

PhD-Thesis

**Role of Adipose Triglyceride Lipase (ATGL) in
Non-alcoholic Fatty Liver Disease (NAFLD)**

submitted by

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1. Zusammenfassung

Die nicht-alkoholische Fettlebererkrankung (NAFLE) betrifft bis zu 45% der westlichen Bevölkerung mit einer Prävalenz bis zu 75% bei Patienten mit Diabetes und Adipositas. Unser Verständnis über die Pathogenese und die Mechanismen der Progression der NAFLE zur nicht-alkoholischen Steatohepatitis (NASH), die letztendlich zu lebensbedrohlichen Komplikationen wie der Entwicklung einer Leberzirrhose und/oder eines hepatozellulären Karzinoms führen kann, ist bislang limitiert. Inflammatorische Prozesse nehmen eine Schlüsselrolle in der Progression der NASH ein. Polymorphismen im PNPLA3 (Adiponutrin) Gen konnten als wichtige genetische Faktoren in der Entwicklung der NAFLE und Progression zur NASH identifiziert werden. Weiters ist bekannt, dass ein vermehrter Fluss freier Fettsäuren beim Menschen bis zu 59% des hepatischen Triglyceridgehalts bedingt. Nachdem die "Adipose triglyceride lipase" (ATGL) als nächstes Homolog zu PNPLA3 eine der wichtigsten intrazellulären Lipasen im Abbau von Triglyceriden darstellt, war das Ziel dieser Studie, die Rolle der ATGL in der Fettverteilung sowie der hepatischen Lipotoxizität und Inflammation in Mausmodellen für NAFLD und NASH zu untersuchen.

Im Folgenden präsentieren wir eine bislang noch unbekannte antiinflammatorische Rolle der "Adipose triglyceride lipase" (ATGL/PNPLA2) und zeigen, dass die ATGL durch Bereitstellung von Liganden (im speziellen 18:2n6) für die Aktivierung von PPAR α eine Schlüsselrolle in der Modulation der hepatischen Inflammation und Progression zur NASH einnimmt.

Durch Fütterung einer Methionin-Cholin-defizienten (MCD) Diät zur Induktion einer NASH bei ob/ob Mäusen konnten wir zeigen, dass eine vermehrte Aktivität der ATGL und der Hormon sensitive Lipase (HSL) für einen massiven Verlust von Fettgewebe gefolgt von Steatose und NASH verantwortlich ist.

Ein umfassendes lipidomisches Profil zeigte, dass die Fettsäure 18:2n6A, ein wichtiger PPAR α Ligand, insbesondere im Serum und in der Leber von MCD-gefütterten ob/ob Mäusen erhöht war. MCD-gefütterte ATGL-knockout (ATGL-KO) Mäuse waren zum Teil vor dem Verlust von weißem Fettgewebe geschützt,

wiesen jedoch ein abgeschwächtes PPAR α Signal, eine ausgeprägte hepatische Steatose, Inflammation und geringgradige Fibrose auf. Wurden ATGL-KO Mäuse einer Endotoxin Belastung (Lipopolysaccharid (LPS), ein möglicher “second hit” in der Progression der NAFLE zur NASH) ausgesetzt, so wiesen diese aufgrund eines gestörten PPAR α Signalwegs durch eine Verminderung der lipolytischen Produkte von ATGL als Aktivator für PPAR α (im speziellen die Defizienz von 18:2n6 und dem herabgesetzten Fettsäure Transport zum Nukleus) eine noch ausgeprägtere hepatische Inflammation, eine gestörte Thermogenese sowie damit verbundene Inaktivität auf. Im Gegensatz dazu zeigten Mäuse, bei denen die Hormon-sensitive Lipase (HSL) fehlte, einen relativ “gutartigen” Phänotyp der dem von Wildtyp Mäusen unter MCD Fütterung bzw. LPS Injektion ähnelte. Interessanterweise konnte durch eine Behandlung von MCD-gefütterten und LPS-injizierten ATGL-KO Mäusen mit dem PPAR α Agonisten Fenofibrat ein Teil des PPAR α Signalwegs wiederhergestellt werden, was wiederum mit einer verminderten hepatischen Inflammation einherging und daher unsere Schlussfolgerung dahingehend stärkt, dass die ATGL durch Modulation der PPAR α Aktivität eine wichtige antiinflammatorische Rolle in der Progression der NASH zu spielen scheint.

Mithilfe unserer Daten konnte wir zeigen, dass die ATGL eine wichtige Rolle in Hinblick auf (i) die “Empfänglichkeit” für eine NAFLE durch Modulation der Lipolyse im Fettgewebe und (ii) die Progression zur NASH durch Modulation der PPAR α Signalkaskade zu spielen scheint. Weiters zeigen unsere Daten dass die ATGL in der Leber *per se* eine wichtige Rolle in der Entgegenwirkung der Entwicklung einer Steatose und Inflammation einnimmt. Zukünftige Studien werden die Aufgabe haben zu prüfen, ob die ATGL als potentiell therapeutisches Ziel in der Lage ist, der Progression der NASH mitsamt aller ihrer Konsequenzen entgegenzuwirken. Die Tatsache dass ein Fehlen der ATGL zu vermehrter Inflammation führt lässt dahingehend optimistisch in die Zukunft blicken, als dass die ATGL ein vielversprechendes therapeutisches Ziel in der Verhinderung der Progression der NAFLE zur NASH darstellen könnte. Abgesehen von den potentiellen Implikationen in Hinblick auf die NASH erweitern die vorliegenden Daten das Verständnis der Rolle der ATGL in der akuten und chronischen hepatischen Inflammation.

2. Summary

Non-alcoholic fatty liver disease (NAFLD) affects nearly 45% of the Western population with prevalence in diabetic and obese individuals approaching 75%. Our understanding of its pathogenesis and the mechanisms underlying the progression to steatohepatitis (NASH) resulting in life-threatening complications such as cirrhosis and cancer are still very limited. Inflammation is one of the key features of NASH progression. Polymorphisms of PNPLA3 (adiponutrin) have been identified as key genetic determinant for NAFLD and progression to NASH in humans. Additionally, in humans, increased peripheral FA flux has been shown to contribute to ~59% to the hepatic TG content in NAFLD patients. Since adipose triglyceride lipase (ATGL) is the closest homologue of PNPLA3 and a rate limiting intracellular lipase our objective was to study the role of ATGL in lipid partitioning, hepatic lipotoxicity and inflammation in mouse models of NAFLD and NASH.

In the present study, we demonstrate a hitherto unknown anti-inflammatory role of adipose triglyceride lipase (ATGL/PNPLA2) and provide compelling evidence that ATGL exerts a key role in modulating hepatic inflammation and progression to NASH by critically determining the ligand availability (in particular 18:2n6) for PPAR α activation.

Using methionine choline deficient diet (MCD) to induce NASH in ob/ob mice we demonstrate that increased ATGL and hormone sensitive lipase (HSL) activity is responsible for massive loss of adipose tissue (AT) followed by hepatic steatosis and inflammation. A comprehensive lipidomic profiling revealed that 18:2n6 fatty acid (FA), an important PPAR α ligand was predominantly elevated in serum and livers of MCD-fed ob/ob mice. MCD-fed ATGL-KO mice were partially protected from WAT loss but showed attenuated hepatic PPAR α signalling, severe hepatic steatosis, inflammation and mild fibrosis. Furthermore, when subjected to endotoxin (LPS, as a potential second hit in progression to NASH), ATGL-KO mice displayed more pronounced hepatic inflammation, defective thermogenesis and torpor due to impaired PPAR α signalling pathway as a result of deficiency in lipolytic products of ATGL.

to activate PPAR α , in particular the deficiency of 18:2n6 and decreased fatty acid transport to the nucleus. On the contrary, mice lacking hormone-sensitive lipase (HSL) had a relatively benign phenotype which resembled that of the WT mice on both MCD feeding and on LPS injection. Notably, PPAR α agonist treatment with fenofibrate partially restored PPAR α signaling and attenuated hepatic inflammation in both MCD-fed and LPS-injected ATGL-KO mice thus strengthening our conclusion that ATGL exerts important anti-inflammatory roles counteracting the progression to NASH *via* the modulation of PPAR α activity.

Our findings suggest that ATGL plays a critical role in (i) susceptibility to fatty liver development by modulating lipolysis in adipose tissue and (ii) in progression to NASH by exerting an anti-inflammatory role in liver by modulating PPAR α signaling. Our data also demonstrates that the role of ATGL in liver *per se* is imperative to counteract steatosis and inflammation. Future studies will have to address whether therapeutic targeting of ATGL is able to counteract hepatic inflammation in progression to NASH and to potentially lethal consequences such as liver cirrhosis and cancer. The demonstration that ATGL deficiency leads to exaggerated inflammatory response fuels optimism that ATGL could represent an exciting therapeutic target for preventing the transition from fatty liver to NASH. Apart from potential implications for NASH, these findings may have general relevance for understanding the role of ATGL in acute and chronic hepatic inflammation.

3. Abbreviations

AT	Adipose tissue
ATGL	Adipose triglyceride lipase
ACC1	Acetyl-CoA carboxylase
ACSL1	long-chain fatty-acid-coenzyme A ligase
Ap2/ FABP4	adipocyte protein 2 / fatty acid binding protein 4
ATP5B	ATP synthase, H ⁺ transporting mitochondrial F1 complex, beta subunit
BAT	Brown adipose tissue
CD11b	Cluster of differentiation 11b
C/EBP α	CCAAT/enhancer-binding protein alpha
CIDEA	Cell death-inducing DFFA –like effector A
Col1a1	Collagen, type I, alpha 1
Col1a2	Collagen, type I, alpha 2
Cox4i1	Cytochrome c oxidase subunit IV isoform 1
CPT	Carnitine palmitoyltransferase
DBA	DNA binding activity
DGAT	Diglyceride acyltransferase
DNL	<i>de novo</i> lipogenesis
FA	Fatty acid
FABP1	Fatty acid binding protein 1
FATP5	Fatty acid transport protein 5
FF	Fenofibrate
FGF21	Fibroblast growth factor 21
GC-MS	Gas chromatography mass spectrometry
H&E	Hematoxylin and eosin
HSL	Hormone sensitive lipase
IL-6	Interleukin 6
IL1 β	Interleukin 1beta
iNOS	Inducible nitric oxide synthase

KO	Knock-out
LPS	Lipopolysaccharide
MCD	Methionine-choline-deficient
Mcp-1	Monocyte chemotactic protein 1
mRNA	Messenger RNA
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
NS	Non-significant
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptors
RXR	Retinoid X receptor
S.D	Standard deviation
SCD1	Stearoyl-CoA desaturase-1
SREBP	Sterol regulatory element-binding protein
TFA	Total fatty acid
TG	Triglyceride
TGF β	Transforming growth factor beta
UCP-1	Uncoupling protein 1
WAT	White adipose tissue
WT	Wild type

Common name of fatty acids: 14:0: Myristic acid; 16:0: Palmitic acid; 18:0: Stearic acid; 20:0: Aracidic acid/ Eicosanoic acid; 24:0: Lignoceric acid; 16:1n7: Palmitoleic acid; 18:1n9: Oleic acid; 18:1n11: Vaccenic acid; 18:2n6: Linoleic acid; 20:4n6: Arachidonic acid; 22:4n6: Adrenic acid; 18:3n6: γ Linolenic acid; 18:3n3: α Linolenic acid; 20:3n6: Dihomo γ linolenic acid; 20:5n3: Eicosapentaenoic acid (EPA); 22:5n3: Docosapentaenoic acid (DPA); 22:6n3: Docosahexaenoic acid

4. Introduction

4.1. NAFLD

Non-alcoholic fatty liver disease (NAFLD) includes a disease spectrum ranging from simple fatty liver (steatosis), to non-alcoholic steatohepatitis (NASH), cirrhosis and ultimately hepatocellular carcinoma (HCC) (Angulo, 2002). NAFLD is commonly observed in obese individuals, diabetics and patients with metabolic syndrome (Adams, 2007). 30-45% of general population and 69% of diabetic patients are affected by NAFLD (Lazo *et al.*, 2008; Trauner *et al.*, 2010; Williams *et al.*, 2011). The prevalence of steatosis and NASH in obese patients undergoing bariatric surgery is as high as 76% and 37%, respectively (Lazo *et al.*, 2008; Machado *et al.*, 2006). This positions NAFLD as the most common liver disease of the 21st century, accounting for 10-29% of liver cirrhosis and cancer in Western countries (Angulo, 2002; Cohen *et al.*, 2011). Figure 1 illustrates the spectrum and progression of NAFLD.

In obese individuals, the increased supply of FAs to the liver from diet, adipose tissue (AT), and through increased *de novo* lipogenesis all serve to promote hepatic steatosis (Cohen *et al.*, 2011). The relative contributions of the three pathways to hepatic steatosis in humans have been defined in only one study in which Donnelly *et al.* (Donnelly *et al.*, 2005) reported that 59% of hepatic fat is derived from circulating FAs, with lesser contributions from *de novo* lipogenesis (26%) and diet (15%). A 2-hit model was proposed for the progression of fatty liver to NASH in which the initial step results in fat accumulation in liver while inflammation acts as a second hit leading to liver injury progression (Lewis *et al.*, 2010). Yet, the mechanisms responsible for further hepatic insults from steatosis to inflammation and progressive fibrosis are not fully understood (Day *et al.*, 1998). Endotoxin or lipopolysaccharide (LPS) from gram-negative bacteria translocates from the intestine through a TLR4-dependent mechanism (Miller *et al.*, 2005; Neal *et al.*, 2006) and could act as the second hit favouring progression to NASH. Altered lipid composition in both plasma and liver has been shown in patients with NAFLD and NASH and hence has been implicated

in triggering inflammation and serving as endogenous danger signals (Puri *et al.*, 2007; Puri *et al.*, 2009). Additionally, hepatic lipotoxicity may ensue when hepatic capacity to utilize (oxidize), store and export fatty acids (FAs) as very low density lipoprotein (VLDL) is overwhelmed by FA flux from the periphery or hepatic *de novo* lipogenesis (DNL) (Cohen *et al.*, 2011; Trauner *et al.*, 2010). According to this concept, pure fatty liver can progress to NASH when adaptive mechanisms protecting hepatocytes from FA-mediated lipotoxicity are overwhelmed (Cohen *et al.*, 2011; Neuschwander-Tetri, 2010).

MCD is a commonly used rodent model of NASH, resulting in steatosis, endotoxin translocation, oxidative stress, inflammatory infiltrate, liver damage and fibrosis (Hebbard *et al.*, 2011; Henao-Mejia *et al.*, 2012; Jha *et al.*, 2013; Rinella *et al.*, 2008). The onset of the disease may be attributed to the lack of methionine and choline in the diet. Choline is essential for *de novo* synthesis of phosphatidylcholine, required for the export of triglycerides (TG) *via* VLDL packaging, therefore its deficiency may cause steatosis (Caballero *et al.*, 2010; Vance, 2008). Methionine is an essential amino acid that plays a key role in protein synthesis and is an intermediate in S-adenosylmethionine (SAM) and glutathione (GSH) synthesis, two important metabolites in cellular homeostasis and hepatocyte function (Mato *et al.*, 2008). Therefore methionine deficiency causes, inflammation, oxidative stress, and fibrosis (Caballero *et al.*, 2010). Most studies on MCD induced NASH model have focused largely on the factors pertaining to liver while the role of adipose tissue in the pathogenesis of NASH has not been well understood.

Notably, genetic variants of adiponutrin (PNPLA3), the closest homologue of ATGL (PNLPA2) have recently emerged as key determinants for NAFLD and progression to NASH in humans (Romeo *et al.*, 2008; Santoro *et al.*, 2010; Sookoian *et al.*) and reduced ATGL expression has been reported in patients with NAFLD (Kato *et al.*, 2008). Importantly, hepatic steatosis is a feature of neutral lipid storage disease (NLSD) caused by mutations of ATGL/PNPLA2 (NLSD with myopathy) or its co-activator - CGI-58 (NLSD with ichthyosis/Chanarin-Dorfman syndrome) (Fischer *et al.*, 2007; Lefevre *et al.*, 2001).

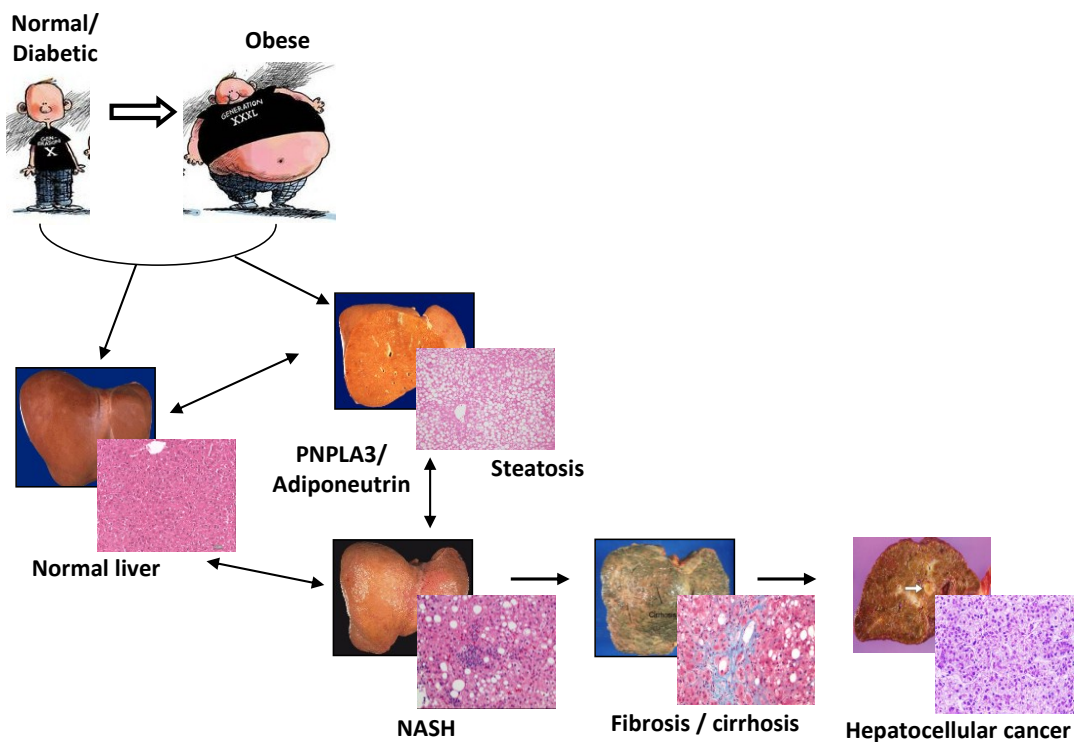


Figure 1. Schematic illustration of nonalcoholic fatty liver disease progression. (Original pictures are from Medscape, American journal of Roentgenology and (Jha et al., 2013)).

In obese or metabolic syndrome patients, there is a massive spillover of fat in the body which gets accumulated in various organs including liver wherein the condition is called steatosis and when inflammation along with fibrosis sets in, the condition is called NASH, which can progress to cirrhosis and then hepatocellular cancer. Polymorphisms of PNPLA3/ adiponeutrin have been implicated as a major genetic determinant of NAFLD/NASH.

4.2. Lipases

AT is the main source of FAs in circulation. Obesity, insulin resistance, NAFLD are all associated with increased adipose tissue lipolysis (Cohen *et al.*, 2011; Donnelly *et al.*, 2005). Additionally, rapid weight loss and bariatric surgery

induces lipolysis in the AT (Duncan *et al.*, 2007; Johansson *et al.*, 2008). Thus understating the factors influencing lipolysis may be of significant importance.

Hydrolysis of neutral lipids (TGs) to FAs and glycerol requires three consecutive steps that involve at least three different enzymes: ATGL catalyzes the initial step of lipolysis and is the rate-limiting enzyme in triglyceride hydrolysis (Haemmerle *et al.*, 2006; Zimmermann *et al.*, 2004), converting TGs to diacylglycerols (DGs). HSL is mainly responsible for the hydrolysis of DGs to monoacylglycerols (MGs) and MG lipase (MGL) hydrolyzes MGs to FA and glycerol (Zechner *et al.*, 2012) (Figure 2). In adipose tissue, ATGL and HSL are responsible for more than 90% of TG hydrolysis (Schweiger *et al.*, 2006). Although most nonadipose tissues, including liver also express ATGL and HSL, their expression levels are rather low compared to adipose tissue, suggesting the presence of additional lipases. In liver, HSL expression is very low compared to ATGL, suggesting a rather benign role of HSL in hepatic lipid metabolism. Importantly, increased hepatic steatosis was shown in liver specific ATGL-KO mice (Wu *et al.*, 2011) and by shRNA mediated ATGL knock-down (Ong *et al.*, 2010). Additionally, severe hepatic steatosis has been also observed in mice lacking the co-activator of ATGL -CGI-58 (Radner *et al.*, 2010). Subsequent work by Brown (Brown *et al.*, 2010) also showed high fat diet-induced severe hepatic steatosis in ASO-mediated knockdown of CGI-58 in mice. These findings strongly suggest a very crucial role of ATGL in hepatic TG metabolism. Furthermore, enzyme activity of ATGL can be enhanced by its coactivator -cgi-58 and be inhibited by G0S2 without changes in protein levels. Additionally, ATGL and HSL activities can be regulated by posttranslational modification by phosphorylation of different serine residues (Zechner *et al.*, 2012).

