

Dissertation

Lipases and bile acids are novel key players in the pathogenesis of hepatic lipotoxicity

Submitted by

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**for the Academic Degree of
Doctor of Philosophie (Ph.D.)**

**at the Medical University of Graz
Department of Internal Medicine**

**Under Supervision of
Univ.Prof. Dr. Michael Trauner**

2012

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”

The part called “Role of ATGL in hepatic Lipotoxicity” is published in HEPATOLOGY under the title: “Absence of Adipose Triglyceride Lipase Protects From Hepatic Endoplasmic Reticulum Stress in Mice”.

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Graz, 16th April 2012

Acknowledgements

Many people have given me valuable encouragement, support and constructive criticism during the last four years. Now it is time to say thank you:

First and foremost I would like to thank all the people from my lab, particularly Univ.Prof. Dr Michael Trauner for giving me the opportunity to do my Ph.D. Thesis in his laboratory and Dr. Thierry Claudel, to whom I am very grateful. He was an excellent co-supervisor and I hope – although my student-life is over now – I still can come to him with the one or another question.

Also many thanks to Dr. Emina Halilbasic, Dr. Marion Pollheimer, Dr. Robert McMahon, Msc. Pooja Kumari, Msc. Nicole Bachhofner, Dagmar Silbert, Judith Gumhold, Bianca Maurer and Angelika Tremmel for their encouragement.

Moreover I would like to thank our co-workers Dr. Harald Koefeler and his team from the Core Facility for Mass Spectrometry, Center for Medical Research (ZMF), Medical University of Graz, Graz, Austria;
as well as Dr. Tatjana Stojakovic and Dr. Hubert Scharnagl with their team from the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria;
and Dr. Günter Haemmerle with his team from the Institute of Molecular Bioscience, Karl-Franzens University, Graz, Austria

I am also very thankful to Rudolf Zechner from the Institute of Molecular Bioscience, Karl-Franzens University, Graz, Austria; Frank Gonzalez from the Laboratory of Metabolism, National Cancer Institute, Bethesda, MD, USA and Renxue Wang and Victor Ling from the British Columbia Cancer Research Centre, British Columbia Cancer Agency, Vancouver, British Columbia, Canada V5Z 1L3 for sharing the ATGL KO mouse, the FXR KO mouse and the BSEP KO mouse, respectively.

I dedicate this work to my family, especially to my boyfriend Hans-Jürgen. From the bottom of my heart I would like to thank him for the patience and the understanding he showed me during the last years.

„Zwei Dinge sind zu unserer Arbeit nötig:

Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen.“

Albert Einstein (1879-1955)

Zusammenfassung

Das Hauptmerkmal der nicht alkoholischen Fettlebererkrankung (NAFLD) ist die Akkumulation von Lipiden in der Leber (Steatose). Die relativ gutartige Steatose kann sich zu einer Steatohepatitis (NASH), Fibrose, Cirrhose und in weiterer Folge zu Krebs entwickeln. Im Vergleich zu Fettgewebe haben andere Organe, wie etwa die Leber nur eine limitierte Aufnahmefähigkeit für Lipide. Ist diese Kapazität erschöpft führt der Überschuss an Lipiden zu Zellschädigungen die unter dem Begriff Lipotoxizität zusammengefasst werden. Metabolische Lipasen und Gallensäuren stellen wichtige Regulatoren im hepatischen Lipid-Metabolismus dar. Ziel dieser Untersuchungen war es die Rolle dieser beiden Faktoren in der Pathogenese der hepatischen Lipotoxizität und der Progression der NAFLD/NASH zu identifizieren.

Der erste Teil befasst sich mit der Rolle der ATGL in hepatischer Lipotoxizität. Vorangegangene Studien zeigten bereits auf dass, durch freie gesättigte Fettsäuren ein endoplasmatischer Reticulum (ER) Stress induziert werden kann und bei verschiedenen Mausmodellen für Steatose und in NAFLD Patienten vorhanden ist. ER Stress könnte somit ein wichtiger Faktor in der Entwicklung der NAFLD sein. Da ATGL die limitierende Lipase im Abbau von Triglyceriden ist stellten wir die Hypothese auf, dass das Fehlen von ATGL und die damit einhergehende Verminderung der vorhandenen Fettsäuren, zu einer erniedrigten Anfälligkeit für hepatischen ER Stress führt. Als experimentelles Model wurde die ATGL KO Maus verwendet. Zur Induktion von ER Stress wurden WT und ATGL KO Mäuse mit Tunicamycin (TM), einem bekanntem Auslöser von ER stress, injiziert. Wir konnten dabei feststellen dass mit TM behandelte ATGL KO Mäuse im Vergleich zu WT Mäuse keinen ER Stress entwickelten. Grund dafür ist der Unterschied in der hepatischen Fettsäurekomposition zwischen WT und ATGL KO Maus. Die WT Maus weist einen höheren Anteil an (potentiell toxischer) Palmitinsäure (PA) verglichen mit der Menge an nicht toxischer Ölsäure (OA) in der Leber auf, während diese beiden Fettsäuren zu gleichen Teilen in der Leber der ATGL KO Maus vorliegen. Unter ER Stress Bedingungen ist die mRNA Expression von

Stearoyl-Coenzym A Desaturase (Scd1) – dem Enzym welches für die Synthese von OA aus PA verantwortlich ist - merklich verringert. Somit ist die WT Maus unter Stressbedingungen nicht in der Lage, die toxische PA in nicht-toxische OA umzubauen. Weiters wurde durch vorangegangene Studien gezeigt, dass der toxische Effekt von PA durch das Vorhandensein von (mindestens der gleichen Menge an) OA aufgehoben werden kann. Die Tatsache dass die WT Maus basal mehr PA als OA in der Leber aufweist und die Expression von Scd1 unter ER Stress Bedingungen erniedrigt ist, führt dazu, dass die WT Maus, im Gegensatz zur ATGL KO Maus nicht vor hepatischem ER stress geschützt ist.

Der zweite Teil befasst sich mit der Rolle des nukleären Gallensäurerezeptors FXR in hepatischer Lipotoxizität. Vorangegangene Studien haben bereits gezeigt, daß der nukleäre Transkriptionsfaktor FXR in den hepatischen Lipidmetabolismus involviert ist. Um die Hypothese zu testen, dass FXR eine Schlüsselrolle in der Pathogenese der NASH spielt, wurden FXR KO Tiere über 5 Wochen mit Methionin – Cholin – defizienter Diet gefüttert. Interessanterweise konnten wir zeigen, dass WT Mäuse unter MCD Fütterung mehr Fett in der Leber akkumulieren als FXR KO Mäuse. Weiters konnten wir zeigen, dass unter Methionin – Cholin – defizienten Bedingungen C/ebp α , ein Masterregulator im hepatischen Lipidmetabolismus, über einen Chop abhängigen Signalweg durch FXR negativ reguliert wird. Die verminderte mRNA expression von C/ebp α führt zu verringerter Sekretion von very low density lipoprotein (VLDL), was wiederum die erhöhte Lipidakkumulierung in der Leber erklärt.

Im dritten Teil wird die Rolle von bile salt export pump (BSEP) in der Entwicklung von Lipotoxizität aufgezeigt. BSEP ist der Haupttransporter von Gallensäuren aus dem Hepatozyten ins Blut und beeinflusst damit die Signalmolekülfunktion von Gallensäuren in Relation zum Grad der Expression von BSEP. Deshalb stellten wir die Hypothese auf, dass der Verlust von BSEP vor NAFLD und der Weiterentwicklung zu NASH schützen könnte. Wir konnten zeigen, dass aufgrund von Änderungen in der Expression von Gallensäuretransportern und den daraus resultierenden Veränderungen im hepatischen Gallensäuren "Flux" BSEP KO Mäuse unter MCD Diät weniger Fett

akkumulieren als die WT Tiere. Weiters war die BSEP KO MCD Maus möglicherweise über die Vermittlung von anti-inflammatorischen Signaleffekten durch Gallensäuren in nicht-toxischer Konzentration vor Inflammation geschützt.

Zusammenfassend lässt sich sagen, dass die metabolischen Schlüsselfaktoren ATGL, FXR und BSEP potentielle pharmakologische Angriffspunkte für die Behandlung der Fettlebererkrankung bieten.

Summary

The pathogenic hallmark of non-alcoholic fatty liver disease (NAFLD) is the accumulation of lipids in the liver, also known as steatosis. Relatively 'benign' steatosis can progress to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and liver cancer. In contrast to adipose tissue, non-adipose cells such as hepatocytes have a limited capacity to store lipids in excess. When this capacity is exceeded, the resultant cellular dysfunction is called lipotoxicity. Lipases and bile acids are known to be important metabolic regulators of the hepatic lipid metabolism. Therefore, this study was designed to identify the role of lipases, and bile acids in the pathogenesis of hepatic lipotoxicity and progression to NASH.

Part 1 aimed to identify the role of ATGL in hepatic lipotoxicity. Several studies have demonstrated that endoplasmic reticulum (ER) stress is induced via free saturated fatty acids and is observed in different animal models for steatosis and in NAFLD patients. Therefore, ER stress may appear as an important event in lipotoxicity. Since adipose triglyceride lipase (ATGL) is the main lipase in triglyceride (TG) breakdown, we hypothesized that lack of ATGL and subsequent reduction of free fatty acid availability may attenuate hepatic ER stress. As experimental model, WT and total body ATGL KO mouse were injected with Tunicamycin (TM) (a known ER stress inducer) for 48h hours. Indeed, ATGL KO TM injected mice were protected from hepatic ER stress.

This protection was based on the differences in hepatic fatty acid composition between WT and ATGL KO mice. At baseline, WT mice consisted of more (potential toxic) Palmitic acid (PA) than non-toxic Oleic acid (OA), whereas ATGL KO mouse displayed the same levels of PA and OA. Moreover, mRNA expression of stearoyl-coenzyme A desaturase (Scd1) – the enzyme responsible for PA modification into OA – was markedly decreased under TM challenging. Previous studies showed that in a ratio of at least 1:1, OA is able to protect from PA induced toxicity. Due to the basal higher amounts of hepatic PA compared to OA and the decrease in Scd1 mRNA expression, WT TM challenged mice

were not protected from hepatic ER stress induction, in contrast to ATGL KO TM mouse.

Part 2 describes the role of the nuclear receptor FXR in hepatic lipotoxicity. It has been shown previously that FXR is involved in hepatic lipid metabolism. To test the hypothesis that FXR play a key role in progression to NASH, WT and FXR KO mice were challenged with a methionine choline deficient (MCD) diet – as a model for NASH – for 5 weeks. Interestingly, we found a more pronounced lipid accumulation in WT mice upon MCD compared to challenged FXR KO mice. We could establish that under MCD challenge – which leads to low serum glucose and high bile acid levels – FXR negatively regulates C/ebp α - a key player in hepatic lipid metabolism – in a Chop dependent manner. The decrease in c/ebp α mRNA expression led to impaired very low density lipoprotein (VLDL) secretion in WT MCD challenged mice, therefore explaining the increased hepatic lipid accumulation.

Part 3 focuses on the role of bile salt export pump (BSEP) in the development of hepatic lipotoxicity. Bile acids are the end product of cholesterol catabolism and thus bile secretion represents the ultimate step in reverse cholesterol transport. BSEP is the main canalicular bile acid transporter, excreting bile acids into the bile. Hence we hypothesized that lack of BSEP could protect from NAFLD and progression to NASH.

We were able to show that altered expression of hepatic bile acid transporters together with modifications in bile acid composition, protected MCD fed BSEP KO mice from hepatic inflammation and lipotoxicity.

In summary, the metabolic keyplayers ATGL, FXR and BSEP are potential pharmacological targets in the defense against the progression of NAFLD and lipotoxicity.

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1. INTRODUCTION

Triglycerides (TGs) provide a higher caloric density (9kcal/g) than carbohydrates (4,5kcal/g) or proteins (4kcal/g) and being water insoluble do not generate intracellular osmotic or colloidal side effects. Under normal conditions, in mammals fatty acids (FA) are stored in adipocytes and released in the periphery after TG lipolysis. However, FA can also be stored in non-adipose tissue such as the liver [1].

In non-adipose tissue, increase in levels of non-esterified fatty acids disrupt the intracellular homeostasis and entails the formation of deleterious lipid derivatives such as ceramides. This leads to ER stress induction, mitochondrial dysfunction, inflammation, impairment of cell membranes and potentially to cell death. This succession of events is called lipotoxicity [2, 3].

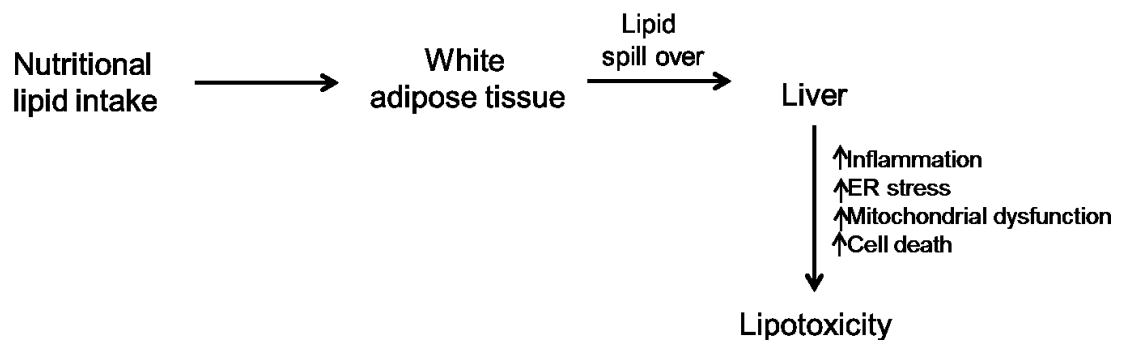


Fig.1 Development of Lipotoxicity.

Until a certain concentration of free fatty acids (FFA), cells are able to counteract lipotoxicity by storing FFA as TGs. The balance between TG formation and hydrolysis provides a physiological FA flux without toxic consequences [3]. Therefore deregulation of lipid turnover can cause metabolic diseases, such as fatty liver.

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of lipids in the liver in the absence of other factors such as alcohol consumption, hepatitis virus infection, or auto immune disease [4, 5]. NAFLD is typically diagnosed by ultra sound, computed tomography, magnetic resonance imaging and elevated serum levels of liver enzymes such as alanine-amino-transferase

(ALT), aspartate-amino-transferase (AST) and gamma glutamyl-transferase (γ GT). Epidemiological studies identified NAFLD as the most common liver disease in the world [6] affecting about 30% of the population [6-9]. Non-alcoholic fatty liver disease (NAFLD) varies from simple steatosis to steatohepatitis, fibrosis, cirrhosis and cancer [5] (Fig.2). Whereas the clinical course of steatosis is relatively benign, steatohepatitis (NASH) patients are at higher risk to develop cirrhosis and/or hepatocellular carcinoma (HCC) [10, 11]. Since up to 30% of patients with NAFLD have NASH [12], NASH development represent the most likely reason for the increased mortality in NAFLD patients [13-15].

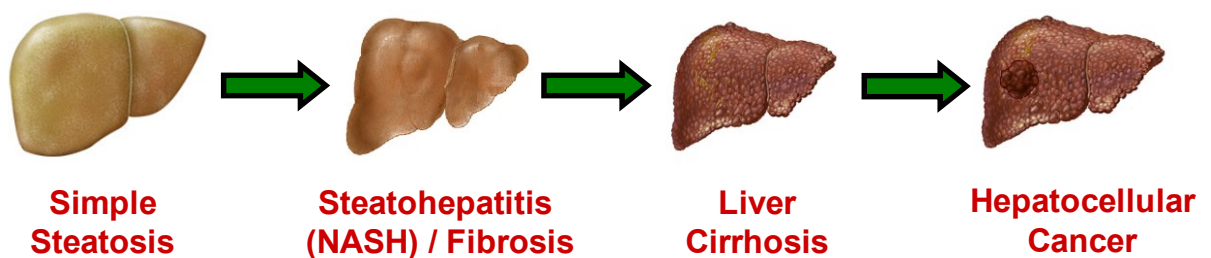


Fig.2: NAFLD progression. Benign steatosis can progress to severe steatohepatitis, fibrosis, cirrhosis and cancer. (Figure modified after Trauner et al. [16])

1.1 Epidemiologic Features

Classically in Western societies, NAFLD is associated with metabolic syndrome (MS) [17]. MS patients display three out of five factors among a category which includes obesity, hypertension, dyslipidaemia, inflammation or insulin resistance. NAFLD, which is characterized by hepatic lipid accumulation and a pro-inflammatory state, can be considered as the liver manifestation of the metabolic syndrome [18].

Indeed, obesity, type 2 diabetes mellitus and hyperlipidaemia are comorbid conditions associated with NAFLD [19]. Obesity correlates with the presence of NAFLD and the prevalence of NAFLD increases by 4.6 times in obese people [20]. In addition, type 2 diabetes increases NAFLD prevalence independently of obesity. [21, 22]. Since patients at high cardiovascular risk usually suffer from MS, it is not surprising that NAFLD correlate with cardiovascular risk factors [23-27].

NAFLD arises in almost all ethnic groups and affect people at any age [28] but – as shown by the Dallas Heart study – at different rates [9]. Interestingly, the higher prevalence of hepatic steatosis in Hispanics (45%) is probably due to higher rates of insulin resistance and obesity in this population. However, the low prevalence to hepatic steatosis found in African Americans (24%) could not be explained by differences in insulin resistance, medication use, or ethnic differences in BMI.

Interestingly, before the age of 60 years, men are more prone to fatty liver development than women, whereas, after 60, women have a higher fatty liver prevalence. The reasons for these gender specific discrepancies are currently unknown [9].

1.2 Liver Histology in NAFLD

The histologic features of non-alcoholic fatty liver diseases are steatosis, inflammatory cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory's hyaline and fibrosis [20]. About 67% of NAFLD patients display fibrosis at the time of diagnosis [29, 30]. Whereas fibrosis can progress to cirrhosis, cirrhosis is found in about 14% of NAFLD patients [29, 30]. Interestingly, livers of cirrhotic patients may no longer display steatosis and/or inflammation [20]. Currently, there is no general agreement about the minimum amount of histological features which have to be present in order to diagnose NASH, even though the presence of steatosis, inflammatory cell infiltration, hepatocyte ballooning and necrosis are required.

1.3 Molecular mechanisms favouring NAFLD development

According to M.G. Sanal [17] there are 4 factors contributing to fatty liver development:

- Increased lipid intake
- Increased de novo lipogenesis
- Decreased lipid outflow
- Decreased oxidation

1.3.1 Increased lipid intake

White adipose tissue acts as an energy accumulator, where energy can be stored as TGs the most concentrated and non-cellular toxic form of fatty acids. After food consumption, the blood carries lipophilic nutrients in chylomicrons and as free fatty acids. Adipose tissue and muscle take up lipids from chylomicrons after LPL hydrolysis and/or fatty acid import. During chronic over nutrition state, adipocytes are no longer able to import serum lipids and glucose [17]. Therefore, TGs are available for storage by non-adipose tissues such as the liver which therefore induces fatty liver development [17]. About 60% of triglycerides stored in liver under NAFLD conditions are derived from circulating non-esterified fatty acids (NEFA) [31]. Insulin resistance [32] and defects in fatty acid transport in adipocytes can increase serum FFA levels and thus favour extra-adipose tissue FA storage [17].

The mass of adipose tissue fat correlates with hepatic fat mass, hence intrahepatic lipids increase by 22% for every 1% increase in total fat mass, by 21% for every 1% increase in subcutaneous adipose tissue and by 104% for every 1% increase in intra-abdominal adipose tissue [33]. Therefore, the liver can be considered as the second lipid storage organ when the adipocyte capacity is overloaded. As soon as the buffering capacity of liver is exceeded, lipids enter the cardiovascular system and other organs and tissue such as skeletal muscle (Fig.3).

Macrophages try to limit the lipid overload by phagocytosis and oxidize them to free toxic radicals and immunogenic compounds. Macrophages storing lipids become “foam cells” [34], which can lead to “fibrous cap” development in vessels, a process called atherosclerosis, which is the homologue to cirrhosis in the liver. Atherosclerosis increase the cardiovascular related mortality [23-25].

Lipoproteins are lipid carriers in the circulatory system. Lipid transfer across the hepatocyte membrane is mediated by several transporters such as fatty acid transport proteins (FATP), fatty acid translocase (FAT/CD36), and Caveolin-1 [17], while fatty acid binding protein (FABP) act as intracellular shuttle systems. The FATP family consists of 5 (known) isotypes. FATP1 is expressed in adipose tissue and heart, while FATP2 and FATP5 are expressed in the liver and FATP4 in the intestine [35].

Overexpression of FATP5 *in vitro* leads to increase FFA uptake, whereas FATP5 knock-down decreased hepatic lipid accumulation and conjugated bile acids production [36-38], indicating that FATP5 is a key player in hepatic fatty acid uptake and bile acid synthesis.

Fatty acids taken up by hepatocytes are either oxidized or esterified for storage or secretion as triglycerides. The preferred substrate for esterification and subsequent TG formation at the ER are monounsaturated fatty acids like oleic acid [39].

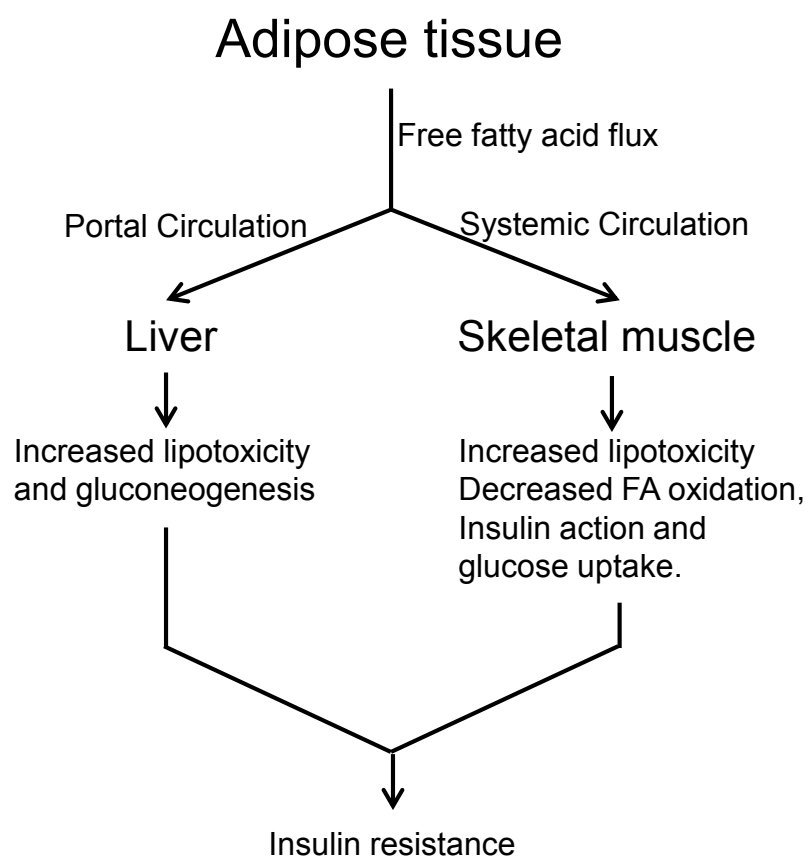


Fig.3: Adipose tissue functions as lipid storage. As soon as the lipid storage capacity of adipocytes is exceeded, other organs such as liver have to buffer the excess of lipids. Lipid overload of the liver causes infiltration in various other organs like skeletal muscle. The consequences of excess lipid infiltration in non-adipose tissue are designated as lipotoxicity which can lead to insulin resistance

1.3.2 Increased de novo lipogenesis

In NAFLD about 25% of the accumulated lipid arises from *de novo* synthesis [31]. The two major enzymes involved in hepatic *de novo* lipogenesis are acetyl CoA carboxylase (ACC) and fatty acid synthase (FasN) [40]. In the liver, and particularly in insulin sensitive tissues, the sterol response element binding protein 1c (SREBP1c) is the master regulator of fatty acid synthesis and also transduces insulin signal, necessary for *de novo* lipogenesis [41, 42].

Under physiological conditions, SREBP1c is transiently induced in the liver by insulin through IRS-2; subsequently causing a switch from glycogen to lipid synthesis. In addition, SREBP1c represses IRS-2 transcription in a negative feedback loop. Under certain pathogenic conditions expression of SREBP1c in the liver remains elevated which in turn increases lipid synthesis resulting in lipid accumulation [43].

Obesity and insulin resistance are pro-inflammatory conditions leading to expression of cytokines such as TNF α and IL6 [34, 44]. Hepatic inflammation causes insulin resistance in addition to activation of SREBP1c and thus fatty acid synthesis [17].

The liver plays not only a role in lipid but also in glucose metabolism. External glucose supply suppresses hepatic glucose synthesis and glucose in excess is converted into glycogen or fatty acids for storage in adipocytes. These mechanism are regulated via insulin [17].

Moreover, Watanabe et al. [45] discovered an important role of bile acids on hepatic *de novo* lipogenesis. They showed that SREBP1c and subsequent FA synthesis is down-regulated via the bile acid activated nuclear receptor farnesoid X receptor (FXR) in a short heteromer protein (SHP) dependent manner.

1.3.3 Decreased lipid outflow

Increased hepatic lipid uptake is followed by increased hepatic lipid secretion in the form of very low density lipoproteins (VLDL). The main apolipoprotein involved in VLDL synthesis is ApoB100, even though other apolipoproteins such as ApoE, ApoC1, CII, CIII are also incorporated into VLDL [46]. SREBPs are the

main regulators of mammalian lipid homeostasis. In the liver, 3 SREBPs regulate lipid production to produce lipoproteins in the plasma or bile [17]. The nuclear receptor FXR is a central regulator of hepatic lipid and bile acid metabolism. FXR represses bile acid synthesis and therefore protects the liver from bile acid overload. FXR transcriptionally regulates several apolipoprotein genes, [47, 48]. VLDL excretion is transiently suppressed via bile acids, after lowering of microsomal triglyceride transport protein (MTTP) activity, a key gene involved in VLDL formation [49]. Hepatic fatty acid levels are sensed by PPAR α and genes involved in lipid secretion such as FABP, MTTP and ApoB are up regulated [50-52]. Thus selective hepatic nuclear transcription factors may be new potential treatments of NAFLD [17].

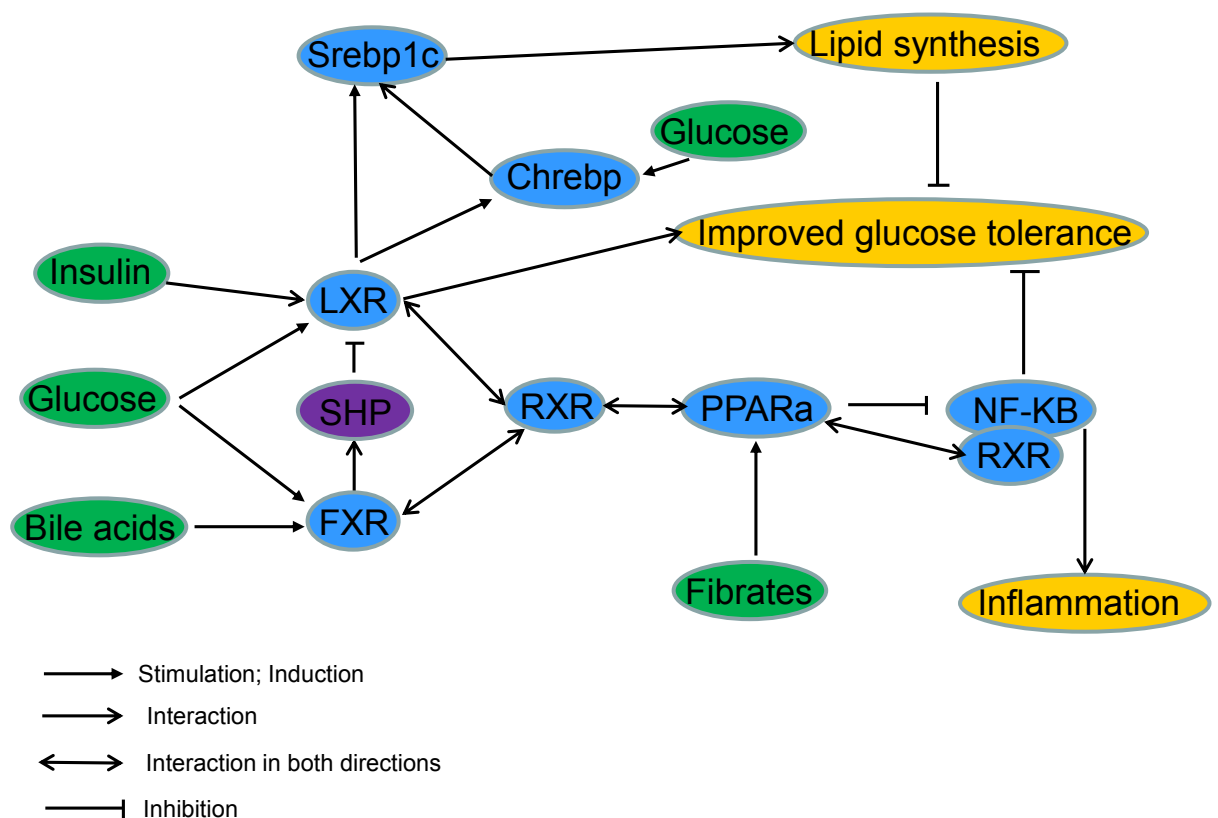


Fig.4: Interaction between nuclear receptors involved in carbohydrate-lipid-bile acid homeostasis. This homeostasis determines the hepatic fat accumulation.

