* 2014, 2014; Guidotti and Chisari 2006; Zahner *et al.* 2017). In a similar line, our study also showed steatosis in HBs mice compared to WT. Interestingly, the steatosis in HBs mice was reduced in HBs/Abcb4$^{-/-}$. On the contrary, hepatic cholesterol (CHOL) did not altered in HBs/Abcb4$^{-/-}$. Additionaly, the serum TAG and CHO showed a reduction in HBs/Abcb4$^{-/-}$ mice as compared to WT. Cholestatic liver disease with toxic accumulation of bile components can disturb many aspects of lipid absorption and metabolism (Watanabe *et al.* 2004). The observed decreased serum CHOL and TAG might be due to excessive increased of serum toxic bile acids in HBs/Abcb4$^{-/-}$ as reported earlier (Zahner *et al.* 2017). Hepatic free fatty acid levels in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ mice were increased. The phenomenon of increased hepatic free fatty acid with reduction of TAGs esterification and storage observed in HBs/Abcb4$^{-/-}$ mice are in the same line as reported in Abcb4$^{-/-}$ mice (Moustafa *et al.* 2012), suggesting an effect of Abcb4 knockout induced cholestasis mediated dysregulation of lipids in HBs mice.

The reduction of hepatic fat is associated with liver weight reduction and improvement of serum aminotransferases (Hayashi *et al.* 2000). Very surprisingly, our results showed rather an increased body and liver weight in both Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ mice, which is in agreement with previous reports (Ehlken *et al.* 2011; Lammert *et al.* 2004). Increased liver and liver to body weight ratio without gross abnormality is a hallmark feature observed in Abcb4$^{-/-}$ mice, which might be due to extensive fibrosis and tumor formation (Ehlken *et al.* 2011; Lammert *et al.* 2004). On the contrary, the tumor formation in Abcb4$^{-/-}$ mice is rather dependent on the genetic background of these mice. For instance Abcb4$^{-/-}$ mice on FVB/N genetic background develop hepatocellular carcinoma with 100% penetrance by the age of 16 months (Ehlken *et al.* 2011) which is not the case in BABL/c mice, as they develop tumor at later stages of life (Zahner *et al.* 2017). The reduction of liver fat was neither associated with liver weight reduction nor the improvement of liver injury. Rather ALT levels were enhanced the in HBs/Abcb4$^{-/-}$, which indicates aggravation of liver injury in this combined model.

Liver is the central core for lipid metabolism. AMP-activated protein kinase (AMPK) is an energy sensor, that regulates various transcription factors and cofactors involved in various metabolic pathways (Carling *et al.* 2012; Garcia and Shaw 2017; Steinberg and Kemp 2009).

Abcb4 deficiency results in activation of AMPK in HBs mice. Activated AMPK can phosphorylate specific transcription factors such as SREBP1c and ACC1 resulting in the suppression of other genes involved in lipogenesis such as FASN, HMGCO-A and PPARγ (C and Grahame 2007; Hardie 2007). In the same line, our results also showed the downregulation of SREBP1c, ACC1, FASN, HMGCoA, Scd1 and PPARγ in HBs/Abcb4$^{-/-}$ mice, suggesting suppression of lipogenesis pathways. Interestingly, not only lipogenesis gene but also genes involved in lipid uptake and transport such as CD36, ApoE, MTTP, and PCTP were decreased hinting towards an overall dysregulation in lipid metabolism.

Previous studies showed that activated AMPK can phosphorylate its downstream target CREB and other members of the CREB family in skeletal muscle and further tissues (Cantó and Auwerx 2010; Thomson *et al.* 2008). Notably, our findings also demonstrated enhanced activation of CREB in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ compared to WT and HBs. Mice lacking hepatic CREB show a fatty liver phenotype with elevated expression of the lipogenic gene PPARγ (Herzig *et al.* 2003). Here, our data showed increased hepatic CREB expression with decreased PPARγ in HBs/Abcb4$^{-/-}$ indicating suppression of lipogenesis. CREB blocks or suppresses lipogenesis indirectly via PPARγ transcription and directly via induction of the E-box repressor Hairy Enhancer of Split (HES-1), a component of the Notch signaling pathway, which is a *bona fide* target for CREB action in liver (Han *et al.* 2015). The effect of HES-1 in liver of HBs/Abcb4 needs further investigations. Similarly, our *in vitro* cells culture data also showed activation of both AMPK, CREB with concomitant reduction of FAS, PLIN2 in HepG2 cells after treatment with bile salts. Thus, suggesting a possible role of toxic bile acid in altering lipid metabolism in Abcb4$^{-/-}$ induced cholestatic liver injury.

FFAs have been shown to activate PPARα (Montagner *et al.* 2016). Intriguingly, our results also showed increased activation of PPARα in Abcb4$^{-/-}$ with no differences observed between HBs and HBs/Abcb4$^{-/-}$. Neither total lipase activity nor the PPARα activation was enhanced in the HBs/Abcb4$^{-/-}$, which doesn't associates with the increased FFAs. However, activated PPARα itself can regulate all the sequential steps of TAGs catabolism including lipases, ATGL (Deng *et al.* 2006). PPARα target gene, lipoprotein lipase (LPL) was found to be regulated in HBs/Abcb4$^{-/-}$ mice. Thus, increased expression of LPL may be associated with an enhancement of TAGs catabolism. In normal physiological states, free fatty acids serve as a preferential substrate for

TAG esterification and storage (Ahmadian *et al.* 2007). Here, higher levels of FFAs parallel with low levels of TAG in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ indicating either suppression of the enzyme machinery involved in TAGs synthesis pathways or excessive enhancement of lipases and/or lipolysis. Interestingly, our findings showed suppression of AGPAT1, GPAT1, MGAT1, and DGAT2 involved in TAG synthesis pathways. Reduced TAG synthesis might be an indication for a lack of esterification of FFA to TAGs, suggesting a possible mechanism for non-utilization of FFAs for TAGs synthesis and storage in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ mice. In this context several studies suggested that the beneficial effects of lipid storage and loss of TAG storage capacity is critically linked to lipotoxicity and shown to exacerbate liver injury (Yamaguchi *et al.* 2007). Therefore, increased FFAs with suppression of TAGs synthesis and storage along with enhanced lipolysis pathways might be linked to acceleration of liver injury by Abcb4$^{-/-}$ in HBs mice (Zahner *et al.* 2017).

TAGs are sequestered and stored as lipid droplets (LDs). Perilipins are the major cytoplasmic lipid droplet-associated proteins which are involved in intracellular lipid droplet formation and stability (Sztalryd *et al.* 2006).The reduction of PLIN2 expression by antisense oligonucleotide treatment led to a decreased hepatic lipid accumulation (Imai *et al.* 2007). Our data also showed the decreased reduction of PLIN2 in HBs/Abcb4$^{-/-}$ and associated with reduction of LDs. The relation between Abcb4$^{-/-}$ induced cholestasis mediated TAGs reduction and PLIN2 has not been reported so far. Our cell culture data further support the BA-mediated reduction of PLIN2 protein expression in HepG2 cells. PLIN2 provides a “shielding effect” on LDs and its reduction is considered as a critical step to promote lipolysis of LDs (Kaushik and Cuervo 2015). Our findings also showed increased expression of cytoplasmic lipoprotein lipases in Abcb4$^{-/-}$ and HBs/Abcb4$^{-/-}$ mice compared to WT and HBs, suggesting a possible mechanism for increased FFAs and reduction of hepatic TAG.

Conclusion: Abcb4 knockout mediates suppression of hepatic lipogenesis and lipid storage in HBs mice via activation of AMPK-CREB signaling pathways. Excessive free fatty acids with blockade of esterification to TAG and further storage might be a confounding factor in acceleration of cholestasis liver injury as observed in this combined model. A schematic diagram (Fig. 25) illustrates the major findings from the studies as shown below.

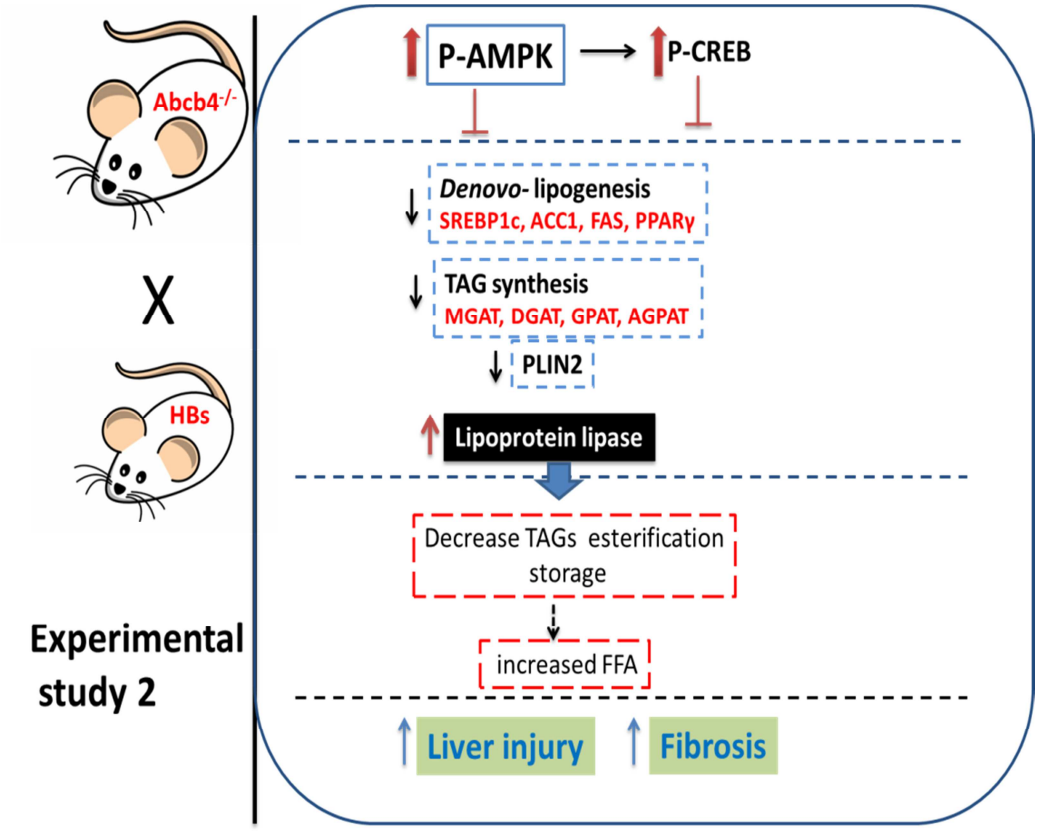


Figure 25: Abcb4$^{-/-}$ mediates the reduction in hepatic synthesis and storage of triacylglyceride in HBs mice via activation of AMPK-CREB signaling pathways. The reduction of liver TAGs with the enhancement of FFA might be associated with liver injury and enhancement of chronic liver disease progression in the combined model.

REFERENCES:

Aarsland, A., Chinkes, D. and Wolfe, R. R. (1997): Hepatic and whole-body fat synthesis in humans during carbohydrate overfeeding. – *The American journal of clinical nutrition* **65**(6): 1774–1782. http://dx.doi.org/10.1093/ajcn/65.6.1774

Abu-Elheiga, L., Brinkley, W. R., Zhong, L., Chirala, S. S., Woldegiorgis, G. and Wakil, S. J. (2000): The subcellular localization of acetyl-CoA carboxylase 2. – *Proceedings of the National Academy of Sciences of the United States of America* **97**(4): 1444–1449. http://dx.doi.org/10.1073/pnas.97.4.1444

Ahmadian, M., Duncan, R. E., Jaworski, K., Sarkadi-Nagy, E. and Sul, H. S. (2007): Triacylglycerol metabolism in adipose tissue. – *Future Lipidol* **2**(2): 229–237. http://dx.doi.org/10.2217/17460875.2.2.229

Alisi, A., Cianfarani, S., Manco, M., Agostoni, C. and Nobili, V. (2012): Non-alcoholic fatty liver disease and metabolic syndrome in adolescents: pathogenetic role of genetic background and intrauterine environment. – *Annals of medicine* **44**(1): 29–40. http://dx.doi.org/10.3109/07853890.2010.547869

Altarejos, J. Y. and Montminy, M. (2011): CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. – *Nature reviews. Molecular cell biology* **12**(3): 141–151. http://dx.doi.org/10.1038/nrm3072

Alves-Bezerra, M. and Cohen, D. E. (2017): Triglyceride Metabolism in the Liver. – *Compr Physiol* **8**(1): 1–8. http://dx.doi.org/10.1002/cphy.c170012

Amini, M., Saboory, E., Pourheydar, B., Bagheri, M. and Naderi, R. (2020): Involvement of endocannabinoid system, inflammation and apoptosis in diabetes induced liver injury: Role of 5-HT3 receptor antagonist. – *International immunopharmacology* **79**: 106158. http://dx.doi.org/10.1016/j.intimp.2019.106158