Other enzymes that have been described to contribute to hepatic TG metabolism include adiponutrin/patatin-like phospholipase domain containing 3 (PNPLA3), some members of the carboxylesterase [Ces (mouse) or CES (human)] family (mouse Ces3/TGH, and its human ortholog CES1/TGH; mouse Ces1/Es-x), arylacetamide deacetylase (AADA or AADAC), hepatic lipase (HL) and lysosomal acid lipase (LAL) (Quiroga *et al.*, 2012). However, it appears that

FAs released from cytosolic lipid droplets by cytosolic lipases (ATGL, HSL, MGL) are exclusively utilized for mitochondrial FA oxidation and perhaps as ligands for nuclear receptors (PPARs). Whereas, the other lipid hydrolases seem to play a role in lipoprotein assembly, modulation of lipid storage and hydrolysis of lipoproteins (Quiroga *et al.*, 2012).

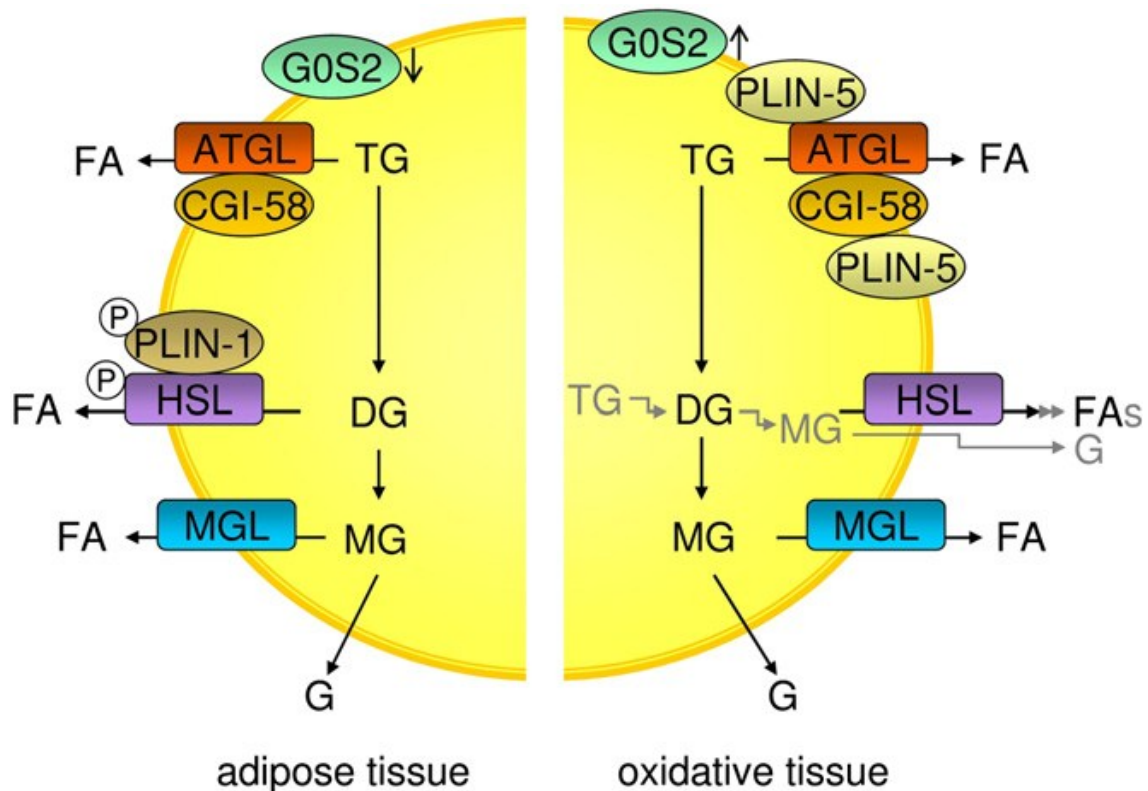


Figure2. Lipolysis in Adipose and Oxidative Tissues during Fasting (Zechner *et al.*, 2012).

In adipose tissues, beta-adrenergic stimulation of lipolysis leads to the consecutive hydrolysis of TG and the formation of FAs and glycerol. The process requires three enzymes: ATGL cleaves the first ester bond in TGs, HSL hydrolyzes DGs, and MGL MGs. For full hydrolytic activity, ATGL interacts with its co-activator protein CGI-58, whereas HSL is phosphorylated, translocates to the lipid droplet (LD), and interacts with phosphorylated PLIN-1. Expression of the ATGL inhibitor G0S2 during fasting is low in adipose and high in oxidative tissues (e.g., liver). In oxidative tissues PLIN-1 is not present on LDs. Instead, PLIN-5 is expressed and interacts with both ATGL and CGI-58, facilitating LD localization of these proteins. ATGL, adipose triglyceride lipase; CGI-58,

comparative gene identification-58; DG, diacylglycerol; FA, fatty acid; G, glycerol; G0S2, G0/G1 switch gene 2; HSL, hormone-sensitive lipase; MG, monoacylglycerol; MGL, monoglyceride lipase; PLIN-1, perilipin-1; PLIN-5, perilipin-5; TG, triacylglycerol.

4.3. Peroxisome proliferator-activated receptors (PPARs)

4.3.1 Overview

PPARs belong to a superfamily of nuclear hormone receptors consisting of 3 isotypes: PPAR α , PPAR β/δ and PPAR γ . They are ligand dependent transcription factors. Upon ligand binding, PPARs form a heterodimer with retinoid X receptor, interact with PPAR response elements in the target genes, and regulate their expression(Berger *et al.*, 2002).

Tissue distribution of PPARs: PPAR α and PPAR δ are highly expressed in oxidative tissues (liver, kidney, heart and muscle) and regulate genes involved in substrate delivery, substrate oxidation, and oxidative phosphorylation. In contrast, PPAR γ is more important in lipogenesis and lipid synthesis, with highest expression levels in WAT. PPAR δ is expressed in a wide range of tissues and cells, with relatively higher levels of expression in brain, adipose, and skin. So far, no PPAR δ -specific gene targets have been identified. Additionally, PPARs are expressed in immunological and vascular wall cell types(Chinetti *et al.*, 2000).

PPAR ligands: PPARs are activated by fatty acids, fatty acid-derived molecules and a number of pharmacological compounds like hypolipidemic drugs of fibrate class, antidiabetic drugs of the thiazolidinedione class etc. As such, the synthetic ligands of PPARs are used to treat diabetes and dyslipidemia. All three PPAR subtypes are able to significantly respond to fatty acids with different yet overlapping specificities. The ligand binding pocket of PPARs appears to be quite large in comparison with other nuclear receptors. This difference may

allow the PPARs to interact with a broad range of structurally distinct natural and synthetic ligands(Lemberger *et al.*, 1996).

PPAR α is activated by a broad array of medium- to long-chain unsaturated fatty acids (C10–C22) which are natural ligands of PPAR α (Forman *et al.*, 1997; Kliewer *et al.*, 1997) and are taken up from the diet(Patsouris *et al.*, 2006; Ziouzenkova *et al.*, 2003)or derived from *de novo* synthesis(Chakravarthy *et al.*, 2005). It has been proposed that fat derived from peripheral mobilization of adipose stores constitutes a different hepatic compartment ('old fat') that does not activate PPAR α as effectively as fat from *de novo* synthesis ('new fat') (Chakravarthy *et al.*, 2005). PPAR α expression in liver is increased by stress or fasting, and its level cycles according to the circadian rhythm of circulating glucocorticoids (Lemberger *et al.*, 1996).

Role of PPAR α in liver. PPAR α is consistently and predominantly expressed in liver which is the major tissue showing peroxisome proliferation in rodents. In liver PPAR α regulates FA synthesis, oxidation, gluconeogenesis, ketogenesis, lipoprotein assembly and represses inflammation(Berger *et al.*, 2002; Devchand *et al.*, 1996). PPAR α null mice are resistant to all of the pleiotropic effects of peroxisome proliferators, including cell proliferation and hepatocarcinogenesis. Remarkably, the toxic effects of PPAR α agonists are lost in humans. This would suggest that the PPAR α signaling potential is much more robust in mice than in humans. To investigate the mechanism of carcinogenesis and species differences in response to peroxisome proliferators, PPAR α -humanized mouse models were generated. The mechanism of hepatocellular proliferation involves downregulation of the microRNA let-7c gene by PPAR α . Let-7c controls levels of proliferative c-myc by destabilizing its mRNA. Thus, upon suppression of let-7c, c-myc mRNA and protein are elevated resulting in enhanced hepatocellular proliferation. In contrast, PPAR α humanized mice are resistant to peroxisome proliferator-induced cell proliferation and cancer. These mice do not exhibit downregulation of let-7c gene expression thus forming the basis for the resistance to hepatocellular carcinogenesis. This suggests that the species differences in response to peroxisome proliferators could be due in part to a differential ability of the mouse and human PPAR α to suppress let-7c gene

expression. The mechanism for suppression of this gene and the molecular differences between m PPAR α and hPPAR α is the subject of future investigations (Gonzalez *et al.*, 2008).

4.3.2. Role of PPAR α in hepatic FA metabolism

Fatty acids (Fas) taken up by the liver originate mainly from non-esterified fattyacids (NEFA) carried by serum albumin and from the hydrolysis of triglycerides conveyed in chylomicrons. Triglycerides are hydrolyzed extracellularly into glycerol and FAs by the lipoprotein lipase (LPL), whose gene is stimulated by PPARs. Once imported into hepatocytes, FAs are activated into acyl-CoA thioesters by various acyl-CoA synthetases (ACS), one of which, the long-chain fatty acid ACS, is regulated by PPAR α at the transcriptional level. Acyl-CoA may then either be esterified or, alternatively, enter the pathway of β -oxidation. In liver, very long-chain fatty acyl-CoA (>C20) is β -oxidized in peroxisomes. All three enzymes involved in the peroxisomal β -oxidation pathway, e.g. acyl-CoA oxidase (ACO), enoyl-CoA hydratase/dehydrogenase bifunctional enzyme (HD), and keto-acyl-CoA thiolase, are direct PPAR α target genes. Long- (C14 to C20) and medium-chain (C8 to C12) fatty acids can be oxidized either in peroxisomes or in mitochondria. The flux of fatty acids into mitochondria is controlled by carnitine palmitoyl transferase I (CPT I), which is strongly induced by peroxisome proliferators and fatty acids. PPAR α further directly regulates the mitochondrial β -oxidation by stimulating the expression of the medium-chain acyl-CoA dehydrogenase (MCAD) gene. Finally, in liver, most of the acetyl-CoA derived from fatty acid β -oxidation is converted into ketone bodies. The rate-limiting enzyme of this last pathway, the hydroxymethylglutaryl-CoA synthetase, is controlled by PPAR α . Fasting or stress situations stimulate adipose lipolysis, which leads to an increase of the plasma levels of non-esterified fatty acids. In turn, the rates of fatty acid uptake, fatty acid β -oxidation, and ketogenesis are activated in liver. Thus fasting and stress represent physiological situations in which PPAR α is likely to be activated by fatty acids and to stimulate the oxidative pathway (Lemberger *et al.*, 1996).

4.3.3. Lipolysis derived fatty acids and PPAR α signaling

FAs involved in signalling originate from import of exogenous FAs (from circulating FA-albumin complexes or from LPL-mediated hydrolysis of plasma VLDL and chylomicrons) or from endogenous *de novo* synthesis. Recently it was shown that neither source of FA can generate PPAR ligands directly; rather, a cycle of FA esterification and rehydrolysis is required. As a consequence, lipolysis impaired ATGL-deficient mice exhibit a severe defect in PPAR α signaling in oxidative tissues such as liver, macrophages and BAT. The most dramatic phenotype is observed in cardiac muscle. The reduced expression of PPAR α target genes in ATGL knockout animals causes severe mitochondrial dysfunction, decreased rates of substrate oxidation and OXPHOS, massive cardiac lipid accumulation, and lethal cardiomyopathy within a few months after birth. HSL deficiency is also associated with moderately decreased PPAR α target-gene expression but does not generate a comparable cardiac phenotype, indicating the specific importance of ATGL activity in the generation of PPAR α ligands or ligand precursors (Zechner *et al.*, 2012).

4.3.4. Anti-inflammatory role of PPAR α

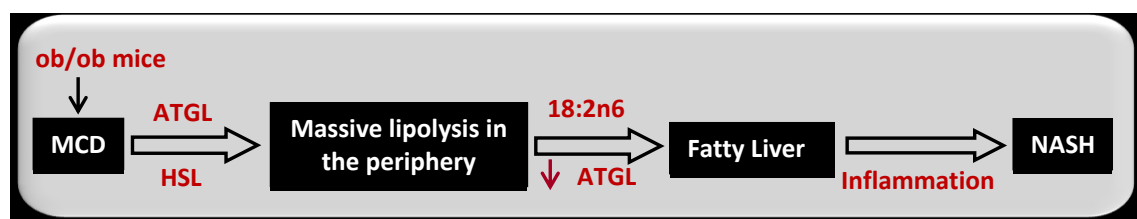
PPAR α was first proposed to be a modulator of inflammation when leukotrieneB₄ (LTB₄), a potent chemotactic agent, was found to be a ligand and agonist for the receptor. It was suggested that activation of PPAR α inhibited the inflammatory action of such eicosanoids by augmenting expression of hepatic enzymes involved in their metabolism (Devchand *et al.*, 1996). This was further proven by the observation that PPAR α null mice have more extended inflammatory responses than their wild-type littermates in response to LTB₄ or its precursor arachidonic acid. Other, nonhepatic, anti-inflammatory mechanisms have been described for PPAR α ligands that may be important in maintaining vascular health. In addition, PPAR α decreases inflammation, mainly through direct interaction with NF- κ B, causing inhibition of its signaling pathway or reducing the activated levels of NF- κ B and subsequent inflammation (Berger *et al.*, 2002). Additionally, lack of functional PPAR α

contributes to increased susceptibility to the development of NAFLD and NASH upon high fat diet or MCD feeding (nutritional model of NASH in rodents) through increased steatosis, oxidative stress and inflammatory response (Abdelmegeed *et al.*, 2011; Ip *et al.*, 2003). This effect can further be ameliorated by treatment with PPAR agonists (Ip *et al.*, 2004). Additionally, it has been shown that the anti-inflammatory effect of PPAR α fails in the fatty liver on increased pro-inflammatory pressure. Thus, an imbalance between PPAR-mediated anti-inflammatory and NF- κ B-mediated pro-inflammatory signals may contribute to increased inflammation in the fatty liver (Romics *et al.*, 2004). Additionally, lack of functional PPAR α contributes to increased susceptibility to hepatic inflammation *per se* and acute phase response (APR) (Gervois *et al.*, 2004; Yoo *et al.*, 2011; Zambon *et al.*, 2006).

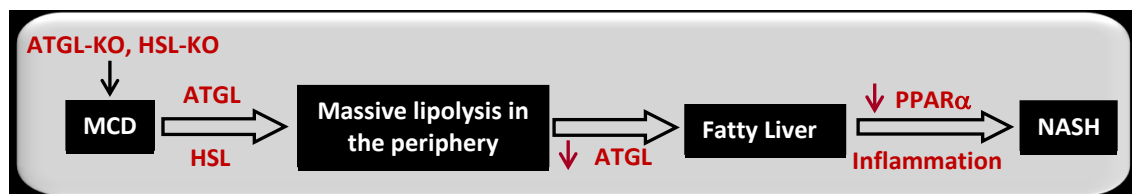
5. AIM AND APPROACH

The aim of the research described in this thesis was to elucidate the role of metabolic lipases (ATGL and HSL) in modulating hepatic inflammation in mouse models of NASH/NAFLD and endotoxemia. In order to address this, the following approaches were taken:

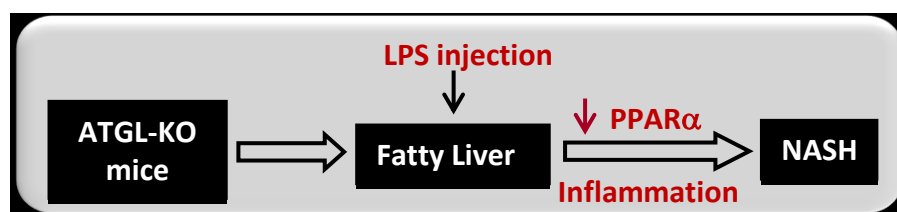
1. ob/ob mice were fed MCD diet to study the role of ATGL and hormone-sensitive lipase (HSL) as main intracellular metabolic lipases in a nutritional model of NASH.



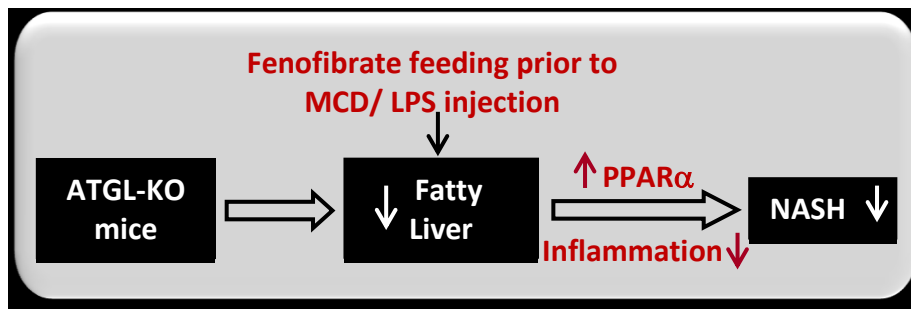
2. To unravel the role of intracellular lipases for liver injury, mice lacking ATGL or HSL were challenged with MCD.



3. To study the role of lipases in acute inflammation ATGL- and HSL-KO mice were challenged with LPS as a potential second hit in progression to NASH.



4. Finally, we tested whether PPAR α agonist (fenofibrate) treatment recovered hepatic inflammation in MCD- or LPS-challenged ATGL-KO mice.



6. Methods

Methods are partially reused from Jha et al., 2013 with modifications. The material is reproduced with permission of John Wiley & Sons, Inc.

6.1. Animal care and use

ob/ob Mice were obtained from Charles River, Germany. ATGL-deficient and HSL-deficient mice were generated by targeted homologous recombination as previously described (Haemmerle *et al.*, 2006; Haemmerle *et al.*, 2002). All animals were housed under a 12:12-hour light/dark cycle and permitted *ad libitum* consumption of water and diet. All 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).

6.2. Feeding and injection protocols

MCD and its corresponding control diet was obtained from Safe-diets (Augy, France). The diets were identical in all nutrients except methionine (2.8g/kg) and choline (1,6/kg), which were present in the control diet only. Both diets provided 64.2% kcal as carbohydrate, 25.8% kcal as protein, and 10% kcal as fat. 8 weeks old ob/ob mice were fed with MCD or its corresponding control diet for 5 weeks. 7-8 week old WT, ATGL-KO and HSL-KO mice were fed with MCD or control diet for 2 weeks. Mice were harvested between 1-4 pm without fasting. 8-10 week old WT, ATGL-KO and HSL-KO mice were injected with a single dose of LPS (4µg/g body weight) intraperitoneally (i.p.) for 12h (4am-4pm). Control mice were injected with saline. Both groups were fasted after injection since LPS is known to induce anorexia (Elander *et al.*, 2007). In order to avoid the effects of fasting prior to LPS, mice were injected in the morning (4am) when they are fully fed. For survival rate analysis, male mice were injected with a single dose of 5µg/g LPS. For fenofibrate (FF) treatment- 8-9 week old WT and ATGL-KO mice were fed with MCD supplemented with 0.1%

FF for 2 weeks and compared it to mice receiving MCD diet alone. Additionally, 8-9 week old WT and ATGL-KO mice received 0.2% FF enriched diet for 6 days followed by 12h LPS injection on the 7th day to the FF-fed or chow-fed mice. Unless otherwise indicated, all experiments were performed on female mice. All data are representative of 2-4 independent experiments with 4-7 animals per group. Further details about animal care and use are described in detail in supplementary methods.

6.3. Serum Analysis

Blood was collected by retro-orbital puncture under anaesthesia and centrifuged for 15 min at 4500 rpm. Serum was stored at -80°C until analysis. ALT, AST, bile acids and bilirubin were analyzed on a Hitachi 917 analyzer (Boehringer Mannheim, Mannheim, Germany). Serum FFAs and ketone bodies were determined using commercial kits (Wako Chemicals, Neuss, Germany). Serum cytokines – TNF α and IL6 were measured using Platinum ELISA kit (eBioscience). Serum FGF21 was measured using ELISA kit (Millipore).