1.3.4 Decreased oxidation

Fatty acid oxidation involves three different organelles, where beta oxidation takes place in mitochondria and peroxisomes, while omega oxidation occurs in the ER [17]. Fatty acids with a carbohydrate chain up to 20 C atoms are oxidized in mitochondria, whereas longer fatty acids and C27 bile acid intermediates are oxidized in peroxisomes [53]. At the ER, the omega oxidation system hydroxylates saturated and unsaturated fatty acids, which then may be oxidized in mitochondria or peroxisomes [53]. Some genes involved in fatty acid oxidation are regulated by PPAR α . PPAR α is the master regulator of mitochondrial beta oxidation and act as heterodimer with retinoid x receptor (RXR) [17].

MCD fed mice (a model for fatty liver development) treated with a PPAR α agonist displayed markedly less steatohepatitis and lower serum ALT levels, indicating that PPAR α activity increased fatty acid oxidation and protects from hepatic fat accumulation [54]. During mitochondrial oxidation, fatty acids are shortened. Subsequently, the Acetyl CoA generated is either converted into ketone bodies and used as energy substrate for extra hepatic tissues (especially during starvation) or enters the three carboxylic fatty acid cycle. Carnitine palmitoyl transferase 1 alpha (CPT1 α) regulates mitochondrial fatty acid oxidation. Carnitine increases the activity of CPT1 α , whereas malonyl CoA is an inhibitor [55]. Inhibition of fatty acid oxidation is an intrahepatic cause of fatty liver development [17]. Mutations in the genes involved in fatty acid oxidation induces different metabolic disorders: malfunction of long-chain-3-hydroxyacyl-CoA dehydrogenase (LCHAD) triggers acute fatty liver in pregnancy (AFLP), while a defect in middle chain acyl CoA dehydrogenase (MCAD) predisposes to sudden infant death syndrome (SIDS) [56]. Little is known about the impact of mitochondrial fatty acid oxidation during NAFLD development, but mice lacking the mitochondrial trifunctional protein (MTP) – which catalyzes long chain fatty acid oxidation – suffer from neonatal death. Heterozygotes develop NAFLD and insulin resistance during aging, indicating a major role for mitochondrial fatty acid oxidation in the pathogenesis of NAFLD [57]. Interestingly, a diet containing 2-5% of energy in form of polyunsaturated fatty acids (PUFA) (n-3 and n-6) suppresses the expression of genes involved

in the glycolytic pathway and *de novo* lipogenesis. Long chain n-3 PUFAs up regulate PPAR α and increase transcription of genes responsible for fatty acid oxidation like CPT1 α and AOX [58, 59]. These findings suggest that PUFAs could be used as a treatment against fatty liver development.

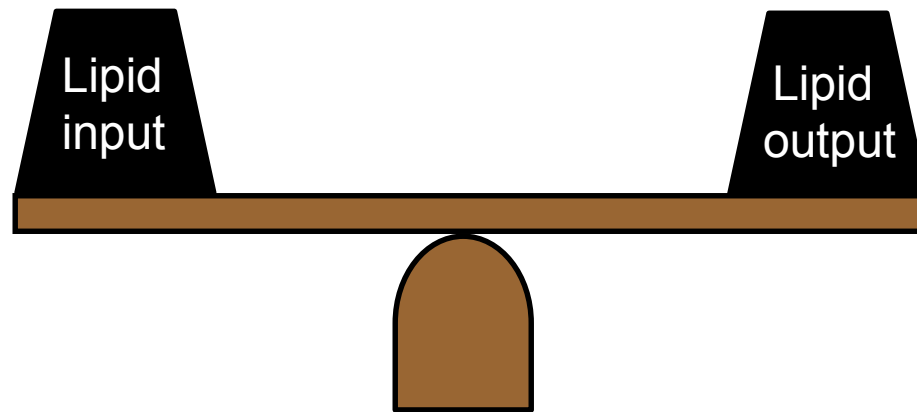


Fig.5: Homeostasis between lipid intake and outflow.

Lipids accumulate in the liver when there is an imbalance between input and output.

1.4 Pathogenesis of NAFLD

The infiltration and accumulation of lipids in the liver is the characteristic for NAFLD, but the mechanism behind is poorly understood [60]. TGs are formed by linking three free fatty acids (FA) to a glycerol backbone. The free fatty acids used to form TG can derive from three different sources: diet; *de novo* lipogenesis or adipose tissue after lipolysis.

Dietary fatty acids are absorbed via the intestine and packed into chylomicrons. They are then hydrolyzed by lipoprotein lipase (LPL) and taken up by muscles and adipose tissue, while about 20% of the remaining FAs enter the liver.

Carbohydrate rich diet promotes *de novo* synthesis of fatty acids, by increasing insulin levels. Insulin activates SREBP1c – the master-regulator of *de novo* lipogenesis – which in turn activates Fasn [41].

Hyperinsulinemia results in insulin resistance [61] (IR) [62-64] which together with *de novo* lipogenesis [64, 65] are the two major factors leading to triglyceride (TG) accumulation in the liver. Other pathways, such as FA oxidation, VLDL synthesis and secretion are also involved in fatty liver development, but to a lesser degree [63]. Furthermore, it is also very likely that

adipokines such as adiponectin, $Tnf\alpha$, leptin, resistin, visfatin and apelin play an important role in fatty liver development [66, 67].

Evidences are accumulating that pro inflammatory cytokines like tumor necrosis factor alpha ($Tnf\alpha$) and interleukin 6 (IL6) are involved in the development of NASH [68]. NASH patients display increased serum levels of these inflammatory markers, whereas their inhibition in rodents led to attenuation of NAFLD [69]. In addition, several studies showed that free fatty acids are also important factors in the progression from steatosis to steatohepatitis. NASH patients display elevated levels of serum free fatty acids which positively correlate with the disease severity [70, 71]. Together with the fact that suppression of free fatty acids improved liver enzyme levels in healthy humans, these data suggest that free fatty acids and the subsequent products of free fatty acid metabolism rather than triglycerides are toxic [72, 73].

A major health concern is the progression from steatosis to NASH. According to the so-called “two hit hypothesis”, a “first hit” induces lipid accumulation in the liver, when a “second hit” is required to progress from steatosis to NASH. However, emerging evidences suggest that some patients directly developed NASH [74]. In line with this observation, other data established that hepatic steatosis may even protect against the development of advanced liver disease. This has led to the concept of “good and bad fat storers”. A good fat storer is able to store fat in form of TG in hepatocytes, whereas the bad fat storer cannot and therefore develop NASH as a consequence of hepatic lipotoxicity.

Non adipocyte cells – like hepatocytes – have a limited capacity to store lipids. When this capacity is exceeded, due to increased flux of free FA from the periphery to the liver, results in cellular dysfunction called lipotoxicity [75] and is probably driven by accumulating FFA but not TG [76-79], therefore identifying intracellular hydrolysis of TG as a new potential mechanism leading to lipotoxicity [80]. Adipose tissue triglyceride lipase (ATGL) was discovered as the main lipase involved in intracellular lipolysis [81]. Although the liver contains several other lipases such as triglyceride lipase 1 and 2 (and also low levels of hormone sensitive lipase) [81, 82], ATGL seems to exert a critical function in hepatic FA metabolism, since ATGL-deficient mice develop hepatic steatosis

[83], indicating that ATGL could prevent lipotoxicity. Since saturated free fatty acids induce endoplasmic reticulum (ER) stress [84, 85], ER stress should be considered as an important condition in lipotoxicity and during NAFLD development. However, the precise role of ATGL during hepatic ER stress is still unknown.

1.5 Genetic risk factors for hepatic steatosis

One genetic variant was recently implicated in the pathogenesis of NAFLD: the missense mutation (Ile¹⁴⁸ → Met¹⁴⁸ (I148M)) in the patatin-like phospholipase domain-containing (PNPLA) 3 gene (*PNPLA3*), also known as adiponutrin [86]. Homozygotes for this allele display a 2 fold increase in hepatic TG content, with the effect being strongly related to adiposity and insulin sensitivity [1]. Like ATGL, its closest homologue, PNPLA 3 is a member of the PNPLA family and is highly expressed in adipose tissue and liver. Insulin transcriptionally regulates PNPLA 3 expression *via* a pathway including LXR and SREBP1c. Under fasting conditions PNPLA 3 mRNA expression was drastically reduced and increased by about 80% after refeeding [87]. However, additional studies are required to understand the molecular impact of PNPLA 3 during fatty liver development.

1.6 ER stress and Unfolded Protein Response

Evidence is emerging that activation of ER stress contributes to the saturated FA induced lipotoxicity [88]. Several pathways could be activated via ER stress which could lead to intracellular inflammation and/or cell death and liver damage. ER is accountable for the correct folding of proteins and for the degradation of incorrectly folded proteins [89]. When the capacity of the ER is exceeded, the resultant cellular dysfunction is called ER stress. Since unfolded or incorrectly folded proteins can impair cellular functions, the ER developed a rescue pathway called unfolded protein response (UPR) [90].

In response to ER stress, three main pathways are activated, which in turn mediate UPR [91] (Fig.5). Pancreatic ER eukaryotic translational initiation factor (eIF)-2 α kinase (PERK) and inositol-requiring enzyme (IRE)-1 are trans-membrane kinases leading to phosphorylation of eIF2 α , which inhibits the

translation and production of the X-box binding protein (XBP)-1 transcription factor by a splicing mechanism. Concomitantly, the activating transcription factor (ATF)-6, a trans-membrane transcription factor released by stress, regulates intra membrane proteolysis. Each pathway activates the transcriptional regulation of genes and contributes to the preservation of cellular integrity during ER stress [92]. Moreover, XBP1 [93] and the translational regulator eIF2 α [94], have also been proposed to directly regulate lipid metabolic pathways. ATF6 α up regulates chaperones such as BiP/Grp78 and the ER associated protein degradation (ERAD) machinery, therefore protecting ER function [95-97]. The deletion of ATF6 α leads to persistent ER stress and fatty liver indicating a link between ER stress and hepatic lipid metabolism [96, 98].

Monounsaturated FAs are the preferential substrate to form triglycerides and are better tolerated by cells compared to saturated FA. Furthermore, monounsaturated FAs are able to rescue saturated FA-induced lipotoxicity [99]. Both saturated and monounsaturated FAs might derive from TG hydrolysis through lipases. Accordingly, the TG hydrolytic capacity and probably the selective release of certain FA species from the TG pool may affect the induction or protection of ER stress. Since ATGL performs the initial step in TG catabolism and ATGL deficiency provokes TG accumulation in many organs including the liver [83, 100] it is relevant to investigate the role of ATGL during hepatic ER stress response.

It was previously shown that unsaturated fatty acids are not harmful to liver cells and its function [84, 101, 102]. Wang et al. fed mice with a high saturated fat and with a high unsaturated fat diet. While high saturated fat led to liver damage, high unsaturated fat diet did not, although both diets increased hepatic TG accumulation at the same levels, establishing the hepatotoxic effects of saturated fatty acids [103]. Evidence is emerging that the low affinity of saturated FAs to build TG causes the noxious effect to the liver. Listenberger et al. [99] showed that in CHO cells the unsaturated FA oleic acid (OA) is able to counteract the toxic effect of saturated FA palmitic acid (PA), perhaps via channelling PA into non-toxic TGs.

It seems that incorporation of saturated fatty acids into TG is a crucial point to disrupt the progression from innocuous steatosis to harmful steatohepatitis.

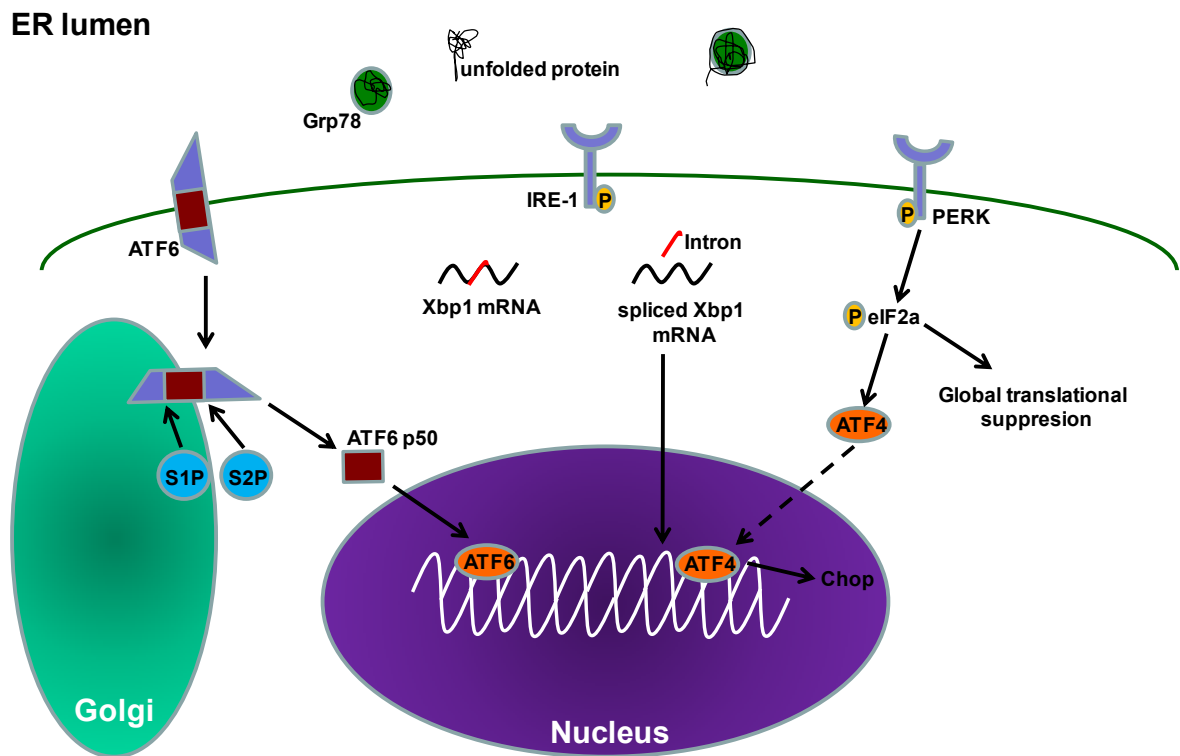


Fig.6: Unfolded Protein response. Three trans-membrane ER- sensors IRE1, PERK and ATF6 regulate the UPR through their respective signaling cascades. Under non-stressed conditions, Grp78 binds to the luminal domains of IRE1, ATF6, and PERK. Upon accumulation of unfolded proteins in the ER lumen, Grp78 is released from IRE1 which dimerizes to activate its kinase and RNase activities to initiate XBP1 mRNA splicing thereby creating a potent transcriptional activator. Primary targets that require IRE1/XBP1 pathway for induction include genes encoding functions in ERAD. Similarly, Grp78 is released from ATF6. ATF6 transits to the Golgi compartment where it is cleaved by S1P and S2P proteases generating a cytosolic fragment that migrates to the nucleus to further activate transcription of UPR-responsive genes. Finally, PERK - released from BiP- dimerizes, autophosphorylates, and phosphorylates eIF2α, leading to general attenuation of mRNA translational initiation. Paradoxically, eIF2α phosphorylation induces translation of ATF4 mRNA. The PERK/eIF2α/ATF4 regulatory axis also induces expression of anti-oxidative stress response genes and expression of genes encoding proteins with pro-apoptotic functions, such as CHOP.

1.7 Triglyceride hydrolysis

Regulation of TG lipolysis is under hormonal control. Under fasting conditions, catecholamines and glucocorticoids activate lipolysis, whereas during feeding insulin inhibits triglyceride breakdown [104-106]. Catecholamines activate the lipolytic pathway *via* the beta adrenergic receptors [107].

The lipolysis pathway consists of three lipases (Fig.7). The first step – breakdown from triglycerides to diacylglycerides – is catalyzed by ATGL/Desnutrin/PNPLA2. Diacylglycerols are converted into monoacylglycerols via HSL. The last step, hydrolysis of monoacylglycerols is done by Monoacylglycerol lipase (MGL).

Whereas MGL is not regulated by any cofactor [82], several regulatory cofactors of HSL and ATGL are known.

1.7.1 Regulation of triglyceride hydrolysis

Catecholamines levels increases during the state of low energy and activate adenylate cyclase which causes increased intracellular cAMP concentration and subsequent activation of cAMP dependent PKA. Activated PKA phosphorylates HSL which increases its hydrolytic activity and translocate HSL from the cytosol to the lipid droplet [82].

Perilipin A is also phosphorylated by PKA, and phosphorylated Perilipin A is proposed to induce HSL hydrolase activity. In addition, a recent study showed that phosphorylation of Perilipin A enhances the interaction between HSL and Perilipin A, but does not impact on HSL translocation [108].

Perilipin-adipophilin-TIP74 (PAT) proteins belong to the lipid droplet associated proteins [109, 110]. However, PAT proteins are not only associated to the lipid droplet but also carry fatty acids from the plasma membrane to the lipid droplet [111]. It seems that lipid droplets recruit their associated proteins by interacting with the plasma membrane [111]. Cohen et al [109] showed that a complex of Perilipin A, Caveolin-1 and the catalytic subunit of PKA is activated via beta adrenergic receptor agonist treatment. In this context the comparative gene identification 58 (CGI-58) interacts with Perilipin and localizes to lipid droplets [112]. Lass et al [113] discovered that CGI-58 regulates ATGL but not HSL.

Futurstudies regarding CGI-58 and its regulation of lipolysis may also provide new insights into the function of other lipid droplet associated proteins.

Regulation of ATGL seems to be quite different from HSL. It has been theorized that ATGL like HSL may be a phosphoprotein but its phosphorylation is not catalyzed via PKA. Furthermore, ATGL does not require translocation from the cytosol to the lipid droplet to get activated, since ATGL is constitutively localized at the lipid droplet in both, unactive and active form [100]. Therefore, additional studies will have to establish whether the phosphorylation of ATGL is required for its activation and localisation. Little is also known about the interactions between ATGL and perilipin.

In addition, ATGL expression is positively regulated by glucocorticoids which are elevated during fasting and inhibited by high insulin levels occurring during feeding conditions [106, 114].

Fatty acids obtained after lipolysis in white adipose tissue are delivered to the periphery for further metabolism. In case of fatty acid in excess, peripheral tissues like liver and skeletal muscles are able to form triglycerides and store.

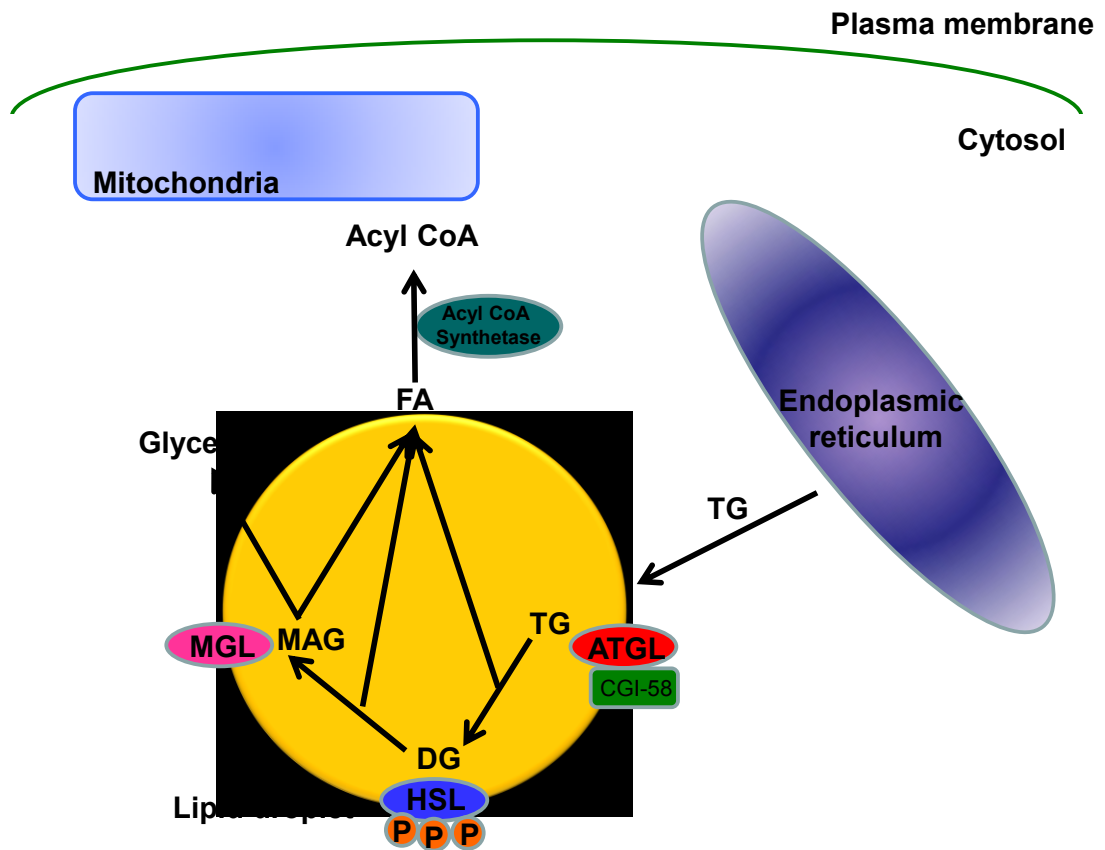


Fig.7: Triglyceride hydrolysis. Adipose triglyceride lipase (ATGL) cleaves the first fatty acid from the triglyceride to form a diacylglycerol (DAG). DAG is hydrolysed to monoacylglycerol (MAG) by hormone sensitive lipase (HSL). MAG is hydrolysed by Monoacylglycerol lipase (MGL) to form glycerol and an additional free fatty acid.

1.8 Triglyceride formation

Triglyceride synthesis is a four-step pathway involving two key enzymes (Fig.8) and takes place at the cytosolic surface of mitochondria and the endoplasmic reticulum (ER). Glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) are catalyzing the first and the last step of TG formation. GPAT catalyzes the acylation of glycerol-3-phosphate to lysophosphatidic acid. There are four isoforms of GPAT. GPAT1 and 2 are localized at the mitochondria, whereas GPAT3 and 4 reside at the ER. Lysophosphatidic acid is converted into phosphatidic acid. At the ER lipin create diacylglycerol from phosphatidic acid. DGAT1 and DGAT2 acylate diacylglycerols to form triglycerols [115].

1.8.1 Regulation of triglyceride formation

Since the main regulation points of TG synthesis take place at the ER, the mitochondrial location of GPAT1 and 2 is unexpected; however lysophosphatidic acid (LPA) the product of GPAT1&2 is used for TG synthesis at the ER. *In vitro*, liver fatty acid binding protein (L-FABP) mediates the transport of LPA to the ER, where it is converted into phosphatidic acid (PA) [116].

Fatty acids have to be converted into acyl-CoA FA to enter a metabolic pathway. After activation, FA can be oxidized *via* carnitine palmitoyltransferase 1 or further used for elongation and TG synthesis *via* the glycerolipid synthesis pathway. Fatty acid activation as well as fatty acid oxidation and glycerolipid synthesis takes place in the mitochondria. Therefore, it is generally assumed that CPT1 α and GPAT1 compete for the same pool of acyl CoA substrate [117]. This hypothesis was confirmed by several studies, which showed that overexpression of GPAT1 in hepatocytes diminishes fatty acid oxidation, while conversely fatty acids were directed into beta oxidation in GPAT1 KO mice [118-120].

The distribution of acyl CoA substrate between oxidation and TG synthesis is regulated by AMP-activated protein kinase (AMPK) [116]. AMPK is activated when energy is low and promotes ATP generation. Therefore, active AMPK inhibits acetyl CoA-carboxylase (ACC) and decreases malonyl-CoA

concentration, which is a potent inhibitor of CPT1 α . GPAT1 activity is diminished by AMPK [116, 117].

The enzymes involved in TG synthesis are regulated via insulin and glucagon. Insulin elevates the activity of lipogenic genes including GPAT1, whereas fasting or diabetic states reduce its activity [116]. The insulin derived activation of GPAT1 is mediated by the induction of SREBP1c at the transcriptional level, indicated by the 6.7 fold GPAT1 mRNA expression level increase in 3T3-L1 adipocytes which ectopically express SREBP1c [116, 117].

DGAT enzymes are also regulated at the transcriptional level, by currently unknown transcription factor(s). mRNA levels of DGAT1 and 2 increase drastically during the differentiation of 3T3-L1 fibroblasts to adipocytes, leading to an increase of DGAT activity during adipogenesis. This finding suggests an involvement of C/EBP α and/or PPAR γ in the regulation of DGAT activity [121-124]. Although SREBP1c regulates several lipogenic genes, it is not implicated in the regulation of DGAT enzymes, whereas XBP1, known to be a key regulator during UPR, regulates DGAT2 expression in the liver [125]. Moreover insulin and glucose activate DGAT additively [123]. Inhibition of DGAT activity takes place during cell division, which may promote the flow of FA to the phospholipid synthesis for membrane building instead of TG formation [126]. Several studies support the assumption that DGAT is also post-transcriptionally regulated by phosphorylation [127-129]. DGAT activity may also be hormonally regulated. As such, a decrease in DGAT activity was observed in rats after exposure to glucagon and epinephrine [129-131]. However, it is not known yet whether these substrates affect DGAT1 or 2 and whether they interfere at transcriptional or post-transcriptional level [126].

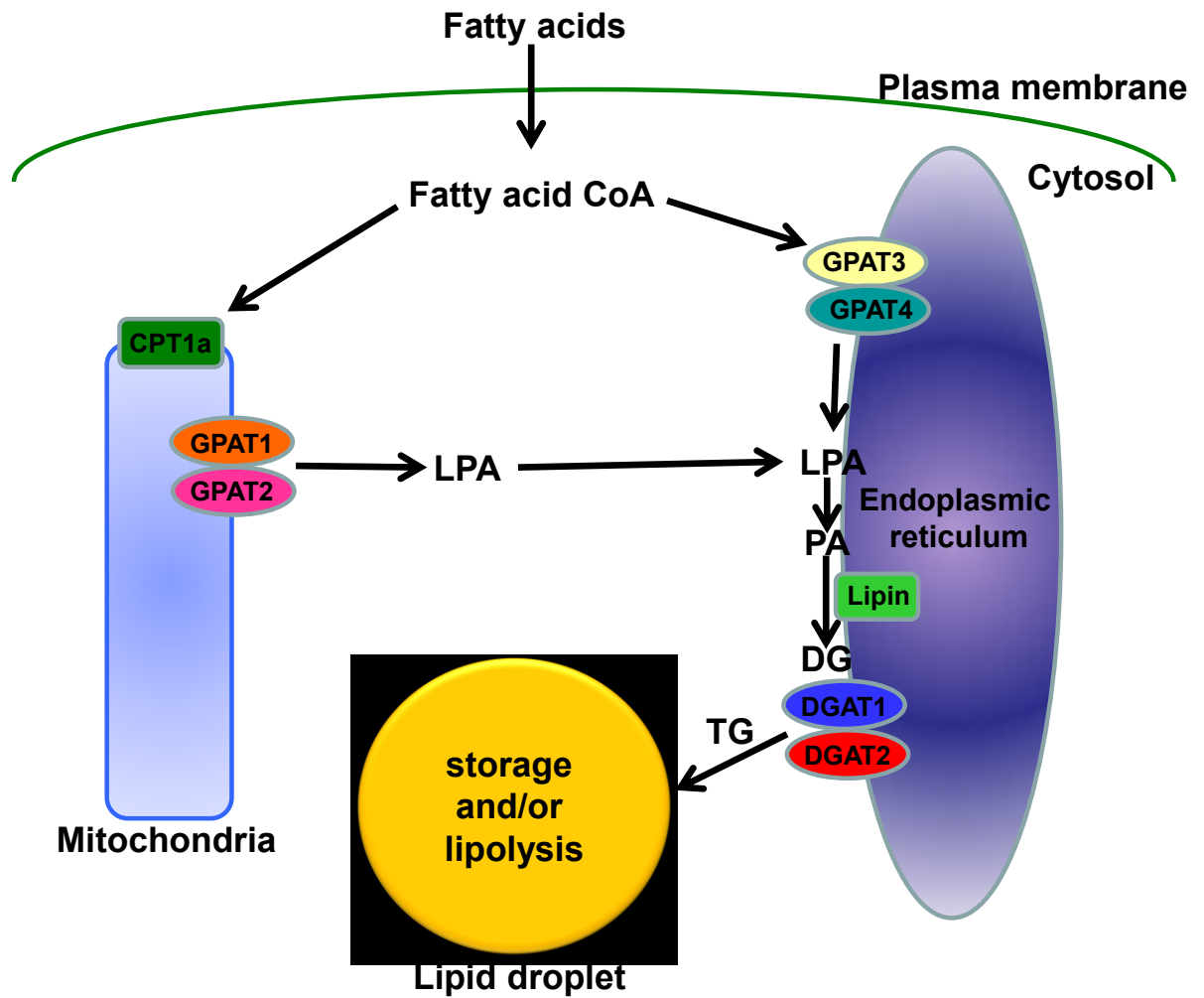


Fig.8: Triglyceride formation. TG formation is initiated by several glycerol-3-phosphate acyltransferase (GPAT1-4). GPATs synthesise lysophosphatidic acid (LPA) out of fatty acid acyl CoA and glycerol-3-phosphate substrates. LPA is acylated to build phosphatidic acid (PA) and lipin converts PA into Diacylglycerols (DAG). Diacylglycerol acyltransferase (DGAT) 1 and 2 form TGs out of DAGs.