Angelin, B., Hershon, K. S. and Brunzell, J. D. (1987): Bile acid metabolism in hereditary forms of hypertriglyceridemia: evidence for an increased synthesis rate in monogenic familial hypertriglyceridemia. – *Proceedings of the National Academy of Sciences of the United States of America* **84**(15): 5434–5438. http://dx.doi.org/10.1073/pnas.84.15.5434

Araújo, A. R., Rosso, N., Bedogni, G., Tiribelli, C. and Bellentani, S. (2018): Global epidemiology of non-alcoholic fatty liver disease/non-alcoholic steatohepatitis: What we need in the future. – *Liver Int* **38**(S1): 47–51. http://dx.doi.org/10.1111/liv.13643

Arisqueta, L., Navarro-Imaz, H., Labiano, I., Rueda, Y. and Fresnedo, O. (2018): High-fat diet overfeeding promotes nondetrimental liver steatosis in female mice. – *American journal of physiology. Gastrointestinal and liver physiology* **315**(5): G772-G780. http://dx.doi.org/10.1152/ajpgi.00022.2018

Auguet, T., Berlanga, A., Guiu-Jurado, E., Terra, X., Martinez, S., Aguilar, C., Filiu, E., Alibalic, A., Sabench, F., Hernandez, M., Del Castillo, D. and Richart, C. (2014): Endocannabinoid receptors gene expression in morbidly obese women with nonalcoholic fatty liver disease. – *BioMed research international* **2014**: 502542. http://dx.doi.org/10.1155/2014/502542

Ayonrinde, O. T., Olynyk, J. K., Marsh, J. A., Beilin, L. J., Mori, T. A., Oddy, W. H. and Adams, L. A. (2015): Childhood adiposity trajectories and risk of nonalcoholic fatty liver disease in adolescents. – *Journal of gastroenterology and hepatology* **30**(1): 163–171. http://dx.doi.org/10.1111/jgh.12666

Azuma, H., Paulk, N., Ranade, A., Dorrell, C., Al-Dhalimy, M., Ellis, E., Strom, S., Kay, M. A., Finegold, M. and Grompe, M. (2007): Robust expansion of human hepatocytes in Fah-/-/Rag2-/-/Il2rg-/- mice. – *Nat Biotechnol* **25**(8): 903–910. http://dx.doi.org/10.1038/nbt1326

Baclig, M. O., Reyes, K. G., Liles, V. R., Mapua, C. A., Dimamay, M. P. S. and Gopez-Cervantes, J. (2018): Hepatic steatosis in chronic hepatitis B: a study of metabolic and genetic factors. – *International journal of molecular epidemiology and genetics* **9**(2): 13–19.

Baghdasaryan, A., Fuchs, C. D., Osterreicher, C. H., Lemberger, U. J., Halilbasic, E., Pahlman, I., Graffner, H., Krones, E., Fickert, P., Wahlstrom, A., Stahlman, M., Paumgartner, G., Marschall, H.-U. and Trauner, M. (2016): Inhibition of intestinal bile acid absorption improves cholestatic liver and bile duct injury in a mouse model of sclerosing cholangitis. – *Journal of hepatology* **64**(3): 674–681. http://dx.doi.org/10.1016/j.jhep.2015.10.024

Baiceanu, A., Mesdom, P., Lagouge, M. and Foufelle, F. (2016): Endoplasmic reticulum proteostasis in hepatic steatosis. – *Nat Rev Endocrinol* **12**(12): 710–722. http://dx.doi.org/10.1038/nrendo.2016.124

Baraona, E. and Lieber, C. S. (1979): Effects of ethanol on lipid metabolism. – *Journal of lipid research* **20**(3): 289–315.

Barbero, P., Buell, E., Zulley, S. and Pfeffer, S. R. (2001): TIP47 is not a component of lipid droplets. – *The Journal of biological chemistry* **276**(26): 24348–24351. http://dx.doi.org/10.1074/jbc.M102468200

Bartenschlager, R. and Schaller, H. (1988): The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. – *EMBO J* **7**(13): 4185–4192.

Begriche, K., Massart, J., Robin, M.-A., Bonnet, F. and Fromenty, B. (2013): Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. – *Hepatology (Baltimore, Md.)* **58**(4): 1497–1507. http://dx.doi.org/10.1002/hep.26226

Ben Loos, André du Toit and Jan-Hendrik S Hofmeyr (2014): Defining and measuring autophagosome flux—concept and reality. – *Autophagy* **10**(11): 2087–2096. http://dx.doi.org/10.4161/15548627.2014.973338

Berge, K. E., Tian, H., Graf, G. A., Yu, L., Grishin, N. V., Schultz, J., Kwiterovich, P., Shan, B., Barnes, R. and Hobbs, H. H. (2000): Accumulation of Dietary Cholesterol in Sitosterolemia Caused by Mutations in Adjacent ABC Transporters. – *Science (New York, N.Y.)* **290**(5497): 1771. http://dx.doi.org/10.1126/science.290.5497.1771

Berglund, E. D., Lee-Young, R. S., Lustig, D. G., Lynes, S. E., Donahue, E. P., Camacho, R. C., Meredith, M. E., Magnuson, M. A., Charron, M. J. and Wasserman, D. H. (2009): Hepatic energy state is regulated by glucagon receptor signaling in mice. – *The Journal of clinical investigation* **119**(8): 2412–2422. http://dx.doi.org/10.1172/jci38650

Bility, M. T., Cheng, L., Zhang, Z., Luan, Y., Li, F., Chi, L., Zhang, L., Tu, Z., Gao, Y., Fu, Y., Niu, J., Wang, F. and Su, L. (2014): Hepatitis B Virus Infection and Immunopathogenesis in a Humanized Mouse Model: Induction of Human-Specific Liver Fibrosis and M2-Like Macrophages. – *PLOS Pathogens* **10**(3): e1004032. http://dx.doi.org/10.1371/journal.ppat.1004032

Bissig, K.-D., Wieland, S. F., Tran, P., Isogawa, M., Le, T. T., Chisari, F. V. and Verma, I. M. (2010): Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. – *The Journal of clinical investigation* **120**(3): 924–930. http://dx.doi.org/10.1172/JCI40094

Bouchard, M. J. and Schneider, R. J. (2004): The enigmatic X gene of hepatitis B virus. – *Journal of virology* **78**(23): 12725–12734. http://dx.doi.org/10.1128/JVI.78.23.12725-12734.2004

Brasaemle, D. L. (2007): Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. – *Journal of lipid research* **48**(12): 2547–2559. http://dx.doi.org/10.1194/jlr.R700014-JLR200

Breiner, K. M., Urban, S. and Schaller, H. (1998): Carboxypeptidase D (gp180), a Golgi-resident protein, functions in the attachment and entry of avian hepatitis B viruses. – *Journal of virology* **72**(10): 8098–8104.

Brownsey, R. W., Boone, A. N., Elliott, J. E., Kulpa, J. E. and Lee, W. M. (2006): Regulation of acetyl-CoA carboxylase. – *Biochemical Society transactions* **34**(Pt 2): 223–227. http://dx.doi.org/10.1042/BST20060223

C, T. M. and Grahame, H. D. (2007): AMP-Activated Protein Kinase in Metabolic Control and Insulin Signaling. – *Circulation Research* **100**(3): 328–341. http://dx.doi.org/10.1161/01.RES.0000256090.42690.05

Cantó, C. and Auwerx, J. (2010): AMP-activated protein kinase and its downstream transcriptional pathways. – Cellular and molecular life sciences : CMLS 67(20): 3407–3423. http://dx.doi.org/10.1007/s00018-010-0454-z

Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J. and Shoelson, S. E. (2005): Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. – *Nature medicine* **11**(2): 183–190. http://dx.doi.org/10.1038/nm1166

Carey, A. N., Zhang, W., Setchell, K. D. R., Simmons, J. R., Shi, T., Lages, C. S., Mullen, M., Carroll, K., Karns, R., Bessho, K., Sheridan, R., Zhao, X., Weber, S. N. and Miethke, A. G. (2017): Hepatic MDR3 expression impacts lipid homeostasis and susceptibility to inflammatory bile duct obstruction in neonates. – *Pediatric research* **82**(1): 122–132. http://dx.doi.org/10.1038/pr.2017.78

Cariello, M., Peres, C., Zerlotin, R., Porru, E., Sabbà, C., Roda, A. and Moschetta, A. (2017): Long-term Administration of Nuclear Bile Acid Receptor FXR Agonist Prevents Spontaneous Hepatocarcinogenesis in Abcb4−/− Mice. – *Scientific reports* **7**(1): 11203. http://dx.doi.org/10.1038/s41598-017-11549-7

Carling, D., Thornton, C., Woods, A. and Sanders, M. J. (2012): AMP-activated protein kinase: new regulation, new roles? – *The Biochemical journal* **445**(1): 11–27. http://dx.doi.org/10.1042/BJ20120546

Cheng, C., Geng, F., Cheng, X. and Guo, D. (2018): Lipid metabolism reprogramming and its potential targets in cancer. – *Cancer Commun (Lond)* **38**(1): 27. http://dx.doi.org/10.1186/s40880-018-0301-4

Chiang, J. Y. L. and Ferrell, J. M. (2018): Bile Acid Metabolism in Liver Pathobiology. – *Gene Expr* **18**(2): 71–87. http://dx.doi.org/10.3727/105221618X15156018385515

Chisari, F. V., Filippi, P., Buras, J., McLachlan, A., Popper, H. and Pinkert, C. A. (1987): Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. – *Proc Natl Acad Sci USA* **84**. http://dx.doi.org/10.1073/pnas.84.19.6909

Chung, Y.L. and Wu, M.L. (2018*):* The Role of Promyelocytic Leukemia Protein in Steatosis-Associated Hepatic Tumors Related to Chronic Hepatitis B virus Infection. – *Transl Oncol* **11**(3): 743–754. http://dx.doi.org/10.1016/j.tranon.2018.03.013

Churin, Y., Roderfeld, M. and Roeb, E. (2015): Hepatitis B virus large surface protein: function and fame. – *Hepatobiliary Surg Nutr* **4**(1): 1–10. http://dx.doi.org/10.3978/j.issn.2304-3881.2014.12.08

Churin, Y., Roderfeld, M., Stiefel, J., Würger, T., Schröder, D., Matono, T., Mollenkopf, H.-J., Montalbano, R., Pompaiah, M., Reifenberg, K., Zahner, D., Ocker, M., Gerlich, W., Glebe, D. and Roeb, E. (2014): Pathological Impact of Hepatitis B Virus Surface Proteins on the Liver Is Associated with the Host Genetic Background. – *PloS one* **9**(3): e90608. http://dx.doi.org/10.1371/journal.pone.0090608

Cicerchi, C., Li, N., Kratzer, J., Garcia, G., Roncal-Jimenez, C. A., Tanabe, K., Hunter, B., Rivard, C. J., Sautin, Y. Y., Gaucher, E. A., Johnson, R. J. and Lanaspa, M. A. (2014): Uric acid-dependent inhibition of AMP kinase induces hepatic glucose production in diabetes and starvation: evolutionary implications of the uricase loss in hominids. – *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **28**(8): 3339–3350. http://dx.doi.org/10.1096/fj.13-243634

Clemente, M. G., Mandato, C., Poeta, M. and Vajro, P. (2016): Pediatric non-alcoholic fatty liver disease: Recent solutions, unresolved issues, and future research directions. – *World journal of gastroenterology* **22**(36): 8078–8093. http://dx.doi.org/10.3748/wjg.v22.i36.8078

Cota, D. (2007): CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. – *Diabetes Metab Res Rev* **23**. http://dx.doi.org/10.1002/dmrr.764

Cota, D., Marsicano, G., Tschop, M., Grubler, Y., Flachskamm, C., Schubert, M., Auer, D., Yassouridis, A., Thone-Reineke, C., Ortmann, S., Tomassoni, F., Cervino, C., Nisoli, E., Linthorst, A. C. E., Pasquali, R., Lutz, B., Stalla, G. K. and Pagotto, U. (2003): The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. – *The Journal of clinical investigation* **112**(3): 423–431. http://dx.doi.org/10.1172/JCI17725

Cota, D., Proulx, K., Smith, K. A. B., Kozma, S. C., Thomas, G., Woods, S. C. and Seeley, R. J. (2006): Hypothalamic mTOR signaling regulates food intake. – *Science (New York, N.Y.)* **312**(5775): 927–930. http://dx.doi.org/10.1126/science.1124147

Covington, J. D., Galgani, J. E., Moro, C., LaGrange, J. M., Zhang, Z., Rustan, A. C., Ravussin, E. and Bajpeyi, S. (2014): Skeletal muscle perilipin 3 and coatomer proteins are increased following exercise and are associated with fat oxidation. – *PloS one* **9**(3): e91675-e91675. http://dx.doi.org/10.1371/journal.pone.0091675

Cui, Y. J., Cheng, X., Weaver, Y. M. and Klaassen, C. D. (2009): Tissue Distribution, Gender-Divergent Expression, Ontogeny, and Chemical Induction of Multidrug Resistance Transporter Genes (Mdr1a, Mdr1b, Mdr2) in Mice. – *Drug Metabolism and Disposition* **37**(1): 203. http://dx.doi.org/10.1124/dmd.108.023721