6.4. RNA isolation and PCR analysis

Tissues were snap frozen in prechilled 2-methylbutane and store in liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA was quantified using NanoDrop 1000 Spectrophotometer (Thermo Scientific). 1.5 μ g of RNA was used for complementary DNA synthesis using random hexamer primer (Applied Biosystems) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. 1:20 dilution of the cDNA was used for qRT-PCR using SYBR Green Master Mix (Applied Biosystems) and was performed using AB7900 Real-Time PCR system (Applied Biosystems). Reactions were performed in duplicates and relative mRNA levels were quantified using a calibration dilution curve normalized to the housekeeping genes.

mRNA levels were normalized to 36b4 or 18S as housekeeping gene which did not vary between groups. 36b4 was used for a) MCD-fed ob/ob mice, b) LPS or

Saline injected WT and ATGL-KO mice and c) Fenofibrate (FF) fed and LPS/Saline injected WT and ATGL-KO mice. 18S was used for a) MCD/control fed WT and ATGL-KO mice and b) MCD or MCD supplemented with FF experiment.

6.5. Assay for tissue TG hydrolase activity

TG hydrolase assay was performed as previously described with modifications (Jha *et al.*, 2013; Schweiger *et al.*, 2006). In brief, WAT and liver samples of mice were surgically removed and washed in phosphate-buffered saline (PBS), containing 1mM EDTA and 5U/ml heparin. Tissues were stored in liquid nitrogen until use. Homogenisation was performed on ice in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1:2000 dilution of protease inhibitor cocktail, pH 7.0) using MagNA Lyser (Roche). Tissue lysates were centrifuged twice at 13,000 rpm, 4 °C. The lipid free infranatant (cytosolic fraction) was collected and used for TG hydrolase assays. The substrate for the measurement of TG hydrolase activity containing triolein and [9,10-³H]triolein (Hartmann analytic GMBH, Vienna, Austria) as radioactive tracer was emulsified with phosphatidylcholine and phosphatidylinositol in 100mM potassium phosphate buffer (pH7) using a sonicator, followed by addition of 5% FA free bovine serum albumin. Final substrate concentration was 167 nmol of triolein/assay (8000 cpm of [9, 10-³H]triolein/nmol). 20-30µg of WAT and 50-150µg of liver cytosolic fractions (0.1 ml) supplemented with or without specific inhibitor for HSL (10µM) were incubated with 0.1 ml of the substrate in a water bath at 37 °C for 60 min. The reaction was terminated by adding 3.25 ml methanol/Chloroform/heptane (10:9:7) and 1 ml 0.1 M potassium carbonate, 0.1 M boric acid, (pH 10.5). After centrifugation at 2,300rpm for 15 min, radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting in LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA). ATGL activity was defined as remaining activity not inhibited by the HSL inhibitor (activity in the presence of the HSL inhibitor). HSL activity was calculated by subtracting the activity obtained in the presence of the HSL inhibitor from total activity.

Ex-vivo lipolysis of isolated gonadal WAT: Free FA release by *ex-vivo* lipolysis of isolated gonadal WAT was performed as previously described (Schweiger *et al.*, 2006).

6.6. DNA binding activity of PPAR α

PPAR α activity was assayed using an enzyme-linked immunosorbent assay-based PPAR α Transcription Factor Assay Kit (Cayman Chemical) according to the manufacturer's instructions. Nuclear proteins were isolated from liver using NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions with minor modifications. Protein concentration was determined by Lowry method. Nuclear extracts were snap frozen and later used for the assay, without any freeze-thaw cycle. Clarified cell lysate was supplied for PPAR α which acted as effective positive controls for PPAR α DNA binding. Specificity of binding was also demonstrated using wells with no nuclear protein added. In these wells, no binding was detected (data not shown). Binding activity was measured at 450 nm (minus the blank) and calculated as relative activity to the control.

6.7. Hepatic TG determination and histology

Liver samples were washed in phosphate-buffered saline, weighed (50-70 mg) and frozen. Total lipids were extracted by the method of Folch (Folch *et al.*, 1957) with modifications:

Tissues were homogenized in 1ml methanol in MagNA Lyser (Roche), transferred in glass pyrex tubes containing 2ml chloroform and 30 μ l acetic acid (chloroform: methanol: acetic acid in the ratio of 66/33/1 v/v/v). Tubes were then placed on a rotator for 2-3 hrs. 600 μ l of water was added to each tube for phase separation (1/5th of methanol and chcl₃) followed by 30 sec Vortexing. Tubes were then centrifuged for 15mins 2000g, 4°C. The lower organic phase was collected in 2ml tubes, from which 200-300 μ l was evaporated. After drying, 1% triton X (in water) was added to the samples. Amount varies depending on WT, KO, fasted or fed. The tubes were kept on heating block 60° for around 1hr and then sonicated. For protein determination, the protein cake in the pyrex tubes

are dried and then dissolved O/N in 2ml, 0,3N NAOH/0, 1% SDS at 60°C, 1400 rpm. TG content was measured with a commercial kit from Diagnostic Systems International (Holzheim, Germany) and normalized to the protein content. For histology livers were fixed in 4% neutral buffered formaldehyde solution for 24 h and embedded in paraffin. 2 µm thick sections were cut and stained with haematoxylin and eosin (H&E).

6.8. Immunohistochemistry (CD11b and F4/80)

CD11b immunohistochemistry was done with support of Dr. Anna Baghdasaryan.

For detection of CD11b positive cells, 2µm thick cryosections of liver tissue were fixed in 4% paraformaldehyde solution in PBS for 10 minutes, rinsed 3 times in PBS, treated with pre-cooled methanol for 5 mins followed by pre-cooled acetone for 3 mins in -20 °C. Sections were then washed 3 times in PBS followed by 1hr incubation with anti-mouse CD11b antibody (BD PharMingen, 550282, dilution 1:100 in antibody diluent (DAKO)). Sections were washed 3 times in PBS followed by 1hr incubation with the polyclonal rabbit-anti-rat secondary antibody (DAKO, E0468, dilution 1:100 in antibody diluent (DAKO)). Sections were washed 3 times in PBS followed by incubation with rabbit on rodent HRP-polymer (catalog no. RMR622; Biocare Medical) for 30 min. Subsequently, detection was carried out using AEC Substrate Chromogen ready-to-use (Dako K3464) under microscopic control. Negative controls were performed in the absence of primary antibody. Nuclei were counter-stained in Hemalaun nach Mayer for 30 sec, followed by washing in running water. Aquatex as glue was used for fixing the cover slips onto the slide. For quantification of CD11b positive cells, the slides were scanned at 40x magnification using scanscope (Aperio technologies, CA). The positive cells were then manually quantified in 60 power fields.

For detection of F4/80 positive cells, 3µm and 2 µm thick paraffin sections of gonadal WAT and BAT respectively were mounted on charged glass slides, deparaffinized in xylene, rehydrated in ethanol and treated with 0.1% protease

XXIV, followed by 1% hydrogen peroxide. Sections were incubated overnight at 4°C with rat-anti-mouse F4/80 antibody (AbD Serotec, MCA497GA; 1:50 dilution in antibody diluent (DAKO)). Subsequently, sections were incubated with polyclonal rabbit-anti-rat secondary antibody (DAKO, E0468, dilution 1:100 in antibody diluent (DAKO)) for 30 min followed by incubation with rabbit on rodent HRP-polymer (catalog no. RMR622; Biocare Medical) for 30 min. Subsequently, detection was carried out as stated above. For quantification of F4/80 positive cells, slides were scanned at 40x magnification using scanscope (Aperio technologies, CA). The positive cells were then manually quantified in 10, 40x power fields.

6.9. GC-NICI/MS of free fatty acids

This was done with support of the core facility of mass spectrometry, ZMF, Graz by Harald Köfeler and Astrid Knopf.

Mouse livers (50-70 mg) were homogenized in 0.5 ml of PBS and 0.5 ml of methanol. Each sample was spiked with 1.2 nmol of 15:0 fatty acid as internal standard immediately. For serum, 2.5 nmol of 15:0 was added to 50 µl of the sample followed by 0.5 ml of methanol. Then, lipids were extracted according to Bligh and Dyer method (Bligh *et al.*, 1959). Lipid extracts were dried and dissolved in 50 µl of a pentafluoro benzyl bromide solution (3.4 % in acetonitrile) and 10 µl of N, N-diisopropyl ethanolamine. After 10 min of incubation at room temperature samples were evaporated under a stream of nitrogen and resuspended in 50 µl of hexane. A Trace-DSQ GC-MS (Thermo Scientific, Austin, TX) equipped with a TR-FAME 30m column was used in splitless mode with 1 ml/min helium as carrier gas and 300°C injector temperature. The initial oven temperature of 150°C was held for 1min and then increased to 200°C at a rate of 25°C/min followed by 325°C at a rate of 12.5°C/min and held for 2min. The mass spectrometer was run in negative ion chemical ionization (NICI) mode. Fatty acids were detected in full scan as carboxylates after loss of the pentafluoro benzyl moiety. Methane was used as CI gas. Source temperature was set to 250°C and the transfer line temperature to 330°C.

6.10. GC-El/MS of total fatty acids (free and esterified)

This was done with support of the core facility of mass spectrometry, ZMF, Graz by Harald Köfeler and Astrid Knopf.

Lipids were extracted according to Bligh & Dyer method as described above. For liver, each sample was spiked with 375 nmol of 15:0 fatty acid as internal standard. For serum, 22.5 nmol of 15:0 was added to 50µl of the sample. Then lipids were dried and suspended in 1 ml methanol NaOH. After 10 min incubation at 80°C, samples were cooled for 5 min on ice. Then, 1 ml BF₃ was added and incubated for 10 min at 80°C. Fatty acid methyl esters were extracted with 1 ml saturated NaCl and 2 ml hexane. The hexane phase was dried and methyl esters dissolved in 1.5 ml hexane. Trace-DSQ GC-MS equipped with a TR-FAME 30m column was used for analysis. Helium was used as carrier gas at a flow of 1.3 ml/min, in split mode, at 250°C injector temperature. Initial oven temperature of 150°C was held for 0.5 min and then temperature was increased to 180°C at a rate of 10°C/min. This was followed by a further increase to 190°C at a rate of 0.5°C/min and then increased to 250°C at a rate of 40°C/min and kept for 3 min. The mass spectrometer was run in electron impact mode and fatty acids were detected in full scan of m/z 80 - 400. Source temperature was set to 250°C and the transfer line temperature to 200°C.

6.11. Western blot analysis

For nuclear FABP1, NE-PER nuclear and cytoplasmic extraction kit (Pierce) was used for nuclear protein isolation. Proteins were separated as described previously with minor modifications (Trauner *et al.*, 1997). Blots were probed with antibodies specific to FABP1 (Santa Cruz, cat. no. sc-50380; 1:3.000) and β-Actin (Sigma-Aldrich, cat. no. A5441) as primary antibodies and a peroxidase-conjugated affinity-purified secondary antibody obtained from Cell Signaling (cat. no. 7074; 1:3000) for FABP1 and from Dako (cat. no. P0260; 1:1000) for β-Actin. 15µg of nuclear extract was used for LPS treatment and 10µg was used for fenofibrate treatment.

For ATGL and HSL Western blots, cytosolic extracts were used as described for TG hydrolase activity. Blots were probed with antibodies specific to ATGL (Cell Signaling, cat. no. 2439S; 1:1,000), HSL (Cell signaling, cat. no. 4107S; 1:500) and β -Actin (Sigma-Aldrich, cat. no. A5441) as primary antibodies and a peroxidase-conjugated, affinity-purified secondary antibody obtained from Cell signaling (cat. no. 7074; 1:3000) for ATGL, and HSL and from Dako (cat. no. P0260; 1:1000) for β -Actin. 20 μ g and 30 μ g of cytosolic extract was used for ATGL and HSL blots for WAT and liver, respectively.

For quantification of relative expression levels, blots were scanned using a densitometer (BioRad) and quantified with ImageJ soft-ware (NIH).

6.12. Statistics

Data are presented as mean $\pm$ SD of reported animals in each group (on average 4-7 animals unless stated otherwise). Statistical analysis was performed using SPSS V.14.0. Statistical significance was determined by the Student's unpaired two-tailed *t* test. Group difference were considered significant for $p < 0.05$ (*, #, §), $p < 0.01$ (**, ##, §§), $p < 0.001$ (***, ###, §§§).

7. Results

7.1. Role of adipose tissue in MCD deficient model of NASH in ob/ob mice

7.1.1. Effect of MCD diet on adipose depots and serum non-esterified fatty acid (NEFA) levels. To delineate the role of adipose tissue in steatohepatitis in a mouse model, which combines key features of obesity and insulin resistance, we challenged ob/ob mice with MCD diet for 5 weeks to promote progression of steatosis to NASH. MCD-fed ob/ob mice lost ~38% of their body weight at the end of 5 weeks (Figure. 3A) due to substantial reduction of all adipose depots: gonadal (~48%), perirenal (~44%), visceral (~53%) and BAT (~60%) (Figure. 3B) in line with the previous findings in MCD-fed db/db mice (Rinella *et al.*, 2008). The expression of transcription factors known to promote adipogenesis, including, PPAR γ 2, C/EBP α , ap2/ FABP4 and SREBP1c were similar in both control and MCD-fed mice (Figure. 3C) suggesting that defective adipogenesis was not responsible for WAT loss. Additionally, expression of genes involved in (i) FA synthesis, including ACC1, SCD1, ACSL1, (ii) FA storage, including DGAT1, DGAT2, LIPIN1 and (iii) FA degradation, including PPAR α and CPT1 β were similar in both groups which suggest that these processes of FA metabolism were not responsible for WAT loss in MCD-fed mice. Despite substantial WAT loss, serum NEFA concentrations were not elevated (Figure. 3D). This apparent discrepancy may be attributed to the reduced amount of WAT in the MCD-fed group since NEFA levels were 1.5-fold elevated when normalized to the WAT weight (Figure. 3E). With disease progression, MCD-fed ob-ob mice were no longer hyperphagic as also previously shown in db/db mice (Rinella *et al.*, 2008) (Figure. 3F).

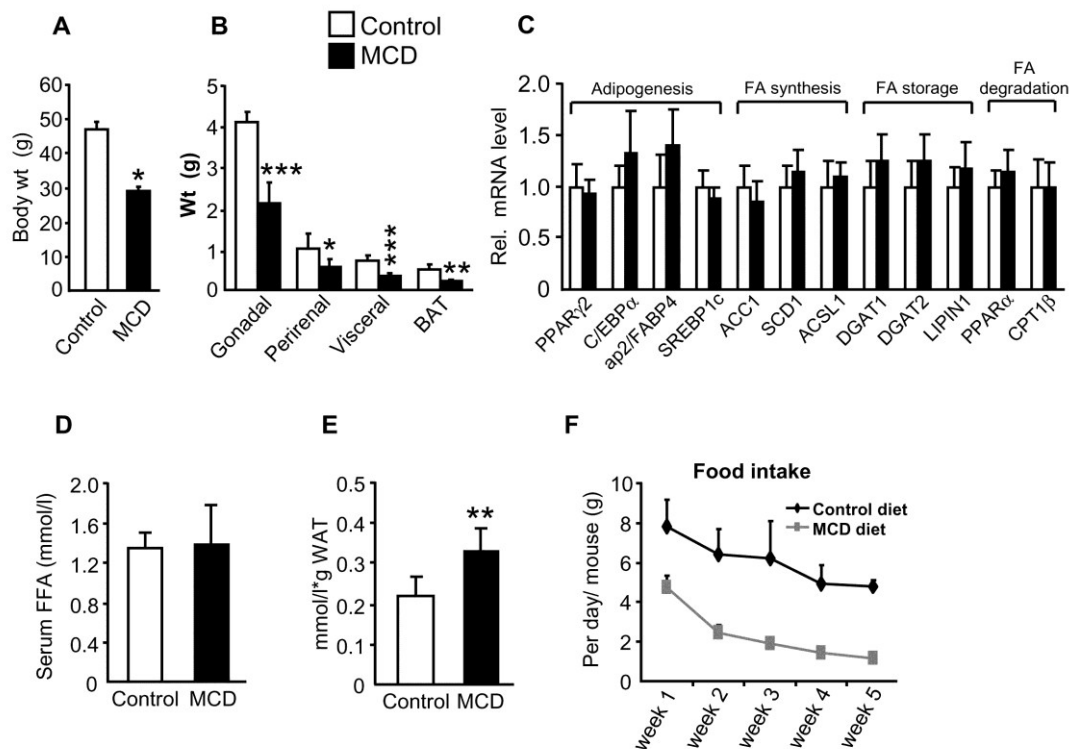


Figure 3. Effect of MCD diet on adipose tissue depots.

ob/ob Mice were fed with control or methionine-choline-deficient (MCD) diet for 5 weeks. Mice were harvested in non-fasted state. (A) Final body weight. (B) Fat depot analysis of gonadal, perirenal, visceral WAT and BAT. (C) mRNA expression of markers of adipogenesis, fatty acid (FA) synthesis, storage and degradation in gonadal WAT. (D) Serum NEFA concentration. (E) Serum NEFA concentration normalized to the total WAT weight. (F) Food intake. $n = 6$ per group. White bars indicate control diet fed mice, black bars MCD-fed mice. Bars represent mean $\pm$ SD. *** $p < 0.001$ ** $p < 0.01$, * $p < 0.05$, versus *ob/ob* control group.

7.1.2. Increased peripheral lipolysis in MCD-fed *ob/ob* mice is due to increased ATGL and HSL activities in adipose depots. Loss of adipose depots and increased ratio of plasma free FAs per gram fat mass indicated an elevated lipolytic rate under non-fasting state. Indeed, as shown in Figure. 4(A-E, white bars), total TG hydrolase activities increased in all adipose depots with the highest increase observed in visceral (~130%), followed by perirenal (~83%), subcutaneous (~82%), gonadal WAT (63%) and BAT (~86%). The

maximal increase of ATGL activity (HSL-inhibited TG hydrolase activity) was seen in visceral (~204%), followed by subcutaneous WAT (~112%), BAT (~93%), perirenal (~82%) and gonadal WAT (~78%) (Figure. 4 A-E, black bars). Compared to ATGL, HSL activity (determined by subtraction of HSL inhibited lipase activity from total lipase activity) was induced to a lesser extent by ~108% in visceral, ~71% in subcutaneous, ~84% in BAT, ~85% in perirenal and ~65% in gonadal WAT (Figure. 4 A-E, grey bars). Western blot analysis also showed increased ATGL and to a lesser extent HSL protein expression in MCD-fed ob/ob mice (Figure. 4F). Collectively, these data show that MCD induces ATGL and HSL activities, resulting in loss of WAT and BAT depots in MCD-fed ob/ob mice.

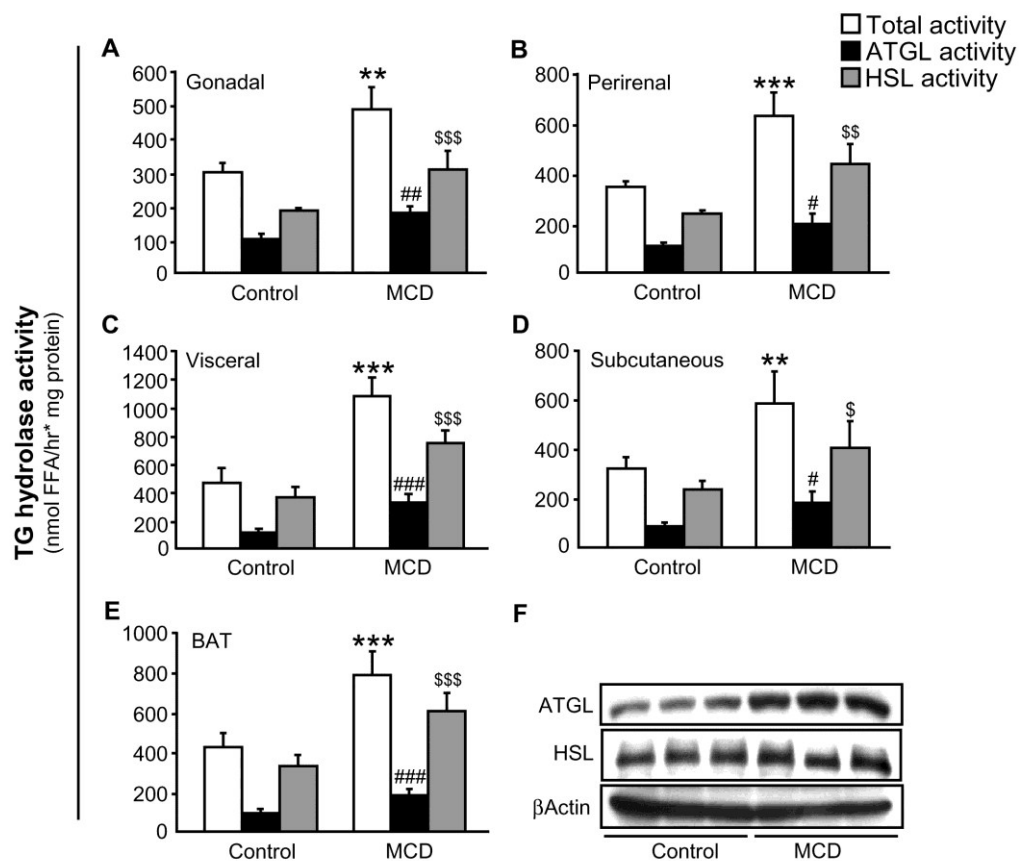


Figure 4. TG hydrolase activities in control and MCD fed ob/ob mice.