1.9 Nuclear receptors in hepatic lipid metabolism and NAFLD

Nuclear receptors are a superfamily of transcription factors which can be activated by natural and synthetic ligands as well as by endogenous compounds such as steroid hormones, fatty acids, bile acids, vitamins and cholesterol [132]. The estrogen receptor alpha and the glucocorticoid receptors were the first nuclear receptors to be cloned [133]. Together with other steroid receptors, thyroid hormone receptor and vitamin D receptor they represent the group of endocrine receptors [134] and ligands for these receptors have been used clinically for decades [135]. In the absence of a ligand, nuclear receptors are inactive and localized either in the cytosol or in the nucleus. They are bound to their DNA hormone response element or to a co-repressor complex. Most of the nuclear receptors act as dimers, either as a homodimer or as a heterodimer with retinoid X receptor (RXR) [136].

Upon ligand binding, the co-repressor complex are substituted for co-activator complexes that activate the transcriptional machinery [137].

A large number of metabolic pathways are under the control of nuclear transcription factors such as hepatic lipid and glucose metabolism, drug disposition, bile acid homeostasis, embryonic development, reproduction, inflammation, cell differentiation and various aspects of tissue repair including liver regeneration, fibrosis and tumour formation [138, 139]. Therefore, nuclear receptors are attractive targets for the development of treatments and therapies against several liver diseases.

1.9.1 Nuclear receptors and NAFLD

The flux of free fatty acids from white adipose tissue to the liver and several critical pathways involved in the progression from fatty liver disease such as lipid storage, lipolysis, export, uptake and oxidation are under the control of nuclear receptors. However, little is known about the role of nuclear receptors in the progression from NAFLD to NASH. [134].

In healthy livers, PPAR α is highly expressed and activates expression of genes involved in fatty acid oxidation. The major role of PPAR α in hepatic lipid metabolism was further demonstrated by the fact that PPAR α null mice develop hepatic steatosis [53, 140]. In contrast, PPAR γ null mice are protected from fatty

liver development [141, 142]. In patients suffering from NASH, PPAR γ expression is reduced and treatment with PPAR γ agonists ameliorates liver enzymes and histological features. In addition, activation of PPAR α and γ can also be anti-inflammatory [134]. In addition, PPAR δ was suggested to reduce liver fat content via elevation of hepatic glucose catabolism and muscle beta oxidation and lowering hepatic SREBP1c activation [143]. FXR deficiency results in development of hepatic steatosis and hypertriglyceridemia, indicating for a critical function of FXR in the regulation of hepatic lipid metabolism [134, 144, 145]. SHP, a downstream target of FXR plays a major role in hepatic fatty liver disease. SHP expression is up-regulated in leptin deficient (ob/ob) mice as well as in high sucrose/high fat diet models of NAFLD, whereas in ob/ob/shp double knock-out mice lack of SHP expression protects from fatty liver development [146]. SHP deficiency results in elevated serum TG levels *via* up-regulation of the hepatic VLDL triglycerides secretion due to increased MTP expression [146]. Notably, ob/ob/SHP double knock-out mice have decreased expression of genes involved in fatty acid uptake and *de novo* lipogenesis. This could contribute to protect against NAFLD [146, 147]. However, in human an association of SHP defects and elevation of body weight were reported [147].

1.9.2 FXR – a nuclear receptors activated via bile acids

Originally, FXR was cloned because of its interaction with RXR and was also named RXR-interacting protein 14 (RIP-14) [148]. Recent studies have demonstrated the primary bile acids such as CDCA and CA are able to bind to FXR *in vitro*. This interaction takes place at physiological bile acid concentrations ($EC_{50} = 10-15\mu M$) and results in cofactors binding to FXR and subsequent increase in mRNA expression of FXR target genes [149-151]. Notably, these *in vitro* findings are supporting the hypothesis that CDCA and CA are directly interacting with FXR *in vivo*. Also polyunsaturated fatty acids such as arachidonic, linoleic or docosahexaenoic acid [152] were identified as ligands for FXR. FXR is able to function either as an activator or a suppressor [153, 154] in form of a monomer [153, 154] or a heterodimer with RXR [155, 156]. Several studies [156, 157] showed that FXR deficient mice developed hypertriglyceridemia, indicating an important function of FXR in triglyceride

metabolism. FXR was identified as a regulator of lipoprotein lipase, an enzyme involved in lipolysis of triglyceride rich lipoprotein particles like very low density lipoprotein and chylomicrons [158-162],.

Moreover, FXR is also identified as a regulator of PPAR α in man [163] PPAR α is known to be the master regulator of fatty acid oxidation and is activated by fatty acids [164] Since PPAR α activation increases FA oxidation and affect also bile acid synthesis [165], one can assume that FXR is able to control bile acid and lipid metabolism in both direct and indirect manner [47].

Forman et al [166] showed that FXR transcripts are present in liver, kidney, intestine, colon and adrenals and it was proposed that FXR was involved in cholesterol metabolism [166], whereas the function of FXR in adrenals is still not known, since this organ is – to the current state of knowledge – not involved in bile acid metabolism [167].

1.9.3 Hepatic lipid/glucose metabolism and fatty liver disease

A broad range of pathways involved in lipid homeostasis such as lipid and lipoprotein metabolism are regulated via nuclear receptors. Several lipids like cholesterol and free fatty acids function as ligands for nuclear receptors. PPAR α , δ , γ and hepatocyte nuclear factor 4 α (HNF4 α) are activated via fatty acids [168, 169]. LXR is activated by oxysterols [170, 171] and FXR by bile acids (cholesterol metabolites) [149-151].

Both, hepatic TG synthesis and clearance are regulated by nuclear receptors. Lipoprotein lipase (LPL) activity, which is responsible for TG lipolysis in the serum, is also modulated by apolipoproteins. Hence, activation of PPAR α results in reduction of serum TG levels due to various pathways: 1. ApoCIII – an inhibitor of LPL – is repressed and ApoAV – a coactivator of LPL – is activated [172]. 2. Intracellular increase of fatty acid oxidation. 3. Decrease in VLDL secretion [173]. Bile acids activate FXR and the G-coupled receptor TGR5 [174]. LPL activity is controlled by FXR which induces of the expression of the LPL co-activator ApoCII and apoAV while repressing ApoCIII, [47]. Furthermore, FXR activated SHP decreases transiently SREBP1c expression [134]. Finally, FXR activates PPAR α expression in human but not in mice [163].

Beside the direct hepatic effects FXR plays also an important role in the intestine, where it activates FGF15, which is the homologue to human FGF19 [134]. FGF15 negatively regulates bile acid synthesis [175] and controls hepatic metabolism [176]. As such, FGF19 transgenic mice have a higher metabolic rate and a decrease in obesity due to better fatty acid oxidation [134].

Bile acid activated FXR and FXR downstream pathways are also involved in the regulation of hepatic gluconeogenesis, glycogen synthesis and insulin sensitivity [134, 177, 178]. Loss of FXR causes glucose intolerance and insulin resistance, while treatment with FXR agonists, or adenoviral over-expression of FXR, decreases blood sugar levels after repression of gluconeogenesis and increase in glycogen synthesis and storage [134]. In summary lack of FXR leads to insulin resistance and glucose intolerance [134, 177].

1.10 Bile acids in NAFLD

1.10.1 Bile acid signalling in the pathogenesis of NAFLD

Dysregulation of bile acid homeostasis and signalling could account for liver injury and lipotoxicity in NAFLD. Therefore, bile acid activated nuclear transcription factors as well as bile acid transporters are potential attractive targets to treat metabolic diseases such as NAFLD, obesity, type II diabetes, hyperlipidemia and atherosclerosis [47, 145]. Little is known about the role of bile acid stimulated nuclear receptors in NAFLD [179]. FXR as well as its downstream target SHP plays a major role in NAFLD progression, as shown by the fatty liver development in FXR null mice [179] and the protection against fatty liver in ob/ob SHP null mice [146]. Ob/ob SHP double knock-out mice had elevated serum TG levels because of increased VLDL-TG secretion due to up-regulated MTP activity [146]. Moreover, double knock-out mice had reduced expression of genes involved in FA uptake and fatty acid synthesis, whereas beta oxidation was not affected [146, 147]. However, the role of SHP in humans is controversial since patients with SHP polymorphisms have increase body weight without fatty liver [147]. Activation of FXR and/or TGR5 via pharmacological ligands improves fatty liver development as well as related insulin resistance in various rodent models of obesity and NAFLD [145, 174]. Ursodeoxycholic acid (UDCA) has been shown to be a very weak FXR and

TGR5 ligand [145]. However, UDCA ameliorates hepatic ER stress and insulin resistance in mice [180].

1.10.2 Bile acids as signalling molecules

Bile acids are cholesterol end-degradation products and thus BA formation represents the predominant metabolic pathway for cholesterol breakdown in humans [181]. The conversion of cholesterol into bile acids transforms a hydrophobic substance into amphiphatic molecules that functions as detergents to absorb and transport lipophilic nutrients, but additionally act as signalling molecules activating nuclear and membrane receptors. Free bile acids, as well as conjugated bile acids activate FXR, which then form heterodimers with RXR [149-151]. FXR has a critical function in the regulation of several metabolic pathways such as bile acid synthesis, excretion, transport [182, 183] lipid and glucose metabolism [45, 144, 145, 178, 183]. The most efficient FXR ligand is the hydrophobic bile acid CDCA, while hydrophilic bile acids such as UDCA and muricholic acid activate FXR very weakly. Moreover, PXR and VDR are activated via bile acids [184, 185], therefore contributing to BA detoxification [184, 186, 187]. Moreover, other signalling pathways are affected by bile acid including calcium modification, cyclic AMP synthesis and protein kinase C activation [188]. Furthermore bile acids stimulate the secretion of pro-inflammatory cytokines, $\text{TNF}\alpha$ and interleukin 1b from Kupffer cells. This in turn activates the TNF receptor signalling and the mitogen activated protein kinase (MAPK)/ JNK pathway [189, 190]. The mitochondrial reactive oxidizing species activate the epidermal growth factor receptor, while Raf-1/MEK/ERK and PI3K/AKT can be stimulated by conjugated bile acids [191, 192] [193, 194]. Secondary, BA such as DCA activates the Fas receptor and the JNK pathway via ceramides in rat primary hepatocytes [195]. Insulin receptor signalling is also induced by bile acids [196]. Apart from the liver, bile acids activate TGR5, a G protein coupled receptor in the brown adipose tissue (BAT) and in macrophages [197, 198]. TGR5 activates production of cAMP, which leads to upregulation of iodothyronine deiodinase 2 (D2) and production of thyroid hormone T3. Intracellular T3 increase leads to higher energy expenditure and improves glucose tolerance and insulin sensitivity [45, 144, 145]. TGR5 is not found in

hepatocytes, but in the sinusoidal endothelial cells, cholangiocytes as well as in the entero-endocrine cells where it has antidiabetic function by controlling the release of the glucagon like peptide 1 (GLP1) [199, 200].

1.10.3 Regulation of the enterohepatic circulation of bile acids

About 0,2-0,6g bile acids are produced daily in the human liver. In the hepatocyte bile acids are conjugated and subsequently excreted into the bile via the canalicular bile salt export pump (BSEP/ABCB11/SPGP) and stored in the gallbladder. When hepatocyte BA concentrations are too high, BA can be exported into the blood and eliminated in urine.

Upon meal digestion bile acids are delivered from the gallbladder into the intestine, where about 95% are reabsorbed in the ileum by apical sodium dependent bile acid transporter (ASBT). Bile acids diffuse through the enterocyte to the basolateral membrane where the organic solute transporter a and b heterodimer (OSTa/OSTb) effluxes bile acids into the portal blood stream where they can reach again the hepatocytes after being taken up via the sodium dependent taurocholate cotransport peptide (NTCP) [181].

About 3g of bile acids are recycled 4-12 times per day. The loss of bile acids via the feces (0,2-0,6g/day) are replaced via hepatic de novo synthesis to assure a constant bile acid pool [181].

FXR is a key player in regulating the enterohepatic circulation of bile acids. As such, the canalicular BA transporter BSEP is stimulated via FXR and the driving force for BA formation. Re-absorption of bile acids into the intestine functions via ASBT, this statement is underlined by the fact that in ASBT null mice the enterohepatic circulation is interrupted and results in bile acid malabsorption [201]. Murine ASBT is down-regulated via FXR, in contrast to human [202]. Moreover, FXR also regulates expression of IBABP. IBABP is proposed to transport bile acids to the sinusoidal membrane of the enterocyte where they are excreted via OSTa/b [203, 204]. OSTa/b are also expressed at the hepatocyte sinusoidal membrane [205]. Bile acids are transported back to sinusoidal membrane of the hepatocytes (by the portal blood stream) where they enter the cells via NTCP. FXR inhibits NTCP expression in a SHP dependent manner [206]. Together these observations argue for a critical role of

FXR in the coordination of bile acid formation, biliary bile acid secretion, intestinal bile acid reabsorption and secretion and bile acid uptake into hepatocytes. Defects in FXR regulation of enterohepatic circulation of bile acids could lead to cholestasis [207-210].

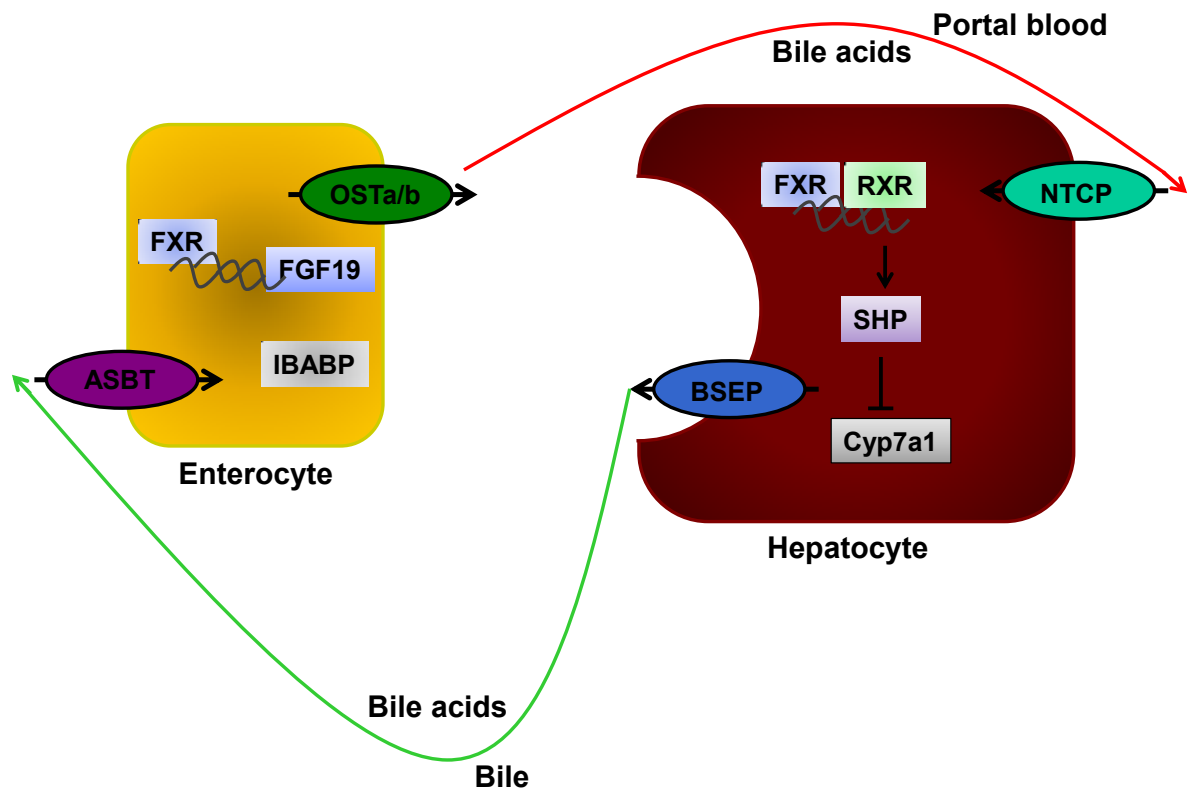


Fig.9: Enterohepatic circulation. Bile acids are synthesized in the hepatocyte and released in the bile via the bile salt export pump (BSEP). Bile acids are then secreted to the intestine. Bile acids are transported back to the liver via the portal blood and taken up by NTCP. Bile acids activate the nuclear receptor FXR. Activation of FXR leads via a SHP dependent pathway to inhibition of Cyp7a1 activity – the limiting enzyme in bile acid synthesis.

1.10.4 Bile salt export pump (BSEP)

Genetic defects of BSEP can lead to harmful liver diseases in human. BSEP deficiency causes a nearly absent canalicular bile salt secretion [211]. Currently two different clinical entities are known: benign recurrent intrahepatic cholestasis type 2 (BRIC2) and the more aggressive form called progressive familial intrahepatic cholestasis (PFIC2) [211-213]. Interestingly, compared to human, mice with inactive BSEP do not develop progressive cholestasis [214]. BSEP KO mice do not show abnormal liver parameters and their bile flow is slightly (but not significantly) decreased, whereas the biliary total bile salt output as well as the liver bile salt concentration are increased.

In addition, BSEP null mice display a decrease in biliary secretion of cholates and its conjugates by 94%, which underlines the importance of BSEP in canalicular taurocholate secretion. Moreover, these mice secrete a tetrahydroxylated bile acid into the bile [215].

Interestingly, BSEP KO mice fed a cholate supplemented diet secrete more cholate conjugate into bile, suggesting the presence of an alternative canalicular bile acid transport system [213]. Gene expression analysis revealed a remarkable increase in *Mdr1a* expression in BSEP KO mice after cholate feeding, indicating for the involvement of mouse *Mdr1a* in bile acid transport under the conditions of high intracellular bile acid levels [213]. Since mRNA expression analysis in PFIC2 patients did not show any significant increase in *MDR1* expression [216] the situation in human differs from the situation in mouse.

BSEP overexpression leads to an increase in biliary bile salt output, bile flow rate and biliary lipid secretion [217]. Of particular interest: Under lithogenic diet, BSEP transgenic mice do not develop steatosis, whereas the WT mouse does [217]. These results illustrate the importance of BSEP in the preservation of hepatic lipid metabolism.

2. AIM OF THE STUDY

These studies were designed to understand the role of lipases (adipose triglyceride lipase; ATGL), hepatobiliary bile acid transporters (bile salt export pump; BSEP) and the bile acid-activated nuclear receptor FXR in the control of hepatic lipid metabolism and the cellular defense against lipotoxicity

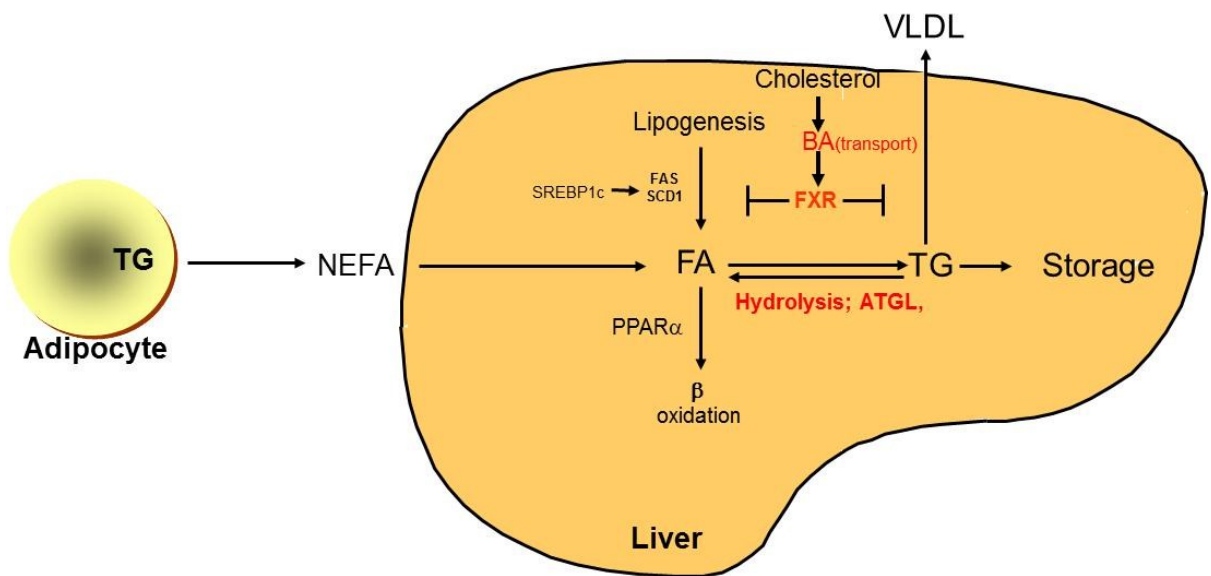


Fig.10: Hepatic lipid metabolism. Bile acid regulated nuclear receptors such as FXR and also lipases such as ATGL are major players in the regulation of hepatic lipid metabolism. Therefore this study was designed to determine the role of bile acids, FXR as well as ATGL in hepatic lipotoxicity.

3. MATERIALS AND METHODS

3.1 Role of ATGL in hepatic lipotoxicity

Animals. Gender and age matched C57/BL6 wild-type (WT) and ATGL knockout (KO) littermates were intraperitoneally injected with 1mg/kg body weight of a 0.1mg/ml suspension of tunicamycin (TM) in saline. Mice were re-injected after 24h. At 48h after the starting point, mice were killed by cervical dislocation. The experimental protocols were approved by the local Animal Care and Use Committees according to criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985). The animals were kindly provided by Rudolf Zechner, Institute of Molecular Biosciences, Karl-Franzens University, Graz, Austria, and generated as described previously [83].

Feeding protocols. Animals were fed a standard rodent chow and housed in a controlled environment with 12h light dark cycles.

Chemicals. Tunicamycin, sodium oleate and sodium palmitate were from Sigma Aldrich (Vienna, Austria).

Serum parameters. Blood was collected at harvesting and centrifuged for 20min at 4500rpm. Serum was stored at -80°C until analysis. Enzymatic assays were used to measure serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), cholesterol (CHOL), triglycerides (TG) (Roche Diagnostics, Mannheim, Germany) and free fatty acids (FAs) (Wako Chemical, Neuss, Germany). Lipoprotein subfractions were determined by quantitative agarose gel electrophoresis (Helena Biosciences, Gateshead, UK).

Liver histology. For conventional light microscopy livers were fixed in 4% neutral buffered formaldehyde solution for 24h, embedded in paraffin and stained with haematoxylin and eosin (H&E) or sirius red. Frozen tissue was embedded in tissue tec (Sakura Finetek Europe B.V.), sections of 2µm were then stained with oil red o.

Immunohistochemistry. Double immunofluorescence staining for cleaved caspase-3 and Cytokeratin 18 was performed as described previously [218].

Messenger RNA analysis and Polymerase Chain Reaction (PCR). RNA isolation, complementary DNA synthesis, and real-time PCR were performed as described previously [219]. All mRNA expression data were normalized to 36b4. Oligonucleotide sequences are available upon request.

Cell culture experiments. Hepa1.6 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% streptomycin/penicillin, sodium pyruvate, glutamine and non-essential amino acids at 37°C in a humidified 5% CO₂ atmosphere. Knock-down for ATGL was performed by using a lentivirus consisting shRNA against ATGL. Plasmid shRNA and lentivirus packaging mix were from Sigma Aldrich and used according to the manufacturer's instructions. The medium was changed every 48 hours.

For palmitic acid (PA) or oleic acid (OA) treatment Hepa1.6 cells were precultured overnight in DMEM without fetal calf serum and treated with oleate 500µM, palmitate 500µM and TM 1µg/ml and harvested at the indicated time points. Experiments were repeated in triplicates.

5mM stock solutions of OA and PA were prepared by dissolving the substances in a mixture of 0.01M NaOH (25%) and 150mM NaCl (40%) while heating. After heating a 24% FA free BSA solution (in 150mM NaCl) was added (18%). Finally PBS (10%) was added and filled up with distilled sterile water to 100%.

Western blotting. Hepatic nuclei were extracted with the NE-PER Nuclear and Cytoplasmic Extraction kit from Pierce Biotechnology. Srebp1c protein levels were determined using the polyclonal antibody Srebp-1(K10): sc-367 (Santa Cruz, Biotechnology, Inc.)

Hepatic triglyceride content. Total lipids were extracted from frozen liver tissue (100mg) homogenates with chloroform/methanol/glacial acetic acid (66:33:1, v/v) and phase separation was achieved by the addition of water.

Dried lipids were re-dissolved in 1% (v/v) Triton X-100, and TG content was measured using the reagent for quantitative TG measurement (DiaSys, Holzheim, Germany).

Hepatic total fatty acid pool composition – Sample Preparation and Derivatization. Mouse liver was homogenized in 0.5ml of phosphate buffered saline and 0.5ml of methanol. Each sample was spiked with 375nmol of 15:0

fatty acid as internal standard immediately. Lipids were then extracted according to Bligh and Dyer. Lipid extracts were taken to dryness and resuspended in 1ml methanolic NaOH. After 10min at 80°C and 5min on ice 1ml BF₃ was added followed by another 10min at 80°C. FA methyl esters were extracted with 1ml of saturated NaCl solution and 2ml of hexane. The hexane phase was taken to dryness and re-dissolved in 1.5ml hexane.

Free fatty acids - Sample Preparation and Derivatization. About 50mg mouse liver was homogenized in 0.5ml of phosphate buffered saline and 0.5ml of methanol. For serum free fatty acid determination 50µl serum were used. Each sample was spiked with 1.2mol of 15:0 fatty acid as internal standard immediately. Following that, lipids were extracted according to Bligh and Dyer. Lipid extracts were taken to dryness and redissolved in 50µl of a pentafluoro benzyl bromide solution (3.4 % in acetonitrile) and 10µl of N,N-diisopropyl ethanolamine. After 10min of incubation at room temperature samples were evaporated under a gentle stream of nitrogen and resuspended in 50µl of hexane.

GC-EI/MS. A Trace-DSQ GC-MS (Thermo Scientific, Austin, TX) equipped with a TR-FAME 30m column was used. Helium was used as carrier gas at 1.3ml/min in split mode at 250°C injector temperature. The initial oven temperature of 150°C was held for 0.5min and then the temperature first was ramped up to 180°C at a rate of 10°C/min. This was followed by a ramp of 0.5°C/min up to 190°C and 40°C/min up to 250°C where the temperature was held for another 3min. The mass spectrometer was run in electron impact (EI) mode where the FAs were detected in full scan of m/z 80 - 400. The source temperature was set to 250°C and the transfer line temperature was 200°C. Peak areas for FAs were calculated by Xcalibur QuanBrowser and related to the internal standard peak area.

GC-NICI/MS. A Trace-DSQ GC-MS (Thermo Scientific) equipped with a TR-FAME 30m column was used in splitless mode with 1ml/min helium as carrier gas and 300°C injector temperature. The initial oven temperature of 150°C was held for 1min and then the temperature first was ramped up to 200°C at a rate of 25°C/min. This was followed by a ramp of 12.5°C/min up to 325°C where the temperature was held for another 2min. The mass spectrometer was run in

negative ion chemical ionization (NICI) mode where the fatty acids were detected in full scan as carboxylates after loss of the pentafluoro benzyl moiety. Methane was used as CI gas, the source temperature was set to 250°C and the transfer line temperature was 330°C.

Statistical analysis. Statistical analysis was performed using SPSS V.18.0. using unpaired Student's t test. Data are reported as means +/- SD. Unless otherwise noted animal numbers were: WT Ctrl: n = 5; ATGL KO Ctrl: n = 5; WT TM treatment: n = 5; ATGL KO TM treatment: n = 6. Cell culture experiments were performed in triplicates. A p-value ≤ 0.05 was considered significant.

3.2 Role of FXR in hepatic lipotoxicity

Animal experiments. Age matched female C57BL/6 *ob/ob* mice, C57BL/6 WT and FXR KO mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed with a 12:12 hour light/dark cycle with water and a methionine choline deficient (MCD) diet for 5weeks. Experimental protocols were approved by the local Animal Care and Use Committee according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by US National Academy of Sciences (National Institutes of Health publication 86e23, revised 1985).

Animal diet. Control and Methionine choline deficient (MCD) diet was obtained from SAFE (Scientific Animal Food & Engineering).

Routine serum biochemistry. Blood was collected at harvesting and centrifuged for 20 min at 4500 rpm. Serum was stored at -80°C until analysis. Assays for alanine aminotransferase, alkaline phosphatase, Cholesterol and Triglyceride were routinely measured, whereas serum bile acid (BA) concentration was determined by using Bile Acid Kit (Ecoline S+ from DiaSys Diagnostic Systems GmbH, Holzheim, Germany) in a Hitachi 917 analyser (Boehringer Mannheim, Mannheim, Germany). Lipoprotein subfractions were determined by quantitative agarose gel electrophoresis (Helena Biosciences, Gateshead, UK). Glucose levels were assessed with an ACCU-Check Active analyzer (Roche, Mannheim Germany).

Chromatin Immunoprecipitation. Chromatin immunoprecipitation kit was from Epigentek Group Inc. (Brooklyn, NY 11226) and used according to the

manufacturer's instructions. For immunoprecipitation Antibodies for farnesoid X receptor (FXR) (sc-1204; Santa Cruz Biotechnology.) and retinoid X receptor (RXR) (sc-553; Santa Cruz biotechnology;) were used.

Messenger RNA analysis and Polymerase Chain Reaction (PCR). RNA isolation, complementary DNA synthesis, and real-time PCR were performed as described previously [219]. All data were normalized to 18sRNA. Oligonucleotides sequences are available upon request.

Cell culture. HepG2 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 1% streptomycin/penicillin, sodium pyruvate, glutamine, and nonessential amino acids (PAA) at 37°C in a humidified 5% CO₂ atmosphere. The medium was changed every 48 hours. HepG2 cells were treated with low and high levels of glucose with or without CDCA at the indicated concentrations for 12 hours in the absence of serum.

