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***Exploring the effects
of different FXR agonists
on progression of
Non-Alcoholic Steatohepatitis
and its transition
to Hepatocellular Carcinoma***

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1. Abbreviations

3D	Three dimensional
<i>Abca1</i>	ATP binding cassette subfamily A member 1
ABCG5/8	ATP-binding cassette sub-family G member 5/8
<i>Acac</i>	Acetyl-CoA carboxylase
<i>Acad10</i>	Acyl-CoA dehydrogenase family member 10
<i>Acat2-3</i>	Acetyl-CoA C-acetyltransferase 2-3
Acc	Acceleration
ACC	Acetyl-CoA carboxylase
ACE	Angiotensin-converting-enzyme
<i>Acly</i>	ATP citrate lyase
<i>Acot2</i>	Acyl-CoA thioesterase 2
<i>Acox3</i>	Acyl-CoA oxidase 3, pristanoyl
<i>Acs11</i>	Acyl-CoA synthetase long chain family member 1
ACSLs	Long-chain acyl-CoA synthetase
<i>Acsm2</i>	Acyl-CoA synthetase medium-chain family member 2
<i>Acsm2</i>	Acyl-CoA synthetase medium-chain family member 2
<i>Acta2/Asma</i>	Actin alpha 2, smooth muscle, aorta
ACVR2A	Activin A Receptor Type 2A
AF	Alexa Fluor
a-Gal	Glycolipid alpha-galactosyl ceramide
AhR	Aryl hydrocarbon receptor
Akt	Protein kinase B
ALD	Alcoholic Liver Disease
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
AMLN	Amylin Liver NASH Diet
AMPK	5' AMP-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Allophycocyanin
Apo	Apo lipoprotein
ARB	Angiotensin receptor blocker
ASBT	Apical sodium-dependent bile acid transporter
AST	Aspartate Transaminase
AST	Aspartate Aminotransferase
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BAAT	Amino acid N-acetyltransferase
BACS	Bile acid CoA synthase
BAR	Bile acid Receptor
BAs	Bile acids
BCA	Bicinchoninic acid
BIP	Binding immunoglobulin protein
BMI	Body mass index
BSEP	Bile salt export pump
BV	Brilliant Violet
C/EBP- α/β	CCAAT/enhancer-binding proteins alpha and beta

CA	Cholic acid
CBP	CREB Binding Protein
<i>Ccl2-5</i>	C-C motif chemokine ligand 2-5
CCl ₄	Carbon tetrachloride
CD	Cluster of differentiation
CDAHFD	Choline-deficient, L-amino acid-defined, high-fat diet
CDCA	Chenodeoxycholic acid
cDCs	Classical dendritic cells
CD-HFD	Choline-deficient, high-fat diet
cDNA	Complementary DNA
CHOP	C/EBP Homologous Protein
ChREBP	Carbohydrate response element binding protein
CIDEB	Cell death-inducing DFFA-like effector b
CLD	Chronic liver disease
CO ₂	Carbon dioxide
Co-A	Coenzyme-A
<i>Colla1</i>	Collagen type I alpha 1 chain
<i>Cpt1</i>	diacylglycerol choline phosphotransferase
CREB	cAMP Response Element-Binding Protein
CRTC2	CREB Regulated Transcription Coactivator 2
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CTLs	Cytotoxic T-lymphocytes
Cy-7	Cyanine-7
CYP27A1	Cytochrome p450 family 27 subfamily A member 1
CYP2C70	Cytochrome p450 family 2 subfamily C member 70
CYP7A1	Cytochrome p450 family 1 subfamily A member 1
CYP8B1	Cytochrome p450 family 8 subfamily B member 1
DAMP	Damage-associated molecular pattern
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA Binding Domain
Dcc	Deceleration
DEN	N-Diethyl nitrosamine
DIAMOND	Diet-Induced Animal Model Of Non-alcoholic fatty liver Disease
DNA	Deoxyribonucleic acid
DNL	De novo lipogenesis
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eIF2 α	Eukaryotic translation initiation factor 2-alpha
<i>El</i>	lipase G, endothelial type
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
eWAT	Epididymal white adipose tissue
f.c.	Final concentration
F1,6P	Fructose 1,6-bisphosphate
F6P	Fructose-6-phosphate
FAs	Fatty acids
FABP	Fatty acid binding protein