(A-E) TG hydrolase activities in cytosolic extracts of gonadal (A), perirenal (B), visceral (C), subcutaneous (D) WAT and BAT (E). Total activity (white bars),

ATGL activity (TG hydrolase activity after inhibition with 10mM HSL inhibitor, black bars), HSL activity (determined by subtraction of HSL inhibited lipase activity from total lipase activity, grey bar). (F) Representative Western blots for protein levels of ATGL and HSL in subcutaneous WAT. $n = 5 - 6$ per group. Bars represent mean $\pm$ SD. $^{\#}\$p<0.05$, $^{**}\#\$\$p<0.01$, $^{***}\#\#\$\$p<0.001$, versus corresponding ob/ob control group.

7.1.3. Analysis of NEFA composition in serum and liver. Next, we determined free/non-esterified fatty acids (NEFA) composition in serum and liver of MCD- and chow-fed ob/ob mice by gas chromatographic/negative ion chemical ionisation mass spectrometry (GC-NICI/MS). 18:2n6 (linoleic acid), was the major free FA species found to be significantly elevated (~89%) in serum of MCD-fed mice. While there was a modest decrease in fatty acids – 18:0, 18:1n11 and 20:4n6 (Figure. 5A). GC-NICI/MS of NEFAs in liver showed a marginal but significant increase in the saturated fatty acids- 14:0 (~17%), 16:0 (~28%), and 18:0 (~30%), (Figure. 5B). With respect to the monounsaturated fatty acid (MUFA), only 16:1n7 was significantly increased (~27%) in MCD-fed mice while there was no significant change in the levels of 18:1n9 and 18:1n11. While the polyunsaturated fatty (PUFA) acid- 18:2n6 showed the maximal increase (~195%) followed by 20:4n6 (~90%) in MCD-fed ob/ob mice (Figure. 5B).

We next analysed whether the increased level of these fatty acids in liver were due to increased lipolytic flux from the adipose tissue or due to increase in DNL or elongation and desaturation of fatty acids in the liver. Our analysis of mRNA expression revealed markedly reduced expression of genes involved in DNL and MUFA synthesis including, sterol regulatory element-binding protein 1c (SREBP1c, ~55%), acetyl CoA carboxylase 1 (Acc1, ~81%), fatty acid synthase (FASN, ~85%), stearoyl-CoA desaturase-1 (SCD1, ~98%) and fatty acid elongase 6 (ELOVL6, ~76%) in MCD-fed ob/ob mice (Figure. 5C), suggesting that the increased levels of FAs- 14:0, 16:0, 18:0 and 16:1n7 was the contribution from WAT. Increased levels of 18:2n6 in the liver was the direct contribution from AT since it is an essential FA. 20:4n6 is also the elongation

and desaturation product of 18:2n6 by fatty acid elongase- ELOVL5 and fatty acid desaturases- FADS2 and FADS1. However, the expression of these genes was all down-regulated in MCD-fed ob/ob mice by 48-82%. (Figure. 5D), suggesting that increased levels of 20:4n6 was also coming from WAT. Additionally, mRNA levels of lipoprotein lipase (LPL) was markedly elevated (~79%) and Apo-CIII, which is the inhibitor of LPL was reduced (~50%) in MCD-fed ob/ob livers, which also suggests an increase in LPL- mediated FA uptake by the liver (Figure. 5E).

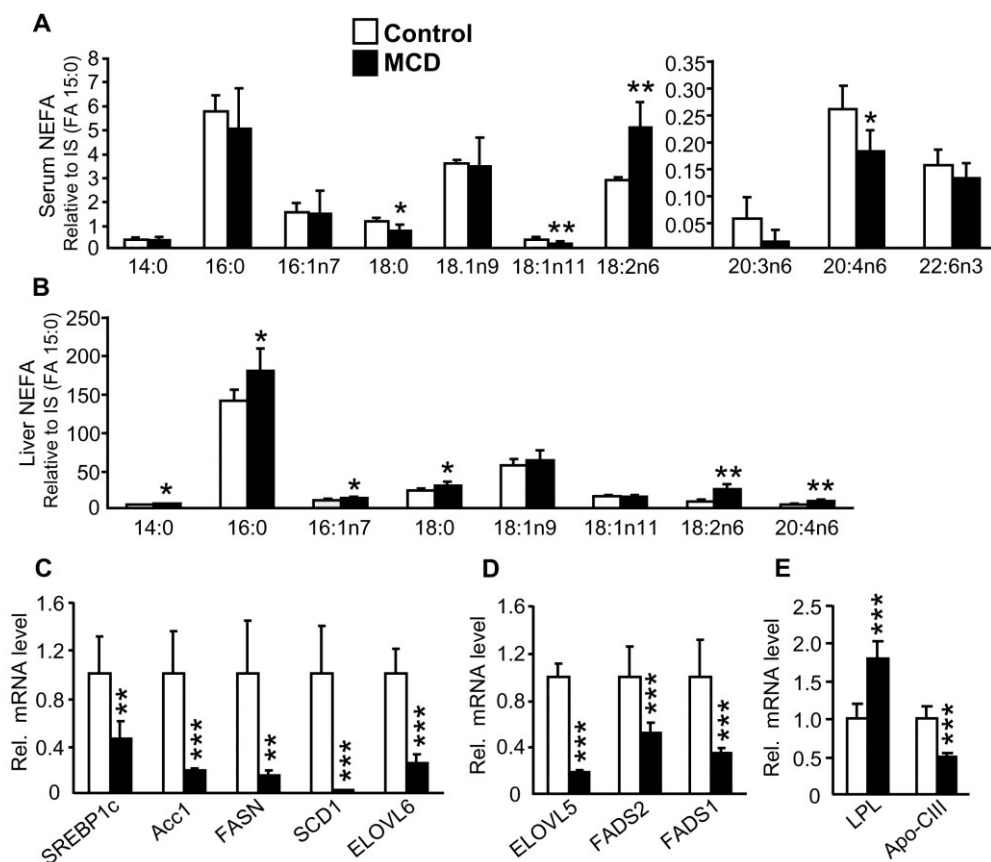


Figure 5. MCD-feeding causes selective increase of NEFA species in serum and liver due to lipolytic flux from adipose tissue.

(A and B) GC-NICI/MS profile of non-esterified fatty acids (NEFA) in serum (A) and liver (B). (C) mRNA expression of genes involved in de novo lipogenesis and monounsaturated fatty acid synthesis. (D) mRNA expression of genes involved in elongation and desaturation of polyunsaturated fatty acids. (D)

*mRNA expression of LPL and Apo-CIII. n = 6 per group. Bars represent mean $\pm$ SD. *** $p < 0.001$ ** $p < 0.01$, * $p < 0.05$, versus ob/ob control group.*

7.1.4. Analysis of TFA composition in serum and liver. GC-MS analysis of TFA (all FAs from triglyceride, cholesterol ester or phospholipid pool) composition in the liver, showed overall reduced TFA composition in the serum (Figure. 6A) whereas, analysis of TFA composition in the liver showed an overall increase of all PUFAs, while 18:0 was the only saturated FA elevated in ob/ob MCD-fed livers (Figure. 6B). In line, our analysis of mRNA expression in liver revealed reduced expression of genes involved in fatty acid oxidation including, PPAR α (~51%), carnitine palmitoyltransferase 1 α (CPT1 α , ~33%), acyl-CoA oxidase (ACO, ~57%), long-chain acyl Co-A dehydrogenase (LCAD, ~27%) and very long-chain acyl Co-A dehydrogenase (VLCAD, ~23%) (Figure. 6C) and liver TG content was increased by 2-fold in MCD-fed ob/ob mice (Figure. 6D). In contrast to the increased total lipase activity in WAT of MCD-fed ob/ob mice, in liver, total lipase activity decreased by ~17% (Figure. 6E, white bar). ATGL activity was not significantly decreased in MCD-fed ob/ob mice, while no change in HSL activity was observed. (Figure. 6E, black and grey bars respectively). Western blot analysis showed significant decrease in protein expression of ATGL whereas HSL expression remained unchanged (Figure. 6F). Collectively, these findings suggest that in addition to reduced VLDL synthesis and secretion from the liver (data not shown) (Yao *et al.*, 1988) hepatic fat accumulation in MCD-fed ob/ob mice may result from the combined effects of flux of free FAs from adipose tissue depots to the liver, reduced TG hydrolase activity and FA oxidation in the liver.

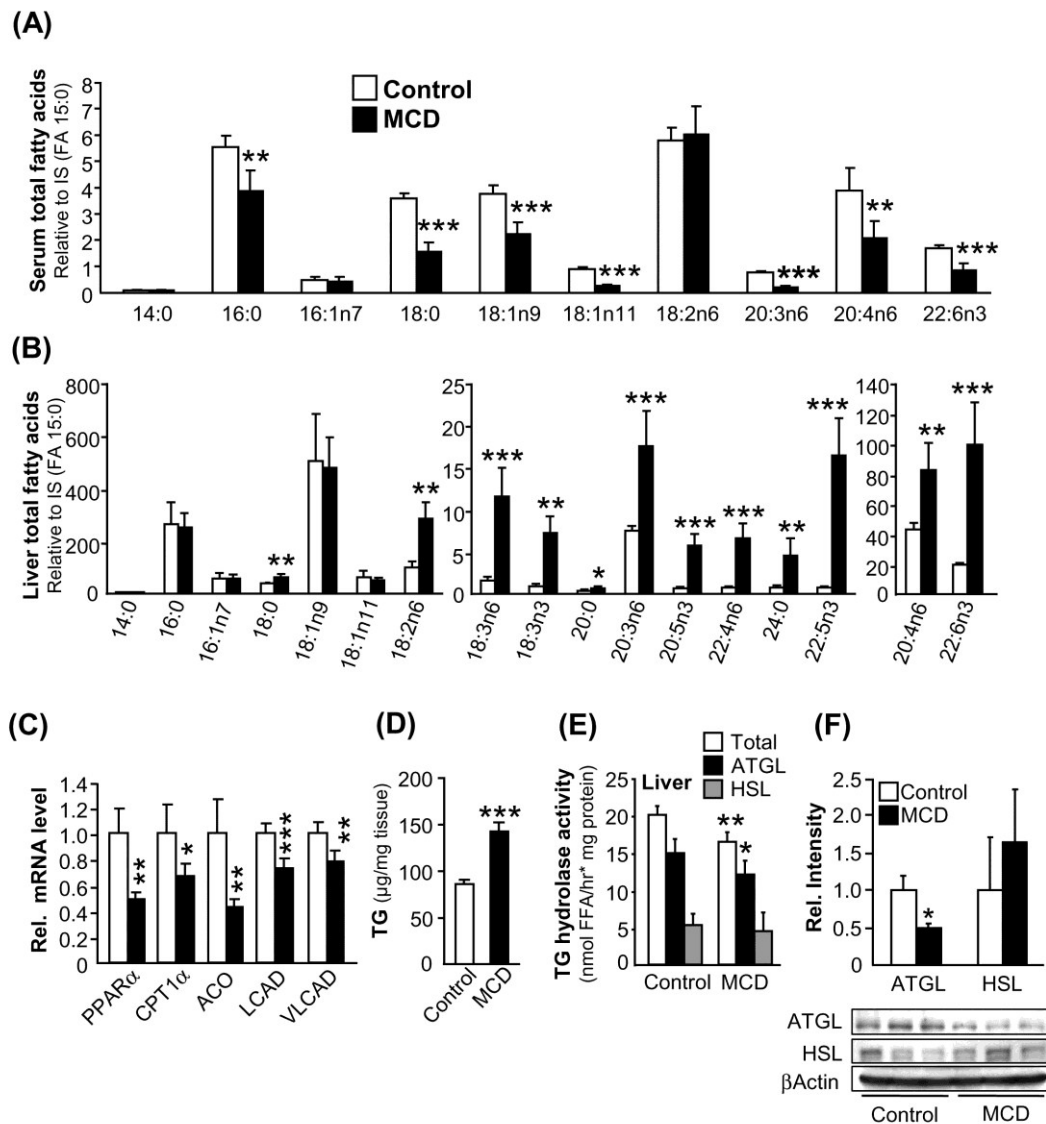


Figure 6. MCD feeding changes the homeostasis of total fatty acid (TFA) profile in serum and liver.

(A and B) GC /MS profile of TFA in serum (A) and liver (B). (C) mRNA expression of genes involved in FA oxidation. (D) Hepatic triglyceride quantification. (E) TG hydrolase activity in liver. Total activity (white bar), ATGL activity (black bar), HSL activity (grey bar). $n = 6$ per group. (F) Western blot analysis of ATGL and HSL protein levels in liver of control and MCD-fed *ob/ob* mice. $n=3$. Bars represent mean $\pm$ SD. *** $p<0.001$ ** $p<0.01$, * $p<0.05$, versus *ob/ob* control group.

7.1.5. Effect of MCD-diet on inflammation in Liver, WAT and BAT.

Following massive fatty acid flux from adipose tissue, MCD feeding resulted in pronounced liver injury as reflected by increased serum levels of bilirubin, bile acids (BAs), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Figure. 7A, left) together with hepatic inflammation reflected by increased mRNA expression of tumour-necrosis factor-alpha ($\text{TNF}\alpha$), inducible nitric oxide synthase (iNOS), monocyte chemotactic protein-1 (MCP-1), and interleukin 1beta ($\text{IL1}\beta$) (Figure. 7A, right). mRNA expression analysis from WAT did not show any difference in inflammatory markers between the two groups (Fig.7B, left). To determine whether these changes of inflammatory markers were due to alteration in gene expression or to a change in macrophage number, we performed immunohistochemistry using an antibody that recognizes the macrophage antigen F4/80. Consistent with the gene expression studies, macrophage number in gonadal adipose tissue remained unchanged in both the groups (Fig.7B, middle and right),

In contrast to WAT, BAT showed even reduced expression of inflammatory markers in MCD-fed ob/ob mice (Fig.7C, left). In line, the macrophage number (F4/80) decreased by -63% in MCD-fed ob/ob mice (Fig 7C, middle and right). Our analysis of mRNA expression in BAT revealed markedly reduced levels of WAT enriched genes including dermatopontin (DPT, -43%), homeobox protein C9 (Hoxc9, -65%) and transcription factor 21 (Tcf21, -67%) in MCD-fed mice (Fig.5D). Furthermore, expression of genes involved in energy expenditure, thermogenesis, mitochondrial and peroxisomal FA oxidation, such as CIDEA, UCP-1, ATP5B, COXIV, CPT1 β , PPAR α and PHYH were all increased by 35-100% in BAT of MCD-fed ob/ob mice (Fig.7E). Together, these data in addition to the increased lipase activity in BAT (Fig 4E) suggest that MCD diet reduces adiposity in BAT and enhances its activity and oxidative capacity which may be responsible for reduced inflammation in BAT.

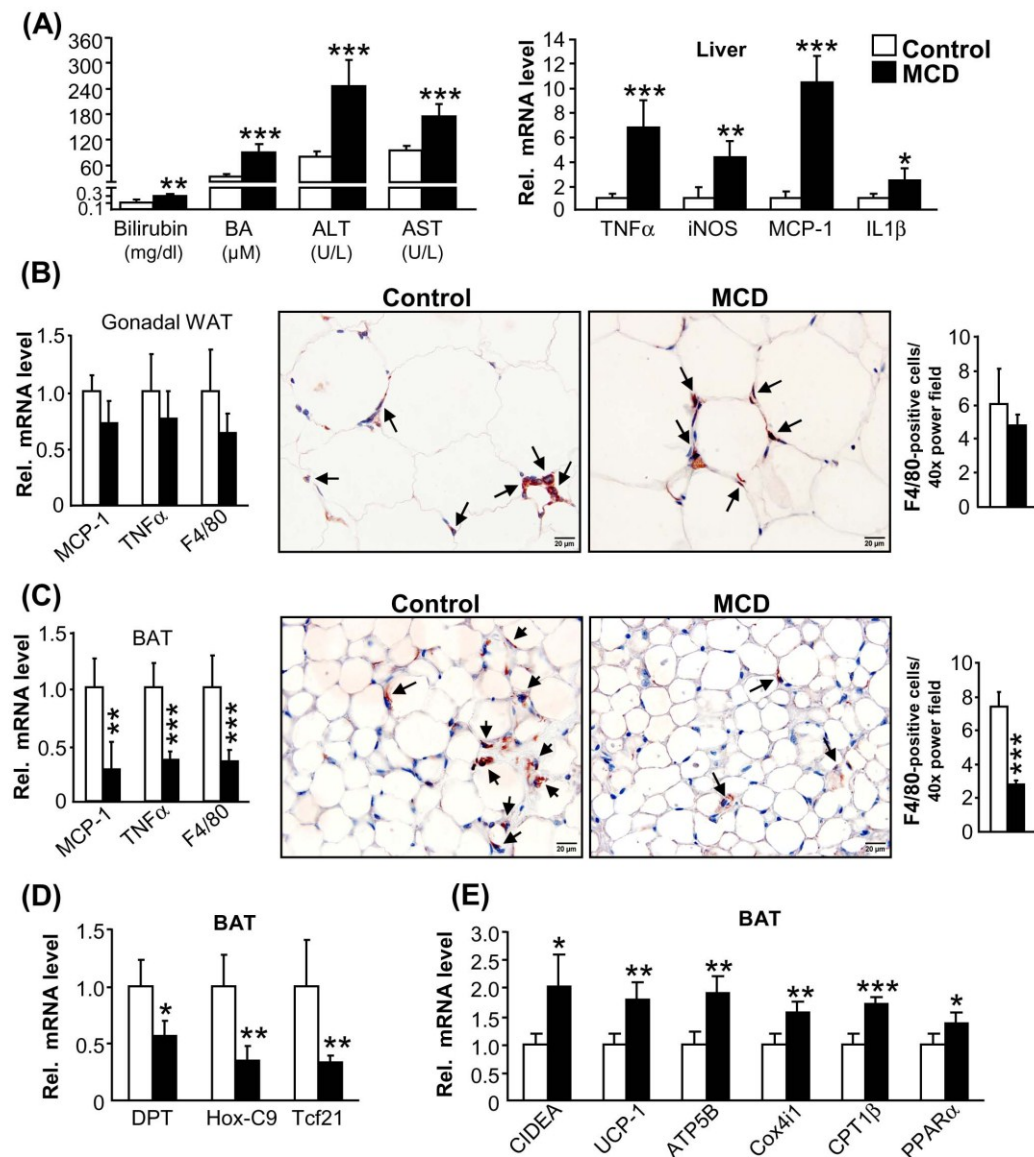


Figure 7. MCD feeding causes enhanced inflammation in liver, whereas unchanged and reduced inflammation in WAT and BAT respectively.

(A) Serum parameters of liver injury (left). mRNA expression of pro-inflammatory markers in liver (right). (B) mRNA expression of pro-inflammatory markers in gonadal WAT (left); Representative F4/80 immunohistochemistry (middle) and its quantification (right) shows similar number of positive cells in gonadal WAT of control and MCD-fed mice. (C) mRNA expression of pro-inflammatory markers in BAT (left); Representative F4/80 immunohistochemistry (middle) and its quantification (right) shows decrease in number of positive cells in MCD-fed ob/ob mice. Magnification, 60x; Scale bar,

20 μ m. (D) mRNA expression of WAT enriched genes in BAT. (E) mRNA expression of markers of energy expenditure, thermogenesis and FA oxidation. $n = 6$ per group. Bars represent mean $\pm$ SD. *** $p < 0.001$ ** $p < 0.01$, * $p < 0.05$, versus ob/ob control group.

7.2. Role of intracellular lipases for liver injury in MCD challenged ATGL- and HSL-KO mice

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7.2.1. ATGL-KO mice show induction of lipolysis upon MCD feeding suggesting activation of other lipases. Since in NASH and obese patients the flux of FAs to the liver increases (Donnelly *et al.*, 2005; Nielsen *et al.*, 2004) and lipolytic activity in WAT of MCD-fed ob/ob mice were increased, we also speculated that ATGL-KO mice could be protected from MCD induced lipolysis and subsequently NASH. We therefore fed ATGL-KO and WT with MCD or control diet only for 2 weeks in order to prevent excessive loss of adipose tissue so as to retain enough for enzyme activity measurements. WT mice on MCD diet lost ~30% of their body weight (Figures 8A and 8B), ~75-85% of WAT depots (gonadal, perirenal and visceral) and ~53% of BAT (Figures 8B and 8C). Surprisingly, ATGL-KO mice on MCD diet also lost substantial amounts of their body weight (~23%) (Figure 8A and 8B) as well as WAT and BAT depots (~51% gonadal, ~52% perirenal, ~44% visceral and ~16% BAT) (Figures 8B and 8C). The substantial loss of WAT depots in the MCD fed ATGL-KO mice indicated that HSL and/or other lipases can partially compensate for the absence of ATGL. We therefore performed TG hydrolase assay in the WAT and BAT depots of WT and ATGL-KO CON or MCD fed mice. Similar to the findings in MCD-fed ob/ob mice, all WAT depots and BAT of WT MCD-fed mice showed increased total, ATGL and HSL lipase activities (Figure 8D). As a result of the excessive loss of visceral WAT, TG hydrolase assays were technically not feasible due to the limited amounts. In MCD-fed ATGL-KO we observed only a marginal increase of total (~18%) and HSL (~20%) lipase activity in gonadal WAT (Figure 8E). No increase was observed in perirenal WAT, visceral WAT and BAT (Figure 5E), despite the loss in their respective weights. These results clearly indicate that other lipases compensated for the absence of ATGL at least in visceral and perirenal WAT.