Immortalized human derived primary hepatocytes (IHH) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 1% streptomycin/penicillin, sodium pyruvate, glutamine, and nonessential amino acids (PAA) at 37°C in a humidified 5% CO₂ atmosphere. The medium was changed every 48 hours. IHH cells were treated with low levels of glucose with or without Int747 at the indicated concentrations for 12 hours in the absence of serum. IHH cells were treated with low levels of glucose with or without Int747 at the indicated concentrations for 12 hours in the absence of serum in a methionine choline deficient (MCD) medium.

Hepatic Triglyceride content. Total lipids were extracted from frozen liver tissue (100 mg) homogenates with chloroform/methanol/glacial acetic acid (66:33:1, v/v), and phase separation was achieved by the addition of water. Dried lipids were re-dissolved in 1% (v/v) Triton X-100, and TG content was measured using the reagent for quantitative TG measurement (DiaSys, Holzheim, Germany)

Statistical analysis. Results were evaluated using SPSS V.18.0. Statistical analysis was performed using student's t test. Data are reported as means of four to six animals per group +/-SD. A p value ≤0.05 was considered significant.

3.3 Role of BSEP in hepatic lipotoxicity

Animal experiments. Male FVB/N wild-type and BSEP KO age matched mice were fed a methionine choline (MCD) deficient diet (obtained from SAFE - Scientific Animal Food & Engineering) for 5 weeks *ad libitum*. Mice were killed by cervical dislocation. Animals were kindly provided by the British Columbia Cancer Research Center, and generated as described previously [214]. The experimental protocols were approved by the local Animal Care and Use Committees according to criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985).

Routine serum biochemistry. Blood was collected at harvesting and centrifuged for 20 min at 4500 rpm. Serum was stored at -80°C until analysis. Assays for alanine aminotransferase, alkaline phosphatase, total cholesterol, triglycerides and free fatty acids were routinely measured, whereas serum bile acid (BA) concentration was determined by using Bile Acid Kit (Ecoline S+ from DiaSys Diagnostic Systems GmbH, Holzheim, Germany) in a Hitachi 917 analyser (Boehringer Mannheim, Mannheim, Germany).

Liver histology. For conventional light microscopy, livers were fixed in 4% neutral buffered formaldehyde solution for 24 h and embedded in paraffin. Sections were cut 2 µm thick and stained with haematoxylin and eosin (H&E).

Messenger RNA analysis and Polymerase Chain Reaction (PCR). RNA isolation, complementary DNA synthesis, and real-time PCR were performed as described previously [219]. All data were normalized to 36b4 and shown as mean +/- SD. All oligonucleotide sequences which were used are available upon request.

Hepatic Triglycerides. Total lipids were extracted from frozen liver tissue (ca 100 mg) homogenates with chloroform/methanol/glacial acetic acid (66:33:1, v/v), and phase separation was achieved by the addition of water.

Dried lipids of murine tissues were re-dissolved in 1% (v/v) Triton X-100, and TG content was measured using the reagent for quantitative TG measurement (DiaSys)

Hepatic bile acid concentrations. Bile acids were extracted from liver homogenate, and analyzed using electrospray ionization mass spectrometry and gas chromatography mass spectrometry as described in [220].

Western blot. Liver membranes were prepared as described previously [221]. Transporter protein levels were determined using polyclonal antibodies against Ntcp (1:2500), kindly provided by Dr. Bruno Stieger, Mrp4 (1: 2000) kindly provided by John Schütz, Mrp3 (1:5000) and β Actin (1:5000) (Sigma Aldrich).

Statistical analysis. Results were evaluated using SPSS V.18.0. Statistical analysis was performed using student's t test. Data are reported as means of four or six animals per group $\pm$ SD. A p value ≤ 0.05 was considered significant.

4. RESULTS

4.1 Role of ATGL in hepatic lipotoxicity

Absence of Adipose Triglyceride Lipase Protects From Hepatic Endoplasmic Reticulum Stress in Mice

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ATGL KO mice show increased hepatic lipid accumulation during tunicamycin induced ER stress. Lipotoxicity is known to induce ER stress *in vitro* [222] and *in vivo* [223]. Therefore, we injected tunicamycin (TM) to induce ER stress in WT and ATGL KO fed mice which have a defect in cellular TG catabolism [83, 100, 224]. Serum alanine aminotransferase (ALT, reflecting hepatocyte damage) levels were not significantly increased in mice injected with TM; alkaline phosphatase (AP) levels reflecting a cholestatic pattern were increased, while total cholesterol, TG and FA were decreased in both genotypes (Fig.11A). Although serum parameters suggested that WT and ATGL KO mice challenged with TM have disturbed lipid metabolism, only ATGL KO mice showed hepatic lipid accumulation (Fig.11B and Fig.12B). Notably, liver/bodyweight ratio was not changed due to TM treatment in both WT and ATGL KO mice (Fig.12A). Liver histology in WT mice was not affected by TM, whereas untreated ATGL KO controls exhibited a moderate lipid infiltration which was further pronounced by TM (Fig.11B and 12B). Biochemical quantification of hepatic lipid content revealed a more than 2-fold increase in hepatic TG accumulation in TM treated ATGL KO mice compared to TM treated WT mice (Fig.11C). Taken together, our data demonstrate that ATGL KO mice show elevated hepatic TG stores after induction of ER stress.

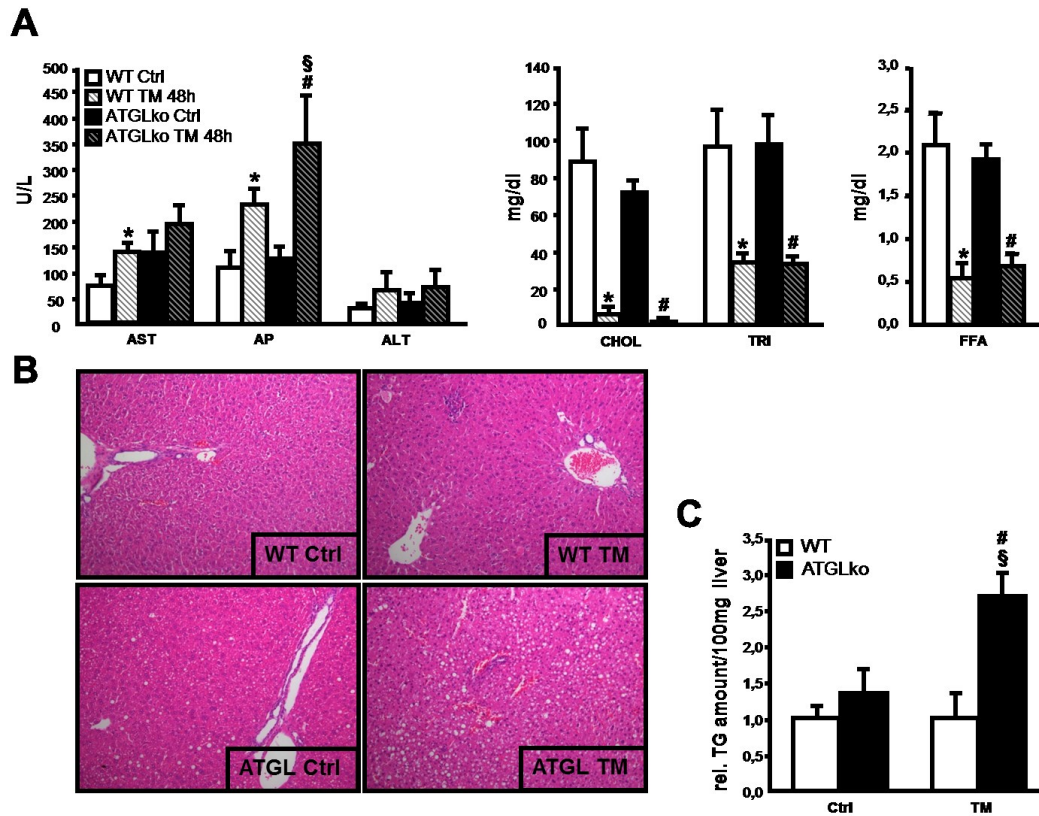


Fig.11: ATGL KO mice display increased lipid droplet accumulation in the liver during tunicamycin induced ER stress. (A) Tunicamycin (TM) injection increased serum alkaline phosphatase (AP) in both WT and ATGL KO mice, aspartate amino transferase (AST) was increased only in WT TM mice, whereas alanine amino transferase (ALT) serum levels were not affected by TM. Serum cholesterol (CHOL), triglycerides (TG) and free fatty acids (FAs) were significantly reduced after TM injection. (B) Hepatic steatosis appeared in ATGL KO mice 48h following TM injection but not in treated WT mice. (H&E staining; 20x magnification). (C) After TM injection, livers of ATGL KO mice showed a significant increase of hepatic TG. * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; § indicates a significant difference from TM treated WT; $P < 0,05$.

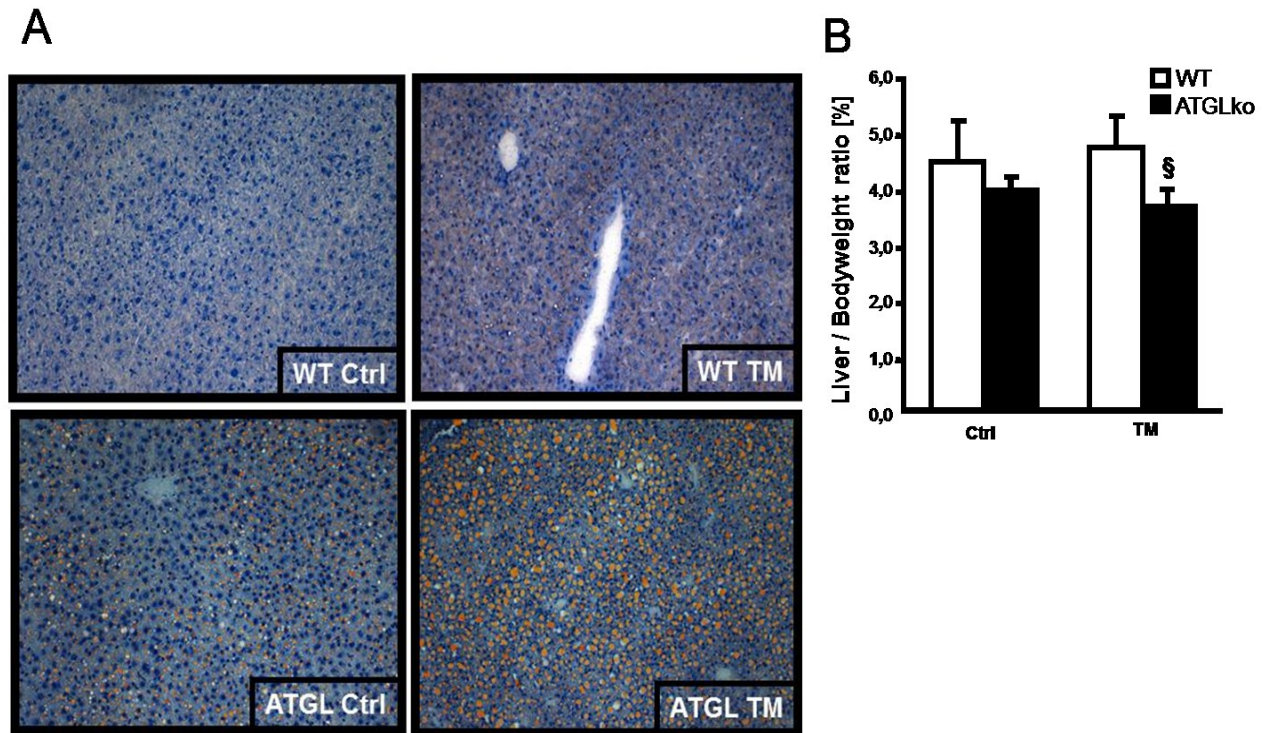


Fig.12: Hepatic lipid accumulation in ATGL KO TM injected mice. (A) Oil red O staining (10x magnification) showed a marked accumulation of lipids in the liver of ATGL KO TM injected mouse. (B) Liver/bodyweight ratio is not changed in WT and ATGL KO mice after Tunicamycin (TM) injection, compared to their respective control groups. § indicates a significant difference from TM treated WT; $P < 0,05$.

Absence of ATGL protects against hepatic ER stress and inflammation in vivo. To further address the role of ATGL in the hepatic ER stress response, we checked mRNA expression levels of ER stress markers in the presence and absence of ATGL *in vivo*. When challenged with TM, WT but not ATGL KO mice showed markedly increased expression levels of spliced Xbp1 (sXbp1) reflecting ER stress (Fig.13A). In line, mRNA expression of the sXbp1 downstream target ERdj4 was exclusively elevated in TM treated WT mice. A similar expression profile was observed for Grp78 – a heat shock chaperone located in the lumen of the ER which activates the UPR – and Chop as downstream target of the integrated stress response (Fig.13A). Notably, gene expression of inflammatory markers Tnf α and iNos in response to TM was suppressed exclusively in ATGL KO mice, while WT mice displayed increased gene expression (Fig.13B). mRNA levels of Col1a1 (indicator for fibrosis) (Fig.14B) and Vcam-1 (Fig.14C) - which is not only correlating with inflammation but also with fibrosis - did not yet reach significant differences 48h after TM treatment. Bcl-2 (anti-apoptosis marker) mRNA expression levels did not differ between untreated and treated WT mice and were even increased in TM injected ATGL KO mice compared to respective controls (Fig.15B). The impression that 48h TM injection did not lead to fibrosis and/or apoptosis given by mRNA levels, was confirmed by Sirius red and cytokeratin 18/caspase 3 double immunostaining, respectively (Fig.15A). These data demonstrate that lack of ATGL protects mice from hepatic ER stress and the subsequent inflammatory response. Notably, kidneys of TM treated ATGL KO mice were not protected from ER stress (Fig.16A), whereas white adipose tissue (WAT) was not affected by TM injection in both, ATGL KO and WT mice (Fig.16B), emphasizing the specificity of the findings for liver.

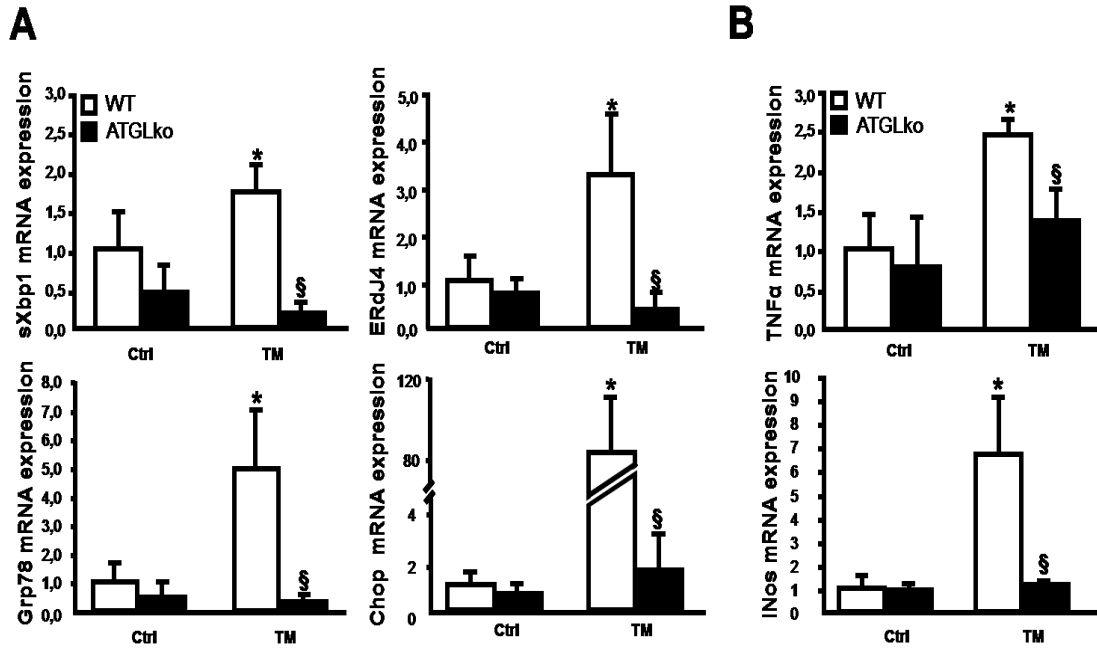


Fig.13: Tunicamycin induces hepatic ER stress and hepatic inflammation in WT mice. Real time PCR was used to measure the expression of (A) sXbp1, ErDj4, Grp78 and Chop as markers for ER stress and (B) the hepatic inflammation markers Tnf α and iNos. In contrast to WT, ATGL KO mice had less ER stress and inflammation. Expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; § indicates a significant difference from TM treated WT; P<0,05.

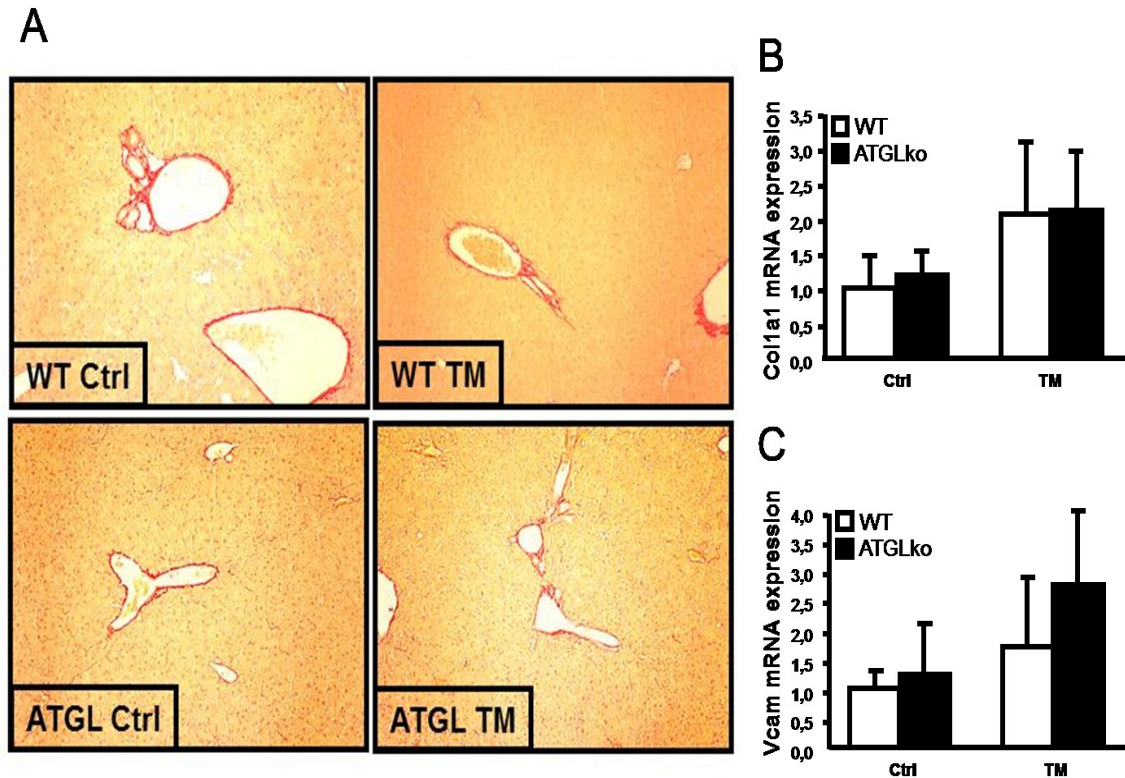
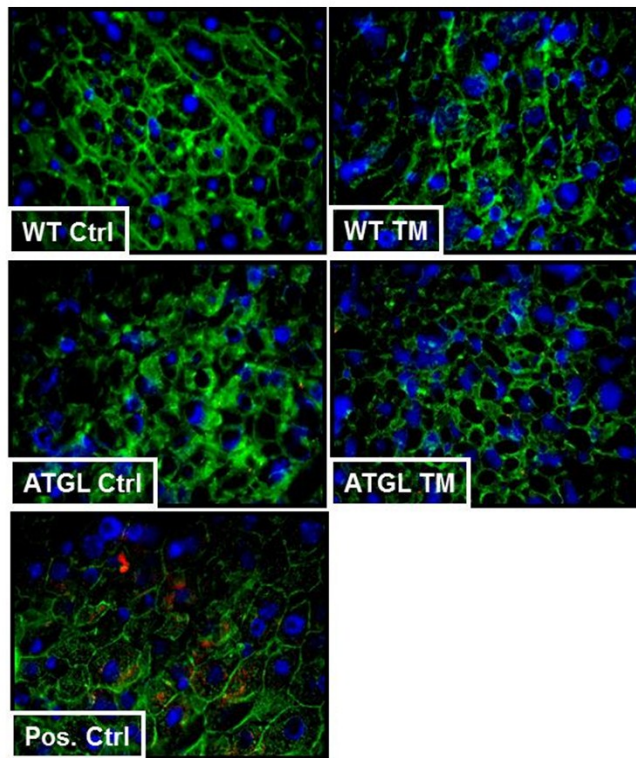


Fig.14: 48h TM injection did not induce fibrosis. (A) Sirius red staining was performed as a marker for fibrosis. No differences were found between control groups and TM exposed animals. (B) Real time PCR was used to measure the expression of Col1a1 and Vcam-1 as markers for fibrosis. Expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. No changes were highlighted in term of Sirius red stainings and mRNA levels of fibrosis marker Col1a1 and Vcam-1 between the groups.

A



B

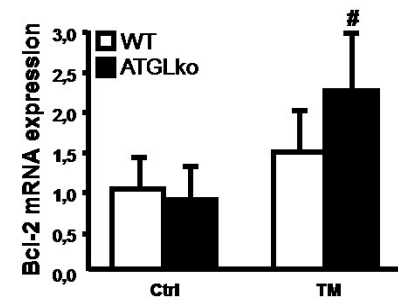


Fig.15: 48h TM injection did not induce apoptosis. (A) Cytokeratin 18 Caspase 3 double immunostaining was assessed to illustrate available apoptosis. No changes were highlighted in term of active Caspase 3 levels between the groups. (B) Real time PCR was used to measure the expression of Bcl-2 as markers for apoptosis. Expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. The anti-apoptotic marker Bcl-2 was up-regulated in ATGL KO mice upon MCD treatment. # indicates a significant difference from ATGL KO Ctrl; $P < 0,05$.

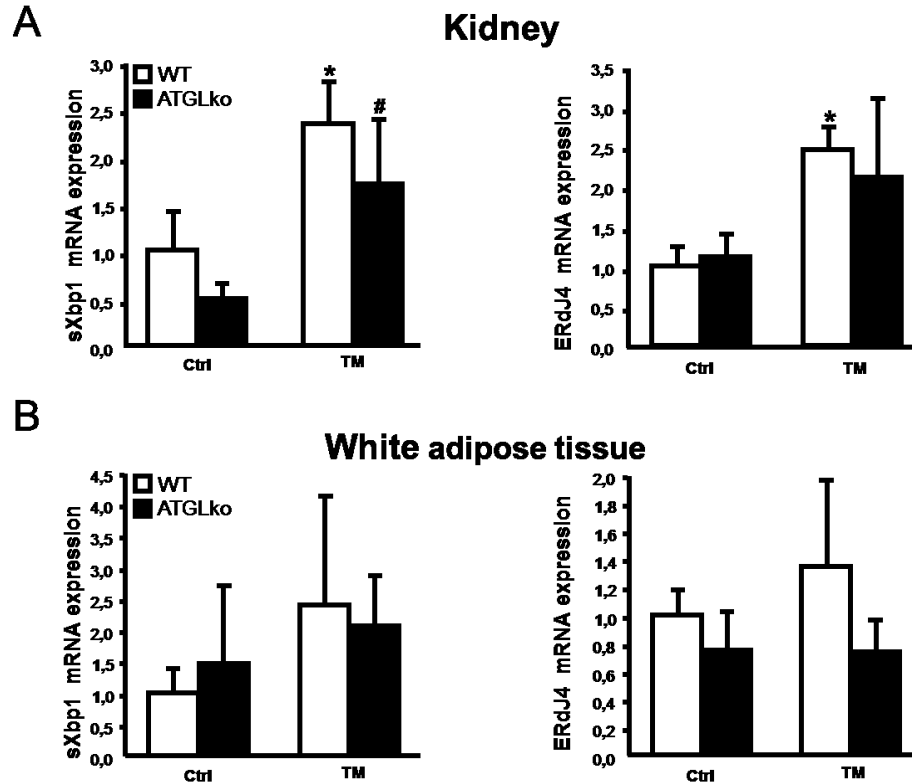


Fig.16: Induction of ER stress markers in kidney but not in white adipose tissue. Real time PCR was used to measure the mRNA expression of markers for ER stress (sXbp1, ERdj4) in (A) kidney and (B) white adipose tissue (WAT). Tunicamycin (TM) treatment led to increase in mRNA expression of both, sXBP1 and ERdj4 in kidney, but not in WAT. Expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; $P < 0,05$. (n(WT Ctrl) = 4; n(ATGL KO Ctrl) = 4; n(WT TM) = 4; n(ATGL KO TM) = 3).

ER stress reduces circulating VLDL-cholesterol, VLDL assembly, endogenous FA synthesis and FA β -oxidation. Since the ER plays a central role in VLDL metabolism, we next investigated whether VLDL and FA metabolism were affected by TM treatment. HDL and VLDL-cholesterol serum levels were drastically reduced in TM challenged mice (Fig.17A). In line with the serum data, mRNA expression of Mttp and ApoB, two key genes involved in VLDL formation, were down-regulated in TM treated mice (Fig.17B). Together, these findings suggest that TM treatment impaired VLDL synthesis in both WT and ATGL KO mice.

To explore whether differences in ER stress and hepatic steatosis after TM application might be due to differences in *de novo* lipogenesis and/or FA β -oxidation, we next assessed hepatic Srebp1c and FasN mRNA (Fig.18A) as well as the nuclear Srebp1c protein levels (Fig.19) as markers for *de novo* lipogenesis and Cpt1 α and Acc2 mRNA levels (Fig.18B) as markers for β -oxidation. mRNA expression levels of Srebp1c – the master regulator of *de novo* lipogenesis – and its downstream target FasN, were down regulated after TM injection in WT and ATGL KO mice, with ATGL KO TM injected mice having slightly higher Srebp1c expression than the WT TM treated mice (Fig.18A). In line with the decrease in Srebp1c mRNA levels in mice challenged with TM the nucleic mature Srebp1c protein expression was also diminished. Both WT and ATGL KO mice challenged with TM showed low mRNA levels for Cpt1 α (Fig.18B), whereas Aox mRNA levels were not changed in mice challenged with TM (data not shown). Moreover, Acc2 expression (responsible for malonyl-CoA generation potentially inhibiting Cpt1 α) was similarly repressed in WT and ATGL KO mice after TM injection. These findings demonstrate that gene expression levels of enzymes implicated in *de novo* lipogenesis and FA β -oxidation are equally lowered in TM exposed mice and therefore cannot explain the differences in hepatic lipid accumulation and ER stress.

Next we explored gene expression levels of key players involved in hepatic TG synthesis. Agpat9 – also known as Gpat3 – and Agpat3 – also known as Lpaat. mRNA expression levels of these genes (Fig.20) were not increased in WT

mice upon TM treatment, whereas TM treated ATGL KO mice showed a marked increase in expression of *Agpat9* (*Gpat3*) (8fold) and *Agpat3* (*Lpaat*) (2,5-fold) compared to untreated ATGL KO mice. Collectively, these findings suggest that an increase in hepatic TG formation in ATGL KO mice challenged with TM may be involved in protection against the induction of ER stress.

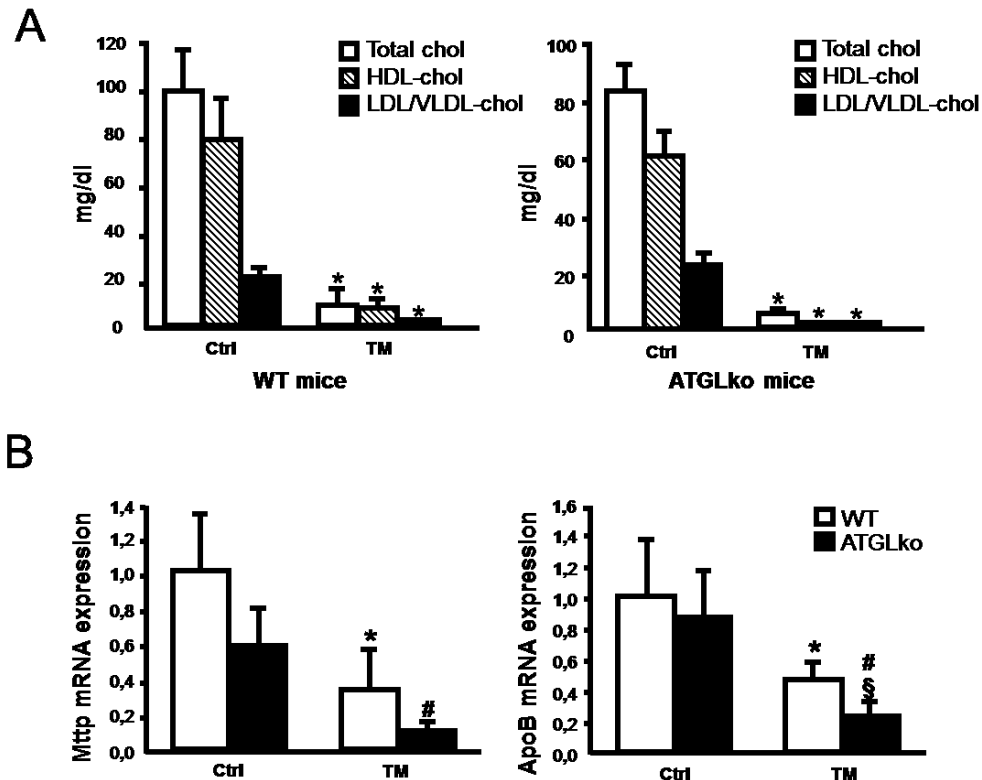


Fig.17: Tunicamycin injection in mice reduces circulating VLDL-cholesterol and expression of genes involved in VLDL assembly. (A) Both, WT and ATGL KO, mice have reduced serum levels of total cholesterol, HDL - and VLDL - cholesterol after tunicamycin (TM) injection. (B) mRNA expression of *Mttp* and *ApoB*, key genes involved in VLDL synthesis were measured by real time PCR. Independently of the genotype, TM injection reduced *Mttp* and *ApoB* gene expression. mRNA expression values were normalized against *36b4* levels and are shown relative to expression level in unchallenged WT animals. (A) * indicates a significant difference from corresponding controls (Ctrl); (B) * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; § indicates a significant difference from TM treated WT; $P < 0,05$.