Cuperus, F. J. C., Claudel, T., Gautherot, J., Halilbasic, E. and Trauner, M. (2014): The Role of Canalicular ABC Transporters in Cholestasis. – *Drug Metabolism and Disposition* **42**(4): 546. http://dx.doi.org/10.1124/dmd.113.056358

Dai, E., Zhang, L., Ye, L., Wan, S., Feng, L., Qi, Q., Yao, F. and Li, Z. (2017): Hepatic expression of cannabinoid receptors CB1 and CB2 correlate with fibrogenesis in patients with chronic hepatitis B. – *International journal of infectious diseases : IJID : official*

publication of the International Society for Infectious Diseases **59**: 124–130. http://dx.doi.org/10.1016/j.ijid.2017.03.008

Dalen, K. T., Dahl, T., Holter, E., Arntsen, B., Londos, C., Sztalryd, C. and Nebb, H. I. (2007): LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. – *Biochimica et biophysica acta* **1771**(2): 210–227. http://dx.doi.org/10.1016/j.bbalip.2006.11.011

Dalen, K. T., Ulven, S. M., Arntsen, B. M., Solaas, K. and Nebb, H. I. (2006): PPARalpha activators and fasting induce the expression of adipose differentiation-related protein in liver. – *Journal of lipid research* **47**. http://dx.doi.org/10.1194/jlr.M500459-JLR200

Dando, I., Donadelli, M., Costanzo, C., Dalla Pozza, E., D'Alessandro, A. and Zolla, L. (2013): Cannabinoids inhibit energetic metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells. – *Cell Death Dis* **4**. http://dx.doi.org/10.1038/cddis.2013.151

Datta, S., Chatterjee, S., Veer, V. and Chakravarty, R. (2012): Molecular biology of the hepatitis B virus for clinicians. – *J Clin Exp Hepatol* **2**(4): 353–365. http://dx.doi.org/10.1016/j.jceh.2012.10.003

Deng, T., Shan, S., Li, P.-P., Shen, Z.-F., Lu, X.-P., Cheng, J. and Ning, Z.-Q. (2006): Peroxisome Proliferator-Activated Receptor-γ Transcriptionally Up-Regulates Hormone-Sensitive Lipase via the Involvement of Specificity Protein-1. – *endo* **147**(2): 875–884. http://dx.doi.org/10.1210/en.2005-0623

Diehl, A. M. and Day, C. (2017): Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. – *The New England journal of medicine* **377**(21): 2063–2072. http://dx.doi.org/10.1056/NEJMra1503519

Doege, H., Baillie, R. A., Ortegon, A. M., Tsang, B., Wu, Q., Punreddy, S., Hirsch, D., Watson, N., Gimeno, R. E. and Stahl, A. (2006): Targeted deletion of FATP5 reveals multiple functions in liver metabolism: alterations in hepatic lipid homeostasis. – *Gastroenterology* **130**(4): 1245–1258. http://dx.doi.org/10.1053/j.gastro.2006.02.006

Dowman, J. K., Tomlinson, J. W. and Newsome, P. N. (2010): Pathogenesis of non-alcoholic fatty liver disease. – *QJM : monthly journal of the Association of Physicians* **103**(2): 71–83. http://dx.doi.org/10.1093/qjmed/hcp158

Ebert, G., Poeck, H., Lucifora, J., Baschuk, N., Esser, K., Esposito, I., Hartmann, G. and Protzer, U. (2011): 5' Triphosphorylated small interfering RNAs control replication of hepatitis B

virus and induce an interferon response in human liver cells and mice. – *Gastroenterology* **141**(2): 696-706, 706.e1-3. http://dx.doi.org/10.1053/j.gastro.2011.05.001

Egan, D. F., Shackelford, D. B., Mihaylova, M. M., Gelino, S., Kohnz, R. A., Mair, W., Vasquez, D. S., Joshi, A., Gwinn, D. M., Taylor, R., Asara, J. M., Fitzpatrick, J., Dillin, A., Viollet, B., Kundu, M., Hansen, M. and Shaw, R. J. (2011): Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. – *Science (New York, N.Y.)* **331**(6016): 456–461. http://dx.doi.org/10.1126/science.1196371

Ehlken, H., Kondylis, V., Heinrichsdorff, J., Ochoa-Callejero, L., Roskams, T. and Pasparakis, M. (2011): Hepatocyte IKK2 protects Mdr2-/- mice from chronic liver failure. – *PLOS ONE* **6**(10): e25942-e25942. http://dx.doi.org/10.1371/journal.pone.0025942

Fabbrini, E., Sullivan, S. and Klein, S. (2010): Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. – *Hepatology (Baltimore, Md.)* **51**(2): 679–689. http://dx.doi.org/10.1002/hep.23280

Fabris, C., Federico, E., Soardo, G., Falleti, E. and Pirisi, M. (1997): Blood lipids of patients with chronic hepatitis: differences related to viral etiology. – *Clinica chimica acta; international journal of clinical chemistry* **261**(2): 159–165. http://dx.doi.org/10.1016/s0009-8981(97)06532-7

Fan, J.-G. and Chitturi, S. (2008): Hepatitis B and fatty liver: causal or coincidental? – *Journal of gastroenterology and hepatology* **23**(5): 679–681. http://dx.doi.org/10.1111/j.1440-1746.2008.05361.x

Fang, Y.-L., Chen, H., Wang, C.-L. and Liang, L. (2018): Pathogenesis of non-alcoholic fatty liver disease in children and adolescence: From "two hit theory" to "multiple hit model". – *World journal of gastroenterology* **24**(27): 2974–2983. http://dx.doi.org/10.3748/wjg.v24.i27.2974

Fried, M. and Crothers, D. M. (1981): Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. – *Nucleic acids research* **9**(23): 6505–6525. http://dx.doi.org/10.1093/nar/9.23.6505

Friedman, S. L., Neuschwander-Tetri, B. A., Rinella, M. and Sanyal, A. J. (2018): Mechanisms of NAFLD development and therapeutic strategies. – *Nature medicine* **24**(7): 908–922. http://dx.doi.org/10.1038/s41591-018-0104-9

Gadaleta, R. M., Scialpi, N., Peres, C., Cariello, M., Ko, B., Luo, J., Porru, E., Roda, A., Sabba, C. and Moschetta, A. (2018): Suppression of Hepatic Bile Acid Synthesis by a non-tumorigenic FGF19 analogue Protects Mice from Fibrosis and Hepatocarcinogenesis. – *Scientific reports* **8**(1): 17210. http://dx.doi.org/10.1038/s41598-018-35496-z

Gao, Q., Jia, Y., Yang, G., Zhang, X., Boddu, P. C., Petersen, B., Narsingam, S., Zhu, Y.-J., Thimmapaya, B., Kanwar, Y. S. and Reddy, J. K. (2015): PPARalpha-Deficient ob/ob Obese Mice Become More Obese and Manifest Severe Hepatic Steatosis Due to Decreased Fatty Acid Oxidation. – *The American journal of pathology* **185**(5): 1396–1408. http://dx.doi.org/10.1016/j.ajpath.2015.01.018

Garcia, D. and Shaw, R. J. (2017): AMPK: Mechanisms of Cellular Energy Sensing and Restoration of Metabolic Balance. – *Molecular cell* **66**(6): 789–800. http://dx.doi.org/10.1016/j.molcel.2017.05.032

Gary-Bobo, M., Elachouri, G., Gallas, J. F., Janiak, P., Marini, P. and Ravinet-Trillou, C. (2007): Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. – *Hepatology (Baltimore, Md.)* **46**. http://dx.doi.org/10.1002/hep.21641

Gerloff, T., Stieger, B., Hagenbuch, B., Madon, J., Landmann, L., Roth, J., Hofmann, A. F. and Meier, P. J. (1998): The Sister of P-glycoprotein Represents the Canalicular Bile Salt Export Pump of Mammalian Liver. – *Journal of Biological Chemistry* **273**(16): 10046–10050. http://dx.doi.org/10.1074/jbc.273.16.10046

Ghonem, N. S., Ananthanarayanan, M., Soroka, C. J. and Boyer, J. L. (2014): Peroxisome proliferator-activated receptor alpha activates human multidrug resistance transporter 3/ATP-binding cassette protein subfamily B4 transcription and increases rat biliary phosphatidylcholine secretion. – *Hepatology (Baltimore, Md.)* **59**(3): 1030–1042. http://dx.doi.org/10.1002/hep.26894

Ginsberg, H. N. (2002): New perspectives on atherogenesis: role of abnormal triglyceride-rich lipoprotein metabolism. – *Circulation* **106**(16): 2137–2142. http://dx.doi.org/10.1161/01.cir.0000035280.64322.31

Ginsberg, H. N. and Fisher, E. A. (2009): The ever-expanding role of degradation in the regulation of apolipoprotein B metabolism. – *Journal of lipid research* **50 Suppl**(Suppl): S162-S166. http://dx.doi.org/10.1194/jlr.R800090-JLR200

Giudice, E. M. d., Grandone, A., Cirillo, G., Santoro, N., Amato, A., Brienza, C., Savarese, P., Marzuillo, P. and Perrone, L. (2011): The association of PNPLA3 variants with liver enzymes in childhood obesity is driven by the interaction with abdominal fat. – *PloS one* **6**(11): e27933. http://dx.doi.org/10.1371/journal.pone.0027933

Glebe, D. and Bremer, C. M. (2013): The molecular virology of hepatitis B virus. – *Seminars in liver disease* **33**(2): 103–112. http://dx.doi.org/10.1055/s-0033-1345717

Gluchowski, N. L., Becuwe, M., Walther, T. C. and Farese, R. V., JR (2017): Lipid droplets and liver disease: from basic biology to clinical implications. – *Nature reviews. Gastroenterology & hepatology* **14**(6): 343–355. http://dx.doi.org/10.1038/nrgastro.2017.32

Gong, J., Sun, Z. and Li, P. (2009): CIDE proteins and metabolic disorders. – *Curr Opin Lipidol* **20**(2): 121–126. http://dx.doi.org/10.1097/MOL.0b013e328328d0bb

Granneman, J. G., Moore, H.-P. H., Krishnamoorthy, R. and Rathod, M. (2009): Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). – *The Journal of biological chemistry* **284**(50): 34538–34544. http://dx.doi.org/10.1074/jbc.M109.068478

Graumann, F., Churin, Y., Tschuschner, A., Reifenberg, K., Glebe, D., Roderfeld, M. and Roeb, E. (2016): Genomic Methylation Inhibits Expression of Hepatitis B Virus Envelope Protein in Transgenic Mice: A Non-Infectious Mouse Model to Study Silencing of HBV Surface Antigen Genes. – *PloS one* **10**(12): e0146099. http://dx.doi.org/10.1371/journal.pone.0146099

Greco, D., Kotronen, A., Westerbacka, J., Puig, O., Arkkila, P., Kiviluoto, T., Laitinen, S., Kolak, M., Fisher, R. M., Hamsten, A., Auvinen, P. and Yki-Jarvinen, H. (2008): Gene expression in human NAFLD. – *American journal of physiology. Gastrointestinal and liver physiology* **294**(5): G1281-7. http://dx.doi.org/10.1152/ajpgi.00074.2008

Green, D. R., Galluzzi, L. and Kroemer, G. (2014): Cell biology. Metabolic control of cell death. – *Science (New York, N.Y.)* **345**(6203): 1250256. http://dx.doi.org/10.1126/science.1250256

Greenberg, A. S., Coleman, R. A., Kraemer, F. B., McManaman, J. L., Obin, M. S., Puri, V., Yan, Q.-W., Miyoshi, H. and Mashek, D. G. (2011): The role of lipid droplets in metabolic disease in rodents and humans. – *The Journal of clinical investigation* **121**(6): 2102–2110. http://dx.doi.org/10.1172/JCI46069

Greenberg, A. S., Egan, J. J., Wek, S. A., Garty, N. B., Blanchette-Mackie, E. J. and Londos, C. (1991): Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. – *The Journal of biological chemistry* **266**(17): 11341–11346.

Gross, D. A. and Silver, D. L. (2014): Cytosolic lipid droplets: from mechanisms of fat storage to disease. – *Critical reviews in biochemistry and molecular biology* **49**(4): 304–326. http://dx.doi.org/10.3109/10409238.2014.931337

Guidotti, L. G. and Chisari, F. V. (2006): IMMUNOBIOLOGY AND PATHOGENESIS OF VIRAL HEPATITIS. – *Annu. Rev. Pathol. Mech. Dis.* **1**(1): 23–61. http://dx.doi.org/10.1146/annurev.pathol.1.110304.100230

Guidotti, L. G., Matzke, B., Schaller, H. and Chisari, F. V. (1995): High-level hepatitis B virus replication in transgenic mice. – *Journal of virology* **69**(10): 6158–6169.