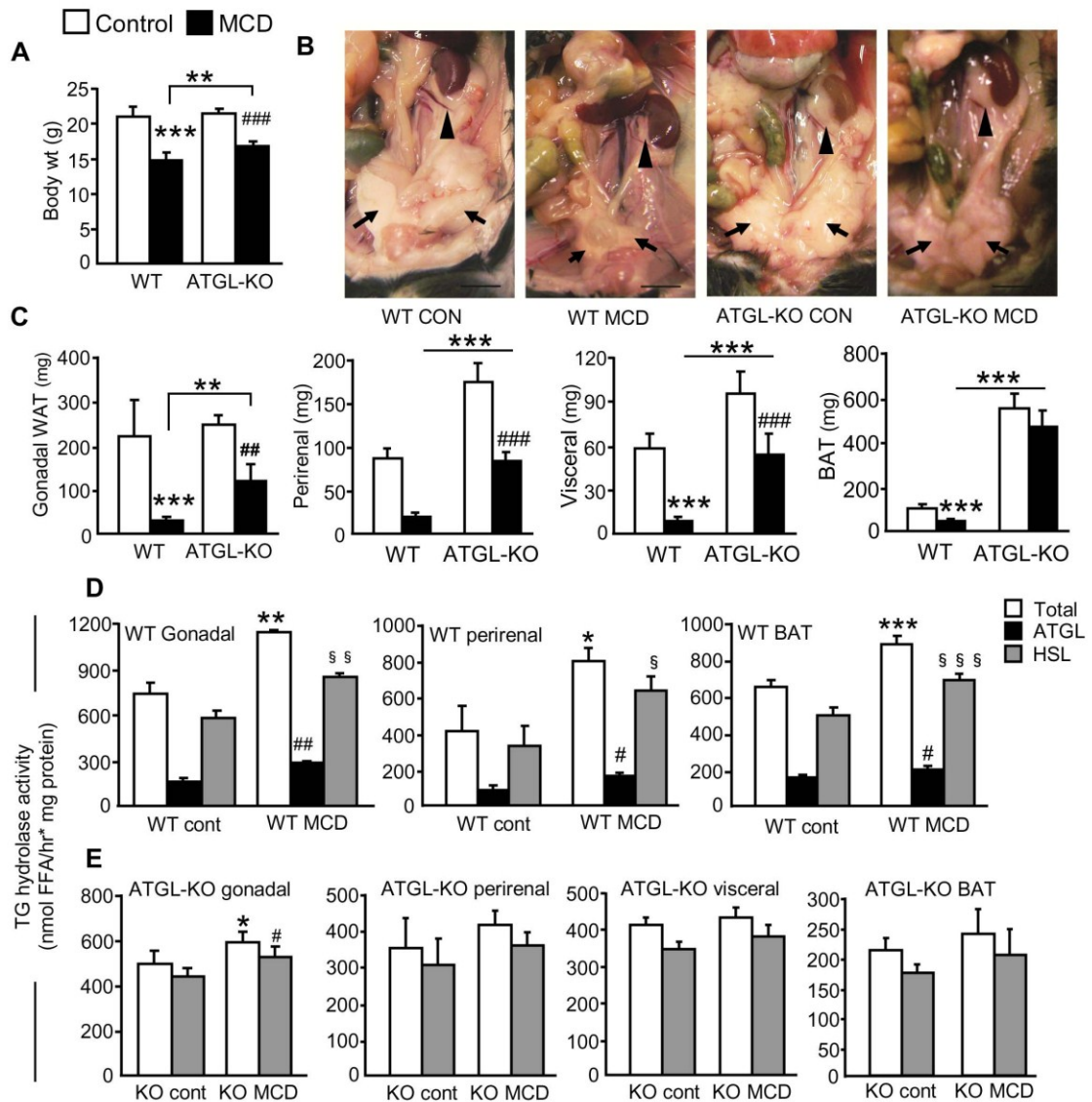


Figure 8. ATGL-KO mice are partially protected from WAT loss upon MCD feeding.

ATGL-KO and WT mice were fed with Control or MCD-diet for 2 weeks. (A) Final body weight. (B) Representative image of non-fasted 11-12 weeks old mice showing ~80% loss of WAT in MCD-fed WT mice and ~50% loss of WAT in MCD-fed ATGL-KO mice. Arrows indicate gonadal WAT and arrow heads perirenal WAT. Scale bars represent a length of 0.5 cm. (C) Fat depot analysis of gonadal, perirenal, Visceral WAT and BAT. (D) TG hydrolase activities in cytosolic extracts of gonadal and perirenal WAT and BAT of WT control or MCD-fed mice. (E) TG hydrolase activities in cytosolic extracts of gonadal,

*perirenal, visceral WAT and BAT of ATGL-KO control or MCD-fed mice. Data are representative of 2 independent experiments. n = 4- 7 per experimental group. Bars represent mean $\pm$ SD. *** p <0.001, ** p <0.01, * p <0.05 versus WT CON diet fed group. ### p <0.001, ## p <0.01, # p <0.05, versus ATGL-KO control diet fed group. For TG hydrolase assay (D and E), comparisons are as follows: *compared to the corresponding total activity (white bars); #, compared to corresponding ATGL activity (black bars); §, compared to corresponding HSL activity (grey bars) of the control group.*

7.2.2. Lack of ATGL aggravates hepatic inflammation, liver injury and fibrosis in MCD-fed mice. PPAR α activation mediates anti-inflammatory effects (Daynes *et al.*, 2002; Devchand *et al.*, 1996) and ATGL deficiency in mice decreases PPAR α and its target gene expression (Haemmerle *et al.*, 2011; Ong *et al.*, 2010). Therefore we hypothesized that ATGL deficiency would result in reduced flux of FAs (potential PPAR α ligands) from adipose tissue to the liver and that absence of ATGL in liver itself could further limit ligand availability for PPAR α activation, resulting in higher susceptibility to inflammatory insults. In WT MCD-fed mice, despite a 20% reduction in PPAR α DNA binding activity (DBA) (Figure 9A) we observed a 3-fold increase in PPAR α expression and a 4- to 15-fold increase in the expression of its target genes (CYP4A14, CPT1 α and FGF21) (Figure 9B) and serum FGF21 concentration (Figure 9C). Notably, MCD-fed ATGL-KO mice showed a blunted induction in the expression of PPAR α and its target genes (Figure 9B) as well as serum FGF21 levels (Figure 9C). Conversely, MCD-fed ATGL-KO mice developed more severe hepatic inflammation compared to MCD-fed WT mice as reflected by increased expression of TNF α , iNOS, MCP-1 and IL1 β (Figures 9D). Quantification of CD11b after immunostaining of liver sections showed increased infiltration by inflammatory cells in MCD-fed ATGL-KO mice (Figure 9E). Liver histology and biochemical analysis revealed a higher degree of steatosis in ATGL-KO which was further exacerbated (~4-fold) by MCD feeding (Figure 9F). In addition to increased inflammation, ATGL-KO mice showed increased expression of fibrosis markers such as collagen1a1 (Col1 α 1), collagen1a2 (Col1 α 2) and transforming growth factor β 1 (TGF- β 1) already at

baseline followed by an increase on MCD diet which was mild due to the short feeding duration (Figure 9G).

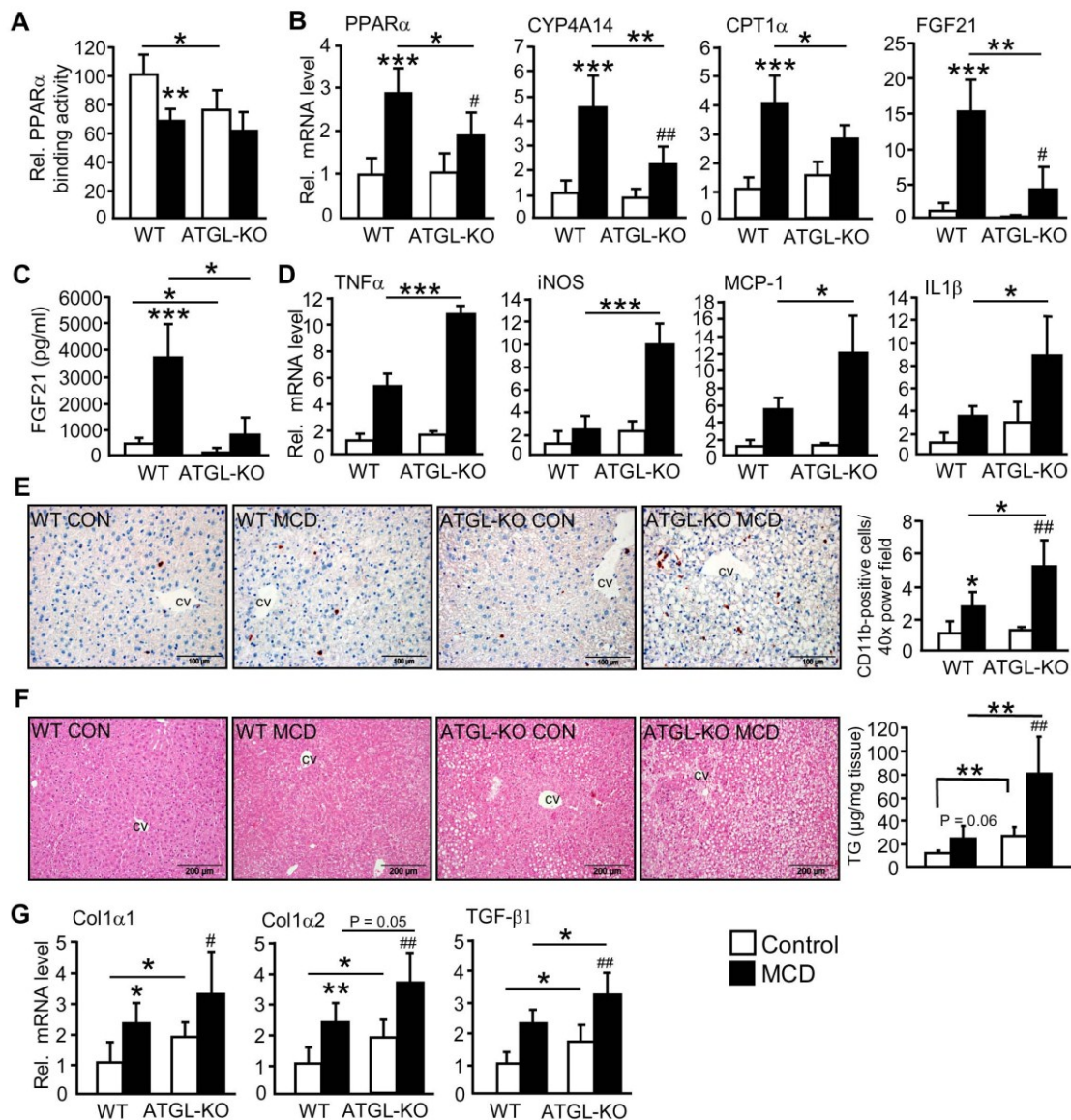


Figure 9. Genetic ablation of ATGL results in aggravated hepatic inflammation upon MCD feeding which may be PPAR α dependent.

WT and ATGL-KO mice were fed with control or MCD-diet for 2 weeks. (A) Hepatic PPAR α DNA binding activity (hereafter referred as DBA) from 20 μ g nuclear extract (NE) (B) Hepatic gene expression of PPAR α and its downstream targets. (C) Serum FGF21 concentration. (D) Hepatic gene expression of pro-inflammatory markers. (E) Representative CD11b immunohistochemistry (IHC) and its quantification (in 60 power fields) shows

increased mononuclear inflammatory cells in MCD-fed ATGL-KO compared to MCD-fed WT mice. Scale bar, 100 μ m. (F) Representative hematoxylin and eosin (H&E) staining show increased steatosis in MCD-fed ATGL-KO mice confirmed by hepatic triglyceride quantification. Scale bar, 200 μ m. Central vein (CV)(G) Gene expression of fibrosis markers. Data are representative of three independent experiments. $n = 4 - 7$ per experimental group. Bars represent mean $\pm$ SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ versus WT control diet fed group. ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$, versus ATGL-KO control diet fed group.

7.2.3. In contrast to ATGL, genetic ablation of HSL does not affect PPAR α signaling and hepatic inflammation upon MCD feeding. In contrast to MCD-fed ATGL-KO mice, the body weight and WAT loss in HSL-KO mice was similar to that of WT mice except for BAT which was ~80% conserved in HSL-KO mice as opposed to ~50% loss in WT mice (Figures 10A and 10B). Similarly, in contrast to MCD-fed ATGL-KO mice, MCD-fed HSL-KO mice had no significant differences to MCD-fed WT mice with regard to PPAR α DBA (Figure 10C), serum FGF21 concentration (Figure 10D) and mRNA expression of PPAR α targets, hepatic pro-inflammatory and fibrotic markers (Figure 10E). Similarly, CD11b quantification (Figure 10F) and TG levels (Figure 10G) did not show any significant difference between MCD-fed WT and HSL-KO mice. Collectively, these results indicate that ATGL-KO and not HSL-KO mice are more susceptible to MCD induced steatohepatitis.

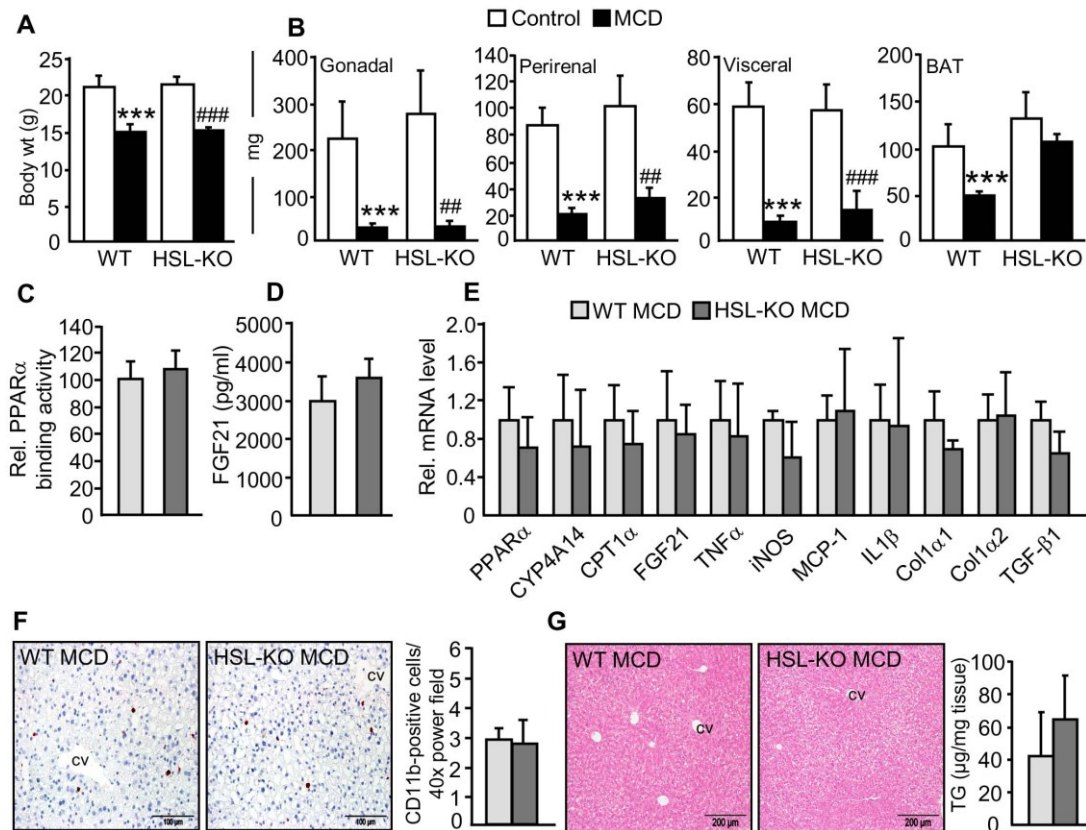


Figure 10. Genetic ablation of HSL does not affect PPARα signaling and hepatic inflammation upon MCD feeding.

WT and HSL-KO mice were fed with MCD-diet for 2 weeks. (A) Final body weight. (B) Fat depot analysis of gonadal, perirenal, Visceral WAT and BAT. (C) Hepatic PPARα DBA from 20μg NE. (D) Serum FGF21 concentration. (E) Hepatic gene expression of PPARα, its target genes, pro-inflammatory and fibrosis markers in liver. (F) Representative CD11b IHC and its quantification. (G) Representative H&E staining and triglyceride quantification. Data are representative of 2 independent experiments. $n = 3-5$ per experimental group. Bars represent mean $\pm$ SD. *** $p < 0.001$ versus WT control diet fed group. ### $p < 0.001$, ## $p < 0.01$, versus HSL-KO control diet fed group.

7.3. Role of intracellular lipases in acute hepatic inflammation in LPS-challenged ATGL- and HSL-KO mice

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7.3.1. Acute endotoxemia causes defective thermogenesis, impaired PPAR α signaling, torpor and higher mortality in LPS-injected ATGL-KO mice. Increased plasma endotoxin levels can be detected in mice with metabolic endotoxemia (Cani *et al.*, 2007) and in humans with NAFLD (Miele *et al.*, 2009). LPS is considered to be a potential second hit required for the progression of steatosis to NASH (Day *et al.*, 1998). Therefore we addressed the role of ATGL in modulating hepatic inflammation after subjecting WT and ATGL-KO mice to acute endotoxemia by injecting LPS compared to saline as control. After a single injection of intraperitoneal (i.p.) LPS, ATGL-KO mice exhibited signs of torpor and showed progressive lowering of their temperature over a period of 12 hrs. In contrast, LPS-injected WT mice recovered rapidly from an initial drop in body temperature (Figure 11A). To explore the potential mechanisms underlying the state of torpor, we measured PPAR α DBA and its mRNA expression in liver. Both were 60% lower at baseline in ATGL-KO and further reduced after LPS injection (Figure 11B). In line, PPAR α targets, FA oxidation genes - CYP4a14 and ACO were already reduced in saline-injected ATGL-KO mice. Their expression was further down-regulated by LPS injection in both WT and ATGL-KO mice (Figure 11C). FGF21, a downstream target of PPAR α and an endocrine regulator of ketogenesis and torpor (Badman *et al.*, 2007) followed the expression pattern of PPAR α (Figure 11D). Furthermore, LPS-injected ATGL-KO mice showed low ketone bodies concentration compared to LPS-injected WT mice, which mirrored the expression pattern of FGF21 (Figure 11E). However, WT LPS-injected mice showed more than 2-fold increase in serum FGF21 levels as also shown by a previous report (Feingold *et al.*, 2012), which was undetectable in ATGL-KO mice (Figure 11F). Furthermore, after a single higher dose of LPS, ATGL-KO mice showed an 80%

mortality rate as opposed to 100% survival in WT mice over a period of 5 days (Figure 11G).