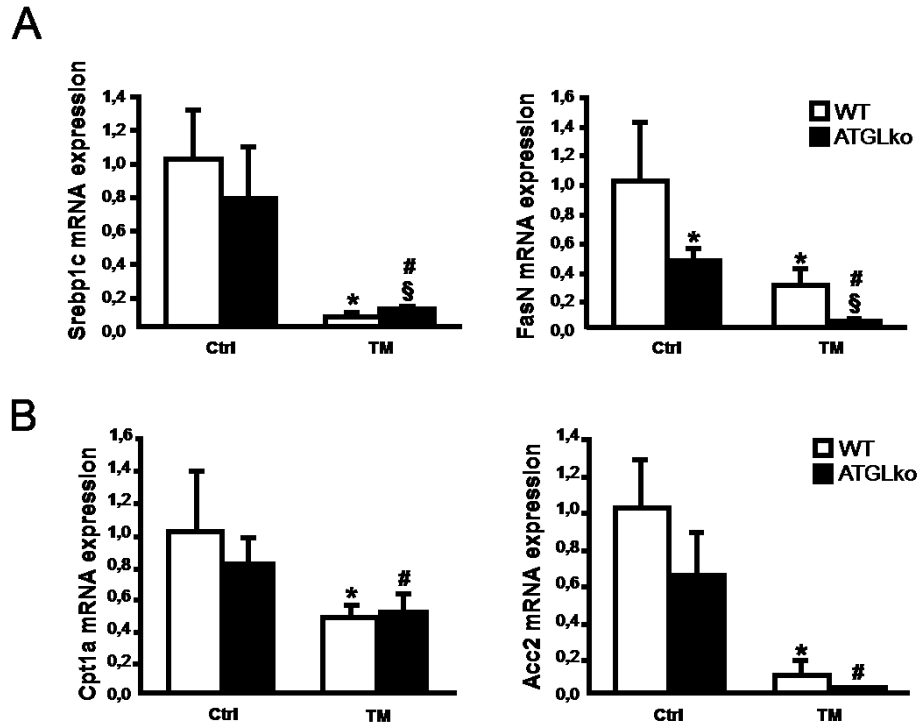


Fig.18: Tunicamycin injection in mice reduces *de novo* lipogenesis and β -oxidation. Real time PCR was used to measure the expression of (A) markers for *de novo* lipogenesis (Srebp1c, FasN) and (B) β -oxidation (Cpt1 α , Acc2). Srebp1c, FasN, Cpt1 α and Acc2 gene expression was down-regulated due to TM challenging. Expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; § indicates a significant difference from TM treated WT; P<0,05.

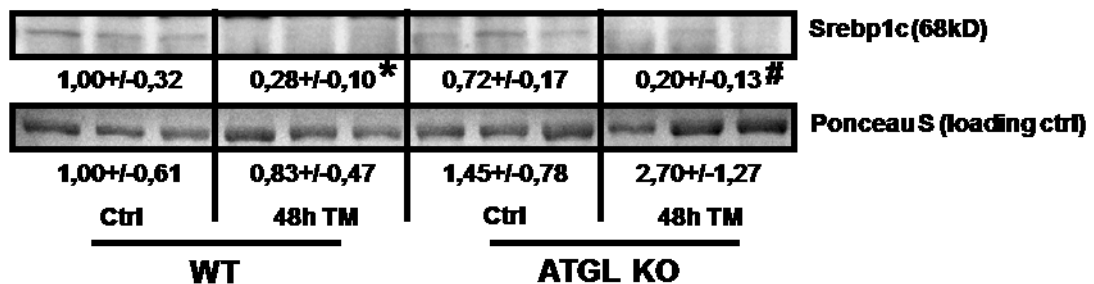


Fig.19: Nuclear hepatic Srebp1c protein levels. Nuclear protein expression levels of Srebp1c were assessed by western blot. Densitometry data are expressed as n-fold change related to unchallenged WT controls. * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; P<0,05

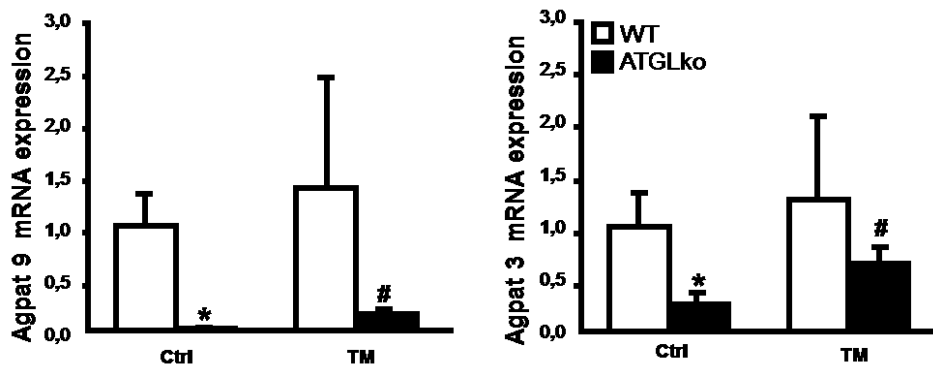


Fig.20: Tunicamycin injection up regulates triglyceride formation in ATGL KO tunicamycin challenged mice. Real time PCR was used to measure the expression of markers for triglyceride (TG) formation (Agpat9/Gpat3, Agpat3/Lpaat). Acyltransferase gene expressions were increased in ATGL KO but not in WT mice injected with tunicamycin (TM). Expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; $P < 0,05$.

Defective hepatic FA and TG metabolism in WT and ATGL KO mice under ER stress. Since TM injected mice exhibited selective fat accumulation in ATGL KO (but not WT) livers, we next addressed the impact of TM treatment on serum and hepatic FA species and their potential role in ER stress induction or protection by measuring free serum, total and free hepatic FA composition in non-fasted mice (Fig.21A, Table1 and Fig.22). Interestingly, TM treatment resulted in an increase of total hepatic palmitic acid (PA, 16:0) and oleic acid (OA, 18:1n9) levels in both WT and ATGL KO mice. However, only WT mice (both untreated and treated) showed higher amounts of total PA related to OA (Fig.21B). In contrast, ATGL KO mouse exhibited higher levels of OA before and after TM injection, reflected by the PA/OA ratio as shown in Fig.6B. Since *Scd1* is down-regulated under TM treatment (Fig.21C), TM-treated WT mice are not able to convert potentially lipotoxic PA into non-toxic – or even protective – OA, in contrast ATGL KO mice exposed to TM, might have been protected by their higher basal amount of OA from PA induced ER stress.

To test whether *Pik3ip1* expression – which is induced by PA *in vitro* [222] thus suggesting an essential role of *Pik3ip1* in PA-induced ER stress – changed after TM injection, we investigated its gene expression levels. Indeed, *Pik3ip1* mRNA was up-regulated in WT but not in ATGL KO mice subjected to TM (Fig.21D). Taken together, these data suggest that saturated FAs, like PA are able to induce hepatic ER stress *via* activation of the Pi3K Inhibitor. Conversely, high hepatic OA contents may counteract *Pik3ip1* activation and therefore prevent ER stress induction in ATGL KO mice.

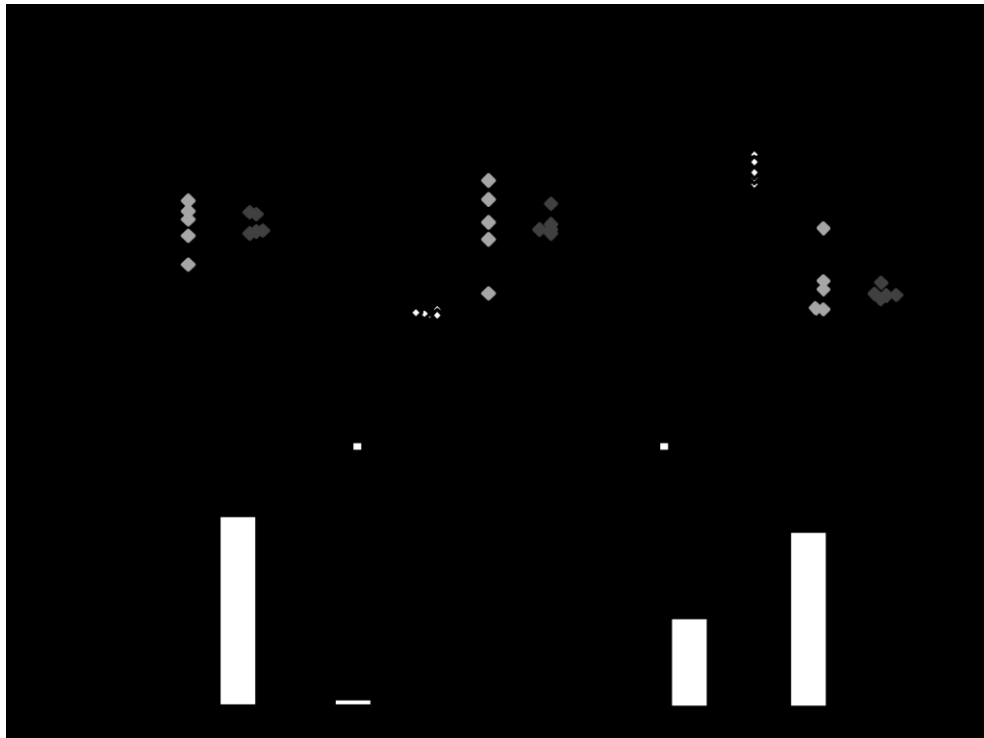


Fig 21: Defective hepatic fatty acid and triglyceride metabolism in WT and ATGL KO mice under ER stress conditions. (A, B) Total hepatic fatty acid composition was measured from non-fasted mice by mass spectrometry as described in M&M. (A) Total Oleic acid (OA; 18:1n9) was significantly higher in ATGL KO mice and increased further after tunicamycin (TM) injection. (B) WT mice have more total palmitic acid (PA; 16:0) than OA at the baseline, shown by the PA/OA ratio. (C, D) Real time PCR was used to measure the expression of Scd1 and Pik3ip1. Scd1 expression was reduced after TM injection, but slightly more in ATGL KO mice. Pik3ip1 levels were increased in WT TM injected mice only. mRNA expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. (A) \$ indicates a significant difference from PA levels of the same group; (A, C, D) * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; § indicates a significant difference from TM treated WT; (B) \$ indicates significant differences from OA/PA ratio in WT controls (Ctrl); P<0,05.

	PA (16:0)	OA (18:1n9)	LA (18:2n6)	AA (20:4n6)	CA (22:6n3)
WT Ctrl	53,23	15,33^{\$}	13,96	2,43	0,49
	2,40	1,13	0,85	0,67	0,19
WT TM	40,93*	17,31*^{\$}	23,29*	2,39	0,59
	4,84	1,36	2,15	0,43	0,23
ATGL KO Ctrl	50,80	17,29^{\$}	11,60*	3,20	0,82
	3,70	1,43	1,30	0,78	0,19
ATGL KO TM	55,12	16,83^{\$}	10,88^{\$}	1,43	0,64[#]
	4,24	1,36	1,53	0,83	0,11

Table1: Hepatic free fatty acid composition in tunicamycin-treated animals in the presence and absence of ATGL. Hepatic free fatty acid composition was measured in non-fasted mice by mass spectrometry as described in M&M. Palmitic acid (PA; 16:0); oleic acid (OA; 18:1n9); linoleic acid (LA; 18:2n6); cervonic acid (CA; 22:6n3); arachidonic acid (AA; 20:4n6). Data represent means (bold) +/- SD (given below). \$ indicates a significant difference from PA levels in the same group. * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; § indicates a significant difference from TM treated WT; P<0,05

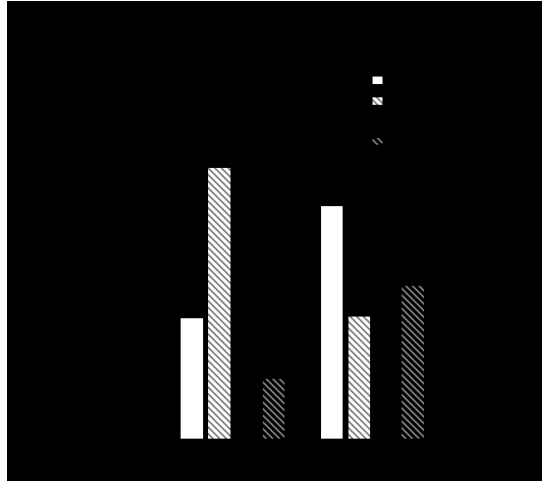


Figure 22: Free Fatty acid composition in serum. Free serum fatty acid composition was measured from non-fasted mice by mass spectrometry as described in M&M. Free serum palmitic acid (PA; 16:0) levels were significantly increased in WT tunicamycin (TM) treated mice, whereas ATGL KO TM mice displayed no differences in PA levels compared to untreated ATGL KO mice. Free serum oleic acid (OA; 18:1n9) was decreased due to TM treatment independent of the genotype. \$ indicates a significant difference from PA levels in the same group; * indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from ATGL KO Ctrl; § indicates a significant difference from TM treated WT; P<0,05.

Oleic acid protects against tunicamycin induced hepatic ER stress. To further test whether absence of ATGL has a direct protective function against TM induced ER stress, we knocked-down ATGL in mouse hepatocytes (Hepa1.6 ATGL KD) by 50% (Fig.23A) and subsequently treated them with TM (Fig.23B). No significant differences in mRNA expression levels of ER stress markers Grp78, Chop (data not shown), sXbp1 and ErDj4 were observed under baseline conditions (Fig.23B). ATGL knock-down markedly attenuated induction of ER stress markers in response to TM (Fig.23B). In order to test whether monounsaturated OA (accumulating *in vivo* in ATGL deficient mice) is able to protect Hepa1.6 ATGL KD cells against TM and/or PA-induced ER stress we co-treated these cells with OA, PA and TM (Fig.23C). Cells treated with PA and TM showed increased expression levels of ER stress markers Chop, sXbp1 and ErDj4 compared to untreated cells, while ER stress marker expression levels in cells treated with OA did not differ from controls (Fig.23C). Notably, cells treated with similar amounts of OA and PA did not show an increase in mRNA expression levels of ER stress markers following TM exposure (Fig.23C), further demonstrating the protective role of monounsaturated OA against PA-induced lipotoxicity and ER stress. Collectively, these data demonstrate that increased cellular concentrations of non-esterified OA in hepatocytes are able to protect from TM induced hepatic ER stress by interfering with Pik3ip1 expression.

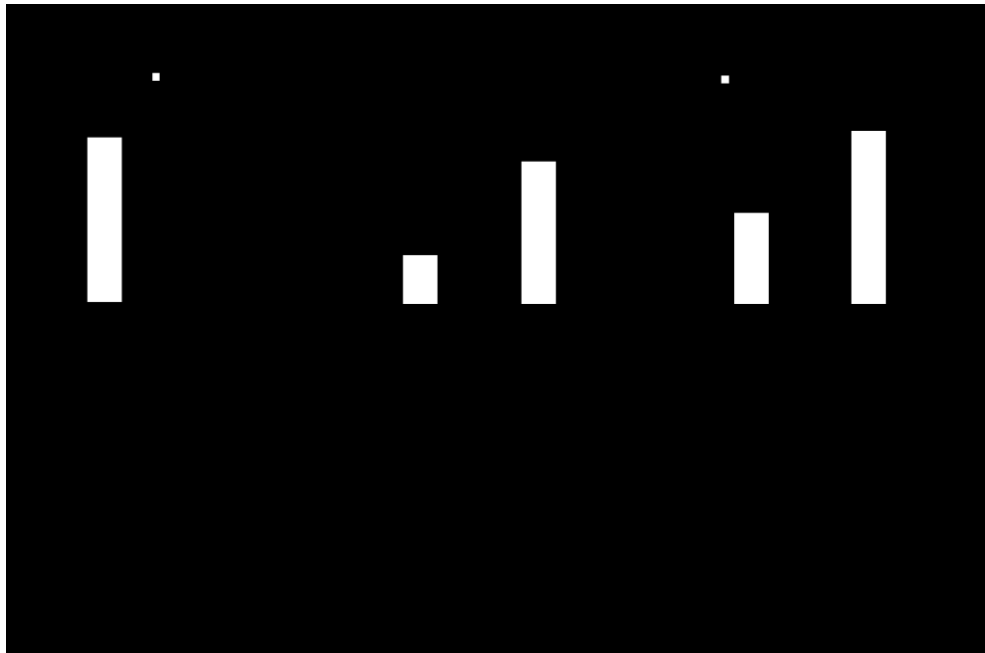


Fig.23: Oleic acid protects against tunicamycin induced hepatic ER stress. mRNA expression levels of markers for ER stress were assessed by real time PCR in Hepa1.6 cells and Hepa1.6 cells knocked-down for ATGL (Hepa1.6 ATGL KD). (A) ATGL mRNA expression levels were repressed in Hepa1.6 ATGL KD cells by 50%. (B) Hepa1.6 cells and Hepa1.6 ATGL KD were treated with tunicamycin (TM) for 7h. Cells exposed to TM showed induction in sXbp1 and ErDj4 mRNA expression levels, but to a greater extent in Hepa1.6 cells compared to Hepa1.6 ATGL KD cells. (C) Hepa1.6 ATGL KD were treated with oleic acid (OA) and/or palmitic acid (PA) for 24h and co-treated with TM for 7h. Cells exposed to OA and PA with and without TM did not show increase in mRNA expression levels of ER stress markers compared to PA or TM treated cells. mRNA expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged Hepa1.6 cells. (A, B) * indicates a significant difference from Hepa1.6 control cells (Ctrl); # indicates a significant difference from Hepa1.6 ATGL KD control cells (Ctrl); § indicates a significant difference from TM treated Hepa1.6 cells (C) * indicates a significant difference from Hepa1.6 ATGL KD control cells (Ctrl); # indicates a significant difference from TM treated Hepa1.6 ATGL KD cells; \$ indicates a significant difference from PA treated Hepa1.6 ATGL KD cells; P<0,05.

4.2 Role of FXR in hepatic lipotoxicity

FXR is a key player in NAFLD development by controlling CHOP expression

(Manuscript in preparation)

Liver steatosis leads to up-regulation of Chop mRNA in human and mice.

Liver samples screening obtained from massive obese patients undergoing bariatric surgery revealed an increase in Chop mRNA expression associated with fatty liver development (Fig.24).

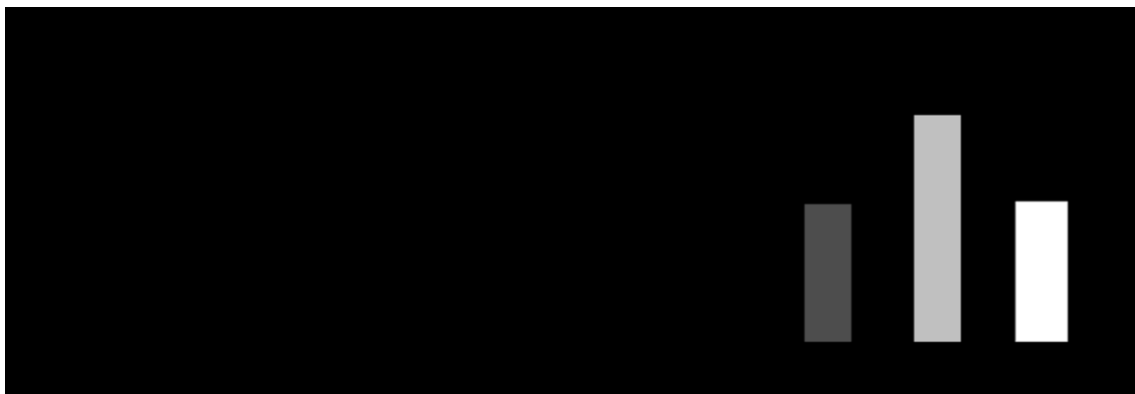


Fig.24: Liver steatosis leads to up-regulation of Chop mRNA in human. Patients with symptoms for NAFL display an increase in Chop mRNA expression levels. a indicates a significant difference from control patients, b indicates a significant difference from NAFLD patients; $P < 0,05$.

Subsequently, we fed ob/ob mice with MCD diet for 3 weeks in order to generate a fatty liver. As expected, mice on MCD diet developed liver injury as shown by ALT levels increase (Fig.25). MCD fed ob/ob mice had lower serum cholesterol and glucose but increased bile acid levels as well as hepatic Chop mRNA expression (Fig.25).

Since Chop is a repressor of C/ebp α , (a key player in hepatic lipid metabolism) and BAs activate FXR, we next investigated the role of FXR in fatty liver development and Chop mRNA regulation.

	<i>ob/ob</i> Control	<i>ob/ob</i> MCD
ALT [U/L]	80+/-12	246+/-59 ^a
Tot. Chol [mg/dl]	226+/-15,73	106+/-13,80 ^a
Glucose [mg/dl]	254,6+/-36,55	147,3+/-38,53 ^a
Bile acid [μmol]	32,40+/-5,95	89,47+/-19,12 ^a

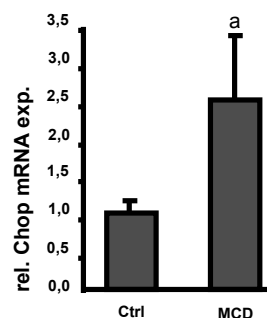


Fig.25: Liver steatosis leads to up-regulation of Chop mRNA in mice *ob/ob* mice fed a MCD diet for 3 weeks showed increased serum levels of ALT and bile acids as well as increase in Chop mRNA expression. Total cholesterol and Glucose were decreased due to MCD feeding in *ob/ob* mice. a indicates a significant difference from untreated control mice (Ctrl); P<0,05

Upregulation of Chop mRNA is abolished in FXR KO mice fed a MCD diet.

Female C56BL/6 and FXR KO mice were fed a MCD diet for 5 weeks to induce fatty liver. Liver injury in MCD fed mice was obtained independently of the genotype as shown by increased levels of serum ALT, AP, AST (Fig.26A). and BA, while cholesterol, free fatty acids (FFA) and glucose were decreased in WT and FXR KO mice upon MCD (Fig.26A).

Compared to control mice, MCD fed WT mice showed a drastic increase in mRNA expression levels of Chop, an effect abolished in MCD fed FXR KO. In line with this results, *C/ebpα* was only down-regulated in WT MCD fed mice but not in FXK KO(Fig.26B).

Moreover, Chop mRNA expression was increased in human immortalized primary hepatocytes cultivated in a MCD medium after incubation with the FXR agonist INT747 (Fig.26C). Taken together these data suggest that Chop mRNA expression is regulated by FXR.

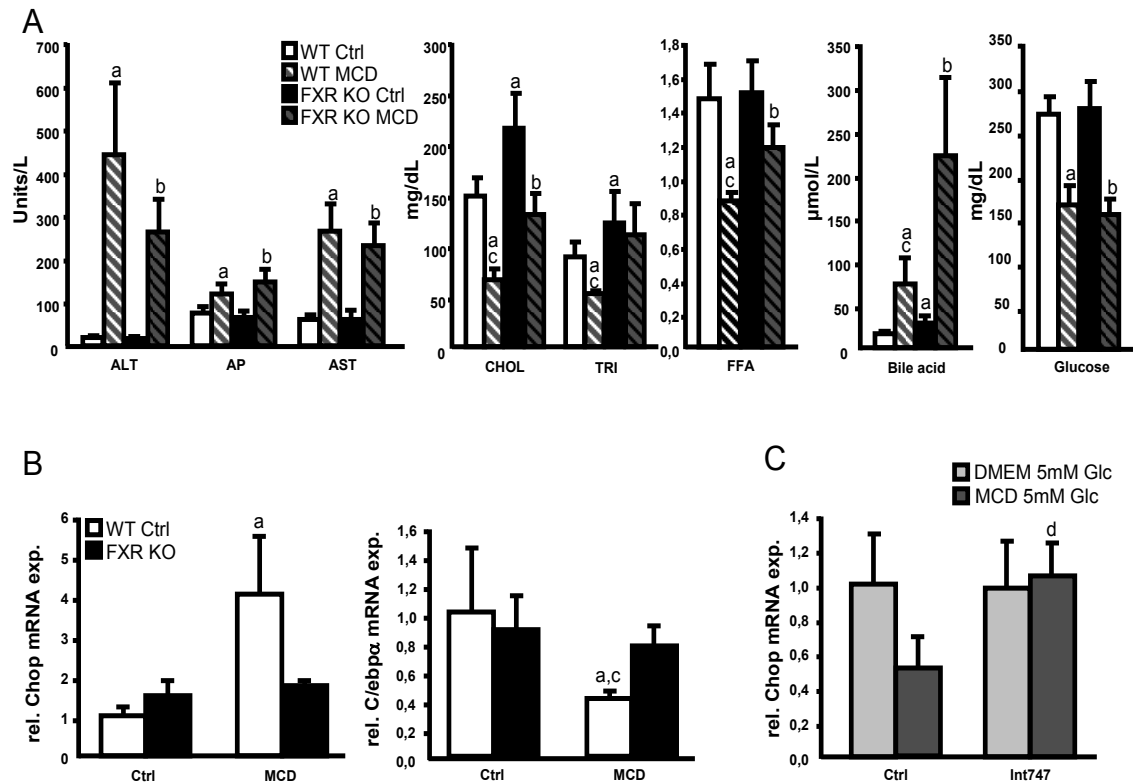


Fig.26: Up-regulation of Chop mRNA is abolished in FXR KO mice fed a MCD diet

(A) WT and FXR KO mice fed a MCD diet for 5 weeks exhibit an increase in serum levels of AST, AP, ALT and bile acids. Total cholesterol and glucose levels were decreased in the presence of MCD independent of the genotype. Serum TG levels were decreased in MCD fed WT mice and not affected in challenged FXR KO mice compared to the control groups. (B) mRNA expression levels of Chop were up-regulated in WT mice fed a MCD diet but not in FXR KO treated mice. C/ebpα mRNA expression levels were decreased in WT mice upon MCD and not changed in fed FXR KO mice compared to control groups. a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; P<0,05. (C) Human immortalized primary hepatocytes (IHH) cells were cultivated either in DMEM with 5mM Glucose (Glc) or in MCD medium with 5mM Glc with or without the FXR agonist Int747 for 12h. mRNA expression levels of Chop were significantly increased in IHH cells treated with MCD medium, 5mM Glc and Int747. *In vitro* experiment was performed in triplicates. d indicates a significant difference from untreated control cells (Ctrl); P<0,05.

Increased hepatic lipid accumulation in MCD fed WT mice. Since *C/ebp α* regulates hepatic lipid homeostasis, we next performed liver histology analysis (Fig.27A) and hepatic triglyceride levels measurement (Fig.27B). Interestingly, only MCD-fed WT mice displayed a significant increase in hep. TG levels compared to control WT mice (Fig.27)

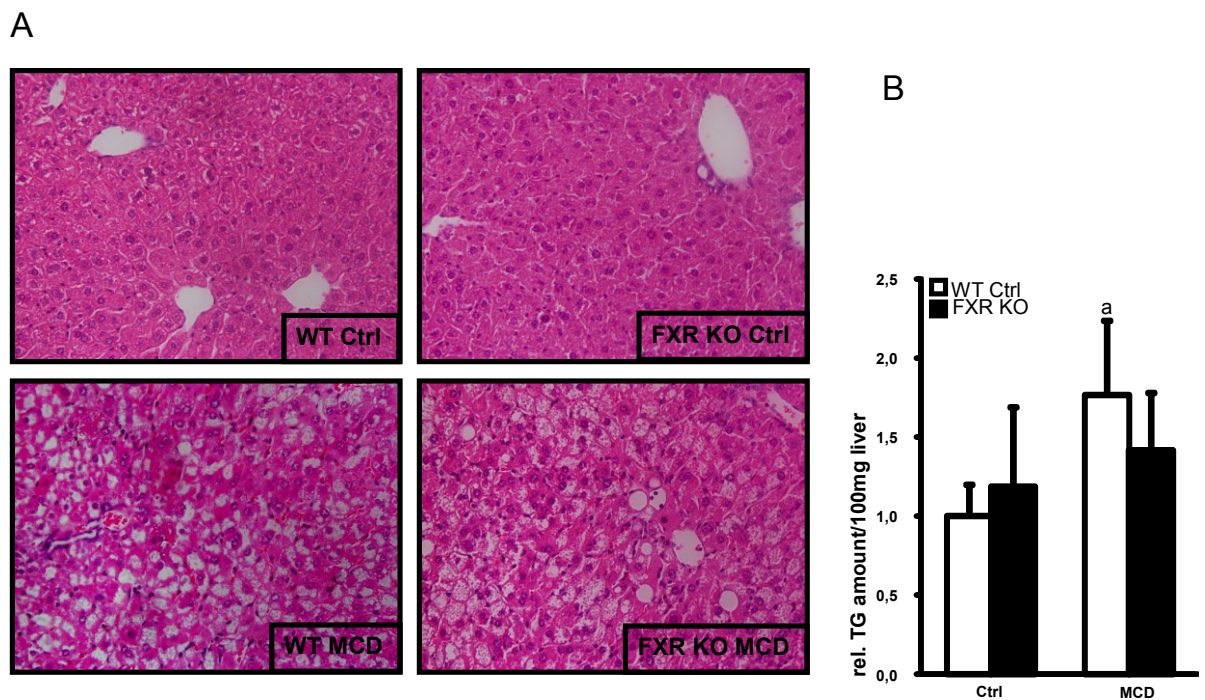


Fig.27: Increased hepatic lipid accumulation in MCD fed WT mice. WT and FXR KO mice were fed a MCD diet for 5 weeks. (A) Hepatic steatosis appeared in WT MCD mice but not in MCD fed FXR KO mice. (H&E staining; 20x magnification). (B) Livers of WT MCD mice showed a significant increase of hepatic TG. A indicates a significant difference from untreated WT controls (Ctrl); $P < 0,05$.