Gupta, S., Todd Stravitz, R., Pandak, W. M., Muller, M., Reno Vlahcevic, Z. and Hylemon, P. B. (2000): Regulation of multidrug resistance 2 P-glycoprotein expression by bile salts in rats and in primary cultures of rat hepatocytes. – *Hepatology (Baltimore, Md.)* **32**(2): 341–347. http://dx.doi.org/10.1053/jhep.2000.9605

Hagenbuch, B. and Meier, P. J. (1994): Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. – *The Journal of clinical investigation* **93**(3): 1326–1331. http://dx.doi.org/10.1172/JCI117091

Han, J., Li, E., Chen, L., Zhang, Y., Wei, F., Liu, J., Deng, H. and Wang, Y. (2015): The CREB coactivator CRTC2 controls hepatic lipid metabolism by regulating SREBP1. – *Nature* **524**(7564): 243–246. http://dx.doi.org/10.1038/nature14557

Hardie, D. G. (2007): AMP-Activated Protein Kinase as a Drug Target. – *Annu. Rev. Pharmacol. Toxicol.* **47**(1): 185–210. http://dx.doi.org/10.1146/annurev.pharmtox.47.120505.105304

Hardie, D. G. (2011): AMP-activated protein kinase-an energy sensor that regulates all aspects of cell function. – *Genes & development* **25**. http://dx.doi.org/10.1101/gad.17420111

Hashimoto, E. and Tokushige, K. (2011): Prevalence, gender, ethnic variations, and prognosis of NASH. – *Journal of gastroenterology* **46 Suppl 1**: 63–69. http://dx.doi.org/10.1007/s00535-010-0311-8

Hayashi, T., Hirshman, M. F., Fujii, N., Habinowski, S. A., Witters, L. A. and Goodyear, L. J. (2000): Metabolic stress and altered glucose transport: activation of AMP-activated protein

kinase as a unifying coupling mechanism. – *Diabetes* **49**. http://dx.doi.org/10.2337/diabetes.49.4.527

Heidrich, F., Schotola, H., Popov, A. F., Sohns, C., Schuenemann, J., Friedrich, M., Coskun, K. O., Lewinski, D., Hinz, J., Bauer, M., Mokashi, S. A., Sossalla, S. and Schmitto, J. D. (2010): AMPK - Activated Protein Kinase and its Role in Energy Metabolism of the Heart. – *Current cardiology reviews* **6**(4): 337–342. http://dx.doi.org/10.2174/157340310793566073

Hellman, L. M. and Fried, M. G. (2007): Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. – *Nature protocols* **2**(8): 1849–1861. http://dx.doi.org/10.1038/nprot.2007.249

Hernandez-Gea, V., Hilscher, M., Rozenfeld, R., Lim, M. P., Nieto, N., Werner, S., Devi, L. A. and Friedman, S. L. (2013): Endoplasmic reticulum stress induces fibrogenic activity in hepatic stellate cells through autophagy. – *Journal of hepatology* **59**(1): 98–104. http://dx.doi.org/10.1016/j.jhep.2013.02.016

Herzig, S., Hedrick, S., Morantte, I., Koo, S.-H., Galimi, F. and Montminy, M. (2003): CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. – *Nature* **426**(6963): 190–193. http://dx.doi.org/10.1038/nature02110

Hézode, C., Zafrani, E. S., Roudot–Thoraval, F., Costentin, C., Hessami, A., Bouvier–Alias, M., Medkour, F., Pawlostky, J.–M., Lotersztajn, S. and Mallat, A. (2008): Daily Cannabis Use: A Novel Risk Factor of Steatosis Severity in Patients With Chronic Hepatitis C. – *Gastroenterology* **134**(2): 432–439. http://dx.doi.org/10.1053/j.gastro.2007.11.039

Hiebel, C., Kromm, T., Stark, M. and Behl, C. (2014): Cannabinoid receptor 1 modulates the autophagic flux independent of mTOR- and BECLIN1-complex. – *J. Neurochem.* **131**. http://dx.doi.org/10.1111/jnc.12839

Hochrath, K., Krawczyk, M., Goebel, R., Langhirt, M., Rathkolb, B., Micklich, K., Rozman, J., Horsch, M., Beckers, J., Klingenspor, M., Fuchs, H., Gailus-Durner, V., Wolf, E., Acalovschi, M., Volmer, D. A., Hrabe de Angelis, M. and Lammert, F. (2012): The hepatic phosphatidylcholine transporter ABCB4 as modulator of glucose homeostasis. – *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **26**(12): 5081–5091. http://dx.doi.org/10.1096/fj.12-209379

Hollands, M. A. and Cawthorne, M. A. (1981): Important sites of lipogenesis in the mouse other than liver and white adipose tissue. – *The Biochemical journal* **196**(2): 645–647. http://dx.doi.org/10.1042/bj1960645

Hotamisligil, G. S. (2006): Inflammation and metabolic disorders. – *Nature* **444**(7121): 860–867. http://dx.doi.org/10.1038/nature05485

Hou, J., Liu, Z. and Gu, F. (2005): Epidemiology and Prevention of Hepatitis B Virus Infection. – *Int J Med Sci* **2**(1): 50–57. http://dx.doi.org/10.7150/ijms.2.50

Howlett, A. C. (2002): The cannabinoid receptors. – *Prostaglandins & other lipid mediators* **68-69**: 619–631. http://dx.doi.org/10.1016/s0090-6980(02)00060-6

Hu, D., Wang, H., Wang, H., Wang, Y., Wan, X., Yan, W., Luo, X. and Ning, Q. (2018): Non-alcoholic hepatic steatosis attenuates hepatitis B virus replication in an HBV-immunocompetent mouse model. – *Hepatology international* **12**(5): 438–446. http://dx.doi.org/10.1007/s12072-018-9877-7

Hu, J. and Liu, K. (2017): Complete and Incomplete Hepatitis B Virus Particles: Formation, Function, and Application. – *Viruses* **9**(3): 56. http://dx.doi.org/10.3390/v9030056

Huang, L.-R., Gabel, Y. A., Graf, S., Arzberger, S., Kurts, C., Heikenwalder, M., Knolle, P. A. and Protzer, U. (2012): Transfer of HBV genomes using low doses of adenovirus vectors leads to persistent infection in immune competent mice. – *Gastroenterology* **142**(7): 1447-50.e3. http://dx.doi.org/10.1053/j.gastro.2012.03.006

Huang, W., Zhang, J., Chua, S. S., Qatanani, M., Han, Y., Granata, R. and Moore, D. D. (2003): Induction of bilirubin clearance by the constitutive androstane receptor (CAR). – *Proceedings of the National Academy of Sciences* **100**(7): 4156. http://dx.doi.org/10.1073/pnas.0630614100

Hussain, M. M., Shi, J. and Dreizen, P. (2003): Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. – *Journal of lipid research* **44**(1): 22–32. http://dx.doi.org/10.1194/jlr.r200014-jlr200

Imai, Y., Varela, G. M., Jackson, M. B., Graham, M. J., Crooke, R. M. and Ahima, R. S. (2007): Reduction of hepatosteatosis and lipid levels by an adipose differentiation-related protein antisense oligonucleotide. – *Gastroenterology* **132**. http://dx.doi.org/10.1053/j.gastro.2007.02.046

Ipsen, D. H., Lykkesfeldt, J. and Tveden-Nyborg, P. (2018): Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. – *Cellular and molecular life sciences : CMLS* **75**(18): 3313–3327. http://dx.doi.org/10.1007/s00018-018-2860-6

Irungbam, K., Churin, Y., Matono, T., Weglage, J., Ocker, M., Glebe, D., Hardt, M., Koeppel, A., Roderfeld, M. and Roeb, E. (2019): Cannabinoid receptor 1 knockout alleviates hepatic steatosis by downregulating perilipin 2. – *Laboratory Investigation*. http://dx.doi.org/10.1038/s41374-019-0327-5

Itabe, H., Yamaguchi, T., Nimura, S. and Sasabe, N. (2017): Perilipins: a diversity of intracellular lipid droplet proteins. – *Lipids Health Dis* **16**(1): 83. http://dx.doi.org/10.1186/s12944-017-0473-y

James, O. F. and Day, C. P. (1998): Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. – *Journal of hepatology* **29**(3): 495–501. http://dx.doi.org/10.1016/s0168-8278(98)80073-1

Jamil, H., Gordon, D. A., Eustice, D. C., Brooks, C. M., Dickson, J. K., JR, Chen, Y., Ricci, B., Chu, C. H., Harrity, T. W., Ciosek, C. P., JR, Biller, S. A., Gregg, R. E. and Wetterau, J. R. (1996): An inhibitor of the microsomal triglyceride transfer protein inhibits apoB secretion from HepG2 cells. – *Proceedings of the National Academy of Sciences of the United States of America* **93**(21): 11991–11995. http://dx.doi.org/10.1073/pnas.93.21.11991

Ji, C. and Kaplowitz, N. (2003): Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. – *Gastroenterology* **124**(5): 1488–1499. http://dx.doi.org/10.1016/s0016-5085(03)00276-2

Jiao, Y., Lu, Y. and Li, X.-y. (2015): Farnesoid X receptor: a master regulator of hepatic triglyceride and glucose homeostasis. – *Acta Pharmacol Sin* **36**(1): 44–50. http://dx.doi.org/10.1038/aps.2014.116

Johansen, T. and Lamark, T. (2011): Selective autophagy mediated by autophagic adapter proteins. – *Autophagy* **7**(3): 279–296. http://dx.doi.org/10.4161/auto.7.3.14487

Julien, B., Grenard, P., Teixeira-Clerc, F., van Nhieu, J. T., Li, L., Karsak, M., Zimmer, A., Mallat, A. and Lotersztajn, S. (2005): Antifibrogenic role of the cannabinoid receptor CB2 in the liver. – *Gastroenterology* **128**(3): 742–755. http://dx.doi.org/10.1053/j.gastro.2004.12.050

Kang, L., Pan, J., Wu, J., Hu, J., Sun, Q. and Tang, J. (2015): Anti-HBV Drugs: Progress, Unmet Needs, and New Hope. – *Viruses* **7**(9): 4960–4977. http://dx.doi.org/10.3390/v7092854

Kaushik, S. and Cuervo, A. M. (2015*a):* AMPK-dependent phosphorylation of lipid droplet protein PLIN2 triggers its degradation by CMA. – *Autophagy* **12**. http://dx.doi.org/10.1080/15548627.2015.1124226

Kawaguchi, T., Osatomi, K., Yamashita, H., Kabashima, T. and Uyeda, K. (2002): Mechanism for fatty acid "sparing" effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. – *The Journal of biological chemistry* **277**(6): 3829–3835. http://dx.doi.org/10.1074/jbc.M107895200

Kersten, S. and Stienstra, R. (2017): The role and regulation of the peroxisome proliferator activated receptor alpha in human liver. – *Biochimie* **136**: 75–84. http://dx.doi.org/10.1016/j.biochi.2016.12.019

Kim, J., Kundu, M., Viollet, B. and Guan, K.-L. (2011): AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. – *Nature cell biology* **13**(2): 132–141. http://dx.doi.org/10.1038/ncb2152

Kim, K. H., Shin, H.-J., Kim, K., Choi, H. M., Rhee, S. H., Moon, H.-B., Kim, H. H., Yang, U. S., Yu, D.-Y. and Cheong, J. (2007): Hepatitis B virus X protein induces hepatic steatosis via transcriptional activation of SREBP1 and PPARgamma. – *Gastroenterology* **132**(5): 1955–1967. http://dx.doi.org/10.1053/j.gastro.2007.03.039

Kim, K. M., Han, C. Y., Kim, J. Y., Cho, S. S., Kim, Y. S., Koo, J. H., Lee, J. M., Lim, S. C., Kang, K. W., Kim, J.-S., Hwang, S. J., Ki, S. H. and Kim, S. G. (2018): Galpha12 overexpression induced by miR-16 dysregulation contributes to liver fibrosis by promoting autophagy in hepatic stellate cells. – *Journal of hepatology* **68**(3): 493–504. http://dx.doi.org/10.1016/j.jhep.2017.10.011

Klingmuller, U. and Schaller, H. (1993): Hepadnavirus infection requires interaction between the viral pre-S domain and a specific hepatocellular receptor. – *Journal of virology* **67**(12): 7414–7422.