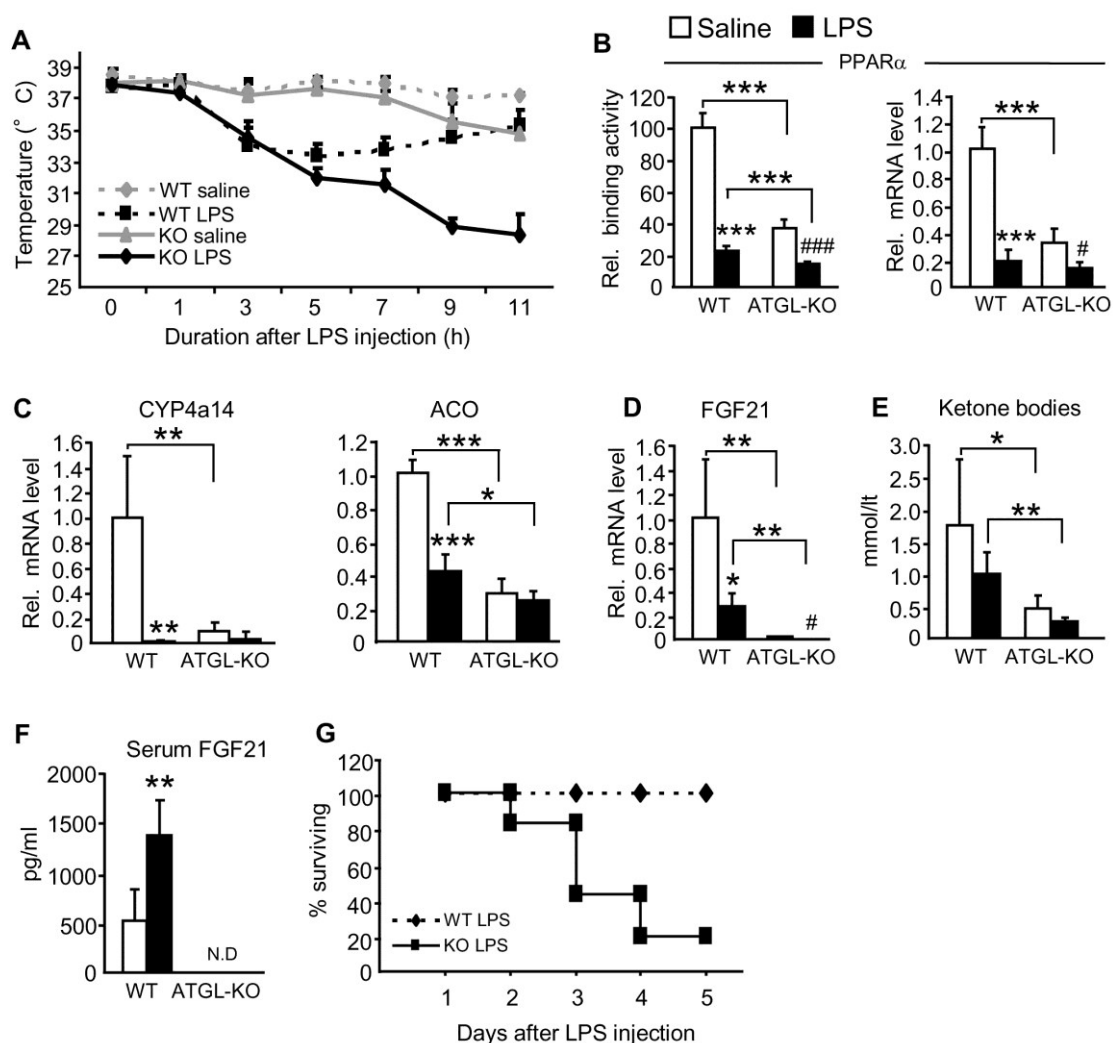


Figure 11. Acute endotoxemia, causes defective thermogenesis, impairs PPAR α signalling and results in higher mortality in ATGL-KO mice

Non-fasted WT and ATGL-KO mice were injected with a single dose of 4 μ g/g body weight LPS or saline for 12h (4am-4pm). To allow better comparison both groups were fasted after the injection because LPS is known to induce anorexia. (A) Rectal temperature of WT and ATGL-KO LPS or saline injected mice (B) Hepatic PPAR α DBA from 30 μ g nuclear extract (left), hepatic PPAR α gene expression (right). (C) mRNA expression of PPAR α target FA oxidation genes - CYP4A14 and acyl-CoA oxidase in liver. (D) FGF21 gene expression. (E) Ketone body (aceto-acetate, 3-hydroxy butyrate) concentration in serum.

(F) FGF21 concentration in serum. (G) Higher mortality rate in ATGL-KO mice when injected with 5µg/g body weight LPS. n= 6 (KO), 11 (WT). Data are representative of 2 independent experiments n= 5-7 per experimental group. Bars represent mean ± SD. ***p<0.001, **p<0.01, *p<0.05 versus WT saline injected group. ###p<0.001, #p<0.05, versus ATGL-KO saline injected group.

7.3.2. ATGL-KO mice are more susceptible to LPS-induced acute hepatic inflammation. We next used a classic inflammatory model (LPS) to understand how ATGL deficiency contributes to acute inflammatory stress. LPS is known to induce anorexia (Elander *et al.*, 2007), therefore all groups were housed without food during the course of treatment. 12h LPS-injected ATGL-KO mice showed marked up-regulation of inflammatory markers in liver compared to the LPS-injected WT mice (Figure. 12A). Additionally, serum TNF α and IL6 levels after 6h and 12h respectively, were higher in LPS challenged ATGL-KO compared to the WT mice (Figure. 12B, 2C). (Serum TNF α and IL6 levels were not detectable in controls). In line, CD11b immunostaining of liver sections showed a marked increase of inflammatory cells in LPS-injected ATGL-KO compared to the WT (Figure. 12D). Furthermore, after a single higher dose of LPS, ATGL-KO mice showed an 80% mortality rate as opposed to 100% survival in WT mice over a period of 5 days (Figure. 12E). Inflammatory gene expression analysis from WAT did not show any significant differences between LPS-injected WT and ATGL-KO mice (Figure. 12F), suggesting a liver specific anti-inflammatory role of ATGL.

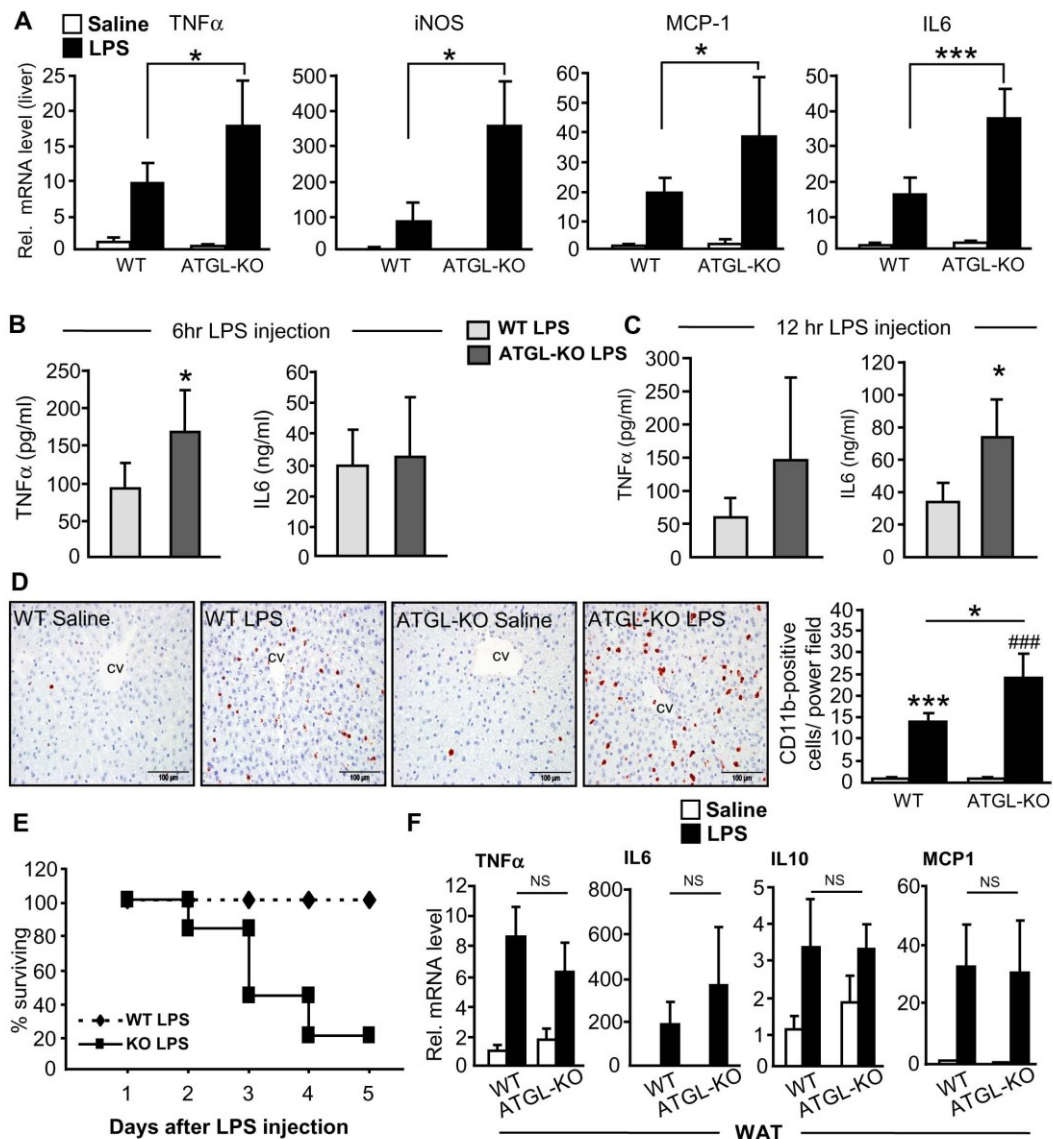


Figure 12. Acute endotoxemia results in, exaggerated inflammatory response in ATGL-KO mice.

Non-fasted WT and ATGL-KO mice were injected with 4 μ g/g body weight LPS or saline for 12h. All groups were fasted during the course of treatment. (A) mRNA expression of hepatic pro-inflammatory markers. (B and C) Serum concentration of TNF α and IL6 after 6h (B) and 12h (C) LPS injection. (D) Representative CD11b IHC and its quantification shows marked increase in mononuclear inflammatory cells in LPS-injected ATGL-KO mice. Scale bar, 100 μ m. (E) Higher mortality rate in ATGL-KO male mice (9-10 weeks old) when injected with 5 μ g/g body weight LPS. n = 6 (KO), 11 (WT). (F) Pro-inflammatory

markers in WAT (NS=non-significant). $n = 5-7$ per group. Bars represent mean $\pm$ SD. *** $p < 0.001$, * $p < 0.05$ versus WT saline injected group. ### $p < 0.001$ versus ATGL-KO saline injected group.

7.3.2. HSL-KO mice do not show increased susceptibility to LPS-induced hepatic inflammation and PPAR α insufficiency. In contrast to the response of ATGL-KO mice, LPS challenge in HSL-KO mice revealed no differences to WT animals for PPAR α DBA, its mRNA expression (Figure 13A), serum FGF21 levels (Figure 13B), mRNA expression of hepatic pro-inflammatory markers (Figure 13C), serum TNF α , IL6 (Figure 13D), and CD11b quantification in liver (Figure 13E). Taken together, these data further indicate a specific role of hepatic ATGL (but not HSL) in protecting the liver during acute inflammation.

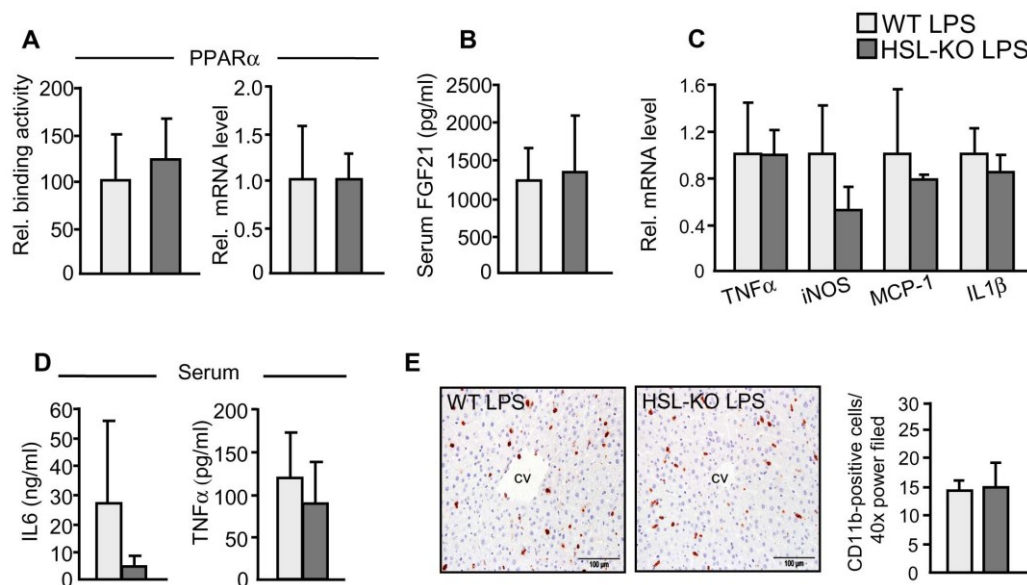


Figure13. HSL-KO mice do not show PPAR α insufficiency and increased susceptibility to LPS-induced hepatic inflammation.

Non-fasted wildtype (WT) and HSL-KO mice were injected with a single dose of 4 μ g/g body weight LPS for 12h (4am-4pm). (A) Hepatic PPAR α DBA from 30 μ g nuclear extract (NE) (left), hepatic PPAR α gene expression (right) (B) Serum FGF21 concentration. (C) mRNA expression of hepatic pro-inflammatory markers in liver. (D) Serum concentration of IL6 and TNF α . (E) Representative

CD11b IHC and its quantification, performed as above. n = 4- 5. Bars represent mean $\pm$ SD.

7.3.3. LPS induced lipolysis is partially preserved in ATGL-deficient mice.

Acute endotoxemia (LPS) causes FA efflux from adipocytes to the bloodstream which is a possible basis for systemic elevation of free FAs and insulin resistance in endotoxemia, obesity or Gram-negative bacterial infection (Khovidhunkit *et al.*, 2004; Zu *et al.*, 2009). We therefore studied the role of ATGL in endotoxemia-induced lipolysis in WAT. Time course experiments revealed that 6h after LPS injection NEFA concentrations reached their maximum in WT mice (Figure 14A). Subsequently, both WT and ATGL-KO non-fasted mice were studied 6 hours after LPS injection. Saline-injected ATGL-KO mice had lower serum NEFA levels compared to the saline-injected WT mice. However, LPS injection raised serum NEFA concentration to a similar level in both WT and ATGL-KO mice (Figures 14B). Subsequently, we explored the serum NEFA composition by GC-NICI/MS. In line with the serum NEFA concentration, saline-injected ATGL-KO mice had low serum NEFA profile compared to the saline-injected WT mice (Figures 14C). However, LPS-injected WT and ATGL-KO mice displayed a similar increase of serum NEFAs across the spectrum with the exception of 18:0 (stearic acid) which was unchanged and 18:3n6 (γ -linolenic acid) as well as 20:4n6 (arachidonic acid) which increased only in LPS-injected ATGL-KO mice (Figure 14C).

To address whether serum NEFA accumulation resulted from increased lipolysis, we performed an *ex vivo* lipolysis assay from gonadal WAT explants obtained 6h after saline or LPS injection in WT and ATGL-KO mice. Compared to WT saline-injected mice, WT LPS-injected mice showed a ~3- fold induction of total lipolysis (Figure 14D, white bars), and a ~4- fold induction of HSL inhibited lipolysis (Figure 14D, dark grey bars). Treatment with forskolin (used as a positive control to induce lipolysis) did not show any difference between the two groups (Figure 14D, light grey bars) and treatment with both HSL inhibitor and forskolin induced lipolysis by 1.8- fold (Figure 14D, black bars). In contrast to LPS-injected WT mice, lipolysis was induced to a lesser extent (~2- 3- fold lower) in LPS-injected ATGL-KO mice in all the WAT explants: 239 ± 37

vs 157 ± 45 nmol/hr*mg protein, respectively for the control explants (Figure 14D and 14E). Taken together, these data suggest that LPS can induce lipolysis not only in WT but also in ATGL-KO mice, although to a lesser degree.

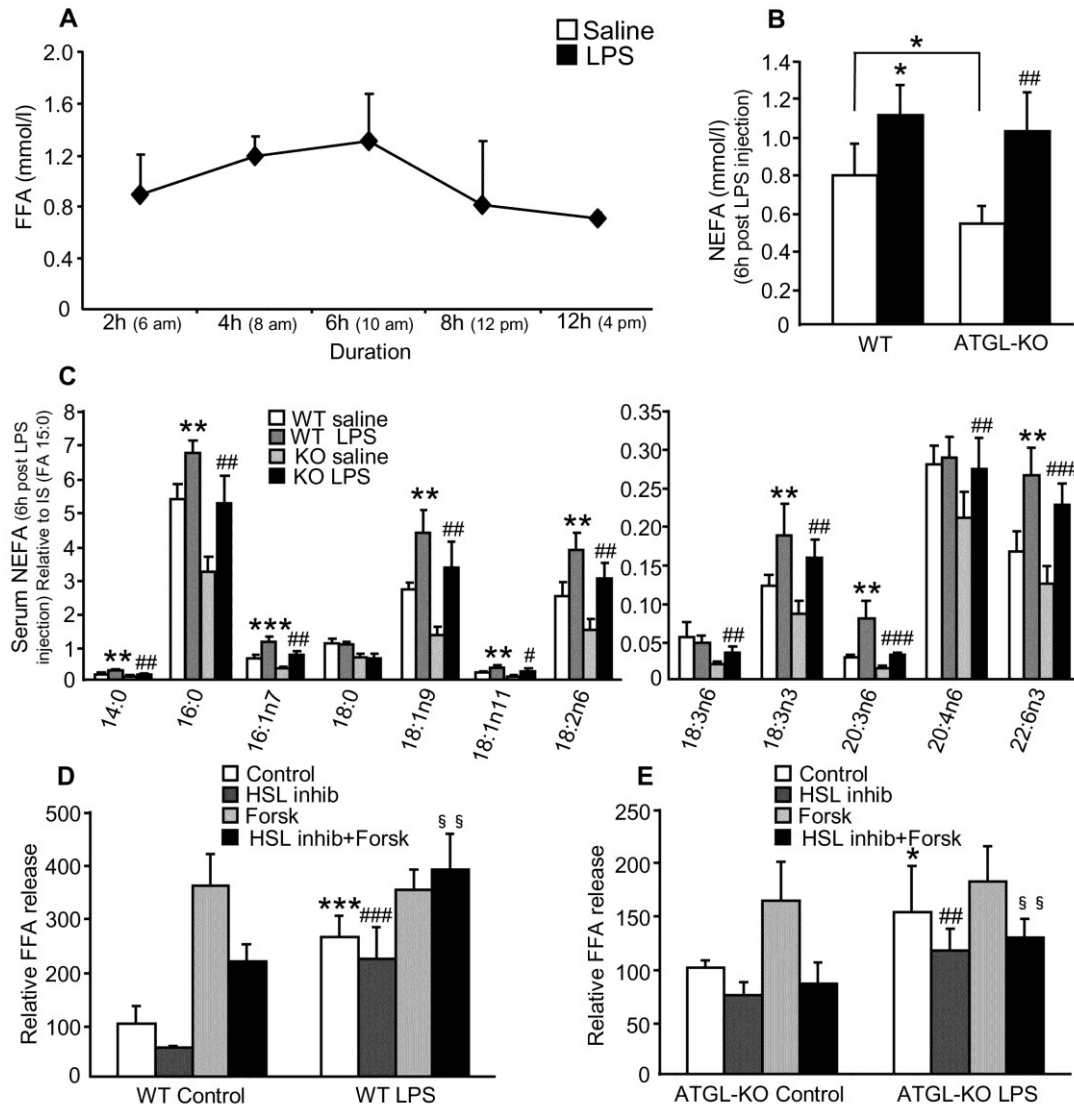


Figure 14. LPS induced lipolysis is partially preserved in ATGL deficient mice.

(A) Time course of NEFA release after LPS injection in WT mice. $n = 3 - 5$ per time point. (B and C) Non-fasted WT and ATGL-KO mice were injected with a single dose of $4\mu\text{g/g}$ body weight LPS or saline for 6h (7am-1pm). Both groups were fasted after injection. (B) Serum NEFA levels. (C) GC-NICI/MS profile of NEFAs in serum. Data are representative of 2 independent experiments. $n = 5$ per experimental group. Bars represent mean $\pm$ SD. *** $p < 0.001$ ** $p < 0.01$

***p<0.05 versus WT saline injected group. ####p<0.001 ##p<0.01, #p<0.05, versus ATGL-KO saline injected group.(D and E) Ex-vivo lipolysis of gonadal WAT explants 6h after LPS/saline injection in WT (D) and ATGL-KO (E) male mice. Each bar represents mean $\pm$ SD of pooled WAT samples from 3-5 mice per group, n=5 for each condition. *p<0.05, ##, §§p<0.001, ***, ####p<0.001 compared to their corresponding controls.*

7.3.4. Impaired FA delivery to hepatocytes and deficiency of 18:2n6 may cause defective PPAR α signaling in LPS-challenged ATGL-KO mice. LPS-injected ATGL-KO mice showed compromised hepatic PPAR α signaling despite induction of lipolysis in adipose tissue and subsequent release of NEFAs that could act as PPAR α ligands. To investigate whether this could reflect a defect of FA transport in hepatocytes which would limit ligand availability for PPAR α , we measured gene expression of proteins involved in hepatic FA binding and transport (Figures 15A and 15B). Notably, FATP5 was lower in ATGL-KO compared to WT. LPS injection significantly lowered FATP5 expression in WT but not in ATGL-KO mice (Figures 15A). FABP1, a PPAR α target which shuttles FAs from cytosol to the nucleus and directly interacts with PPAR α (Wolfrum *et al.*, 2001) showed very low expression in ATGL-KO mice at baseline and its expression was further reduced after LPS injection (Figure 15B). In line, Western blot analysis from the nuclear extracts showed ~50% reduced FABP1 protein content in ATGL-KO compared to WT which was further reduced after LPS injection (Figure 15C). Furthermore, compared to the saline injected mice, LPS-injected WT mice showed reduced serum NEFA concentration as well as NEFA species 12h after LPS injection, while ATGL-KO LPS-injected mice had higher serum NEFA concentrations and NEFA species compared to saline injected ATGL-KO mice (Figures 15D and 15E). This suggests that in ATGL-KO mice, the delivery of FAs into hepatocytes may be compromised which is further aggravated under acute endotoxemia resulting in impairment of PPAR α signaling. In line, detailed analysis of hepatic NEFA composition revealed that among all investigated NEFA species, 18:2n6 (an important PPAR α ligand) (Forman *et al.*, 1997; Kliewer *et al.*, 1997) was the most

strikingly reduced NEFA in livers of saline and LPS-injected ATGL-KO mice both 6 and 12h after LPS/ saline injection (Figures 15F and 15G).