Impaired de novo lipogenesis, β -oxidation and VLDL secretion in FXR WT mice fed MCD diet. To determine how FXR impacts on the regulation of hepatic lipid metabolism, we next measured gene expression of key players controlling *de novo* lipogenesis (Srebp1c, Fasn, Scd1 and Acc2) (Fig.28A).

MCD feeding in both, WT and FXR KO mice reduced their compared to control groups albeit to a greater extent in WT mice (Fig.28A). Ppar α and Acox mRNA expression levels were then measured as indicators for FA oxidation. Both are down-regulated in mice treated with MCD diet independently of the genotype, with Acox expression levels being even more repressed in MCD-fed WT mice (Fig.28B). Taken together, these data suggest that FXR deficiency impacts negatively on the regulation of fatty acid metabolism.

Although Mttp and ApoB – two key genes involved in VLDL synthesis – are not changed at mRNA level under MCD challenge in both the genotypes (Fig.29A), serum VLDL concentration in WT MCD mice was reduced compared to control group as well as compared to FXR KO MCD mice (Fig.29B). This goes in line with the drastic increase of VLDL receptor mRNA levels discovered in WT MCD fed mice (Fig.30).

Together, these data suggest that WT mice exposed to MCD diet accumulate higher amounts of hepatic lipids due to increased uptake of remnant VLDL particles.

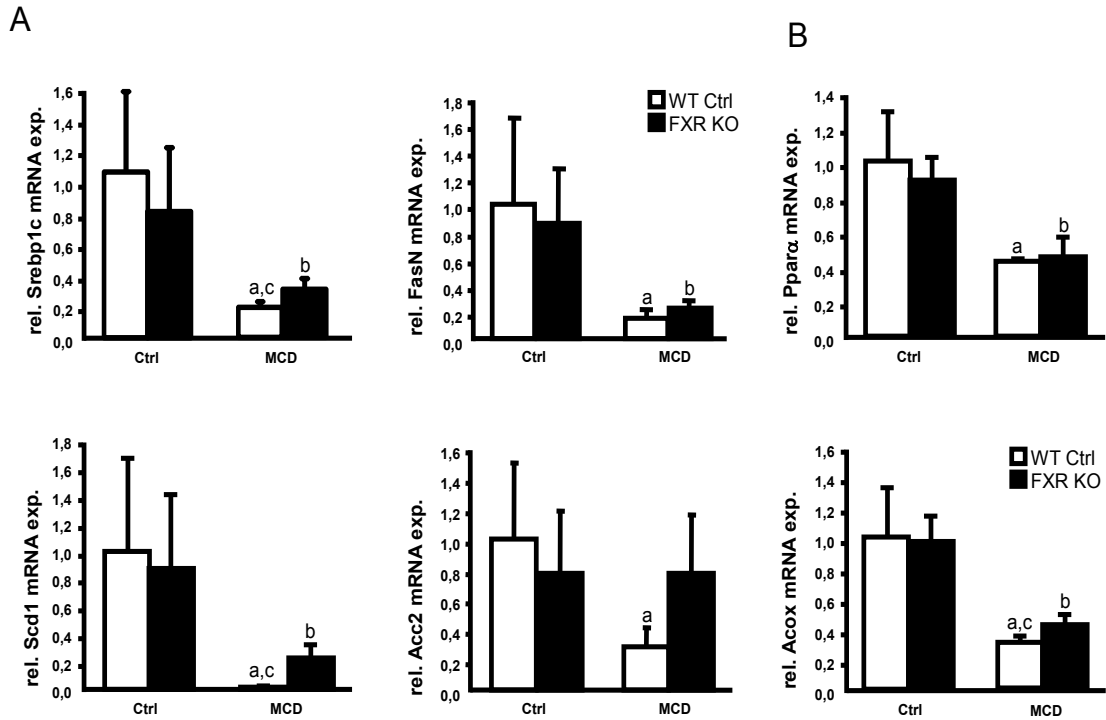


Fig.28: Impaired *de novo* lipogenesis, β -oxidation in FXR WT mice fed MCD diet.

WT and FXR KO mice were fed a MCD diet for 5 weeks. Real Time PCR was used to assess (A) mRNA levels of Srebp1c, FasN, Scd1 and Acc2. (B) Ppar α and Acox. (A) mRNA levels of Srebp1c, FasN, Scd1 and Acc2 were downregulated due to MCD feeding in WT and FXR KO mice upon MCD but to a greater extent in MCD fed WT mice. (B) mRNA levels of Ppar α and Acox were down-regulated in both, WT and FXR KO mice fed a MCD diet for 5 weeks. In term of Acox, mRNA levels of MCD fed WT mice are even more decreased than in MCD fed FXR KO mice. a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; P<0,05.

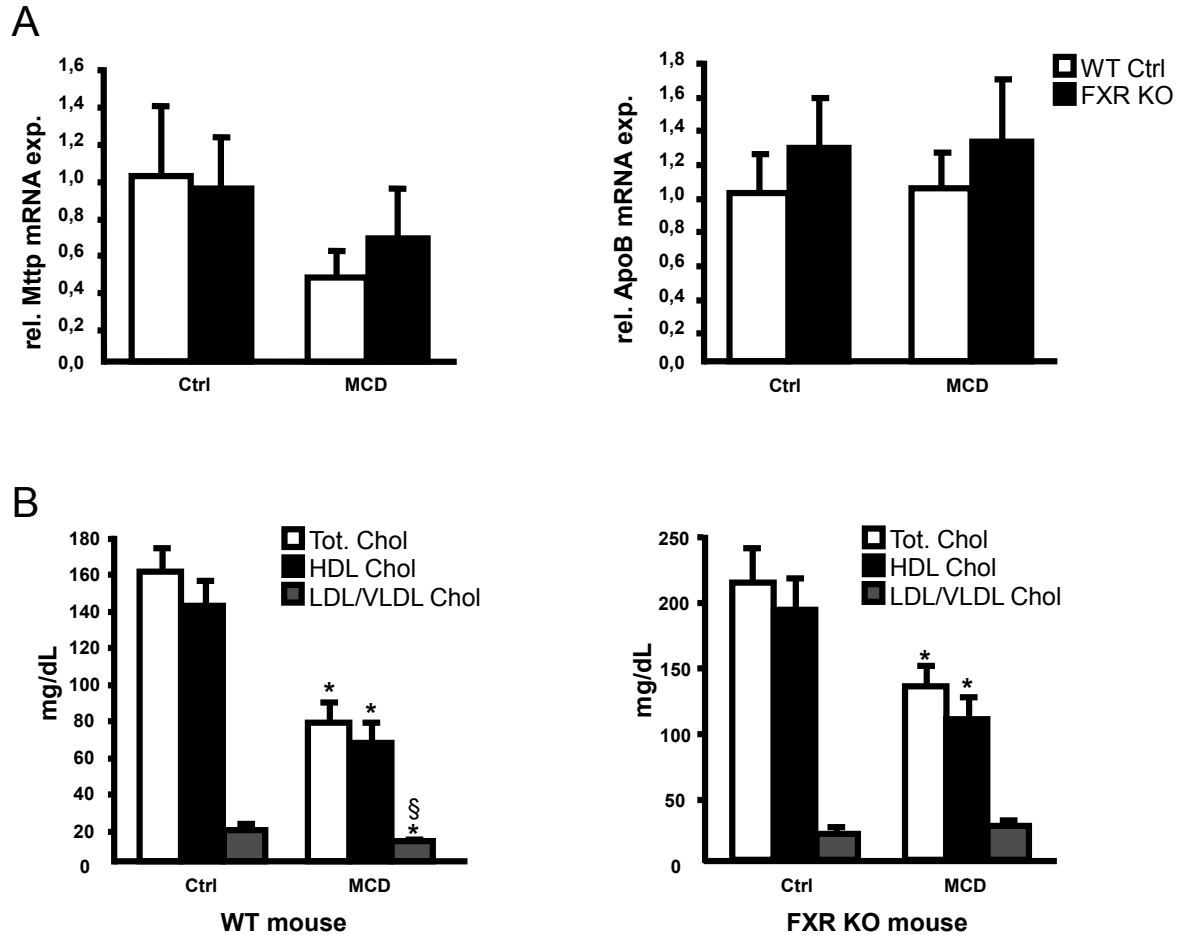


Fig.29: Impaired VLDL synthesis and excretion in FXR WT mice fed MCD diet. WT and FXR KO mice were fed a MCD diet for 5 weeks. Real Time PCR was used to assess (A) mRNA levels of Mttp and ApoB. Both, Mttp and ApoB expression levels were not impaired by MCD feeding in any genotype. Data represent means $\pm$ SD from four to six animals. 18sRNA was used as a housekeeping gene. (B) Serum levels of total Cholesterol, HDL cholesterol and LDL/VLDL cholesterol were assessed. MCD feeding decreased total cholesterol and HDL cholesterol in both, WT and FXR KO mice, whereas VLDL cholesterol were down-regulated in WT MCD fed mice only. * indicates a significant decrease from corresponding control groups; $P < 0,05$.

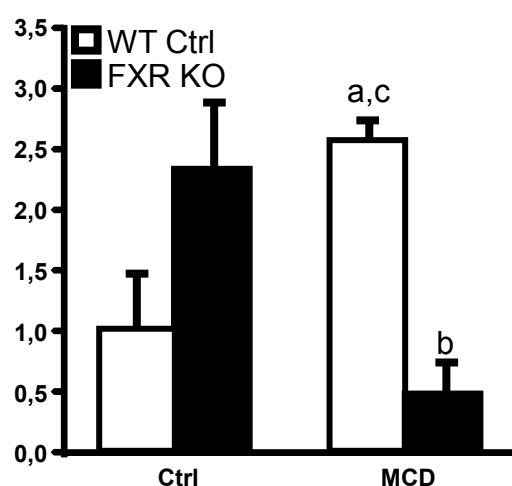


Fig.30: VLDL receptor expression is up-regulated in MCD fed WT mice. Real Time PCR was used to assess mRNA levels VLDL receptor. Expression levels were increased by MCD feeding in WT MCD fed mice. a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; $P < 0.05$.

FXR WT mice challenged with MCD diet do not develop inflammation.

Since hepatic lipid accumulation could initiate inflammation, we next examined whether WT MCD fed mice develop hepatitis. Interestingly, mRNA expression levels of inflammation markers such as F4/80 and $Tnf\alpha$ were up-regulated in FXR KO MCD mice but not changed in WT mice after MCD feeding (Fig.31), therefore suggesting an anti-inflammatory effect of FXR under MCD feeding.

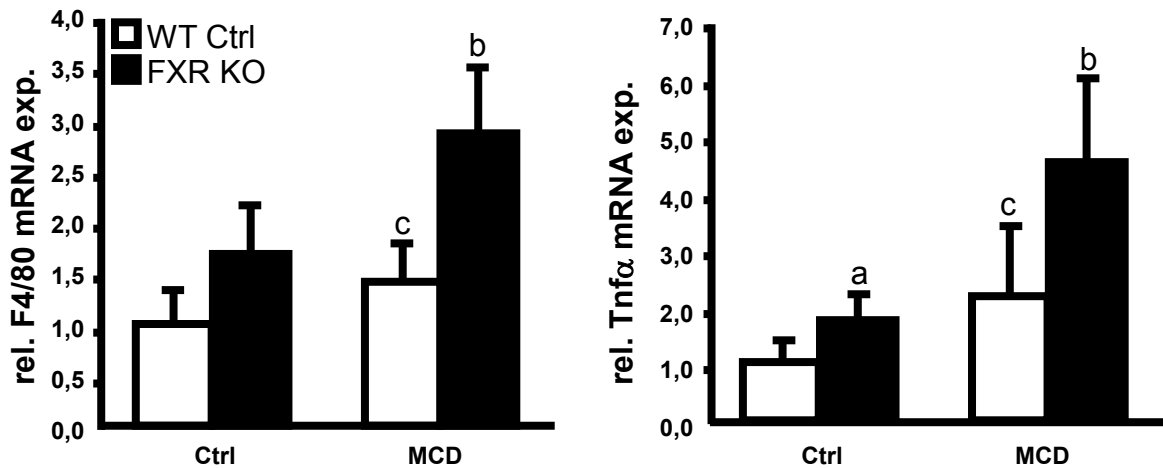


Fig.31: WT MCD fed mice do not develop hepatic inflammation. Real Time PCR was used to assess mRNA levels inflammation markers. Expression levels were increased by MCD feeding in FXR KO MCD fed mice. a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; $P < 0,05$.

Bile acid and low glucose regulate Chop mRNA expression transcriptionally in HepG2 cells via FXR and RXR. To understand how high BA and low glucose levels – which were found in both, ob/ob and FXR mouse models fed a MCD diet – are signalling via FXR and consecutively regulate Chop and C/ebp α mRNA expression, we treated HepG2 cells with low and high dose of glucose and CDCA. Bile acid incubation at low glucose concentrations increased Chop expression, an effect lost at high glucose concentration (Fig.32A).

FXR can form heterodimers RXR or monomers to bind FXR response element. To determine whether FXR regulates Chop expression as heterodimer or monomer, we treated HepG2 cells with the RXR agonist 9-cis-retinoic acid. As shown in Fig.29B RXR activation enhanced Chop mRNA expression levels in absence or presence of CDCA. In line, mRNA levels of C/ebp α were decreased in cells treated with 9-cis-retinoic acid and or CDCA.

To determine whether the regulation involved transcriptional or translational mechanism we co-treated HepG2 cells with low glucose, CDCA and ActinomycinD (ActD) (Fig.32C). Interestingly, Chop mRNA induction was blunted in low glucose, CDCA and ActD treated cells. Next, to understand whether Chop induction by low glucose and CDCA required neosynthesis of protein(s) we treated cells with low glucose, CDCA and Cycloheximide (CHX) (Fig.32C). Chop mRNA expression in CHX co-treated cells was decreased but not abolished indicating that the regulation of Chop was mainly not requiring neosynthesis of protein(s). In silico analysis of the Chop promoter revealed one *bona fide* FXR/RXR response element using MatInspector. Next, we performed a chromatin immunoprecipitation (ChIP) assay to determine where FXR and RXR could bind the promoter of Chop. Fig.34D showed that in low glucose conditions FXR binds in a limited manner the promoter whereas RXR is highly bound to it. After CDCA incubation, FXR binding to the promoter was significantly increased. Taken together, these data showed that FXR/RXR binds to the Chop promoter and mediate transcriptional regulation of Chop expression mainly in a direct manner.

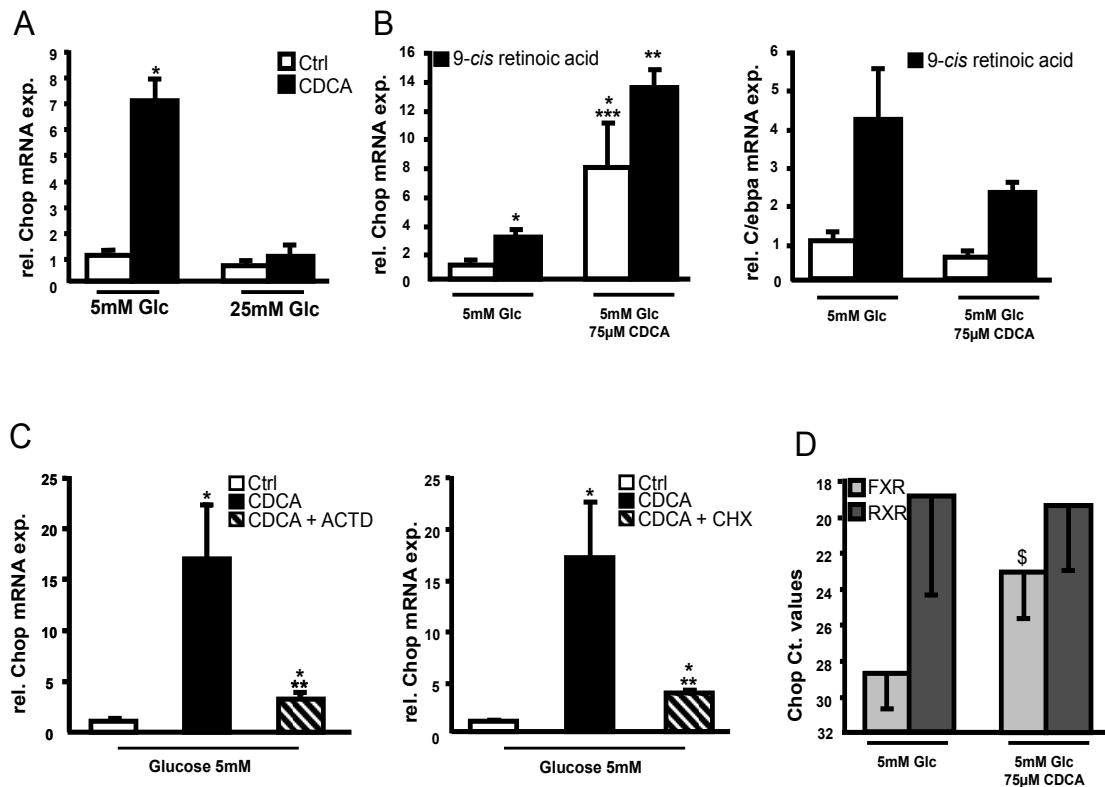


Fig.32: Bile acid and low Glucose regulate Chop mRNA expression transcriptionally in HepG2 cells via FXR and RXR. Human derived HepG2 cells were cultured in DMEM medium without fetal calf serum and 5mM or 25mM Glucose (Glc) with and without 75μM CDCA for 12h. Chop mRNA expression levels were drastically up-regulated in cells treated with 5mM Glc and 75μM CDCA compared to cells treated with 5mM Glc only. Whereas in cells treated with high dose of Glucose with and without CDCA Chop expression was not affected. (B) HepG2 cells were cultured in DMEM medium without fetal calf serum and treated with low dose of Glc (5mM), 75μM CDCA with and without 9-cis retinoic acid for 12h. Chop mRNA levels were upregulated in HepG2 cells treated with 9-cis retinoic acid compared to control groups. C/ebpα expression levels was down-regulated in cells treated with 5mM Glc and 9-cis retinoic acid and in cells treated with 25mM Glc and 75μM CDCA with and without 9-cis retinoic acid. *In vitro* experiments were performed in triplicates. * indicates a significant difference from 5mM Glc treated control cells (Ctrl); ** indicates a significant difference from cells treated with 5mM Glc and 9-cis retinoic acid; *** indicates a significant difference from cells treated with 5mM Glc, CDCA and 9-Cis retinoic acid $P < 0.05$. (C) HepG2 cells were cultured in DMEM without fetal calf serum treated with low dose of Glc (5mM) and 75μM CDCA with and without Actinomycin (ActD) and Cycloheximide (CHX). Chop mRNA levels were upregulated in cells treated with 5mM Glc and CDCA compared to control cells (Ctrl) and decreased in cells treated

with CDCA and ActD or CHX. *In vitro* experiment was performed in triplicates. * indicates a significant difference from 5mM Glc treated control cells (Ctrl); ** indicates a significant difference from cells treated with 5mM Glc and 75 μ M CDCA. $P < 0,05$. (D) HepG2 cells were cultured in DMEM without fetal calf serum treated with 5mM Glc with and without 75 μ M CDCA. Cells harvested after 6h of treatment and Chromatin immunoprecipitation performed with antibodies against FXR and RXR. DNA precipitated was measured by real time PCR. Chop promoter was bound by high levels of RXR and low FXR in samples incubated with low glucose, whereas low glucose and CDCA increased FXR binding. \$ and # indicates a significant difference from corresponding control group; § indicates a significant difference from cells where FXR antibody was used $P < 0,05$.

4.3 Role of BSEP in hepatic lipotoxicity

BSEP KO mice are protected from hepatic inflammation in methionine choline deficient diet induced NASH

(Manuscript in preparation)

RESULTS

Absence of BSEP partially protects against hepatic steatosis. After 5 weeks of feeding, serum aspartate amino transferase (AST) and alanine amino transferase (ALT) levels as well as serum bile acid (BA) concentrations were increased in both genotypes in response to MCD diet (Fig.33A). Notably, serum alkaline phosphatase (AP) levels remained unchanged indicating that MCD feeding induced liver damage and cholestasis with BA accumulation mainly at the hepatocellular and not cholangiocellular level. In addition, serum total cholesterol (CHOL), triglycerides (TG) and free fatty acids (FA) were lowered by MCD feeding, with TG and free FA being most reduced in WT mice (Fig.33A). Liver histology of MCD fed BSEP KO mice showed a milder accumulation of lipids compared to MCD WT mice (Fig.33B). In line, MCD challenged WT mice had more than 1,5-fold increase in hepatic lipid accumulation compared to WT mice on chow diet (Fig.33C), whereas fed BSEP KO mice showed no significant differences in lipid accumulation compared to their control group. Taken together, our data demonstrate that compared to WT mice, BSEP KO are partially protected against fatty liver in response to MCD diet.

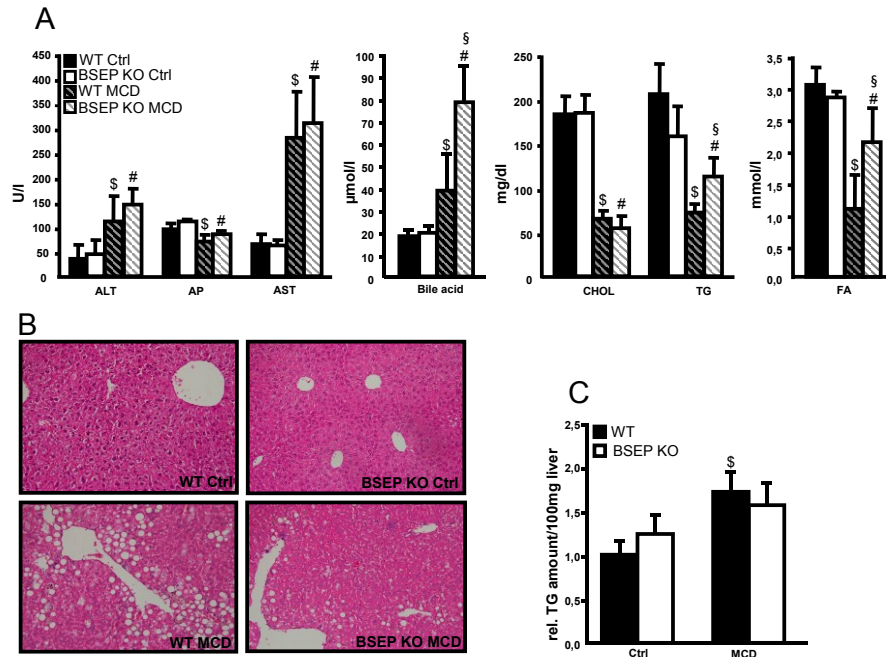


Fig.33: WT and BSEP KO have different liver damage after MCD feeding. FVB/N WT and BSEP KO mice received methionine choline deficient (MCD) diet for 5 weeks. (A) MCD diet induced cholestasis as shown by increased liver enzymes (AST; ALT) in both wild type (WT) and BSEP knockout (KO). Bile acid levels increased markedly more in BSEP KO after MCD feeding than in WT mice. Serum cholesterol (CHOL), TG and FA were significantly reduced after MCD challenging, independently of the genotype. (B) Hepatic steatosis developed in WT and to a lesser extent in BSEP KO mice after 5 weeks of MCD treatment (H&E staining, 20x magnification). (C) After 5 weeks of MCD exposure, livers of FVB/N WT showed a significant increase of hepatic TG. \$ indicates a significant difference from untreated WT controls (Ctrl); § indicates a significant difference from MCD fed WT; # indicates a significant difference from BSEP KO Ctrl; $P < 0,05$

MCD attenuates VLDL synthesis and catabolism. Since hepatic FAs are necessary to generate VLDL and increased VLDL secretion can counteract fatty liver development, we therefore next determined serum VLDL levels. Serum total-, HDL- and VLDL-cholesterol levels were drastically reduced in MCD challenged mice (Fig.34A). We therefore investigated mRNA expression levels of *Mttp* and *ApoB* (Fig.34B), two key genes involved in VLDL synthesis. While *Mttp* levels were not changed upon MCD feeding in WT and BSEP KO mice, *ApoB* mRNA expression was decreased in both the groups (Fig.34B). As VLDL and TG are metabolized *via* the lipoprotein lipase (LPL), we next measured LPL activity together with mRNA expression levels of its activator *ApoCII*. Notably LPL activity and *ApoCII* expression levels were decreased in both WT and BSEP KO MCD fed mice (Fig.34C).

Together these data suggest that MCD treatment impaired VLDL synthesis and catabolism in a similar manner in both WT and BSEP KO mice, therefore not accounting for the mild but selective hepatic lipid accumulation in WT mice exposed to MCD diet.

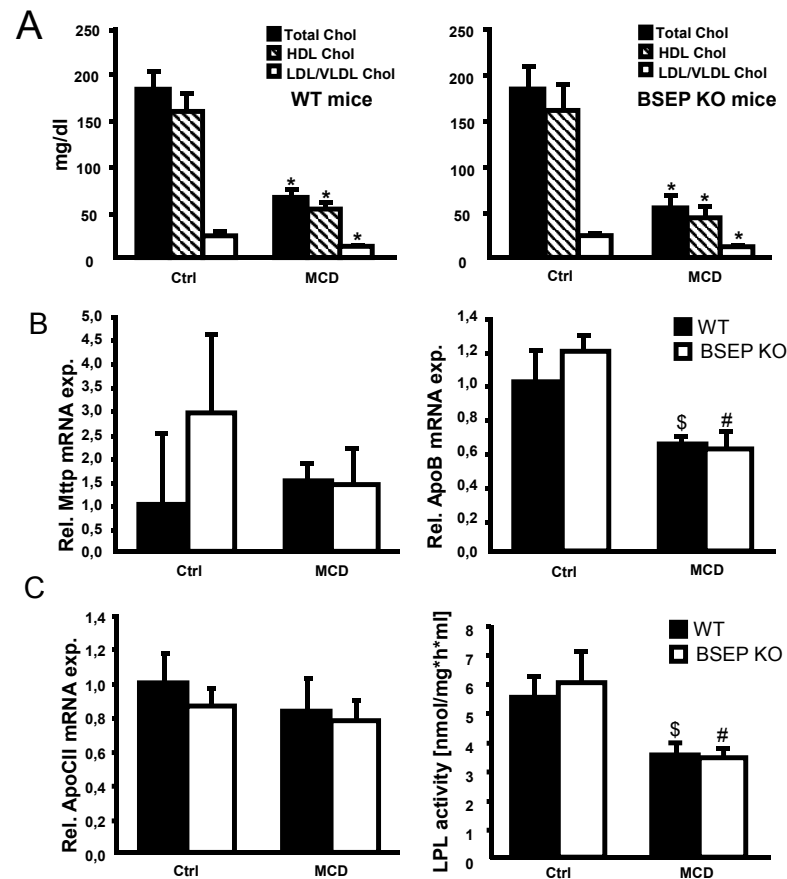


Fig.34: MCD attenuates VLDL synthesis and LPL activity. (A) Both WT and BSEP KO have reduced serum levels of total cholesterol, HDL- and VLDL- cholesterol. Real time PCR was used to measure the expression of (B) Mttp and ApoB – two key genes involved in VLDL synthesis. Whereas Mttp expression was not changed, ApoB gene expression was reduced by half in WT and BSEP KO MCD fed mice. (C) mRNA expression of ApoCII was measured by real time PCR. MCD feeding did not affect ApoCII expression in both, WT and BSEP KO mice. LPL activity was measured as described in Materials & Methods. LPL activity was decreased due to MD challenge, independent of the genotype. Data represent means $\pm$ SD from four to six animals and are shown relative to expression level in wild type unchallenged animals. * indicates a significant difference from correspondve control; \$ indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from BSEP KO Ctrl; $P < 0,05$

Disturbances in hepatic lipid metabolism in mice fed MCD. To determine whether differences in hepatic steatosis after MCD feeding might be due to differences in *de novo* lipogenesis, FA β oxidation and/or fatty acid transport, we measured hepatic Srebp1c, FasN and Scd1 mRNA (Fig.35A) as markers for *de novo* lipogenesis, Ppar α , Aox, Cpt1 α and Acc2 mRNA levels (Fig.35B) as markers for β -oxidation and Fatp5 and Fabp1 mRNA expression (Fig.35C) as indicator of hepatic fatty acid transport. mRNA expression levels of Srebp1c – the master regulator of *de novo* lipogenesis – and its downstream targets FasN and Scd1 were more pronouncedly down-regulated in BSEP KO mice after MCD feeding than in WT. Both, WT and BSEP KO mice challenged with MCD for 5 weeks showed lower mRNA levels for Ppar α , Aox, Cpt1 α and Acc2 (Fig.35B). Although PPAR α expression was significantly more reduced in BSEP KO than in WT MCD fed (Fig.35B), its direct target genes Cpt1 α and Aox were not different reflecting a preserved β -oxidation capacity independent of the genotype. However, as shown in Fig.35C, Fatp5 and Fabp1 were drastically lowered in MCD fed BSEP KO mice compared to their control group and MCD fed WT mice. All together, these findings suggest that MCD fed WT mice develop a higher grade of steatosis compared to BSEP KO mice as a result of elevated fatty acid transport and *de novo* lipogenesis.