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H. and Acevedo Arozena, A. (2016): Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). – *Autophagy* **12**. http://dx.doi.org/10.1080/15548627.2015.1100356

Kloet, A. D. and Woods, S. C. (2009): Minireview: Endocannabinoids and their receptors as targets for obesity therapy. – *endo* **150**(6): 2531–2536. http://dx.doi.org/10.1210/en.2009-0046

Kounakis, K., Chaniotakis, M., Markaki, M. and Tavernarakis, N. (2019): Emerging Roles of Lipophagy in Health and Disease. – *Frontiers in Cell and Developmental Biology* **7**: 185. http://dx.doi.org/10.3389/fcell.2019.00185

Krüger, S., Bürmann, L. and Morlock, G. E. (2015): Comparison and characterization of soybean and sunflower lecithins used for chocolate production by high-performance thin-layer chromatography with fluorescence detection and electrospray mass spectrometry. – *Journal of agricultural and food chemistry* **63**(11): 2893–2901. http://dx.doi.org/10.1021/jf506332f

Kunos, G., Osei-Hyiaman, D., Liu, J., Godlewski, G. and Batkai, S. (2008): Endocannabinoids and the control of energy homeostasis. – *The Journal of biological chemistry* **283**(48): 33021–33025. http://dx.doi.org/10.1074/jbc.R800012200

Laggai, S., Simon, Y., Ranssweiler, T., Kiemer, A. K. and Kessler, S. M. (2013): Rapid chromatographic method to decipher distinct alterations in lipid classes in NAFLD/NASH. – *World J Hepatol* **5**. http://dx.doi.org/10.4254/wjh.v5.i10.558

Lamireau, T., Bouchard, G., Yousef, I. M., Clouzeau-Girard, H., Rosenbaum, J., Desmouliere, A. and Tuchweber, B. (2007): Dietary lecithin protects against cholestatic liver disease in cholic acid-fed Abcb4- deficient mice. – *Pediatric research* **61**(2): 185–190. http://dx.doi.org/10.1203/pdr.0b013e31802d7780

Lammert, F., Wang, D. Q.-H., Hillebrandt, S., Geier, A., Fickert, P., Trauner, M., Matern, S., Paigen, B. and Carey, M. C. (2004): Spontaneous cholecysto- and hepatolithiasis in Mdr2–/– mice: A model for low phospholipid-associated cholelithiasis. – *Hepatology* **39**(1): 117–128. http://dx.doi.org/10.1002/hep.20022

Lanaspa, M. A., Sanchez-Lozada, L. G., Choi, Y.-J., Cicerchi, C., Kanbay, M., Roncal-Jimenez, C. A., Ishimoto, T., Li, N., Marek, G., Duranay, M., Schreiner, G., Rodriguez-Iturbe, B., Nakagawa, T., Kang, D.-H., Sautin, Y. Y. and Johnson, R. J. (2012): Uric acid induces hepatic steatosis by generation of mitochondrial oxidative stress: potential role in fructose-dependent and -independent fatty liver. – *The Journal of biological chemistry* **287**(48): 40732–40744. http://dx.doi.org/10.1074/jbc.M112.399899

Langhi, C., Marquart, T. J., Allen, R. M. and Baldán, A. (2014): Perilipin-5 is regulated by statins and controls triglyceride contents in the hepatocyte. – *Journal of hepatology* **61**. http://dx.doi.org/10.1016/j.jhep.2014.04.009

Lee, H., Jeong, H., Lee, S. Y., Kim, S. S. and Jang, K. L. (2019): Hepatitis B Virus X Protein Stimulates Virus Replication Via DNA Methylation of the C-1619 in Covalently Closed Circular DNA. – *Mol Cells* **42**(1): 67–78. http://dx.doi.org/10.14348/molcells.2018.0255

Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez-Salguero, P. M., Westphal, H. and Gonzalez, F. J. (1995): Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. – *Molecular and cellular biology* **15**(6): 3012–3022. http://dx.doi.org/10.1128/mcb.15.6.3012

Lemasters, J. J. (2005): Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. – *Rejuvenation research* **8**(1): 3–5. http://dx.doi.org/10.1089/rej.2005.8.3

Li, G., Zhu, Y., Shao, D., Chang, H., Zhang, X., Zhou, D., Gao, Y., Lan, K. and Deng, Q. (2018): Recombinant covalently closed circular DNA of hepatitis B virus induces long-term viral persistence with chronic hepatitis in a mouse model. – *Hepatology (Baltimore, Md.)* **67**(1): 56–70. http://dx.doi.org/10.1002/hep.29406

Li, J. Z., Ye, J., Xue, B., Qi, J., Zhang, J., Zhou, Z., Li, Q., Wen, Z. and Li, P. (2007): Cideb regulates diet-induced obesity, liver steatosis, and insulin sensitivity by controlling lipogenesis and fatty acid oxidation. – *Diabetes* **56**(10): 2523–2532. http://dx.doi.org/10.2337/db07-0040

Li, Y., Xu, S., Mihaylova, M. M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J. Y.-J., Gao, B., Wierzbicki, M., Verbeuren, T. J., Shaw, R. J., Cohen, R. A. and Zang, M. (2011): AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. – *Cell metabolism* **13**(4): 376–388. http://dx.doi.org/10.1016/j.cmet.2011.03.009

Liang, T. J. (2009*):* Hepatitis B: The virus and disease. – *Hepatology (Baltimore, Md.)* **49**(S5): S13-S21. http://dx.doi.org/10.1002/hep.22881

Liao, W., Hui, T. Y., Young, S. G. and Davis, R. A. (2003): Blocking microsomal triglyceride transfer protein interferes with apoB secretion without causing retention or stress in the ER. – *Journal of lipid research* **44**(5): 978–985. http://dx.doi.org/10.1194/jlr.M300020-JLR200

Lincke, C. R., Smit, J. J., van der Velde-Koerts, T. and Borst, P. (1991): Structure of the human MDR3 gene and physical mapping of the human MDR locus. – *The Journal of biological chemistry* **266**(8): 5303–5310.

Listenberger, L. L., Ostermeyer-Fay, A. G., Goldberg, E. B., Brown, W. J. and Brown, D. A. (2007): Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. – *Journal of lipid research* **48**(12): 2751–2761. http://dx.doi.org/10.1194/jlr.M700359-JLR200

Liu, K. and Czaja, M. J. (2013): Regulation of lipid stores and metabolism by lipophagy. – *Cell death and differentiation* **20**(1): 3–11. http://dx.doi.org/10.1038/cdd.2012.63

Liu, W. J., Ye, L., Huang, W. F., Guo, L. J., Xu, Z. G., Wu, H. L., Yang, C. and Liu, H. F. (2016): p62 links the autophagy pathway and the ubiqutin–proteasome system upon ubiquitinated protein degradation. – *Cellular & Molecular Biology Letters* **21**(1): 29. http://dx.doi.org/10.1186/s11658-016-0031-z

Liu, Y., Binz, J., Numerick, M. J., Dennis, S., Luo, G., Desai, B., MacKenzie, K. I., Mansfield, T. A., Kliewer, S. A., Goodwin, B. and Jones, S. A. (2003): Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. – *The Journal of clinical investigation* **112**(11): 1678–1687. http://dx.doi.org/10.1172/JCI18945

Livak, K. J. and Schmittgen, T. D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. – *Methods* **25**. http://dx.doi.org/10.1006/meth.2001.1262

Lok, A. S. F. and McMahon, B. J. (2009): Chronic hepatitis B: update 2009. – *Hepatology (Baltimore, Md.)* **50**(3): 661–662. http://dx.doi.org/10.1002/hep.23190

Lord, C. C., Ferguson, D., Thomas, G., Brown, A. L., Schugar, R. C., Burrows, A., Gromovsky, A. D., Betters, J., Neumann, C., Sacks, J., Marshall, S., Watts, R., Schweiger, M., Lee, R. G., Crooke, R. M., Graham, M. J., Lathia, J. D., Sakaguchi, T. F., Lehner, R., Haemmerle, G., Zechner, R. and Brown, J. M. (2016): Regulation of Hepatic Triacylglycerol Metabolism by

CGI-58 Does Not Require ATGL Co-activation. – *Cell reports* **16**(4): 939–949. http://dx.doi.org/10.1016/j.celrep.2016.06.049

Lu, M. and Shyy, J. Y.-J. (2006): Sterol regulatory element-binding protein 1 is negatively modulated by PKA phosphorylation. – *American journal of physiology. Cell physiology* **290**(6): C1477-86. http://dx.doi.org/10.1152/ajpcell.00374.2005

Ludwig, J., Viggiano, T. R., McGill, D. B. and Oh, B. J. (1980): Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. – *Mayo Clinic proceedings* **55**(7): 434–438.

Machado, M. V., Oliveira, A. G. and Cortez-Pinto, H. (2011): Hepatic steatosis in hepatitis B virus infected patients: meta-analysis of risk factors and comparison with hepatitis C infected patients. – *Journal of gastroenterology and hepatology* **26**(9): 1361–1367. http://dx.doi.org/10.1111/j.1440-1746.2011.06801.x

Mack, H. I. D., Zheng, B., Asara, J. M. and Thomas, S. M. (2012): AMPK-dependent phosphorylation of ULK1 regulates ATG9 localization. – *Autophagy* **8**(8): 1197–1214. http://dx.doi.org/10.4161/auto.20586

MacLachlan, J. H. and Cowie, B. C. (2015): Hepatitis B virus epidemiology. – *Cold Spring Harbor perspectives in medicine* **5**(5): a021410-a021410. http://dx.doi.org/10.1101/cshperspect.a021410

Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lustig, K. D., Mangelsdorf, D. J. and Shan, B. (1999): Identification of a Nuclear Receptor for Bile Acids. – *Science (New York, N.Y.)* **284**(5418): 1362. http://dx.doi.org/10.1126/science.284.5418.1362

Mallat, A. and Lotersztajn, S. (2008): Endocannabinoids and liver disease. I. Endocannabinoids and their receptors in the liver. – *American journal of physiology. Gastrointestinal and liver physiology* **294**(1): G9-G12. http://dx.doi.org/10.1152/ajpgi.00467.2007

Mallat, A., Teixeira-Clerc, F., Deveaux, V., Manin, S. and Lotersztajn, S. (2011): The endocannabinoid system as a key mediator during liver diseases: New insights and therapeutic openings. – *Br J Pharmacol* **163**. http://dx.doi.org/10.1111/j.1476-5381.2011.01397.x

Mallat, A., Teixeira-Clerc, F. and Lotersztajn, S. (2013): Cannabinoid signaling and liver therapeutics. – *Journal of hepatology* **59**(4): 891–896. http://dx.doi.org/10.1016/j.jhep.2013.03.032

Martina, J. A., Diab, H. I., Lishu, L., Jeong-A, L., Patange, S., Raben, N. and Puertollano, R. (2014): The nutrient-responsive transcription factor TFE3 promotes autophagy, lysosomal biogenesis, and clearance of cellular debris. – *Sci Signal* **7**(309): ra9-ra9. http://dx.doi.org/10.1126/scisignal.2004754

Martinez-Lopez, N., Garcia-Macia, M., Sahu, S., Athonvarangkul, D., Liebling, E., Merlo, P., Cecconi, F., Schwartz, G. J. and Singh, R. (2016): Autophagy in the CNS and Periphery Coordinate Lipophagy and Lipolysis in the Brown Adipose Tissue and Liver. – *Cell metabolism* **23**(1): 113–127. http://dx.doi.org/10.1016/j.cmet.2015.10.008

Martinez-Lopez, N. and Singh, R. (2015): Autophagy and Lipid Droplets in the Liver. – *Annual Review of Nutrition* **35**(1): 215–237. http://dx.doi.org/10.1146/annurev-nutr-071813-105336

Marzuillo, P., Del Giudice, E. M. and Santoro, N. (2014): Pediatric non-alcoholic fatty liver disease: New insights and future directions. – *World J Hepatol* **6**(4): 217–225. http://dx.doi.org/10.4254/wjh.v6.i4.217

Matias, I., Gonthier, M.-P., Orlando, P., Martiadis, V., Petrocellis, L. de, Cervino, C., Petrosino, S., Hoareau, L., Festy, F., Pasquali, R., Roche, R., Maj, M., Pagotto, U., Monteleone, P. and Di Marzo, V. (2006): Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. – *The Journal of clinical endocrinology and metabolism* **91**(8): 3171–3180. http://dx.doi.org/10.1210/jc.2005-2679

McGarry, J. D. and Foster, D. W. (1977): Hormonal control of ketogenesis. Biochemical considerations. – *Archives of internal medicine* **137**(4): 495–501.

McManaman, J. L., Bales, E. S., Orlicky, D. J., Jackman, M., MacLean, P. S., Cain, S., Crunk, A. E., Mansur, A., Graham, C. E., Bowman, T. A. and Greenberg, A. S. (2013): Perilipin-2-null mice are protected against diet-induced obesity, adipose inflammation, and fatty liver disease. – *Journal of lipid research* **54**(5): 1346–1359. http://dx.doi.org/10.1194/jlr.M035063

Menendez, J. A., Vazquez-Martin, A., Ortega, F. J. and Fernandez-Real, J. M. (2009): Fatty acid synthase: association with insulin resistance, type 2 diabetes, and cancer. – *Clinical chemistry* **55**(3): 425–438. http://dx.doi.org/10.1373/clinchem.2008.115352

Mihaylova, M. M. and Shaw, R. J. (2011): The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. – *Nature cell biology* **13**(9): 1016–1023. http://dx.doi.org/10.1038/ncb2329

Misra, B. and Singh, S. (2012): Nonalcoholic fatty liver disease and hepatitis B virus infection. – *Hep B Annu* **9**. http://dx.doi.org/10.4103/0972-9747.193290

Mittal, S. and El-Serag, H. B. (2013): Epidemiology of hepatocellular carcinoma: consider the population. – *J Clin Gastroenterol* **47 Suppl**(0): S2-S6. http://dx.doi.org/10.1097/MCG.0b013e3182872f29

Montagner, A., Polizzi, A., Fouché, E., Ducheix, S., Lippi, Y., Lasserre, F., Barquissau, V., Régnier, M., Lukowicz, C., Benhamed, F., Iroz, A., Bertrand-Michel, J., Al Saati, T., Cano, P., Mselli-Lakhal, L., Mithieux, G., Rajas, F., Lagarrigue, S., Pineau, T., Loiseau, N., Postic, C., Langin, D., Wahli, W. and Guillou, H. (2016): Liver PPARα is crucial for whole-body fatty acid homeostasis and is protective against NAFLD. – *Gut* **65**(7): 1202. http://dx.doi.org/10.1136/gutjnl-2015-310798

Mouslech, Z. and Valla, V. (2009): Endocannabinoid system: An overview of its potential in current medical practice. – *Neuro endocrinology letters* **30**(2): 153–179.