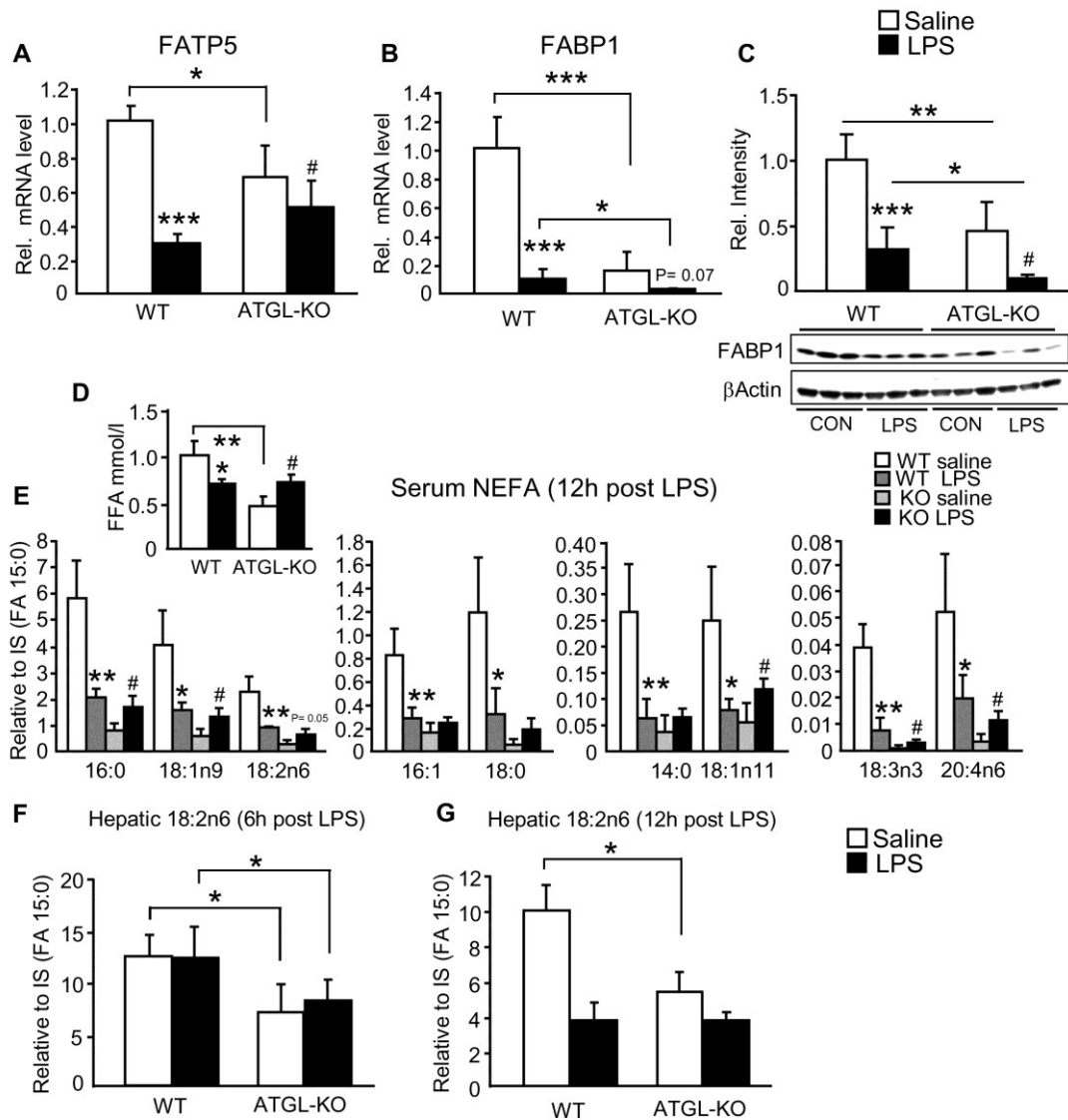


Figure 15. *PPARα* insufficiency in LPS-injected ATGL-KO could be due to decreased FA delivery to the hepatocytes.

(A and B) mRNA expression of FATP5 (A) and FABP1 (B) in liver, 12h post LPS injection. (C) Western blot analysis of nuclear FABP1 protein. (D) Serum NEFA concentration 12h post LPS injection. (E) GC-NICI/MS profile of NEFA in serum 12h after LPS injection. (F and G) GC-NICI/MS of non-esterified 18:2n6 in liver of WT and ATGL-KO mice after 6h (F) and 12h (G) of LPS or saline injection. Data are representative of two independent experiments. $n = 4-5$ per

*experimental group. Bars represent mean $\pm$ SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ versus corresponding saline injected WT mice. # $p < 0.05$ versus corresponding saline injected ATGL-KO mice.*

7.4. Effect of PPAR α agonist treatment in MCD- or LPS-challenged ATGL-KO mice

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7.4.1. Fenofibrate (PPAR α agonist) treatment reverts hepatic steatosis, improves inflammatory response and restores PPAR α signaling in MCD-fed ATGL-KO mice. To further explore the role of compromised PPAR α signalling in ATGL-KO mice for aggravation of inflammation and steatosis, we fed MCD or 0.1% fenofibrate (FF) in addition to MCD diet to WT and ATGL-KO mice for 2 weeks. FF as a model PPAR α ligand, completely normalized hepatic steatosis in ATGL-KO mice and reduced CD11b positive cells in both WT and ATGL-KO mice (Figure. 16A and 16B). MCP-1 expression was significantly reduced in ATGL-KO mice on FF-supplemented MCD diet (Figure. 16C). However, the expression of other inflammatory genes such as iNOS, TNF α and IL1 β was not significantly reduced (Figure 16C). Notably, FF supplementation restored PPAR α DBA (Figure 16D), its mRNA expression and the expression of its target genes – CYP4A14, CPT1 α – in ATGL-KO mice to the level of MCD+FF fed WT mice (Figure 16E). However, mRNA and serum levels of FGF21 did not increase in both the genotypes (Figure 16F). Collectively, these data suggest that PPAR α agonism (using FF as a model compound) reverts hepatic steatosis and partially improves the inflammatory response in MCD-fed ATGL-KO mice.

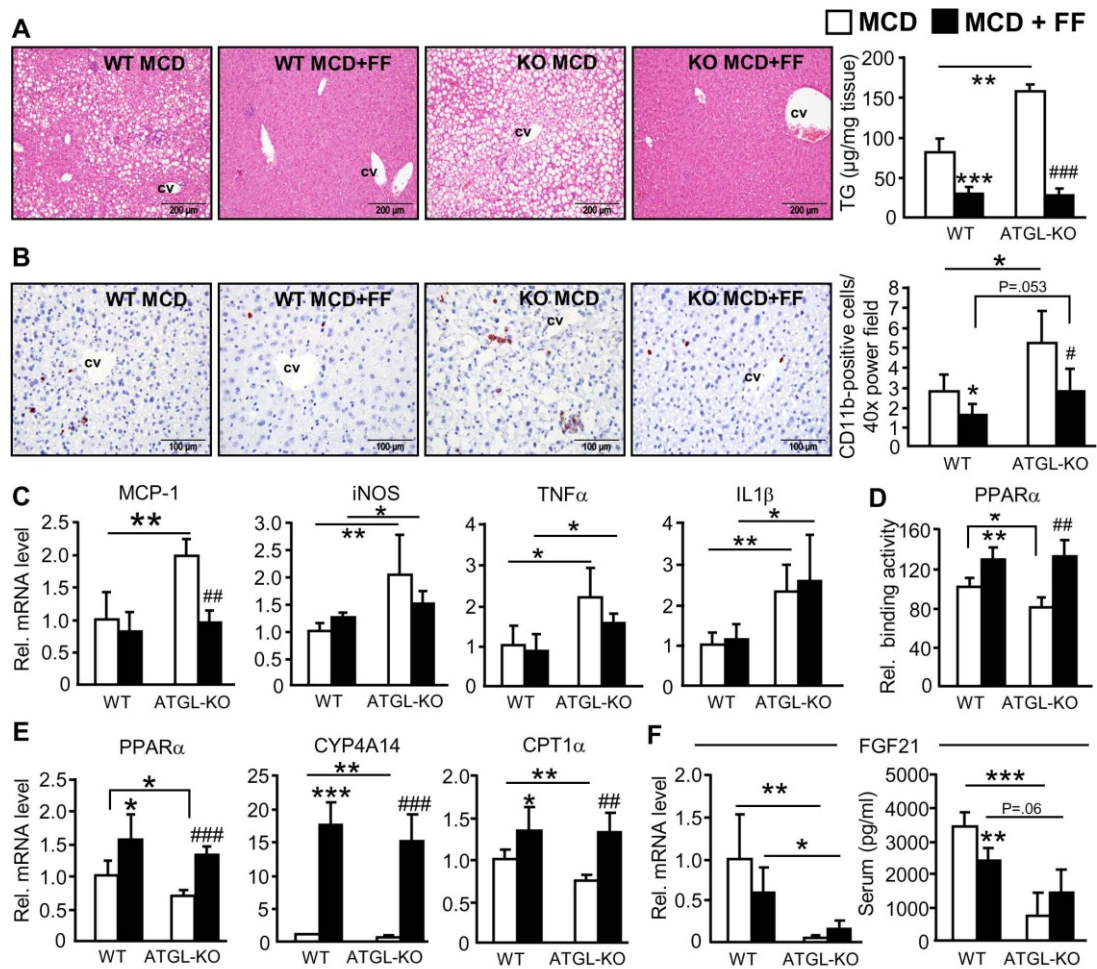


Figure 16. PPAR α agonist fenofibrate (FF) partially improves the hepatic inflammatory response of MCD-fed ATGL-KO mice in vivo

WT and ATGL-KO mice were fed with either MCD or MCD supplemented with 0.1% FF (MCD+FF) for 2 weeks. Thereafter, mice were harvested in the non-fasted state. (A) Representative H&E staining from liver sections and TG quantification. Scale bar, 200µm. (B) Representative CD11b IHC and its quantification in 60 power fields. Scale bar, 100µm. (C) mRNA expression profile of inflammatory markers in liver. (D) Hepatic PPAR α DBA from 20µg NE. (E) Hepatic mRNA expression of PPAR α and its target genes. (F) Hepatic gene expression (left) and serum concentration (right) of FGF21. Data are representative of two independent experiments. $n=5-6$ per experimental group. Bars represent mean $\pm$ SD. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ versus corresponding MCD fed WT mice. #### $p<0.001$, ### $p<0.01$, # $p<0.05$ versus corresponding MCD fed ATGL-KO mice.

7.4.2. Fenofibrate treatment ameliorates the hepatic inflammatory response and restores PPAR α activity in LPS-injected ATGL-KO mice. We next assessed the influence of PPAR α agonist treatment on LPS-induced acute hepatic inflammatory response in WT and ATGL-KO mice. 0.2% FF pre-treatment for 6 days lowered the CD11b positive cells in both WT and ATGL-KO LPS-injected mice, though the levels remained significantly higher in ATGL-KO compared to the WT (Figure. 17A). IL6 mRNA expression as well as serum concentrations were also reduced in both WT and ATGL-KO FF- pre-treated LPS-injected mice (Figure. 17B, left). However other inflammatory markers like TNF α , iNOS, and MCP-1 were not reduced in WT mice while we observed a trend towards decreased expression of these inflammatory markers in ATGL-KO mice (Figure. 17B, right) Moreover, FF-pre-treatment lowered the mortality of ATGL-KO mice from 80% (Figure. 11G) to 40% when injected with a single higher dose of 5 μ g/g LPS (Figure. 17C). Furthermore, FF-pre-treatment restored PPAR α DBA, its mRNA expression and expression of its target genes in ATGL-KO mice to the level of WT FF-pre-treated LPS-injected mice (Figure. 17D and 17E). However, nuclear FABP1 protein levels remained low in FF-pre-treated LPS-injected ATGL-KO mice compared to the corresponding WT mice (Figure. 17F). Further, FGF21 mRNA expression and its serum levels were not restored, though marginally induced in ATGL-KO mice (Figure. 17G) suggesting a highly ATGL dependent regulation of FGF21. Collectively, these results indicate that PPAR α agonist treatment can only partially improve the hepatic phenotype of LPS-injected ATGL-KO mice.

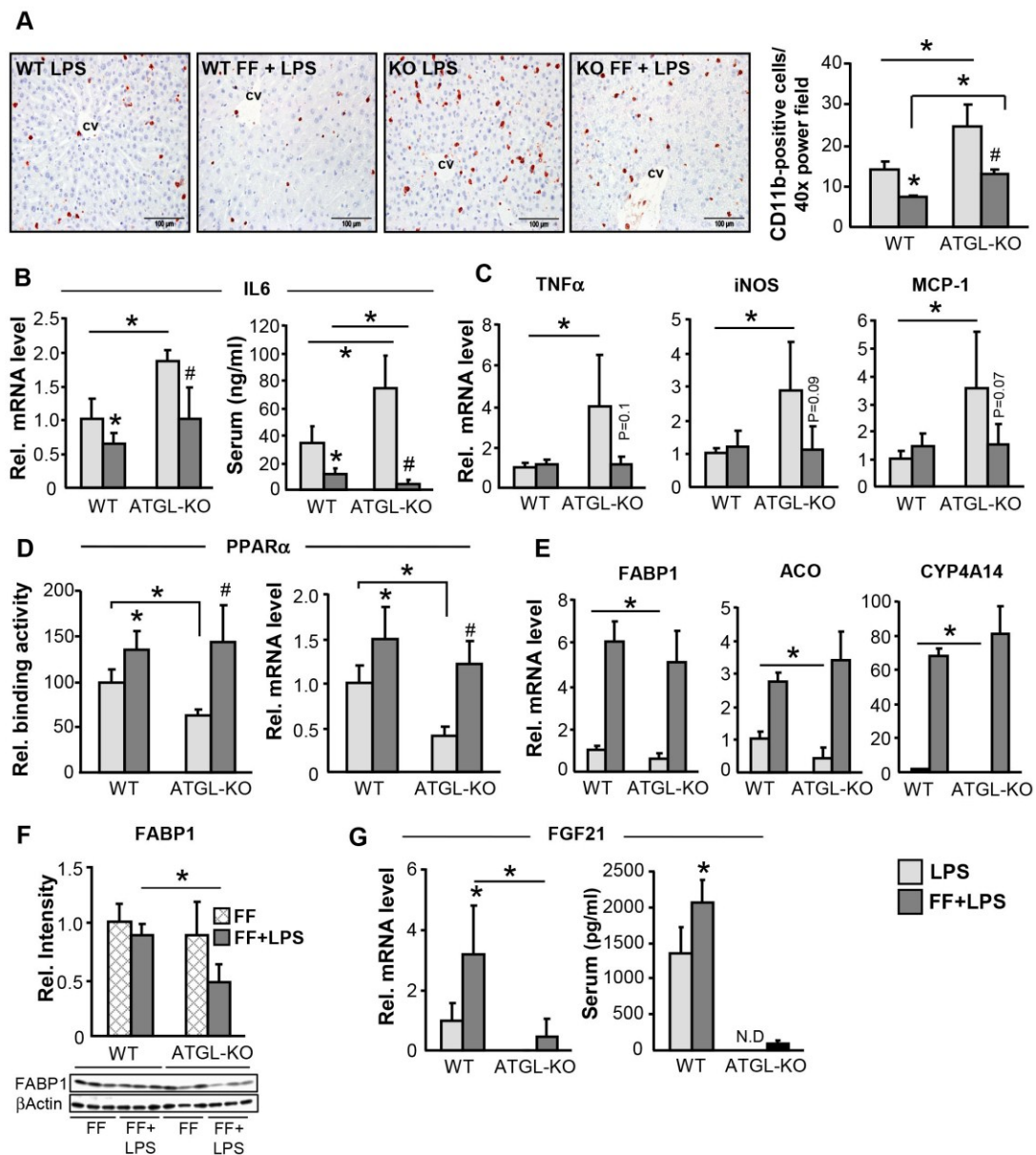


Figure 17. PPAR α agonist fenofibrate (FF) improves PPAR α deficiency and partially restores the hepatic inflammatory response of LPS-injected ATGL-KO mice in vivo.

WT and ATGL-KO mice were fed with 0.2% FF for 6 days followed by LPS injection to chow-fed (LPS) or FF-fed (FF+LPS) mice. Mice were fasted after single LPS injection. (A) Representative CD11b IHC and its quantification showing reduction of inflammatory cells in FF-fed+LPS-injected groups. Scale bar, 100 μ m. (B) mRNA expression (left) and serum concentration (middle)) of IL6. mRNA expression of TNF α , iNOS and MCP-1 (right). (C) Survival rate of male WT and ATGL-KO mice (9-10 weeksold) pre-fed with 0.2% FF for 6 days

*followed by LPS injection (5µg/g). n=5-6 per group. For survival rate, n= 7 (KO), 9 (WT). (D) Hepatic PPAR α DBA from 25µg nuclear extract (left), mRNA expression of PPAR α (right). (E) mRNA expression of PPAR α target genes. (F) Western blot analysis of nuclear FABP1 in liver. (G) Hepatic gene expression (left) and serum concentration (right) of FGF21. n=5-6 per group. Bars represent mean $\pm$ SD. * $p < .05$ versus corresponding LPS-injected WT group. # $p < .05$, versus corresponding LPS-injected ATGL-KO group.*

7.5. Proposed mechanism for enhanced susceptibility to acute and chronic hepatic inflammation in ATGL-KO mice

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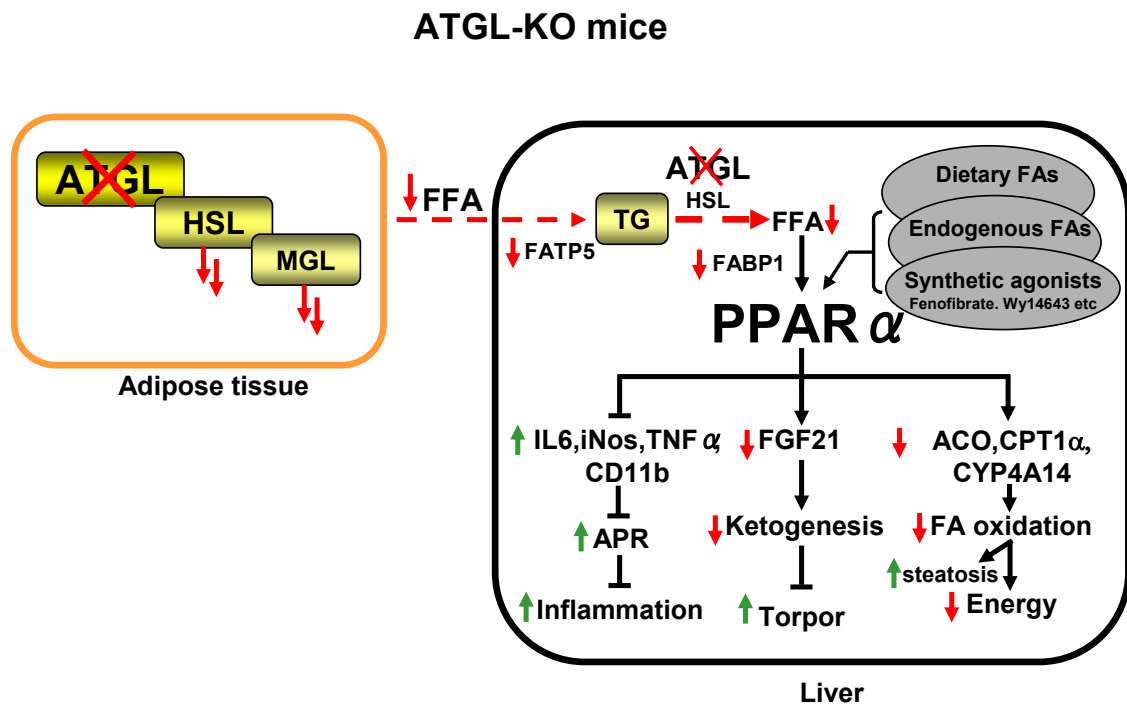


Figure 18. Proposed mechanism for enhanced susceptibility to acute and chronic hepatic inflammation in ATGL-KO mice.