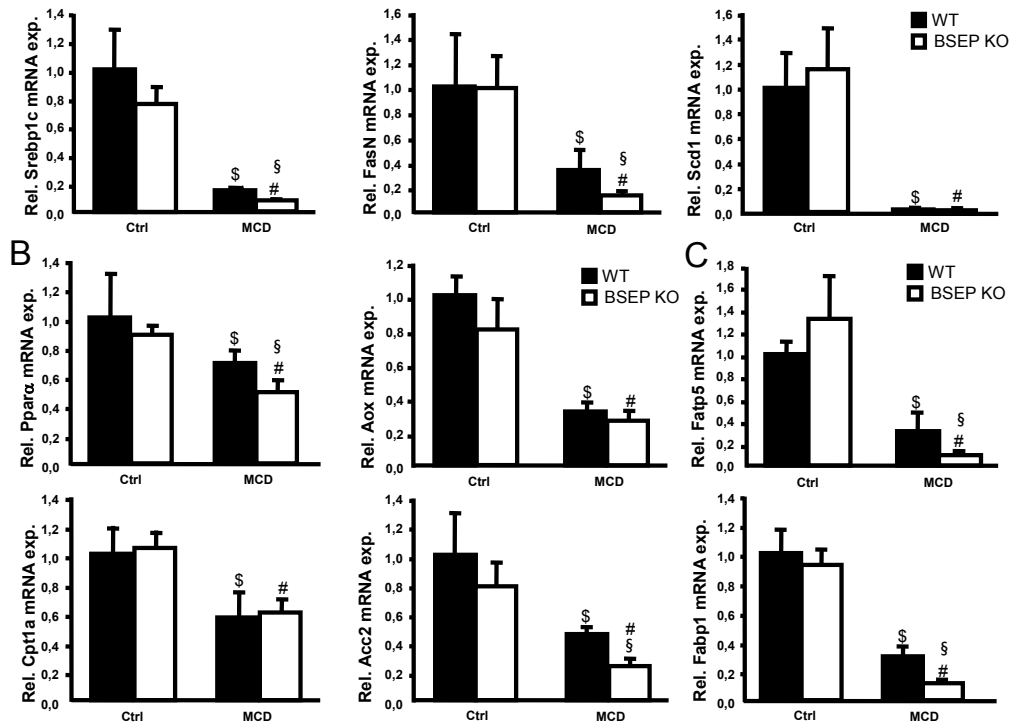


Fig.35: Disturbances in hepatic lipid metabolism in mice fed MCD. Real time PCR was used to measure the expression of (A) the *de novo* lipogenesis genes Srebp1c, FasN and SCD1, (B) β Oxidation markers Ppar α , Aox, Cpt1 α and Acc2 and (C) fatty acid transporters Fatp5 and Fabp1. Independently of the genotype, *de novo* lipogenesis, β -oxidation and fatty acid transport were generally down-regulated in mice exposed to methionine choline deficient (MCD) diet but to a greater extend in BSEP KO mice. \$ indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from MCD fed WT; $ indicates a significant difference from BSEP KO Ctrl; P<0,05

BSEP KO mice are protected from MCD induced inflammation. We next explored the susceptibility of BSEP KO mice challenged with a MCD diet for 5 weeks to hepatic inflammation. In line with our hypothesis, MCD fed BSEP KO mice displayed no changes in mRNA levels of $Tnf\alpha$ and $Mcp1$ compared to control mice (Fig.36A), whereas $Tnf\alpha$ and $Mcp1$ expression were increased in WT mice. Notably, $F4/80$ and $iNos$ mRNA levels even decreased in BSEP KO mice after MCD feeding (Fig.36A). To exclude the possibility that inflammation might result from modifications in apoptosis and/or ER stress, we measured bax mRNA expression, a marker for apoptosis, and the ER stress marker $Grp78$. Both genes were not affected by MCD feeding in WT and BSEP KO mice (Fig.36B). These data demonstrate that BSEP KO mice fed with MCD diet are protected from hepatic inflammation, as indicator of NASH.

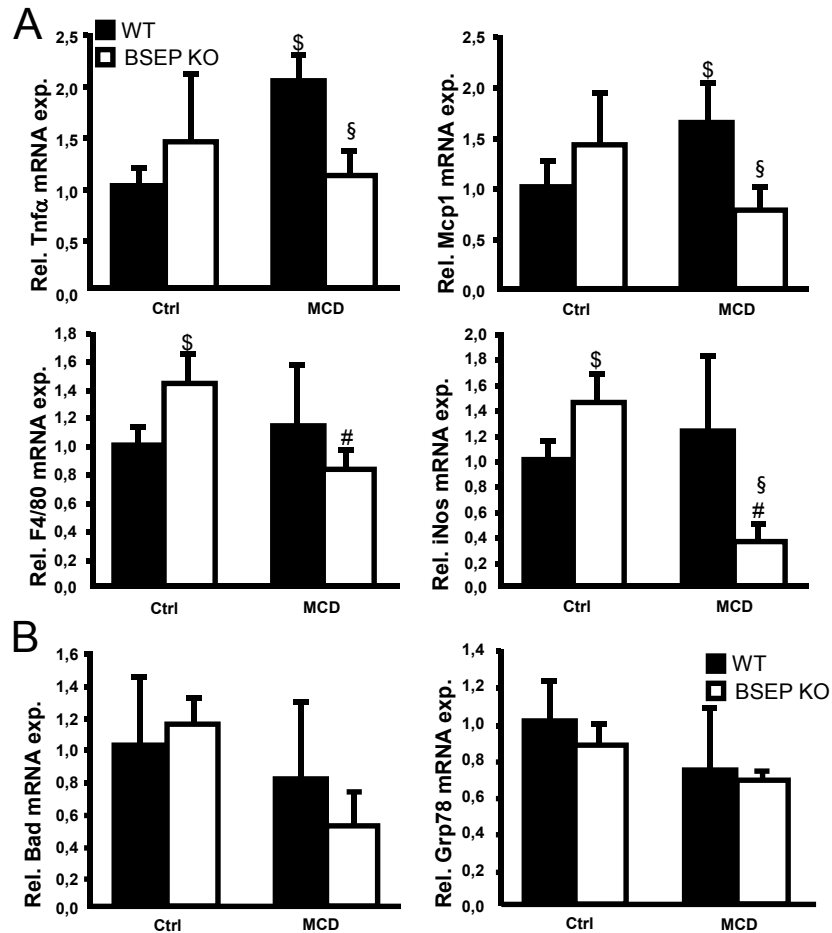


Fig.36: BSEP KO mice are protected from MCD induced inflammation. Real time PCR was used to measure the expression of (A) inflammation markers $TNF\alpha$, $Mcp1$, F4/80 and iNos. (B) and markers for apoptosis and ER stress Bad and Grp78, respectively. WT mice challenged with methionine choline deficient (MCD) diet showed increase in mRNA levels of indicators for inflammation, whereas MCD fed BSEP KO mice did not, despite more iNos and F4/80 in BSEP KO than WT mice at the basal level. MCD feeding did not increase apoptosis and/or ER stress in WT and BSEP KO mice. \$ indicates a significant difference from untreated WT controls (Ctrl); § indicates a significant difference from MCD fed WT; # indicates a significant difference from BSEP KO Ctrl; $P < 0,05$

Down-regulation of BA synthesis in BSEP KO mice fed a MCD diet. We next investigated whether changes in bile acid synthesis could contribute differences in fatty liver injury and inflammation in MCD fed WT and BSEP KO mice. Therefore, we measured intestinal FGF15 and hepatic Cyp7a1 mRNA expression (Fig.37A and B). FGF15 which is known to negatively regulate Cyp7a1, the rate limiting enzyme in BA synthesis, was significantly down-regulated in intestine of BSEP KO mice at the baseline level as well as under MCD feeding. This goes in line with the up-regulation of Cyp7a1 seen in BSEP KO control and MCD mice. The decrease of Cyp7a1 in BSEP KO MCD fed mice compared to their control could be an effect of the MCD treatment and indicates activation of the nuclear transcription factor farnesoid X receptor (FXR). Taken together, these data suggest that in MCD fed BSEP KO mice BA synthesis is up-regulated compared to challenged WT mice and therefore does not explain the hepatic protection observed against inflammation.

The low intrahepatic bile acid concentration found in MCD fed WT mice (Fig.37C) goes in line with the decreased Cyp7a1 expression in WT mice after challenging with MCD. Beside total hepatic bile acid concentration, also the bile composition is changed due to MCD feeding. MCD fed WT mice have more hydrophobic (potentially toxic) bile acids than hydrophilic (non-toxic) bile acids, shown in Fig.37D as ratio between hydrophilic and hydrophobic bile acids. These data suggest that the higher amount of toxic bile acids in livers of WT MCD mice make these mice more susceptible to inflammation.

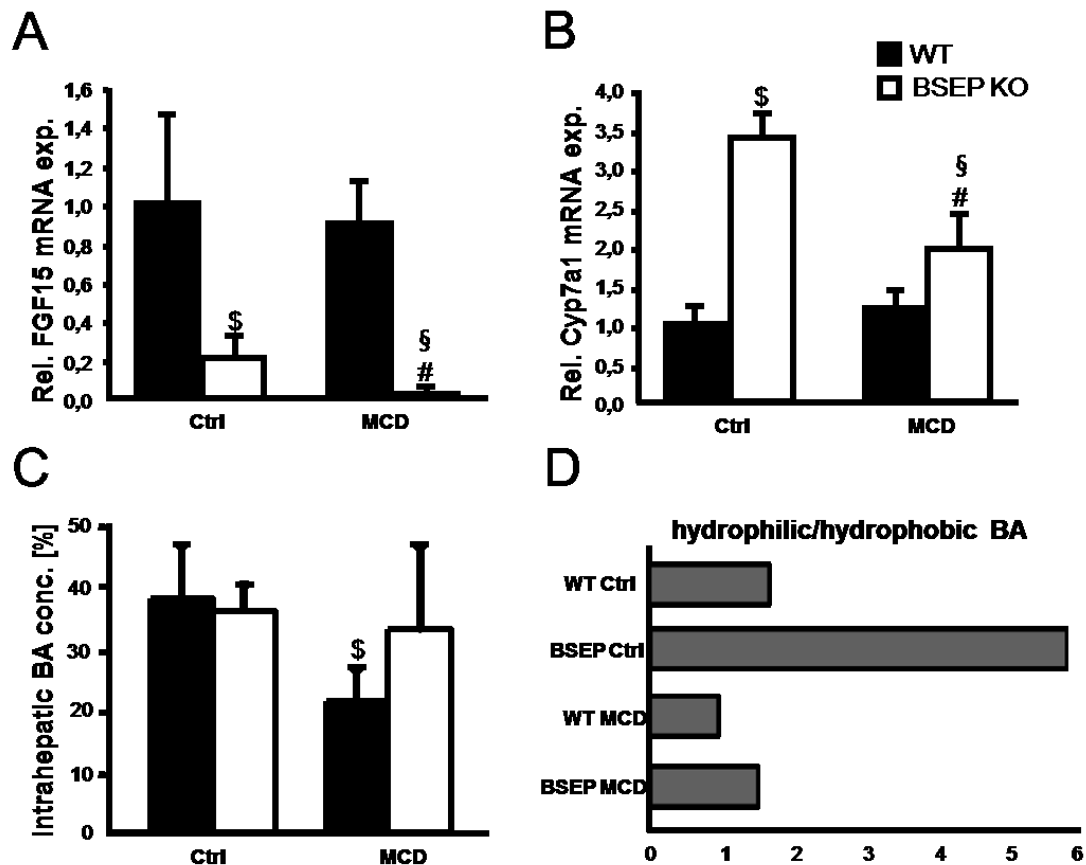


Fig.37: Downregulation of BA synthesis in BSEP KO mice fed an MCD diet. (A) Real time PCR was used to measure the expression of intestinal FGF15 mRNA levels and (B) hepatic Cyp7a1 mRNA levels as markers for bile acid synthesis. Both genes were down-regulated in BSEP KO mice exposed to MCD diet. (C) Intrahepatic bile acid concentration was measured as described in material and methods. While MCD feeding did not impact hepatic bile acid concentration in BSEP KO mice, it lead to a 15% reduction of the hepatic bile acid pool in WT MCD mice compared to corresponding controls. (D) MCD feeding lead also to a change of the hepatic bile composition, shown by the ratio of hydrophobic versus hydrophilic bile acids. \$ indicates a significant difference from untreated WT controls (Ctrl); # indicates a significant difference from MCD fed WT; # indicates a significant difference from BSEP KO Ctrl; P<0,05

Bile acid uptake and alternative sinusoidal transport is changed in BSEP KO mice fed a methionine choline deficient (MCD) diet. To determine whether bile acid transport is changed by MCD feeding, we screened for mRNA expression levels of Ntcp, a bile acid transporter involved in BA uptake, Mrp2 and Mdr1a the canalicular transporters and Mrp4, Ost β and Mrp3 the transporters responsible for bile acid excretion into the blood. In response to MCD feeding Ntcp mRNA expression was more down-regulated in BSEP KO than in WT mice (Fig.38A), whereas Mrp2 and Mdr1a mRNA levels were not affected (Fig.38B). Interestingly, mRNA expression levels of Mrp4 and Ost β were up-regulated in BSEP KO but not in WT mice upon MCD feeding (Fig.38C). Mrp3 was decreased in both WT and BSEP KO MCD fed mice. mRNA data were confirmed at the protein level, Ntcp protein was decreased in BSEP KO mice challenged with MCD compared to WT mice, whereas Mrp4 was increased in MCD fed BSEP KO mice (Fig.38D). Although RNA expression levels of Mrp3 were decreased in challenged BSEP KO mice, protein levels are not affected (Fig.38D). In line with these data, we found a drastic increase of serum bile acid levels in BSEP KO MCD fed mice compared to control group and MCD fed WT mice (Fig.33A). Collectively, these results indicate that BA uptake is decreased in BSEP KO mice treated with MCD diet for 5 weeks, whereas BA excretion into the blood seems to be up-regulated. Consequently, bile acids are found in serum and therefore could contribute to the protection against hepatic inflammation.

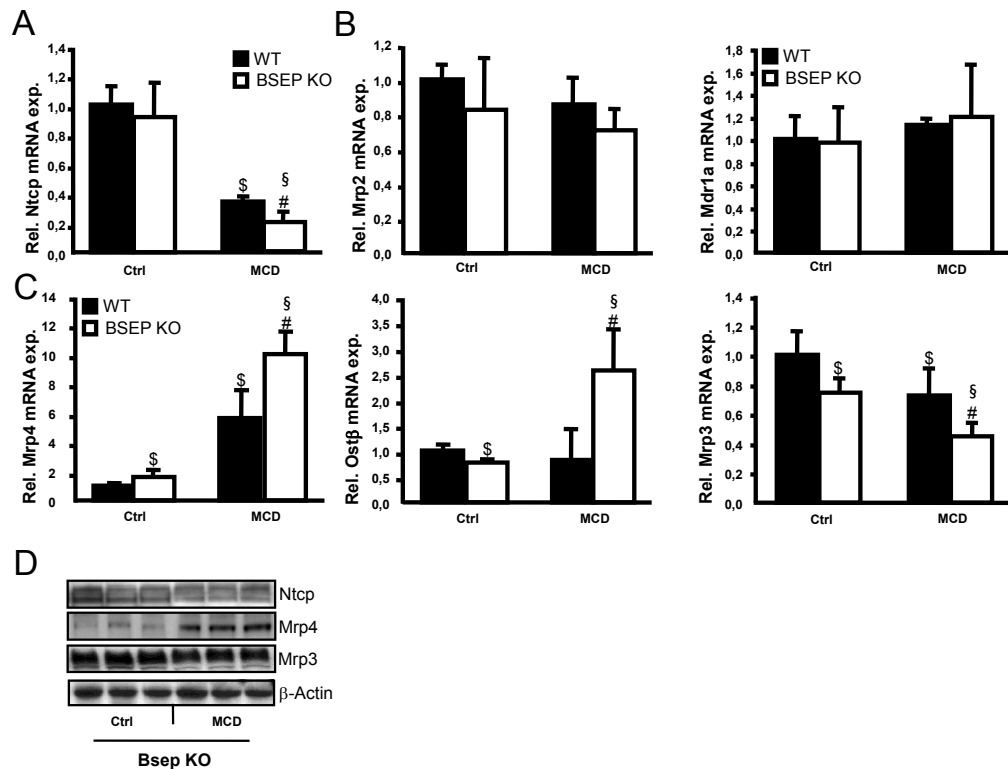


Fig.38: Bile acid uptake and alternative sinusoidal transport is changed in BSEP KO mice fed a methionine choline deficient (MCD) diet. Real time PCR was used to measure the expression of (A) Ntcp, a bile acid transporter involved in bile acid uptake from the blood, (B) Mrp2 and Mdr1a, transporters involved in canalicular export of bile acids and (C) Mrp4, Ost β and Mrp3, bile acid transporters essential for sinusoidal bile acid export. Ntcp mRNA level was down-regulated in WT mice fed a MCD diet for 5 weeks and even to a greater extent in BSEP KO mice. mRNA expression of Mrp2 and Mdr1a were not affected by MCD feeding, independently of the genotype. In contrast, Mrp4 and Ost β mRNA expression were increased in MCD fed BSEP KO compared to their corresponding control group and WT mice upon MCD. Mrp3 mRNA levels were decreased due to MCD feeding and to a greater extent in BSEP KO mice upon MCD. \$ indicates a significant difference from untreated WT controls (Ctrl); § indicates a significant difference from MCD fed WT; # indicates a significant difference from BSEP KO Ctrl; P<0,05

(D) Western blot analysis of membrane proteins using antibodies against MRP3, MRP4 and β actin was performed.

CAR, but not PXR, together with FXR protect from hepatic inflammation induced by MCD feeding. Several other nuclear receptors like CAR and PXR are involved in the regulation of bile acid homeostasis and detoxification [225]. We therefore next checked mRNA levels of downstream targets of these transcription factors. mRNA expression levels of Cyp2b10 – a target of CAR – was increased in BSEP KO mice fed a MCD diet compared to control mice (Fig.39), while there was no significant difference in mRNA levels of treated WT and WT control mice. Cyp3a11 – downstream target of PXR – was not affected by MCD feeding in any genotype (Fig.39). To summarize, these data suggest that FXR may down regulates bile acid uptake *via* Ntcp, while CAR together with FXR could up-regulate bile acid excretion into the blood *via* Mrp4 and Ost β , respectively, in mice lacking BSEP and challenged with MCD diet for 5 weeks.

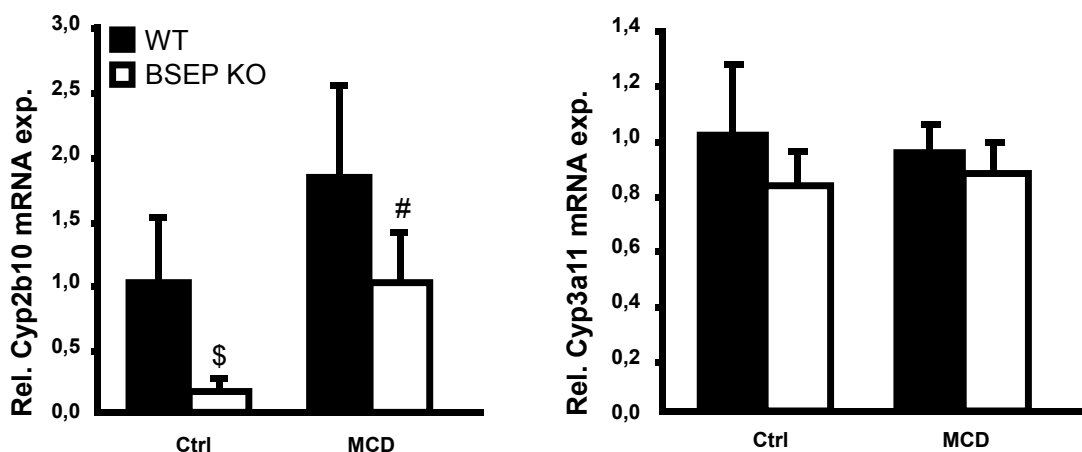


Fig.39: CAR (together with FXR) protect from hepatic inflammation induced by MCD feeding Real time PCR was used to measure the expression of (A) Cyp2b10; downstream target of CAR and Cyp3a11; downstream target of PXR. Whereas Cyp2b10 mRNA expression was increased in BSEP KO mice upon MCD feeding compared to control group and not changed in WT mice exposed to MCD, Cyp3a11 mRNA expression levels were not affected through MCD feeding in both genotypes, indicating an up-regulation of CAR activity in MCD challenged BSEP KO mice. \$ indicates a significant difference from untreated WT controls (Ctrl); § indicates a significant difference from MCD fed WT; # indicates a significant difference from BSEP KO Ctrl; P<0,05

5. DISCUSSION

In this study we investigated the function of ATGL, FXR and BSEP in the development of NAFL and in the defense against hepatic lipotoxicity.

NAFL has arisen as an intense and prevalent obesity-related disorder. The spectrum of NAFLD ranges from simple hepatic steatosis to steatosis attended by inflammation, fibrosis and necrosis.

The most used theory to explain the progression from NAFLD to NASH is the “two-hit-hypothesis”. Thereby the lipid accumulation in the liver is not effectual enough to induce the advancement to steatohepatitis. But it makes the liver more susceptible to a so called “second hit”. This “two-hit” hypothesis was first proposed by Day and James in 1998 [226]. In compliance with this theory hepatic lipid accumulation represents the first hit, which increases the sensitivity of the liver to a second hit, which in turn leads to inflammation, fibrosis and cellular death.

In the first part of this study we explored the impact of ATGL (PNPLA2) in the development of acute hepatic ER stress. The antibiotic TM is a widely used experimental tool to induce ER stress by inhibition of GlcNAc phosphotransferase (GPT), the main enzyme in the first step of glycoprotein synthesis, resulting in the induction of the UPR. Notably, absence of ATGL in KO mice *in vivo* and silencing of ATGL in hepatocytes *in vitro* protected from TM-induced hepatic ER stress and inflammation through alterations of potentially lipotoxic FA profiles and subsequent downstream modulation of phosphoinositide-3-kinase inhibitor (Pik3ip1) (Fig.40).

Serum markers for liver damage (ALT, AST, AP) and lipid parameters (CHOL, TG, FA) were comparable in WT and ATGL KO mice upon TM treatment (Fig.11A). However, hepatic inflammation markers were significantly increased in WT mice challenged with TM compared to ATGL KO TM mice (Fig.13B and Fig.40) indicating a role for ATGL in protection from potentially harmful consequences of ER stress in response to TM.

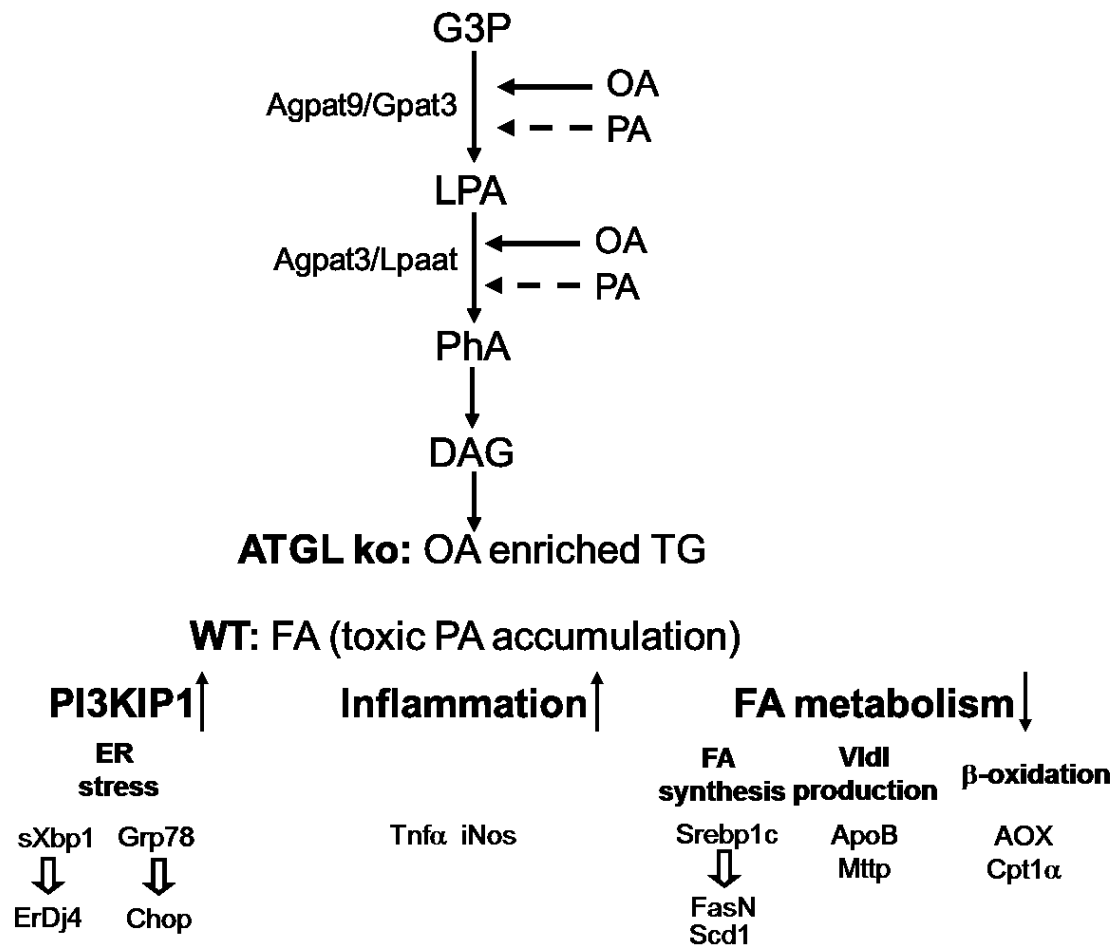


Fig.40: Proposed mechanism for the protection of tunicamycin induced hepatic ER stress of ATGL KO mice in contrast to ER stress in treated WT mice. Monounsaturated fatty acids (FAs), like oleic acid (OA), are efficiently incorporated into triglyceride (TG), therefore protecting from ER stress. In contrast to OA, unsaturated FAs like palmitic acid (PA) are not easily stored as TG and free PA leads to ER stress. The first step of TG formation is catalysed by glycerol-3-phosphate acyltransferase (Gpat3/Agpat9), which converts glycerol-3-phosphate (G3P) into lysophosphatidic acid (LPA). LPA is then converted into phosphatidic acid (PhA) by lysophosphatidic acid acyltransferase (Lpaat/Agpat3) and subsequently TGs are formed. In ATGL KO mice under ER stress conditions, OA rich TGs were formed. In WT mice under tunicamycin (TM), TG formation was prevented by high PA levels, therefore inducing Pik3ip1 that in turn increased ER stress leading to activation of heat shock chaperones (Grp78) and integrated ER stress response (sXbp1). Moreover, WT mice challenged with TM showed increased inflammation. Both, WT and ATGL KO mice treated with TM mice lowered FA synthesis and decreased β -oxidation and VLDL-synthesis. (Open arrows indicate transcriptional up-regulation)

mRNA expression levels for markers of *de novo* lipogenesis (Srebp1c, Fasn, Scd1) (Fig.18A) and β -oxidation (CPT1 α , Acc2) (Fig.18B) were down-regulated in both genotypes challenged with TM. Repression of genes involved in FA synthesis was in line with decreased nuclear Srebp1c protein levels in mice exposed to TM (Fig.19) and a pronounced reduction of Mttp and ApoB mRNA expression levels (Fig.17B). Furthermore, we could show that key players in hepatic TG formation like Agpat9 (Gpat3) and Agpat3 (Lpaat) were up-regulated exclusively in ATGL KO TM challenged mice (Fig.20), suggesting that TG formation could have a protective role against hepatic ER stress (Fig.40). Increased accumulation of hepatic lipids in ATGL KO mice 48h after TM injection is consistent with this hypothesis. Through FA profiling we could further demonstrate that ATGL KO mice had higher levels of total hepatic OA - an “anti-lipotoxic” monounsaturated FA - independent of TM treatment (Fig.21A), whereas untreated as well as treated WT mice contained more total hepatic PA than OA (Fig.21B). Moreover, the high serum levels of free PA which were observed in the WT TM challenged mice (Fig.22) are consistent with higher hepatic PA levels in these mice.