Moustafa, T., Fickert, P., Magnes, C., Guelly, C., Thueringer, A., Frank, S., Kratky, D., Sattler, W., Reicher, H., Sinner, F., Gumhold, J., Silbert, D., Fauler, G., Höfler, G., Lass, A., Zechner, R. and Trauner, M. (2012): Alterations in lipid metabolism mediate inflammation, fibrosis, and proliferation in a mouse model of chronic cholestatic liver injury. – *Gastroenterology* **142**(1): 140-151.e12. http://dx.doi.org/10.1053/j.gastro.2011.09.051

Munro, S., Thomas, K. L. and Abu-Shaar, M. (1993): Molecular characterization of a peripheral receptor for cannabinoids. – *Nature* **365**(6441): 61–65. http://dx.doi.org/10.1038/365061a0

Nassir, F. and Ibdah, J. A. (2014): Role of mitochondria in nonalcoholic fatty liver disease. – *International journal of molecular sciences* **15**(5): 8713–8742. http://dx.doi.org/10.3390/ijms15058713

Nguyen, P., Leray, V., Diez, M., Serisier, S., Le Bloc'h, J., Siliart, B. and Dumon, H. (2008): Liver lipid metabolism. – *Journal of animal physiology and animal nutrition* **92**(3): 272–283. http://dx.doi.org/10.1111/j.1439-0396.2007.00752.x

Nishio, T., Hu, R., Koyama, Y., Liang, S., Rosenthal, S. B., Yamamoto, G., Karin, D., Baglieri, J., Ma, H.-Y., Xu, J., Liu, X., Dhar, D., Iwaisako, K., Taura, K., Brenner, D. A. and Kisseleva, T. (2019): Activated hepatic stellate cells and portal fibroblasts contribute to cholestatic liver fibrosis in MDR2 knockout mice. – *Journal of hepatology* **71**(3): 573–585. http://dx.doi.org/10.1016/j.jhep.2019.04.012

Olsen, J. A., Alam, A., Kowal, J., Stieger, B. and Locher, K. P. (2020): Structure of the human lipid exporter ABCB4 in a lipid environment. – *Nature structural & molecular biology* **27**(1): 62–70. http://dx.doi.org/10.1038/s41594-019-0354-3

Osei-Hyiaman, D., DePetrillo, M., Pacher, P., Liu, J., Radaeva, S., Batkai, S., Harvey-White, J., Mackie, K., Offertaler, L., Wang, L. and Kunos, G. (2005): Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. – *The Journal of clinical investigation* **115**(5): 1298–1305. http://dx.doi.org/10.1172/JCI23057

Osei-Hyiaman, D., Liu, J., Zhou, L., Godlewski, G., Harvey-White, J., Jeong, W.-i., Batkai, S., Marsicano, G., Lutz, B., Buettner, C. and Kunos, G. (2008): Hepatic CB1 receptor is required for development of diet-induced steatosis, dyslipidemia, and insulin and leptin resistance in mice. – *The Journal of clinical investigation* **118**(9): 3160–3169. http://dx.doi.org/10.1172/JCI34827

Ota, T., Gayet, C. and Ginsberg, H. N. (2008): Inhibition of apolipoprotein B100 secretion by lipid-induced hepatic endoplasmic reticulum stress in rodents. – *The Journal of clinical investigation* **118**(1): 316–332. http://dx.doi.org/10.1172/JCI32752

Oude Elferink, Ronald P. J. and Paulusma, C. C. (2007): Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein). – *Pflügers Archiv - European Journal of Physiology* **453**(5): 601–610. http://dx.doi.org/10.1007/s00424-006-0062-9

Paglialunga, S. and Dehn, C. A. (2016): Clinical assessment of hepatic de novo lipogenesis in non-alcoholic fatty liver disease. – *Lipids Health Dis* **15**(1): 159. http://dx.doi.org/10.1186/s12944-016-0321-5

Paulusma, C. C., Kool, M., Bosma, P. J., Scheffer, G. L., ter Borg, F., Scheper, R. J., Tytgat, G. N., Borst, P., Baas, F. and Oude Elferink, R. P. (1997): A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome. – *Hepatology (Baltimore, Md.)* **25**(6): 1539–1542. http://dx.doi.org/10.1002/hep.510250635

Peleg, N., Issachar, A., Sneh Arbib, O., Cohen-Naftaly, M., Braun, M., Leshno, M., Barsheshet, A. and Shlomai, A. (2019): Liver steatosis is a strong predictor of mortality and cancer in chronic hepatitis B regardless of viral load. – *JHEP Reports* **1**(1): 9–16. http://dx.doi.org/10.1016/j.jhepr.2019.02.002

Peverill, W., Powell, L. W. and Skoien, R. (2014): Evolving concepts in the pathogenesis of NASH: beyond steatosis and inflammation. – *International journal of molecular sciences* **15**(5): 8591–8638. http://dx.doi.org/10.3390/ijms15058591

Previsani N, L. D. (2002): Hepatitis B. Department of Communicable Diseases Surveillance and Response,. – *World Health Organisation, Geneva.*

Pugsley, H. R. (2017): Assessing Autophagic Flux by Measuring LC3, p62, and LAMP1 Co-localization Using Multispectral Imaging Flow Cytometry. – *JoVE* (125): e55637. http://dx.doi.org/10.3791/55637

Quaye, O., Amuzu, B. G., Adadey, S. M. and Tagoe, E. A. (2019): Effect of Hepatitis B Virus (HBV) Infection on Lipid Profile in Ghanaian Patients. – *Virology : research and treatment* **10**: 1178122X19827606. http://dx.doi.org/10.1177/1178122X19827606

Rao, M. S. and Reddy, J. K. (2001): Peroxisomal beta-oxidation and steatohepatitis. – *Seminars in liver disease* **21**(1): 43–55. http://dx.doi.org/10.1055/s-2001-12928

Rastogi, A., Sakhuja, P., Kumar, A., Hissar, S., Jain, A., Gondal, R. and Sarin, S. (2011): Steatosis in chronic hepatitis B: Prevalence and correlation with biochemical, histologic, viral, and metabolic parameters. – *Indian Journal of Pathology and Microbiology* **54**(3): 454–459. http://dx.doi.org/10.4103/0377-4929.85074

Ratziu, V., Bellentani, S., Cortez-Pinto, H., Day, C. and Marchesini, G. (2010): A position statement on NAFLD/NASH based on the EASL 2009 special conference. – *Journal of hepatology* **53**(2): 372–384. http://dx.doi.org/10.1016/j.jhep.2010.04.008

Ravinet Trillou, C., Arnone, M., Delgorge, C., Gonalons, N., Keane, P., Maffrand, J.-P. and Soubrie, P. (2003): Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-

induced obese mice. – *American journal of physiology. Regulatory, integrative and comparative physiology* **284**(2): R345-53. http://dx.doi.org/10.1152/ajpregu.00545.2002

Reddy, J. K. and Rao, M. S. (2006): Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. – *American journal of physiology. Gastrointestinal and liver physiology* **290**(5): G852-8. http://dx.doi.org/10.1152/ajpgi.00521.2005

Reichert, M. C. and Lammert, F. (2018): ABCB4 Gene Aberrations in Human Liver Disease: An Evolving Spectrum. – *Seminars in liver disease* **38**(4): 299–307. http://dx.doi.org/10.1055/s-0038-1667299

Reiter, F. P., Wimmer, R., Wottke, L., Artmann, R., Nagel, J. M., Carranza, M. O., Mayr, D., Rust, C., Fickert, P., Trauner, M., Gerbes, A. L., Hohenester, S. and Denk, G. U. (2016): Role of interleukin-1 and its antagonism of hepatic stellate cell proliferation and liver fibrosis in the Abcb4(-/-) mouse model. – *World J Hepatol* **8**(8): 401–410. http://dx.doi.org/10.4254/wjh.v8.i8.401

Roderfeld, M., Rath, T., Voswinckel, R., Dierkes, C., Dietrich, H., Zahner, D., Graf, J. and Roeb, E. (2010): Bone marrow transplantation demonstrates medullar origin of CD34+ fibrocytes and ameliorates hepatic fibrosis in Abcb4-/- mice. – *Hepatology (Baltimore, Md.)* **51**(1): 267–276. http://dx.doi.org/10.1002/hep.23274

Roeb, E., Steffen, H. M., Bantel, H., Baumann, U., Canbay, A., Demir, M., Drebber, U., Geier, A., Hampe, J., Hellerbrand, C., Pathil-Warth, A., Schattenberg, J. M., Schramm, C., Seitz, H. K., Stefan, N., Tacke, F., Tannapfel, A., Lynen Jansen, P. and Bojunga, J. (2015): S2k Guideline non-alcoholic fatty liver disease. – *Z Gastroenterol* **53**(7): 668–723. http://dx.doi.org/10.1055/s-0035-1553193

Romeo, S., Kozlitina, J., Xing, C., Pertsemlidis, A., Cox, D., Pennacchio, L. A., Boerwinkle, E., Cohen, J. C. and Hobbs, H. H. (2008): Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. – *Nature genetics* **40**(12): 1461–1465. http://dx.doi.org/10.1038/ng.257

Rotman, Y. and Sanyal, A. J. (2017): Current and upcoming pharmacotherapy for non-alcoholic fatty liver disease. – *Gut* **66**(1): 180–190. http://dx.doi.org/10.1136/gutjnl-2016-312431

Saka, H. A. and Valdivia, R. (2012): Emerging roles for lipid droplets in immunity and host-pathogen interactions. – *Annual review of cell and developmental biology* **28**: 411–437. http://dx.doi.org/10.1146/annurev-cellbio-092910-153958

Sanchez-Gurmaches, J., Tang, Y., Jespersen, N. Z., Wallace, M., Martinez Calejman, C., Gujja, S., Li, H., Edwards, Y. J. K., Wolfrum, C., Metallo, C. M., Nielsen, S., Scheele, C. and Guertin, D. A. (2018): Brown Fat AKT2 Is a Cold-Induced Kinase that Stimulates ChREBP-Mediated De Novo Lipogenesis to Optimize Fuel Storage and Thermogenesis. – *Cell metabolism* **27**(1): 195-209.e6. http://dx.doi.org/10.1016/j.cmet.2017.10.008

Sanyal, A. J. (2019): Past, present and future perspectives in nonalcoholic fatty liver disease. – *Nature reviews. Gastroenterology & hepatology* **16**(6): 377–386. http://dx.doi.org/10.1038/s41575-019-0144-8

Sathyanarayan, A., Mashek, M. T. and Mashek, D. G. (2017): ATGL Promotes Autophagy/Lipophagy via SIRT1 to Control Hepatic Lipid Droplet Catabolism. – *Cell reports* **19**(1): 1–9. http://dx.doi.org/10.1016/j.celrep.2017.03.026

Sato, K., Cho, Y., Tachibana, S., Chiba, T., Schneider, W. J. and Akiba, Y. (2005): Impairment of VLDL secretion by medium-chain fatty acids in chicken primary hepatocytes is affected by the chain length. – *The Journal of nutrition* **135**(7): 1636–1641. http://dx.doi.org/10.1093/jn/135.7.1636

Schatz, A. R., Lee, M., Condie, R. B., Pulaski, J. T. and Kaminski, N. E. (1997): Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. – *Toxicology and applied pharmacology* **142**(2): 278–287. http://dx.doi.org/10.1006/taap.1996.8034

Schwimmer, J. B., Behling, C., Newbury, R., Deutsch, R., Nievergelt, C., Schork, N. J. and Lavine, J. E. (2005): Histopathology of pediatric nonalcoholic fatty liver disease. – *Hepatology (Baltimore, Md.)* **42**(3): 641–649. http://dx.doi.org/10.1002/hep.20842

Shi, D., Chen, J., Wang, J., Yao, J., Huang, Y., Zhang, G. and Bao, Z. (2019): Circadian Clock Genes in the Metabolism of Non-alcoholic Fatty Liver Disease. – *Frontiers in physiology* **10**: 423. http://dx.doi.org/10.3389/fphys.2019.00423

Shi, D., zhan, X., Yu, X., Jia, M., Zhang, Y. and Yao, J. (2014): Inhibiting CB1 receptors improves lipogenesis in an in vitro non-alcoholic fatty liver disease model. – *Lipids Health Dis* **13**. http://dx.doi.org/10.1186/1476-511X-13-173

Shipovskaya, A. A. and Dudanova, O. P. (2018): Intrahepatic cholestasis in nonalcoholic fatty liver disease. – *Terapevticheskii arkhiv* **90**(2): 69–74. http://dx.doi.org/10.26442/terarkh201890269-74