Under normal conditions (in WT mice) the classical pathway of lipolysis comprising of ATGL, HSL and MGL in adipose tissue and liver provides free fatty acids (FAs) for activation of PPAR α in liver along with endogenously synthesized and dietary FAs. In ATGL-KO mice, the flux of free FAs from this canonical pathway to the liver and ATGL activity in liver itself is compromised. Hence, during an inflammatory stress HSL, other lipases and compensatory mechanisms release free FAs. However, these free FAs do not completely compensate for the absence of lipolytic products provided by ATGL for PPAR α activation. This, together with the deficiency of FATP5 and FABP1 leads to PPAR α insufficiency in ATGL-KO mice. As a result, anti-inflammatory PPAR α signaling is impaired, resulting in an enhanced acute phase response

leading to increased inflammation, while impaired FGF21 signaling pathway leads to impaired ketogenesis and torpor. Decreased FA oxidation further contributes to steatosis and insufficient metabolic fuelling to sustain inflammatory stress. Altogether these series of events render ATGL-KO mice extremely susceptible to acute and chronic inflammation in the pathogenesis of NASH which can be partially rescued by treatment with a PPAR α agonist.

8. Discussion

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This study unravels a hitherto unknown role of ATGL in protection from hepatic inflammation. Our data provides compelling evidence that ATGL - as a rate limiting enzyme for TG breakdown and a major player in lipid partitioning - may play a critical role in protection from hepatic inflammation as an integral feature of steatohepatitis/NASH. We further demonstrate that ATGL deficiency leads to an exaggerated inflammatory response upon endotoxin (LPS) challenge. Additionally, our data indicate that the anti-inflammatory role of ATGL may partially be dependent on PPAR α signalling.

MCD is a commonly used rodent model of NASH, resulting in endotoxin translocation, steatosis and inflammation (Hebbard *et al.*, 2011; Henao-Mejia *et al.*, 2012; Rinella *et al.*, 2008; Sahai *et al.*, 2004). Other models have been described like fast food feeding, intragastric overfeeding or high fat diet feeding which may be more representative of human NAFLD/NASH but take rather long time spans (25-35 weeks) for development of a full metabolic phenotype and lack a major contribution of FA flux from adipose tissue (Charlton *et al.*, 2011; Deng *et al.*, 2005; Hebbard *et al.*, 2011). MCD model recapitulates several key histological and molecular alterations found in patients with NASH, despite some limitations including the absence of obesity and insulin resistance phenotype (Hebbard *et al.*, 2011). Therefore, we combined MCD diet with ob/ob mice as another established model of NAFLD.

Earlier studies have shown weight and WAT loss on MCD feeding (Jha *et al.*, 2013; Rahman *et al.*, 2007; Rinella *et al.*, 2008), but the underlying mechanisms have remained unclear. In humans, increased peripheral FA flux has been shown to contribute to ~59% to the hepatic TG content as opposed to ~26% by DNL and ~15% from the diet in NAFLD patients (Donnelly *et al.*, 2005). Our study now demonstrates that when applied to ob/ob mice on MCD diet as a murine model of NASH, increased ATGL and HSL activities resulted in massive

loss of all WAT depots, thus increasing the flux of FAs from adipose tissue to the liver. As such the MCD model mimics to some extent the hepatic stress resulting from FA flux to the liver, in addition to its direct impact on liver.

We have earlier shown that cancer-induced cachexia increases lipase activity and causes WAT loss in WT and HSL-KO mice and that ATGL-KO mice are partially protected from WAT loss (Das *et al.*, 2011; Jha *et al.*, 2013). Based on these findings, together with our current observations, it is quite conceivable that ATGL may be the rate limiting metabolic lipase responsible for increased FA flux from adipose tissue in human NAFLD/ metabolic syndrome patients also, as it is for patients suffering from cancer-induced cachexia (Das *et al.*, 2011). Additionally, several studies have shown that massive weight loss shortly after bariatric surgery exacerbates fibrosis/NASH (Hafeez *et al.*, 2013; Mathurin *et al.*, 2009). Our findings from this model may also provide a fundamental plausible mechanism of WAT loss after bariatric surgery. Notably, various WAT depots behaved differently in terms of lipolytic activity mediated fat loss, with visceral WAT showing the highest rate of mobilization and subcutaneous the least. These observations in mice are in line with lipolysis from WAT as a major source of FA in humans where visceral WAT also has a higher rate of mobilization compared to subcutaneous WAT (Hoffstedt *et al.*, 1997). Furthermore, venous blood from visceral fat is directly drained to the liver through the portal vein. Therefore visceral fat is metabolically more active and is the major contributing source in the overflow of FAs to the liver (Donnelly *et al.*, 2005). Notably, expansion of visceral but not subcutaneous fat depots has been linked to insulin resistance and NAFLD (van der Poorten *et al.*, 2008).

It has been previously shown that reduction in body weight after MCD feeding is a characteristic of methionine deficiency (Caballero *et al.*, 2010) and was not associated with the biochemical effect of fasting (Leclercq *et al.*, 2000). Moreover, WT mice on MCD diet consumed similar amount of food as their controls (Jha *et al.*, 2013; Leclercq *et al.*, 2000; Rahman *et al.*, 2007; Rizki *et al.*, 2006). Thus it would be incorrect to conclude that adipose tissue loss is related entirely to less food intake. Therefore, WAT loss should be seen as independent of loss of hyperphagia. Additionally the effects of MCD feeding on

WAT loss could be due to increased metabolic rate upon MCD feeding in WT mice (Rizki *et al.*, 2006).

Our data demonstrates the importance of separately analysing different FA pools, i.e. total FA (free and esterified) and free FA/NEFA. Analysis of NEFA in serum revealed that 18:2n6 was preferentially released from adipose tissue into the serum and subsequently absorbed in the liver in MCD-fed ob/ob mice. This indicates that TG-18:2n6 could be one of the preferred substrates of ATGL. Indeed it has been earlier shown that ATGL preferentially hydrolyses long-chain FA esters *in vivo* (upon 8h fasting) (Eichmann *et al.*, 2012). Despite increased levels of important PPAR α ligands - 18:2n6 and 20:4n6 - in the liver, MCD-fed ob/ob mice showed enhanced inflammation. This could be due to the fact that with increased pro-inflammatory pressure the anti-inflammatory effect of PPAR α may fail in a fatty liver due to an imbalance between PPAR α -mediated anti-inflammatory and NF- κ B-mediated pro-inflammatory signals (Romics *et al.*, 2004).

In contrast to the NEFA profile in serum and liver, the TFA profile in serum showed an overall decreased composition of FA species which may be attributed to the reduced lipoprotein synthesis and excretion from the liver (Vance, 2008). The massive increase of TFA composition in the liver of MCD-fed ob/ob mice reflects the combined effect of increased fatty acid flux from adipose tissue, decreased oxidation of fatty acids and reduced TG hydrolase activity in the liver. Excess FA accumulation may further sensitize the liver for injury and inflammation. Further, the lack of FA hydrolysis and oxidation, limits the ligand availability for PPAR α signaling as well as other cellular signaling processes dependent on lipolytic products and their intermediates (Zechner *et al.*, 2012). Since ATGL provides ligands for PPAR α activation and 18:2n6 is a potent PPAR α agonist, the lack of ATGL-hydrolyses TG-18:2n6 in the liver may also be responsible for reduced PPAR α signaling in MCD-fed ob/ob mice. A previous human study has shown that the absence of BAT in adult life is associated with an increased risk of fatty liver and increased BAT activity may

exert a protective effect towards the development of NAFLD (Yilmaz *et al.*, 2011). Our results for the first time show the association between a rodent NASH model and BAT, which is partly in contrast to the (only) human study on association between BAT and NAFLD (Yilmaz *et al.*, 2011). While reduced BAT mass in MCD-fed ob/ob mice is in line with the human data, increased BAT activity is in contrast to this study. Further studies may be required to elucidate these observations in both humans and rodents.

It would be ideal to conduct pair-feeding experiments to match weight loss in control and MCD-fed ob/ob mice, although such is the severity of weight loss in MCD-fed mice that this would be technically very challenging. Further, what triggers the activity of the lipases in this model needs to be further explored. Our study was focused on a relatively late stage of MCD feeding and therefore it is plausible that in the early stages there is more contribution of DNL to hepatic steatosis, however, it remains speculative. Whether blocking peripheral lipolysis could limit hepatic steatosis and further progression to NASH remains to be studied. However, since AT may also provide ligands for PPAR α activation in liver, blocking peripheral lipolysis may also exaggerate the inflammatory response in progression to NASH. Therefore, it may be of crucial importance to elucidate this in future studies.

ATGL-KO mice are insulin sensitive in contrast to the metabolic phenotype typically associated with human NAFLD (Haemmerle *et al.*, 2006). However, we aimed to understand how NAFLD development depends on ATGL activity and to test the specific hypothesis, that lack of ATGL may increase hepatic inflammation in progression to NASH due to altered lipid partitioning and impaired PPAR α signaling. Loss of WAT in MCD-fed WT mice was due to increased ATGL and HSL activities in all WAT depots, while in MCD-fed ATGL-KO mice, despite the substantial loss of all WAT depots (~44-52%), the increase in total TG hydrolase and HSL activity was observed only in gonadal WAT (~18%) suggesting that anabolic processes such as FA uptake or esterification may be defective which might contribute to the loss of perirenal and visceral WAT mass. Following lipolysis in adipose tissue, the flux of FAs to

liver resulted in increased number of ligands for PPAR α and subsequent induction of PPAR α target genes in MCD-fed WT mice. In contrast, induction of PPAR α and its target genes in MCD-fed ATGL-KO mice was quite marginal due to the lack of ATGL generated FAs for PPAR α activation. Despite increase in gene expression of PPAR α and its downstream targets on MCD feeding, we observed a decrease in PPAR α DBA in both the genotypes. This discrepancy could be due to the fact that PPAR α gene expression is under a tight circadian regulation. Moreover, since mice were harvested between 2-3pm, a marginal fasting effect could result in increased PPAR α expression. ATGL-KO mice showed increased steatosis on MCD feeding due to the lack of FA oxidation as also observed by CPT1 inhibition in PPAR α KO mice (Djouadi *et al.*, 1998). The increased hepatic inflammation further reflects aggravation of NASH in ATGL-KO mice. These findings are consistent with lower expression of PPAR α and thus compromised anti-inflammatory effects in the absence of ATGL. In contrast to PPAR α , we observed no significant differences in the expression of PPAR δ or PPAR γ and downstream targets in MCD-fed WT and ATGL-KO mice, nor in controls or MCD fed ob/ob mice (data not shown), which suggests a highly PPAR α dependent regulation of inflammatory response in ATGL-KO mice.

Notably, upon LPS challenge, ATGL-KO mice showed increased inflammation compared to LPS-injected WT mice, indicating that ATGL may be critical for counteracting acute hepatic inflammation. Low levels of ketone bodies in the serum of WT and LPS-injected ATGL-KO mice indicate impaired ATGL-catalyzed lipolysis and as a result decreased FAs as substrate for ketone body synthesis in the liver. Undetectable serum FGF-21 may further explain the torpor state of the mice. Furthermore, binding of LPS to lipoproteins neutralizes LPS *in vitro* and also protects animals from LPS-induced fever, hypotension and death (Harris *et al.*, 1990; Ulevitch *et al.*, 1979). Since ATGL-KO mice have low serum lipoproteins (Haemmerle *et al.*, 2006) this could also contribute to the exaggerated acute phase response and high mortality in LPS-injected ATGL-KO mice. The relatively benign inflammatory response of HSL-KO mice in response to MCD and LPS further argues against a critical role of the lipolytic products of HSL in modulating the inflammatory response.

Several studies have demonstrated *in vitro* induction by cytokines or LPS induced lipolysis from adipocytes and WAT depots (Bezair *et al.*, 2009; Khovidhunkit *et al.*, 2004; Yang *et al.*, 2011; Zu *et al.*, 2009). However, the *in vivo* role of these lipases in the genetic KO mouse model had not been explored so far. We herein show that LPS enhanced serum NEFA concentration as well as NEFA species in both WT and ATGL-KO mice. It is quite conceivable that in the absence of ATGL, HSL may partially compensate for lipolysis, leading to increased serum NEFA. However, *ex vivo* lipolysis from gonadal fat pad explants showed a much smaller induction of LPS induced lipolysis in the ATGL-KO mice compared to the WT mice. Furthermore, compared to ATGL-KO controls only a marginal induction of *ex vivo* lipolysis was observed in LPS-injected ATGL-KO mice when treated with HSL inhibitor and when treated with HSL inhibitor and forskolin together. This suggests that other sources/ compensatory lipase(s)/ pathways could contribute to increased serum NEFA concentrations and species in ATGL-KO mice during an endotoxin induced inflammatory insult. Since ATGL-KO mice showed PPAR α insufficiency despite induction of lipolysis in adipose tissue and release of NEFA species in serum, which are potential PPAR α ligands, we hypothesized that this insufficiency may be due to impaired FA uptake by hepatocytes during acute inflammatory stress. Indeed, we observed diminished expression of hepatic FATP5 and FABP1-mediated FA uptake into the liver and lower levels of nuclear FABP1 in ATGL-KO livers, as well as higher levels of circulating NEFA species in the serum of LPS-challenged ATGL-KO mice. Furthermore, 18:2n6, an important PPAR α ligand (Forman *et al.*, 1997; Kliewer *et al.*, 1997) was selectively reduced in ATGL-KO livers suggesting that this ligand may be a preferred FA cleaved by ATGL and could be an important signaling lipid which needs to be addressed in future studies.

In line with a key role of ATGL in PPAR α signaling, treatment with fenofibrate (FF) as PPAR α agonist was able to partially attenuate MCD-induced NASH and LPS-induced inflammation in ATGL-KO mice. This further indicates that ATGL-catalysed natural ligands for PPAR α may be more effective in completely

reverting the hepatic phenotype of ATGL-KO mice under inflammatory challenge. Importantly, IL6 mRNA expression as well as serum concentrations were reduced in both WT and ATGL-KO FF-pretreated LPS-injected mice (Fig. 6E). This is in line with previous findings which show that IL6 is the principal inducer of acute phase response (APR) gene expression and PPAR α activation by FF reduces IL6 mediated APR gene expression (Gervois *et al.*, 2004; Zambon *et al.*, 2006). Consistent with a previous finding in PPAR α -KO mice fed with Wy-14,643 supplemented MCD diet (Ip *et al.*, 2003), FF also partially improved liver inflammation in ATGL-KO mice on MCD diet. However, unlike failure to reverse hepatic steatosis in PPAR α -KO mice in their study (Ip *et al.*, 2003), we observed a complete normalization of hepatic steatosis in ATGL-KO mice which may be mostly attributed to increased oxidation of exogenous fatty acids due to FF and also the MCD diet itself since MCD feeding increases the expression of FA oxidation genes. Contrary to our findings, a previous study reported that PPAR α agonist did not restore the expression of PPAR α and its target genes to the WT levels in mice injected with ATGL shRNA (Ong *et al.*, 2010). This discrepancy could be due to lower dose of FF and also the overnight fasting performed in their study (Ong *et al.*, 2010).

Additionally, our data suggests a strong ATGL dependent regulation of FGF21. While in untreated ATGL-KO mice serum FGF21 levels were undetectable, FF treatment induced the FGF21 levels although to a much lower degree than in WT mice. It has been previously shown that hepatic mRNA expression and serum FGF21 was significantly elevated in NAFLD patients (Dushay *et al.*, 2010), as also observed in this study in MCD fed WT mice (Figure 6C) and MCD-fed ob/ob mice (data not shown). Therefore the decrease in hepatic mRNA and serum levels of FGF21 in fenofibrate treated mice could be due to the improvement of steatosis. Further, upon LPS injection WT mice showed increase in serum FGF21 levels despite lower levels hepatic FGF21 mRNA levels. This data is consistent with a previous report highlighting the contribution of extra-hepatic tissues, in particular WAT and muscle in FGF21 blood levels (Feingold *et al.*, 2012) (data not shown).

While ATGL ablation enhances the hepatic inflammatory response, its absence may protect from tunicamycin-induced hepatic ER stress as shown previously (Fuchs *et al.*, 2012). These two separate effects of ATGL may be due to the distinct signaling pathways involved in the modulation of MCD or LPS-induced inflammation and tunicamycin-mediated ER stress. Recent studies have shown amelioration of hepatic steatosis in mice by adenoviral over-expression of ATGL and HSL (Reid *et al.*, 2008), while liver specific ATGL-KO (Wu *et al.*, 2011) and shRNA knockdown of ATGL (Ong *et al.*, 2010) resulted in increased hepatic steatosis. While these studies suggest a liver-specific role of ATGL or HSL in hepatic lipid metabolism and signaling, our data add an important additional aspect with regard to the indispensable role of ATGL in regulating hepatic inflammation by providing PPAR α ligands. Figure 15 summarizes the proposed mechanisms, which may result in increased susceptibility for hepatic inflammation and development of NASH in the absence of ATGL.

This study did not address the tissue specific function of ATGL in adipose tissue vs. liver. Whether over-expression of ATGL in liver can normalize the hepatic inflammatory response or liver specific ATGL knockdown exaggerates the response needs to be addressed in future studies. Notably, mice lacking HSL did not show aggravated response to MCD and LPS, supporting a role for ATGL in liver in modulating the severity of NASH. Moreover WAT did not show increased inflammatory response in LPS treated ATGL-KO mice suggesting that absence of ATGL in WAT may also not contribute to hepatic inflammatory response in ATGL-KO mice. While it is clear from the lipase activity data that increased lipase activity in adipose tissue contributes to hepatic steatosis, a detailed study is required to address the contribution of FAs from adipose tissue in modulating hepatic PPAR α signaling. Furthermore, pigment epithelium-derived factor (PEDF) has been shown to regulate hepatic TG content through binding to ATGL (Chung *et al.*, 2008), and deregulation of this pathway in the absence of ATGL may also be a major mechanism underlying fatty liver disease. Importantly, liver steatosis has also been observed in patients with neutral lipid storage disease (NLSD) caused due to mutation in ATGL or CGI-58 (Schweiger *et al.*, 2009), thus may be worthwhile to search for potential genetic variants of PNPLA2 in the pathogenesis of human NAFLD/NASH. Such studies

could add to recent studies that suggest genetic variants of PNPLA3 (the closest homologue to ATGL/PNPLA2) are associated with susceptibility and progression of NAFLD to NASH, although the underlying functional mechanisms are still controversial (Basantani *et al.*, 2011; Romeo *et al.*, 2010; Romeo *et al.*, 2008).

In conclusion, the presented work provides compelling evidence that ATGL may play a key role in protection from hepatic inflammation as an integral feature/component in progression to NASH as well as from acute hepatic inflammation. Apart from potential implications for NASH, these findings may have general relevance for understanding the role of ATGL in acute and chronic hepatic inflammation. Future studies will have to address whether therapeutic targeting of ATGL could counteract hepatic inflammation in general and specifically in progression to NASH to potentially lethal consequences such as liver cirrhosis and cancer.

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11. 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”.

Signature

Date

12. Appendix

Publication (published/ communicated/ in preparation)

Jha P, Claudel T, Baghdasaryan A, Mueller M, Halilbasic E, Das SK, Lass A, Zimmermann R, Zechner R, Hoefler G, Trauner M. Role of adipose triglyceride lipase (PNPLA2) in protection from hepatic inflammation in mouse models of steatohepatitis and endotoxemia. **Hepatology** (2013); Sep 3. doi: 10.1002/hep.26732

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Jennifer M. Collins, Simon Lord, Barbara A. Fielding, Katherine E. Pinnick, Anne Clark, Simon Wigfield, Pooja Jha, Gerald Höfler, Adrian L. Harris, Fredrik Karpe. Anti-proliferative effect of metformin is associated with sequestration of fatty acids in an inaccessible lipid droplet store. (**Submitted in JNCI**)

Pooja Jha, Jianfeng Huang, Ganapathy S Paramasivan, Astrid Knopf, Harald Koefeler, Thierry Claudel, Michael Trauner. Deficiency of ATGL in mice disturbs circadian regulation of hepatic lipid metabolism. (**Manuscript in preparation**)

Pooja Jha, Astrid Knopf, Harald Koefeler, Thierry Claudel, Michael Trauner. Impact of LPS-induced stress on lipolysis and lipidomic profile in Adipose triglyceride lipase deficient mice. (**Manuscript in preparation**)

Pooja Jha, Michael Trauner. Absence of Adipose triglyceride lipase (ATGL) enhances inflammation in brown adipose tissue. (**Manuscript in preparation**)

Claudia Fuchs, Thierry Claudel, Pooja Jha, Tatjana Stojakovic, Walter Spindelboek, Michael Trauner. FXR is a key player in NAFLD development by controlling CHOP expression. (**Manuscript in preparation**)