Listenberger et al. [99] and our *in vitro* studies (Fig.23) showed that (at least an equal concentration of) OA (related to PA concentration) is able to protect against PA induced toxicity. Together these factors suggest that the higher concentration of total OA in the ATGL KO mice compared to total PA concentration could be able to rescue these mice from PA induced hepatic ER stress. In addition, the low levels of free hepatic linoleic acid (Table 1) which has a pro-inflammatory effect, in ATGL KO TM mice compared to treated WT mice are further in line with protection against inflammation as reflected by reduced levels of respective mRNA markers (Tnf α , iNOS; Fig.13B)

The increase in OA after TM injection in ATGL KO mice (Fig.21A) was unexpected, since Scd1, the central enzyme in PA and stearate desaturation to monounsaturated FA [227] was down-regulated during ER stress (Fig.21C). OA is the preferential substrate for glycerol esterification, TG synthesis and lipid droplet formation in the liver. Therefore, liver OA accumulation could be a consequence of ATGL deficiency [228] This assumption is underlined by the

low PA/OA ratio found in ATGL KO mice at baseline (Fig.21B). Ong *et al* [228] suggest that ATGL may be specific for the release of certain FA species including OA. We propose that cellular OA concentrations are determined by a cycle of TG hydrolysis and re-esterification to TG and that ATGL is required to release OA from the TG pool. Future studies will have to address the preference of ATGL for various FA during hydrolysis.

We and others found that TM induces ER stress in liver and kidney [96, 229-231] but not in WAT (Fig.16). This may be surprising since TM is known to be lipophilic, but due to the high capacity of adipocytes to store TG, white adipose tissue might be protected against FA-induced ER stress. Since our mouse model systemically lacks ATGL, it is difficult to differentiate from the *in vivo* findings whether ATGL deficiency in WAT or liver or both provided the protection against TM induced hepatic ER stress. On the one hand, lack of ATGL in WAT reduces the FA flux from WAT to the liver [100] therefore lowering the amount of FA entering the liver. On the other hand, lack of ATGL in the liver reduces hepatic TG hydrolysis and subsequently the release of FA from the hepatic TG pool thereby affecting FA levels in the liver. Notably, protection from TM-induced ER stress in the absence of ATGL was observed in liver but not in kidney. Tissue specific ATGL KO mice will clarify this issue *in vivo*. Since such mice were not yet available at the time of our study, we addressed this question by ATGL knock-down in hepatocytes prior to treatment with OA or PA and TM *in vitro*. This set of experiment clearly showed that knock-down of ATGL and OA protected from ER stress *in vitro* (Fig.23C). Therefore, it is tempting to speculate that ATGL deficiency in the WAT may protect the liver from ER stress by changing the balance between lipotoxic PA and protective OA taken up by the liver.

OA has been shown to prevent PA- induced ER stress [99] *via* down regulation of Pik3ip1 [222] Our data indicate that the baseline amount of OA in ATGL KO mice may have a protective function against PA-derived ER stress. Thus, it is tempting to speculate that the first FA which enters the – nontoxic – TG has to be a monounsaturated FA, *i.e.* that monounsaturated FAs are the preferential

substrate for Agpat9 (Gpat3), whereas Agpat3 (Lpaat) would favour saturated FAs (Fig.40). The low free hepatic PA levels which we found in WT TM mice (Table1) further supports the assumption that under conditions of low concentrations of monounsaturated FAs for TG synthesis saturated FAs are not able to enter the TG and undergo the synthesis of lipid components which are supposed to be toxic.

The impact of FA-induced ER stress on lipogenesis is controversial. While some studies demonstrated that ER stress can activate sterol regulatory element-binding proteins (SREBPs), transcription factors involved in the activation of the lipogenic gene machinery [232, 233], other studies by Rutkowski *et al* [98] showed that ER stress down regulates *de novo* lipogenesis due to the negative regulation of C/ebp α , a transcription factor involved in hepatic gluconeogenesis and lipid homeostasis via Chop. Notably, in our study, WT and ATGL KO mice had reduced mRNA and protein expression levels of Srebp1c the master regulator of *de novo* lipogenesis after TM injection (Fig.18A and Fig.19). Interestingly, although Srebp1c mRNA was significantly down regulated in TM treated WT mice compared to treated ATGL KO, we observed a significantly higher mRNA expression of its downstream targets FasN and Scd1 in TM injected WT compared to ATGL KO mice, suggesting that (at mRNA level) another factor different from Srebp1c may activate expression of genes involved in *de novo* lipogenesis under ER stress conditions. Recently, Lee *et al.* [93] suggested that, besides its function as stress related factor Xbp1, the protein may also have a role in the regulation of *de novo* lipogenesis. Therefore, the higher levels of FasN and Scd1 expression in TM treated WT mice compared to TM challenged ATGL KO mice could be due to increased sXbp1 expression.

Puri *et al.* [234] did not find activation of the ATF4 – CHOP – GADD34 pathway despite increase in phosphorylation of eIF-2 α in NAFLD and NASH patients indicating that there may be considerable differences between our acute ER stress model and the pathogenesis of NAFLD. It appears that during progression from NAFL to NASH the ER trans-membrane proteins PERK and IRE 1 α are already activated, whereas their downstream targets are still

repressed. This may lead to the speculation that the integrated stress response and subsequent acute ER stress could be a rather late event in the progression to more advanced fibrosis or cirrhosis. However, our *in vitro* data (Fig.23) suggest that hepatic fatty acid flux to the liver and fatty acid composition may play a major role in hepatic ER stress development.

It is tempting to speculate that hepatocytes are able to cope with increased fatty acid uptake as long as there is a homeostasis between OA and PA. As such high levels of toxic PA could result in up-regulation of downstream targets of the UPR such as sXBP1, ERdj4 and Chop.

Another member of the PNPLA family, namely PNPLA3 (closest homologue to ATGL/PNPLA2) has recently been implicated in the progression of NAFLD in human. A missense mutation (I148M) in the PNPLA3 gene is associated with increased hepatic TG accumulation in human [1]. Interestingly, recent studies in mice did not reveal an altered susceptibility to NAFLD [235]. This could be due to species specific differences in the regulation and function of PNPLA3 [87]. Since PNPLA3 is the closest homologue to ATGL it is attractive to speculate that ATGL (PNPLA2) variants could also play a role for the progression of NAFLD, perhaps via modulation of ER stress.

Dubuquoy *et al.* [236] showed that Srebp1c directly regulates PNPLA3 in mice. Our study revealed repression of Srebp1c mRNA by TM and a trend for reduced PNPLA3 levels in TM treated WT mice while PNPLA3 expression remained preserved in ATGL KO mice after TM challenge (Fig.41). This finding implicates a potential role for PNPLA3 in the rescue from ER stress in ATGL KO TM mice. Therefore it is tempting to speculate that under conditions with high amounts of OA – the potentially favoured FA for TG formation – PNPLA3 could function as an acyltransferase [237], thus facilitating the TG formation protecting from lipotoxicity [238].

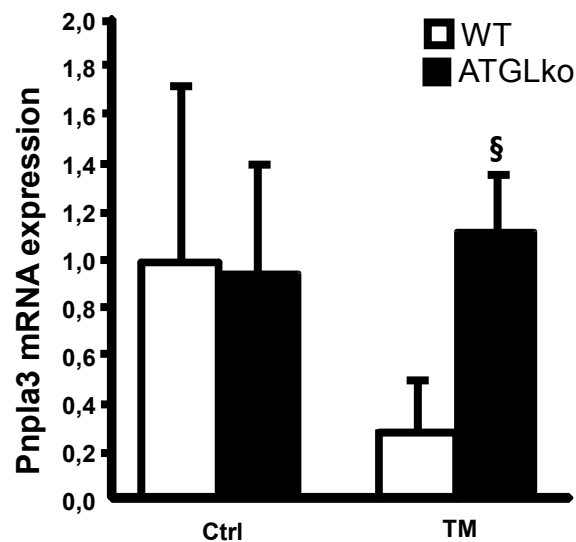


Figure 41: PNPLA3 mRNA expression is preserved in TM treated ATGL KO mice. mRNA expression of PNPLA3 was assessed by real time PCR. While in WT mice TM injection tended to decrease PNPLA3 mRNA levels, ATGL KO mice showed preserved PNPLA3 expression after TM exposure. Expression values were normalized against 36b4 levels and are shown relative to expression level in unchallenged WT animals. § indicates a significant difference from TM treated WT; $P < 0,05$

In summary, our data established that WT mice exposed to ER stress are not able to form TG, as a result of low hepatic OA and high PA levels which furthermore efficiently promotes Pik3ip1 expression and thereby increases ER stress. Conversely, we show that an enrichment of OA in the hepatic TG pool of ATGL KO mice prevents against TM induced hepatic ER stress. Accordingly, ATGL-mediated TG hydrolysis may constitute a novel target in the treatment of ER stress which is typically present in patients suffering from NAFLD and NASH.

In the second part we examined the impact of the farnesoid X receptor (FXR) deficiency on the development of fatty liver disease. To this purpose, we used a methionine choline deficient (MCD) diet. The MCD dietary model was used in previous studies of fatty liver disease since it induces progressive, fibrosing steatohepatitis in rodents [239-244]. Notably, WT mice fed a MCD diet developed a higher grade of steatosis but were protected from hepatic inflammation. Our *in vivo* and *in vitro* data emphasize a potential role of FXR in the development of steatohepatitis via alterations of Chop mRNA levels under conditions of high BA and low glucose concentrations.

Serum levels of ALT, AP, AST and bile acids were increased due to MCD feeding independent of the genotype (Fig.26A), whereas glucose levels were decreased due to MCD feeding in both, WT and FXR KO mice (Fig.26A). Serum lipid parameters (CHOL and TG) were decreased in mice exposed to MCD diet for 5 weeks, but to a higher extend in WT mice (Fig.26A). WT mouse under challenging conditions developed a higher grade of hepatic steatosis than the FXR KO mouse (Fig.27). However, inflammation indicators F4/80 and Tnf α were markedly increased in FXR KO MCD mice and not changed in WT MCD mice compared to corresponding controls (Fig.31), indicating for a role of FXR in protection from potentially harmful consequences of hepatic lipid accumulation in response to MCD diet.

mRNA levels of Chop were drastically increased in WT mice fed a MCD diet and not changed in challenged FXR KO mice (Fig.26B). Chop is supposed to negatively regulate C/ebp α [98] - a well-known master regulator of lipid metabolism [245]. In line with literature we found a decrease of C/ebp α in WT mice fed a MCD diet, whereas FXR KO MCD fed mice did not show significant differences compared to control mice (Fig.26B), indicating that FXR is involved in regulation of C/ebp α via Chop under methionine choline deficiency.

This impression was supported by *in vitro* data. Immortalized human derived primary hepatocytes treated with low levels of glucose and 10 μ M Int747 (6ECDCA) in methionine choline deficient (MCD) medium display an increase in Chop mRNA expression compared to cells treated without Int747 (Fig.26C).

Cells cultured in normal DMEM medium with low levels of glucose, with or without FXR agonist did not show any differences in Chop expression (Fig.26C), indicating for activation of FXR under conditions leading to fatty liver injury.

Moreover, additional cell culture experiments in HepG2 cells under low glucose conditions and CDCA treatment made clear that FXR impacts on Chop mRNA expression (Fig.32A) in absence and presence of glucose and bile acids respectively. Additional treatment with 9-cis retinoic acid as an agonist for retinoic X receptor (RXR) demonstrate that also RXR is involved in regulation of Chop mRNA expression (Fig.32B). Furthermore, our *in vitro* studies reveal that the regulation of Chop via FXR and RXR may happens indirect transcriptional (Fig.32C) and the two transcription factors need to be available as proteins (Fig.32D). Overall, our data propose that under conditions of MCD diet induced fatty liver disease (low glucose, high bile acids) FXR together with RXR activate Chop mRNA expression and subsequent inhibition of C/ebp α , which implicates the lowering in Srebp1c levels in MCD fed WT mice.

The situation of low glucose and high bile acids levels is found after every meal in obese people who already developed insulin resistance. During that stage FXR is able to up-regulate Chop expression, which further counteracts the development of inflammation and progression to NASH. The certainty that we could not find increased Chop expression in NASH patients (Fig.24) gives raise to the assumption that FXR activated Chop is capable to cope with potential inflammation mediators until a dedicated time-point. Thereafter the progression from NAFLD to NASH takes its course.

Although MCD fed WT mice have increased hepatic TG amounts compared to FXR KO MCD fed mice they are protected from hepatic inflammation, notifying anti-inflammatory effects of FXR and/or RXR at that stage of liver disease (Fig.42).

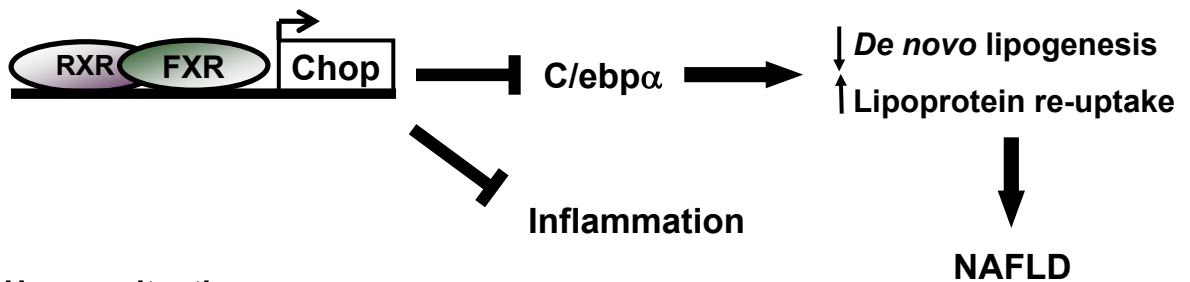
The fact that our MCD fed WT mice did not develop inflammation although they display steatosis, goes in line with the data which were presented from Randal

Kaufman's group at the AASLD meeting 2011 [246]. They suggest that up-regulation of Chop leads to apoptosis of activated macrophages and that the Chop-induced cell death rescues mice from inflammation development. This theory is underlined by the increased F4/80 mRNA levels we found in our FXR KO MCD fed mice (Fig.31) which do not display increased Chop mRNA levels (Fig.26B), indicating protective function of Chop in the development from NAFL to NASH.

FXR KO MCD MOUSE



WT MCD MOUSE



Human situation

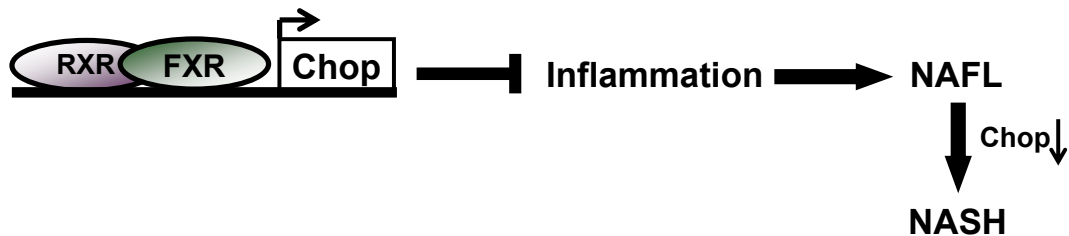


Fig.42: Proposed regulatory mechanism of lipid accumulation via Chop and FXR

In WT MCD mouse FXR together with RXR, up-regulates Chop mRNA expression under conditions of low Glucose and high bile acids. Chop on the one hand down-regulates C/ebp α , which furthermore leads to down-regulation of *de novo* lipogenesis and on the other hand attenuates inflammation. Increased FXR activation induces hepatic lipoprotein re-uptake. Overall activation of FXR under the stage of low glucose and high bile acids causes increased steatosis but no inflammation.

Despite the fact that Watanabe and co-workers suggest that bile acid activated FXR counteracts hepatic TG accumulation [48] due to Srebp1c down-regulation, we see a markedly increase in hepatic lipid accumulation in WT MCD fed mice although these mice exhibit clear reduction in Srebp1c mRNA levels compared to FXR KO MCD fed mice. Due to the fact that WT MCD fed mice showed low serum VLDL levels and increased expression of VLDL receptor someone can assume that MCD-diet induces FXR-dependent increase of hepatic VLDL receptor expression, subsequent hepatic lipoprotein re-uptake and TG accumulation. Yu and coworkers [247] figured out that induction of LPL activity through MCD feeding itself is not strong enough to counteract fatty liver development. This achievement encourages the possibility that WT MCD fed mice develop steatosis because of increased VLDL particle re-uptake.

Furthermore, it has to be mentioned that the inhibitory effect of activated FXR on Srebp1c expression shown by Watanabe et al [48] seems to be an acute response to increased bile acid levels, since after 7 days of cholic acid (CA) feeding mRNA levels of Srebp1c and its downstream targets *Fasn* and *Scd1* went back to normal [48]. Therefore we suggest that in our MCD fed mouse model Srebp1c expression is reduced due to the Chop induced inhibition of *C/ebp α* .

Conclusively this study identifies FXR as a potential pharmacological drug-target to decelerate the progression from NAFLD to NASH.

In the third part we examined the impact of bile salt export pump (BSEP) as critical regulator of hepatic BA flux in the development of fatty liver and NASH. To this purpose, we used a methionine choline deficient (MCD) diet to induce fatty liver/NASH. This MCD dietary model was used in previous studies of fatty liver disease since it induced progressive, fibrosing steatohepatitis in rodents [240, 241, 248-251].

Notably, BSEP KO mice challenged with MCD diet developed milder hepatic steatosis and were protected from liver inflammation. Serum indicators for liver injury (AST and ALT) were up-regulated in mice challenged with MCD diet, the same pattern was found for bile acids (Fig.33A). Interestingly, AP indicating for damage in cholangiocytes was lowered due to MCD feeding, independently of the genotype (Fig.33A). Serum lipid parameters (CHOL, TG and FA) were decreased in mice exposed to MCD diet for 5 weeks, but to a higher extend in WT mice (Fig.33A). Disturbances in hepatic lipid metabolism were confirmed by liver histology and hepatic TG amount. Both, WT and BSEP KO mice fed a MCD diet developed hepatic steatosis. However, WT mice developed a significantly higher grade of steatosis than BSEP KO mice (Fig.33B), which may be attributed to higher *de novo* lipogenesis (Srebp1c, FasN) as well as fatty acid transport (Fatp5 and Fabp1) in WT mice compared to BSEP KO mice which showed more pronounced repression of these genes in comparison to WT mice (Fig.35A and C).

In line with changes in bile acid synthesis in MCD fed BSEP KO mice (Fig.37A and B), we also found changes in hepatic bile acid concentration/composition (Fig.37C and D) and in expression of bile acid transporters (Fig.38). Sinusoidal bile acid uptake *via* Ntcp was drastically reduced in MCD fed BSEP KO mice compared to their control group and fed WT mice (Fig.38A), whereas bile acid excretion into the blood *via* Mrp4 and Ost β was induced in MCD challenged BSEP KO mice (Fig.38C). Although MCD fed WT mice display the lowest concentration of intrahepatic bile acids (Fig.37C) (in line with decreased Cyp7a1 mRNA expression (Fig.37B)) they contain the highest amount of potentially toxic hydrophobic BAs (Fig.37D). mRNA expression levels of downstream targets of the nuclear transcription factors FXR, CAR and PXR (Cyp7a1,

Cyp2b10 and Cyp3a11) (Fig.37B and 39) suggest that the rescuing pathway is regulated *via* FXR and CAR (Fig.43).

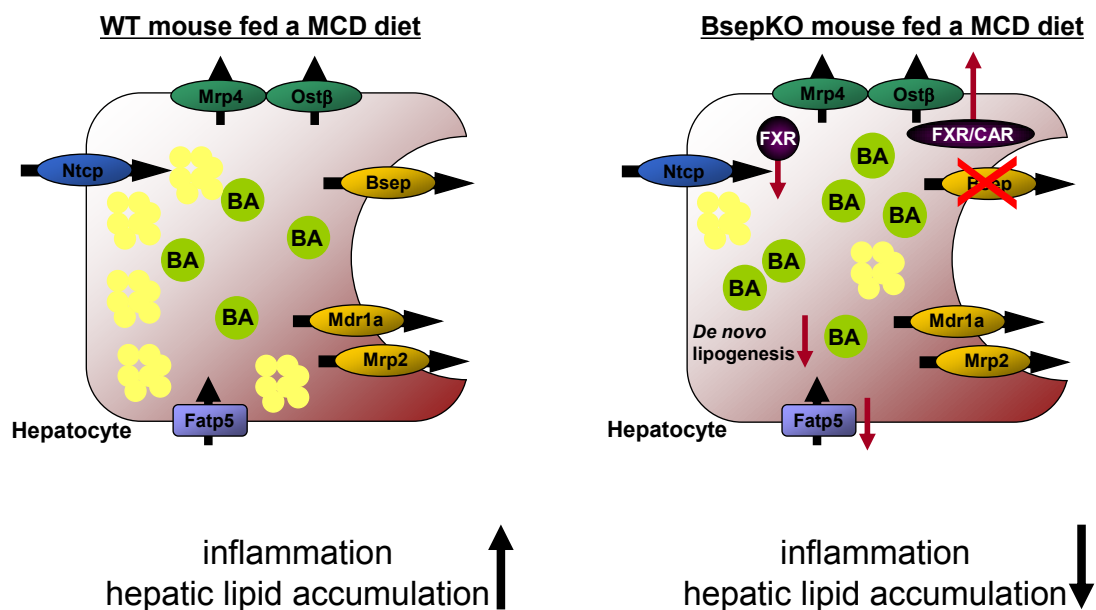


Fig.43: MCD fed BSEP KO mouse display attenuated hepatic lipid accumulation and inflammation Under MCD challenging BSEP KO mouse is able to decrease bile acid uptake from the blood via Ntcp and increase the alternative bile acid export to the blood via Mrp4 and Ostβ. Moreover, *de novo* lipogenesis and fatty acid uptake via Fatp5 are down-regulated, leading to minor steatosis compared to MCD fed WT mice.

Together with the high levels of serum bile acid levels we found in BSEP KO mice upon MCD (Fig.33A), these data suggest that MCD exposed BSEP KO mice export bile acids into the blood thus limiting bile acid induced hepatic inflammation. Moreover the increased expression of CAR raises evidence for increased BA detoxification and subsequent low concentrations of hydrophobic BA in MCD fed BSEP KO mice.

The mechanism how methionine choline deficient (MCD) diet induce fatty liver injury is not known exactly. Sundaram et al [252] suggest that NAFLD induction could be due to impairment of phosphatidylcholine (PC) synthesis, since both, methionine and choline are precursors of PC. Figge et al [217] developed transgenic mice overexpressing BSEP (BSEPtg). Enhanced canalicular expression of BSEP results in increased bile flow and increased biliary lipid

secretion. Particularly, BSEP^{tg} mice display an increase in biliary secretion of bile salts, cholesterol and phospholipids [217]. Since it was shown previously that PC secretion into the bile is very high and that the entire content of hepatic PC is secreted into the bile every day [253, 254], Sundaram et al [252] proposed that mice with increased biliary secretion of PC, like BSEP^{tg} mice, may be more susceptible to development of fatty liver injury induced by MCD diet.

Mice lacking BSEP show an increase in secretion of biliary phospholipids despite lower biliary bile salt excretion compared to WT mice [214] and according to our data these mice are less prone to fatty liver development than WT mice while challenged with MCD diet. Moreover, Wang et. al. [214] showed that BSEP KO mice display lower bile acid concentration in bile than WT mice. The increase in bile flow which was found by Figge and coworkers [217] in mice overexpressing BSEP indicates elevated biliary bile acid levels. Together with our data which display that BSEP KO mice fed a MCD diet are protected from hepatic inflammation, (indicated by mRNA expression levels of $Tnf\alpha$, $Mcp1$, $F4/80$ and $iNos$) (Fig.36A), data from literature [214, 217] suggest and encourage the findings of Allen and coworkers [244] that bile acids have a pro inflammatory effect and could be a driving force (second hit) to progress from steatosis to steatohepatitis. Overall, this issue identifies BSEP as a key player in the development of inflammation during the progression from steatosis to steatohepatitis.

It has been shown that bile acid have also function as signalling molecules in hepatic lipid metabolism. Bile acids are suggested to negatively regulate serum TG levels [255, 256] because of repressing both, hepatic TG production as well as secretion. Watanabe and coworkers [48] show that in liver $Srebp1c$ and its lipogenic downstream targets are downregulated in a FXR – SHP dependent manner. FXR activated SHP represses $Srebp1c$ expression and subsequent hepatic fatty acid and TG formation. This entails a decrease in hepatic TG accumulation and VLDL secretion [48]. These observations are confirmed by our study, where we found lower hepatic BA concentration in MCD fed WT mice

(Fig.37C) which accumulates higher amounts of hepatic TG compared to MCD BSEP KO mice (Fig.33B,C).

Interestingly, we found that BSEP KO mice show significant higher levels of F4/80 and iNOS mRNA expression at the baseline compared to WT mice (and even a tendency for $Tnf\alpha$ and $Mcp1$) (Fig.36A). Since Wang et al [214] discovered that mice lacking BSEP had higher levels of hepatic bile acid concentration than the control mice someone can assume that a hepatocyte is able to store a certain amount of bile acid before delivering it either into bile or into blood. In BSEP KO mice the transport to the bile is less efficient albeit not totally abolished in contrast to human PFIC2 and biliary bile acid concentration is lowered by 4.2 fold compared to control mice, whereas the shuttle system to the blood is not affected [214]. Our data suggest that under MCD challenge, BSEP KO mouse is capable to up regulate the bile acid transport system to the blood (Fig.33A and 38C) and therefore be protected from hepatic inflammation. The increase of bile acid flux from hepatocytes to the blood under the state of BSEP loss was also found by others [257]. Hence Zollner et al [257] showed that bile acid feeding in FXR KO mice led to hepatic bile acid accumulation because of inadequate BSEP expression. The strong induction of $Mrp3$ may explain the significantly higher serum bile acid levels which Zollner and co-workers [257] found in bile acid fed FXR KO mice. In addition, increased $Cyp2b10$ mRNA expression levels found in BSEP KO MCD fed mice (Fig.39) was reminiscent of what was found by Schuetz et al [258] in FXR null mice fed a bile acid enriched diet. The increase in $Cyp2b10$ expression suggests that the hepatic oxidation of “toxic” bile acids is increased in mice lacking BSEP. In addition, our data, and literature [257, 258] propose that loss of BSEP induces the basolateral efflux of bile acids. The up-regulated tetra hydroxylation of bile acids found from Wang et al [214] in BSEP KO mice could be the subsequent detoxification mechanism preventing the toxic effect from bile acids stored in the hepatocyte.

In summary our data propose BSEP as a new target for pharmacological down regulation to attenuate the progression from NAFLD to NASH.

6. CONCLUSION

Several factors are proposed as prospective inducers of cellular toxicity, followed by hepatic inflammation, leading to steatohepatitis.

Literature [84, 102, 259-261] and our data (**Absence of ATGL protects from hepatic ER stress**) suggest that the presence of the increased levels of saturated fatty acids but not (poly)unsaturated fatty acids may promote the development of liver injury. We were able to show that under acute ER stress conditions induced by Tunicamycin injection ATGL KO mouse but not the WT mouse is protected from ER stress and inflammation development. Total hepatic fatty acid composition measurement illustrated that ATGL KO mouse has higher oleic acid contents at the baseline than palmitic acid. Whereas in the WT mouse it is the other way around. Together with the knowledge from literature our data emphasize the implication of saturated fatty acids in the progression from simple benign steatosis to harmful steatohepatitis. Therefore ATGL – the main lipase in TG breakdown – is discovered as a potential driving-point for pharmaceutical treatment against the hastening of NAFL development because of lipotoxicity.

Beside fatty acids also bile acids are implicated in the progression from NAFLD to NASH [244]. Within the bile acid pool someone has to distinguish between the potentially toxic hydrophobic bile acids and the non-toxic hydrophilic bile acids. As shown from Marshall et al [262] naïve WT mice consists of about 22% hydrophilic bile acids in the hepatic bile acid pool. It could be taken into account that the high amount of hydrophobic intrahepatic bile acid gives impulsion to the enhancement of fatty liver disease in an already harmed liver.

Moreover it has to be considered that bile acid function as signalling molecules and be ligands for nuclear transcription factors such as FXR. Therefore it is very likely that bile acids have a direct and/or an indirect effect on the development of inflammation.

Interestingly, we found that FXR WT mice fed a MCD diet as a model for NASH do not develop hepatic inflammation although they accumulate more hepatic

lipids than the MCD fed FXR KO mice (**FXR is a key player in NAFLD development by controlling CHOP expression**). Furthermore, the FXR KO MCD mouse develops inflammation. Since serum bile acids were high due to MCD feeding the reasonable suspicious rise up that activated FXR counteracts bile acid derived hepatic inflammation. Moreover we could show that FXR activates Chop mRNA expression. Randal Kaufmann's group [246] proposes that activated macrophages undergo Chop mediated apoptosis, thus Chop activation mediates hepatic inflammation and injury. Hence bile acids can be seen as a double edged sword, on the one hand they are able to induce hepatic inflammation but on the other hand they induce a rescue pathway via FXR – Chop activation.

Another phenomenon regarding bile acid induced inflammation we found in BSEP KO mice fed a MCD diet (**BSEP KO mice are protected from hepatic inflammation in methionine choline deficient diet induced NASH**). Since the lack of BSEP lead to a decreased bile salt output at the baseline [214], someone could assume that the increased intrahepatic bile acid pool could lead to inflammation under the conditions of MCD feeding.

However, BSEP KO mice have a ratio of hydrophilic versus hydrophobic bile acids of about 6 whereas in WT mice it is about 1,5. This quotient indicates the higher intrahepatic concentration of nontoxic BAs in BSEP KO MCD mice.

Moreover BSEP KO MCD fed mice had a decreased expression of NTCP – the transporter responsible for BA uptake from the blood - and an increased expression of Mrp4 – transporter responsible for BA secretion into the blood. Together with the high serum BA levels this finding indicates that under MCD challenging BSEP KO mice accumulate BA in the blood. Thus BA are not toxic to hepatocytes and BSEP KO MCD fed mice do not develop inflammation.

Conclusively it has to be mentioned that within this study we discovered ATGL, FXR and BSEP as three potentially points of action in term of counteracting the induction of hepatic inflammation and subsequent progression from NAFLD to NASH.

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