Sinal, C. J., Tohkin, M., Miyata, M., Ward, J. M., Lambert, G. and Gonzalez, F. J. (2000): Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. – *Cell* **102**(6): 731–744. http://dx.doi.org/10.1016/s0092-8674(00)00062-3

Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I. and Komatsu, M. (2009): Autophagy regulates lipid metabolism. – *Nature* **458**. http://dx.doi.org/10.1038/nature07976

Smit, J. J., Schinkel, A. H., Oude Elferink, R. P., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A., Ottenhoff, R., van der Lugt, N M and van Roon, M. A. (1993): Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. – *Cell* **75**(3): 451–462. http://dx.doi.org/10.1016/0092-8674(93)90380-9

Solinas, G., Boren, J. and Dulloo, A. G. (2015): De novo lipogenesis in metabolic homeostasis: More friend than foe? – *Molecular metabolism* **4**(5): 367–377. http://dx.doi.org/10.1016/j.molmet.2015.03.004

Sookoian, S., Rosselli, M. S., Gemma, C., Burgueno, A. L., Fernandez Gianotti, T., Castano, G. O. and Pirola, C. J. (2010): Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor gamma coactivator 1alpha promoter. – *Hepatology (Baltimore, Md.)* **52**(6): 1992–2000. http://dx.doi.org/10.1002/hep.23927

Speliotes, E. K., Butler, J. L., Palmer, C. D., Voight, B. F. and Hirschhorn, J. N. (2010): PNPLA3 variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. – *Hepatology (Baltimore, Md.)* **52**(3): 904–912. http://dx.doi.org/10.1002/hep.23768

Stapelbroek, J. M., van Erpecum, K. J., Klomp, L. W. J. and Houwen, R. H. J. (2010): Liver disease associated with canalicular transport defects: current and future therapies. – *Journal of hepatology* **52**(2): 258–271. http://dx.doi.org/10.1016/j.jhep.2009.11.012

Steinberg, G. R. and Kemp, B. E. (2009): AMPK in Health and Disease. – *Physiological reviews* **89**(3): 1025–1078. http://dx.doi.org/10.1152/physrev.00011.2008

Sticova, E. and Jirsa, M. (2019): ABCB4 disease: Many faces of one gene deficiency. – *Ann Hepatol*: S1665-2681(19)32262-8. http://dx.doi.org/10.1016/j.aohep.2019.09.010

Straub, B. K., Stoeffel, P., Heid, H., Zimbelmann, R. and Schirmacher, P. (2008): Differential pattern of lipid droplet-associated proteins and de novo perilipin expression in hepatocyte

steatogenesis. – *Hepatology (Baltimore, Md.)* **47**(6): 1936–1946. http://dx.doi.org/10.1002/hep.22268

Sundaram, M. and Yao, Z. (2010): Recent progress in understanding protein and lipid factors affecting hepatic VLDL assembly and secretion. – *Nutrition & metabolism* **7**: 35. http://dx.doi.org/10.1186/1743-7075-7-35

Sundaram, M., Zhong, S., Bou Khalil, M., Links, P. H., Zhao, Y., Iqbal, J., Hussain, M. M., Parks, R. J., Wang, Y. and Yao, Z. (2010): Expression of apolipoprotein C-III in McA-RH7777 cells enhances VLDL assembly and secretion under lipid-rich conditions. – *Journal of lipid research* **51**(1): 150–161. http://dx.doi.org/10.1194/M900346-JLR200

Sundaram, S. S. and Sokol, R. J. (2007): The Multiple Facets of ABCB4 (MDR3) Deficiency. – *Curr Treat Options Gastroenterol* **10**(6): 495–503. http://dx.doi.org/10.1007/s11938-007-0049-4

Sztalryd, C., Bell, M., Lu, X., Mertz, P., Hickenbottom, S., Chang, B. H.-J., Chan, L., Kimmel, A. R. and Londos, C. (2006): Functional compensation for adipose differentiation-related protein (ADFP) by Tip47 in an ADFP null embryonic cell line. – *The Journal of biological chemistry* **281**(45): 34341–34348. http://dx.doi.org/10.1074/jbc.M602497200

Sztalryd, C. and Brasaemle, D. L. (2017): The perilipin family of lipid droplet proteins: Gatekeepers of intracellular lipolysis. – *Biochimica et biophysica acta. Molecular and cell biology of lipids* **1862**(10, Part B): 1221–1232. http://dx.doi.org/10.1016/j.bbalip.2017.07.009

Taha, M. N., Krawinkel, M. B. and Morlock, G. E. (2015): High-performance thin-layer chromatography linked with (bio)assays and mass spectrometry – A suited method for discovery and quantification of bioactive components? Exemplarily shown for turmeric and milk thistle extracts. – *Journal of Chromatography A* **1394**: 137–147. http://dx.doi.org/10.1016/j.chroma.2015.03.029

Tam, J., Liu, J., Mukhopadhyay, B., Cinar, R., Godlewski, G. and Kunos, G. (2011): Endocannabinoids in liver disease. – *Hepatology (Baltimore, Md.)* **53**(1): 346–355. http://dx.doi.org/10.1002/hep.24077

Tang, L. S. Y., Covert, E., Wilson, E. and Kottilil, S. (2018): Chronic Hepatitis B Infection: A Review. – *JAMA* **319**(17): 1802–1813. http://dx.doi.org/10.1001/jama.2018.3795

Teixeira-Clerc, F., Julien, B., Grenard, P., van Tran Nhieu, J., Deveaux, V., Li, L., Serriere-Lanneau, V., Ledent, C., Mallat, A. and Lotersztajn, S. (2006): CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. – *Nature medicine* **12**(6): 671–676. http://dx.doi.org/10.1038/nm1421

Thakur, P., Lamoke, F., Chaffin, J. M., Bartoli, M., Lee, J. R. and Duncan, M. B. (2014): Dysplastic Hepatocytes Develop Nuclear Inclusions in a Mouse Model of Viral Hepatitis. – *PloS one* **9**(6): e99872. http://dx.doi.org/10.1371/journal.pone.0099872

Thomson, D. M., Herway, S. T., Fillmore, N., Kim, H., Brown, J. D., Barrow, J. R. and Winder, W. W. (2008): AMP-activated protein kinase phosphorylates transcription factors of the CREB family. – Journal of Applied Physiology 104(2): 429–438. http://dx.doi.org/10.1152/japplphysiol.00900.2007

Thoeni, C., Waldherr, R., Scheuerer, J., Schmitteckert, S., Roeth, R., Niesler, B., Cutz, E., Flechtenmacher, C., Goeppert, B., Schirmacher, P. and Lasitschka, F. (2019): Expression Analysis of ATP-Binding Cassette Transporters ABCB11 and ABCB4 in Primary Sclerosing Cholangitis and Variety of Pediatric and Adult Cholestatic and Noncholestatic Liver Diseases. – *Canadian journal of gastroenterology & hepatology* **2019**: 1085717. http://dx.doi.org/10.1155/2019/1085717

Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J. and Schoonjans, K. (2008): Targeting bile-acid signalling for metabolic diseases. – *Nat Rev Drug Discov* **7**(8): 678–693. http://dx.doi.org/10.1038/nrd2619

Thomopoulos, K. C., Arvaniti, V., Tsamantas, A. C., Dimitropoulou, D., Gogos, C. A., Siagris, D., Theocharis, G. J. and Labropoulou-Karatza, C. (2006): Prevalence of liver steatosis in patients with chronic hepatitis B: a study of associated factors and of relationship with fibrosis. – *European Journal of Gastroenterology & Hepatology* **18**(3).

Till, A., Lakhani, R., Burnett, S. F. and Subramani, S. (2012): Pexophagy: the selective degradation of peroxisomes. – *International journal of cell biology* **2012**: 512721. http://dx.doi.org/10.1155/2012/512721

Timothy, J. S. (2002): An Introduction to Lipid Analysis in the Cell Biology Laboratory. – *The American Biology Teacher* **64**(2): 122–129. http://dx.doi.org/10.1662/0002-7685(2002)064[0122:AITLAI]2.0.CO;2

Tiwari, S. and Siddiqi, S. A. (2012): Intracellular trafficking and secretion of VLDL. – *Arteriosclerosis, thrombosis, and vascular biology* **32**(5): 1079–1086. http://dx.doi.org/10.1161/ATVBAHA.111.241471

Towbin, H., Staehelin, T. and Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. – *Proceedings of the National Academy of Sciences of the United States of America* **76**(9): 4350–4354. http://dx.doi.org/10.1073/pnas.76.9.4350

Tran, K., Sun, F., Cui, Z., Thorne-Tjomsland, G., St Germain, C., Lapierre, L. R., McLeod, R. S., Jamieson, J. C. and Yao, Z. (2006): Attenuated secretion of very low density lipoproteins from McA-RH7777 cells treated with eicosapentaenoic acid is associated with impaired utilization of triacylglycerol synthesized via phospholipid remodeling. – *Biochimica et biophysica acta* **1761**(4): 463–473. http://dx.doi.org/10.1016/j.bbalip.2006.03.018

Trauner, M. and Boyer, J. L. (2003): Bile salt transporters: molecular characterization, function, and regulation. – *Physiological reviews* **83**(2): 633–671. http://dx.doi.org/10.1152/physrev.00027.2002

Trauner, M., Fickert, P. and Stauber, R. E. (1999): Inflammation-induced cholestasis. – *Journal of gastroenterology and hepatology* **14**(10): 946–959.

Tsai, T.-H., Chen, E., Li, L., Saha, P., Lee, H.-J., Huang, L.-S., Shelness, G. S., Chan, L. and Chang, B. H.-J. (2017): The constitutive lipid droplet protein PLIN2 regulates autophagy in liver. – *Autophagy* **13**(7): 1130–1144. http://dx.doi.org/10.1080/15548627.2017.1319544

Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y., Ochi, H., Chayama, K., Tateno, C. and Yoshizato, K. (2005): Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. – *Hepatology (Baltimore, Md.)* **42**(5): 1046–1054. http://dx.doi.org/10.1002/hep.20892

Tuohetahuntila, M., Molenaar, M. R., Spee, B., Brouwers, J. F., Wubbolts, R., Houweling, M., Yan, C., Du, H., VanderVen, B. C., Vaandrager, A. B. and Helms, J. B. (2017): Lysosome-mediated degradation of a distinct pool of lipid droplets during hepatic stellate cell activation. – *The Journal of biological chemistry* **292**(30): 12436–12448. http://dx.doi.org/10.1074/jbc.M117.778472

Velkov, S., Ott, J. J., Protzer, U. and Michler, T. (2018): The Global Hepatitis B Virus Genotype Distribution Approximated from Available Genotyping Data. – *Genes (Basel)* **9**(10): 495. http://dx.doi.org/10.3390/genes9100495

Ventura, E. E., Davis, J. N. and Goran, M. I. (2011): Sugar content of popular sweetened beverages based on objective laboratory analysis: focus on fructose content. – *Obesity (Silver Spring, Md.)* **19**(4): 868–874. http://dx.doi.org/10.1038/oby.2010.255

Villanueva-Paz, M., Cotan, D., Garrido-Maraver, J., Oropesa-Avila, M., La Mata, M. de, Delgado-Pavon, A., Lavera, I. de, Alcocer-Gomez, E., Alvarez-Cordoba, M. and Sanchez-Alcazar, J. A. (2016): AMPK Regulation of Cell Growth, Apoptosis, Autophagy, and Bioenergetics. – *Experientia supplementum (2012)* **107**: 45–71. http://dx.doi.org/10.1007/978-3-319-43589-3_3

Walther, T. C. and Farese, R. V. (2012): Lipid droplets and cellular lipid metabolism. – *Annual review of biochemistry* **81**: 687–714. http://dx.doi.org/10.1146/annurev-biochem-061009-102430

Wang, B., Li, W., Fang, H. and Zhou, H. (2019): Hepatitis B virus infection is not associated with fatty liver disease: Evidence from a cohort study and functional analysis. – *Mol Med Rep* **19**(1): 320–326. http://dx.doi.org/10.3892/mmr.2018.9619

Wang, Y., Han, X., Fu, M., Wang, J., Song, Y., Liu, Y., Zhang, J., Zhou, J. and Ge, J. (2018): Qiliqiangxin attenuates hypoxia-induced injury in primary rat cardiac microvascular endothelial cells via promoting HIF-1alpha-dependent glycolysis. – *Journal of cellular and molecular medicine* **22**(5): 2791–2803. http://dx.doi.org/10.1111/jcmm.13572

Wang, Y., Viscarra, J., Kim, S.-J. and Sul, H. S. (2015): Transcriptional regulation of hepatic lipogenesis. – *Nature reviews. Molecular cell biology* **16**(11): 678–689. http://dx.doi.org/10.1038/nrm4074

Washburn, M. L., Bility, M. T., Zhang, L., Kovalev, G. I., Buntzman, A., Frelinger, J. A., Barry, W., Ploss, A., Rice, C. M. and Su, L. (2011): A Humanized Mouse Model to Study Hepatitis C Virus Infection, Immune Response, and Liver Disease. – *Gastroenterology* **140**(4): 1334–1344. http://dx.doi.org/10.1053/j.gastro.2011.01.001

Watanabe, M., Houten, S. M., Wang, L., Moschetta, A., Mangelsdorf, D. J., Heyman, R. A., Moore, D. D. and Auwerx, J. (2004): Bile acids lower triglyceride levels via a pathway

involving FXR, SHP, and SREBP-1c. – *The Journal of clinical investigation* **113**(10): 1408–1418. http://dx.doi.org/10.1172/JCI21025

Welte, M. A. (2015): Expanding roles for lipid droplets. – *Current biology : CB* **25**(11): R470-81. http://dx.doi.org/10.1016/j.cub.2015.04.004

Wetterau, J. R., Combs, K. A., Spinner, S. N. and Joiner, B. J. (1990): Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. – *The Journal of biological chemistry* **265**(17): 9800–9807.

WHO : *Global hepatitis report, 2017.* ISBN: 978-92-4-156545-5.

Wieland, S. F. (2015): The chimpanzee model for hepatitis B virus infection. – *Cold Spring Harbor perspectives in medicine* **5**(6). http://dx.doi.org/10.1101/cshperspect.a021469

Wilson, C. G., Tran, J. L., Erion, D. M., Vera, N. B., Febbraio, M. and Weiss, E. J. (2016): Hepatocyte-Specific Disruption of CD36 Attenuates Fatty Liver and Improves Insulin Sensitivity in HFD-Fed Mice. – *endo* **157**(2): 570–585. http://dx.doi.org/10.1210/en.2015-1866

Wu, Y.-L., Peng, X.-E., Zhu, Y.-B., Yan, X.-L., Chen, W.-N. and Lin, X. (2015): Hepatitis B Virus X Protein Induces Hepatic Steatosis by Enhancing the Expression of Liver Fatty Acid Binding Protein. – *Journal of virology* **90**(4): 1729–1740. http://dx.doi.org/10.1128/JVI.02604-15

Xiao, B., Sanders, M. J., Underwood, E., Heath, R., Mayer, F. V., Carmena, D., Jing, C., Walker, P. A., Eccleston, J. F., Haire, L. F., Saiu, P., Howell, S. A., Aasland, R., Martin, S. R., Carling, D. and Gamblin, S. J. (2011): Structure of mammalian AMPK and its regulation by ADP. – *Nature* **472**(7342): 230–233. http://dx.doi.org/10.1038/nature09932

Xiao, J., Liu, R. and Chen, C.-S. (2017): Tree shrew (Tupaia belangeri) as a novel laboratory disease animal model. – *Zool Res* **38**(3): 127–137. http://dx.doi.org/10.24272/j.issn.2095-8137.2017.033

Xiao, S.-Y., Lu, L. and Wang, H. L. (2008): Fibrosing cholestatic hepatitis: clinicopathologic spectrum, diagnosis and pathogenesis. – *International journal of clinical and experimental pathology* **1**(5): 396–402.

Yamaguchi, K., Yang, L., McCall, S., Huang, J., Yu, X. X., Pandey, S. K., Bhanot, S., Monia, B. P., Li, Y.-X. and Diehl, A. M. (2007): Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic

steatohepatitis. – *Hepatology (Baltimore, Md.)* **45**(6): 1366–1374. http://dx.doi.org/10.1002/hep.21655

Yamashita, A., Hayashi, Y., Matsumoto, N., Nemoto-Sasaki, Y., Oka, S., Tanikawa, T. and Sugiura, T. (2014): Glycerophosphate/Acylglycerophosphate acyltransferases. – *Biology (Basel)* **3**(4): 801–830. http://dx.doi.org/10.3390/biology3040801

Yan, H., Liu, Y., Sui, J. and Li, W. (2015): NTCP opens the door for hepatitis B virus infection. – *Antiviral Research* **121**: 24–30. http://dx.doi.org/10.1016/j.antiviral.2015.06.002

Yang, C., Ruan, P., Ou, C., Su, J., Cao, J., Luo, C., Tang, Y., Wang, Q., Qin, H., Sun, W. and Li, Y. (2015): Chronic hepatitis B virus infection and occurrence of hepatocellular carcinoma in tree shrews (Tupaia belangeri chinensis). – *Virol J* **12**: 26. http://dx.doi.org/10.1186/s12985-015-0256-x

Yang, P. L., Althage, A., Chung, J. and Chisari, F. V. (2002): Hydrodynamic injection of viral DNA: A mouse model of acute hepatitis B virus infection. – *Proceedings of the National Academy of Sciences* **99**(21): 13825. http://dx.doi.org/10.1073/pnas.202398599

Yang, Z., Kim, H., Ali, A., Zheng, Z. and Zhang, K. (2017): Interaction between stress responses and circadian metabolism in metabolic disease. – *Liver research* **1**(3): 156–162. http://dx.doi.org/10.1016/j.livres.2017.11.002

Yen, C.-L. E., Stone, S. J., Koliwad, S., Harris, C. and Farese, R. V. (2008): Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. – *Journal of lipid research* **49**(11): 2283–2301. http://dx.doi.org/10.1194/jlr.R800018-JLR200

Younossi, Z. M., Golabi, P., Avila, L., Paik, J. M., Srishord, M., Fukui, N., Qiu, Y., Burns, L., Afendy, A. and Nader, F. (2019): The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. – *Journal of hepatology* **71**(4): 793–801. http://dx.doi.org/10.1016/j.jhep.2019.06.021

Zahner, D., Glimm, H., Matono, T., Churin, Y., Herebian, D., Mayatepek, E., Köhler, K., Gattenlöhner, S., Stinn, A., Tschuschner, A., Roderfeld, M. and Roeb, E. (2017): Hepatitis B virus surface proteins accelerate cholestatic injury and tumor progression in Abcb4-knockout mice. – *Oncotarget* **8**(32): 52560–52570. http://dx.doi.org/10.18632/oncotarget.15003

Zatloukal, K., Stumptner, C., Fuchsbichler, A., Heid, H., Schnoelzer, M., Kenner, L., Kleinert, R., Prinz, M., Aguzzi, A. and Denk, H. (2002): p62 Is a common component of cytoplasmic

inclusions in protein aggregation diseases. – *The American journal of pathology* **160**(1): 255–263. http://dx.doi.org/10.1016/S0002-9440(10)64369-6

Zhang, Z., Yao, Z., Chen, Y., Qian, L., Jiang, S., Zhou, J., Shao, J., Chen, A., Zhang, F. and Zheng, S. (2018): [Copy] Lipophagy and liver disease: New perspectives to better understanding and therapy. – *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **97**: 339–348. http://dx.doi.org/10.1016/j.biopha.2017.07.168

Zimmermann, R., Strauss, J. G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A. and Zechner, R. (2004): Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. – *Science (New York, N.Y.)* **306**(5700): 1383–1386. http://dx.doi.org/10.1126/science.1100747

Zollner, G., Marschall, H.-U., Wagner, M. and Trauner, M. (2006): Role of nuclear receptors in the adaptive response to bile acids and cholestasis: pathogenetic and therapeutic considerations. – *Molecular pharmaceutics* **3**(3): 231–251. http://dx.doi.org/10.1021/mp060010s

Zollner, G. and Trauner, M. (2008): Mechanisms of cholestasis. – *Clinics in liver disease* **12**(1): 1-26, vii. http://dx.doi.org/10.1016/j.cld.2007.11.010

Zubiete-Franco, I., Garcia-Rodriguez, J. L., Martinez-Una, M., Martinez-Lopez, N., Woodhoo, A., Juan, V. G.-D., Beraza, N., Lage-Medina, S., Andrade, F., Fernandez, M. L., Aldamiz-Echevarria, L., Fernandez-Ramos, D., Falcon-Perez, J. M., Lopitz-Otsoa, F., Fernandez-Tussy, P., Barbier-Torres, L., Luka, Z., Wagner, C., Garcia-Monzon, C., Lu, S. C., Aspichueta, P., Mato, J. M., Martinez-Chantar, M. L. and Varela-Rey, M. (2016): Methionine and S-adenosylmethionine levels are critical regulators of PP2A activity modulating lipophagy during steatosis. – *Journal of hepatology* **64**(2): 409–418. http://dx.doi.org/10.1016/j.jhep.2015.08.037

DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Date: 08-05-2020 Irungbam Karuna Devi

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to Prof Dr. med Elke Roeb for being an ideal mentor. I greatly appreciate the confidence you had in my capabilities. Thanks for giving room for improving and learning the fascinating world of science and working hard on improving my presentation, and writing skills. I am certain that guidance and training you have provided me has laid the foundation for future success.

I would like to extend special thanks to my lab supervisor, PD Dr. Martin Roderfeld, for giving me such a great opportunity to work on this doctoral project and his never-ending support in all forms and inquisitive about my research work. I appreciate and thank you once again for your continuous patience while correcting my paper and thesis, in addition to your heavy load of lab duties. You have created always a warm environment for everyone that makes my stay in the lab enjoyable and stress free. I'm quite inspired from your positivity to research but also to life.

I highly acknowledged the guidance and support received from my co-supervisor Prof. Dr. Reinhard Dammann. This thesis would not come to this form without the support and guidance that I received from Dr. Yuri Churin.Your thought provoking discussion and valuable suggestion for improving my doctoral research work is commendable and acknowlwdged. Your undying boundless interest in making science was my guiding force which keeps me going even in hardest time and making me believed that science is possible even with many limitations.

I'm thankful to Prof. Dr. Getrud Morlock, Food and Science Nutrition Department, for giving space and resources necessary during my lab rotation period. Thanks to her and PD Dr. Imanuel for enriching and integrating HPTLC based lipid analysis into my PhD project and for insightful discussions were immensely beneficial in shaping up my dissertation work.

I'm also grateful to the Graduate School of life sciences (GGL), Giessen for providing many learning platforms and opportunities that help building my scientific skills. I would like to thank IVRI, ICAR, India and DAAD, Germany for providing me all support to carry out this PhD research.

I, extend my sincere appreciation to our lab technician Annette Tschusner and Heike Müller for a great technical support and helping me acquainted during my initial days in the lab. You both have been so caring and helpful. Warm thanks also go to all the AG Roeb lab members' students for making my lab days memorable one. I express special thanks to my friends Hiren Ghosh, Rahul Khatri, Dilraj Kaur, Swapnil and his wife, Sarmisth's family, Lieu and Mussarat Kamal for their constant emotional support all these years. A special thanks to my colleague Dr. Mukesh Kumar for all his constant emotional and scientific supports. Thanks to my senior colleague Dr. Vikram Aditya Upmanyu for believing in me and supporting my PhD.

Giessen would not have a great place to stay far away from home and family without you, Christiane Süβel. Your "warmly space" within your heart and home has been my greatest comfort. The emotional support and the care that I get from you was immense and the only driving force behind my achievement. It was always exciting to exchange Indo-German cultures, and thanks to my wonderful German friends Martin Schneider and family, Frank, Jürgen, Udo Vetter and Linda for having a memorable time spend together. Samantha, you have been my great stressbuster, thanks for making me feel relax even during hard time. Appreciation to my cousin brother Rajiv Irungbam for all the support here in Germany.

At last, I wanted to thank God for giving me strength to face all the challenges in life. Lastly, I owe this dissertation to my mom and dad and my dear family members for their constant support, endless and unconditional love.

List of tables

List of figures

PUBLICATIONS

1. **Karuna Irungbam**, Roderfeld M., Churin Y., **Yüce I.,** Morlock G.E., Roeb E. Cholestasis reduced hepatic lipid storage via AMPK-CREB signaling pathways in hepatitis B virus surface protein transgenic mice (**2020**). **Laboratory investigation (under review).**
2. **Karuna Irungbam**, Yuri Churin, Tomomitsu Matono, Jakob Weglage, Matthias Ocker, Dieter Glebe, Martin Hardt, Alica Koeppel, Martin Roderfeld & Elke Roeb. Cannabinoid receptor 1 knockout alleviates hepatic steatosis by downregulating perilipin 2(**2019**). **Laboratory investigation.** https://doi.org/10.1038/s41374-019-0327-5.
3. Hempel F, Roderfeld M, Savai R, Sydykov A, **Irungbam K**, Schermuly R, Voswinckel R, Köhler K, Churin Y, Kiss L, Bier J, Pons-Kühnemann J, Roeb E **(2019).** Depletion of Bone Marrow-Derived Fibrocytes Attenuates TAA-Induced Liver Fibrosis in Mice. **Cells**. Oct;8(10). doi:10.3390/cells8101210. PMID: 31591328; PMCID: PMC6829877.
4. Roderfeld, M., Padem, S., Lichtenberger, J., Quack, T., Weiskirchen, R., Longerich, T., Schramm, G., Churin, Y., **Irungbam, K**., Tschuschner, A., Windhorst, A., Grevelding, C.G. and Roeb, E **(2018).** *Schistosoma mansoni* Egg–Secreted Antigens Activate Hepatocellular Carcinoma–Associated Transcription Factors c-Jun and STAT3 in Hamster and Human Hepatocytes. **Hepatology.** 2018, .Doi:10.1002/hep.30192.