FACS	Fluorescence activated cell sorting
Fas	Fatty acid synthase
Fas-L	Fas ligand
<i>Fasn</i>	Fatty acid synthase
FATP	Fatty acid transport protein
FBPase	Fructose 1,6 bisphosphatase
FCS	Foetal calf serum
Fex	Fexaramine
FFA	Free fatty acid
FFPE	Formalin fixed, paraffin embedded
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
Fix/Perm	Fixation/Permeabilization
Foxm1b	Forkhead box m1b
FOXO1	Forkhead Box O1
FPP	Farnesyl pyrophosphate
FXR	Farnesoid X receptor
G6P	Glucose 6-phosphate
G6Pase	Glucose-6-phosphatase
GAN	Gubra Amylin NASH Diet
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gbpar-1	G protein-coupled bile acid receptor-1 (TGR5)
GCK	Glucokinase
GGPP	Geranylgeranyl pyrophosphate
GLP-1	Glucagon-like Peptide-1
GLUT2	Glucose transporter 2
G-MCA	Glyco-Muricholic Acid
GP	glycogen phosphorylase
GPAM	Glycerol-3-Phosphate Acyltransferase
<i>Gpat</i>	Glycerol-3-phosphate acyltransferase
GPP	Geranyl pyrophosphate
GSK-3	Glycogen synthase kinase 3
GYS1	Glycogen synthase
GzmB	Granzyme B
H&E	Haematoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HBV	Hepatitis B virus
HCA	Hyocholic acid
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDCA	Hyodeoxycholic acid
HFD	High fat diet
HGF	Hepatocyte growth factor
<i>Hl</i>	Hepatic lipase
HMGB1	High mobility group protein B1
HMG-CoA	β -Hydroxy β -methylglutaryl-CoA
HRP	Horse-radish peroxidase
HSCs	Hepatic Stellate Cells

i.p.	Intraperitoneal
ICAM1-	Intracellular adhesion molecule 1
IFN γ	Interferon-gamma
IHC	immunohistochemistry
IL	Interleukin
ILCs	Innate lymphoid cells
iNKT	Invariant natural killer T
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin resistance
IRS	Insulin receptor substrate
iWAT	Inguinal white adipose tissue
JNK	c-Jun N-terminal kinase
KC	Kupffer cell
LAG-3	lymphocyte-activation gene 3
LBD	Ligand Binding Domain
LCA	Lithocholic acid
LDL	Low density lipoprotein
LDLR	LDL receptor
<i>Light</i>	Tumor necrosis factor (ligand) superfamily, member 14
<i>Lipe</i>	lipase, hormone sensitive
LKB1	Liver Kinase B1
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
IrNK	Liver-resident NK cells
LSECs	Liver sinusoidal endothelial cells
<i>Ltb</i>	Lymphotoxin B
LXR	Liver X receptor
Ly6G	Lymphocyte antigen 6 complex locus G6D
MAPK	Mitogen-activated protein kinase
MCD	Methionine-choline deficient diet
M-CSF	Macrophage colony-stimulating factor
MDCA	Murideoxycholic acid),
MHC I/II	Major histocompatibility complex class I and II
Min	Minutes
mL	Millilitre
MRP2	Multidrug related protein 2
mTORC1	Mammalian target of rapamycin complex 1
MUP-uPA	Major urinary protein-urokinase-type plasminogen activator
<i>Mvd</i>	Mevalonate diphosphate decarboxylase
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NCP1L1	Niemann-Pick C1-Like 1
ND	Normal diet
NDRG2	N-Myc Downstream-Regulated Gene 2 Protein
NEFAs	Non-esterified fatty acids
NF- κ B	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

NK	Natural killer cells
NKG2D	Natural killer group 2D
NKT	Natural killer T-cells
NLRP3	NLR family pyrin domain containing 3
Notch	Neurogenic locus notch homolog protein 1
NQO1	NAD(P)H dehydrogenase (quinone 1)
Nrf2	Nuclear factor erythroid 2-related factor 2
NTCP	Sodium taurocholate co-transporting polypeptide
o.n.	Overnight
OCA	Obeticholic acid
PAMP	Pathogen-associated molecular pattern
PBC	Primary biliary cholangitis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cells
PD-L1	Programmed cell death-ligand 1
PE	Phycoerythrin
PEP	Phosphoenolpyruvate
PEPCK-C	Cytoplasmic phosphoenolpyruvate carboxylase
PerCP-Cy5.5	Peridinin chlorophyll protein – Cyanine 5.5
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
PGC-1 α	Pparg coactivator 1 alpha
PGE2	Prostaglandin E2
PI3 kinase	Phosphoinositide 3-kinase
PI3K	Phosphoinositide 3-kinase
PP1	Protein phosphatase 1
PPAR	Peroxisome proliferator-activated receptor
Ppm	Parts per million
PVDF	Polyvinylidene fluoride
PXR	Pregnane X receptor
qRT-PCR	Quantitative real-time PCR
RA	Retinoic acid
RelA (p65)	Nuclear factor NF-kappa-B p65 subunit
RelB	V-Rel reticuloendotheliosis viral oncogene homolog B
RER	Respiratory exchange ratio
RIPA	Radio immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI medium	Roswell Park Memorial Institute medium
RSK	Ribosomal S6 Kinase
RT	Room temperature
RT	Reverse transcriptase
S1PR2	Sphingosine-1 phosphate receptor 2
SBARMs	Selective bile acid receptor modulators
<i>Scd1</i>	Stearoyl-Coenzyme A desaturase 1
SEM	Standard error of the mean
SGLT2	Sodium-glucose cotransporter-2

SHP	Small heterodimer partner
SIRT	Sirtuin
SOCS3	Suppressor Of Cytokine Signalling 3
Sox9	SRY-box transcription factor 9
<i>Sqle</i>	Squalene epoxidase
SRC-1/2	Steroid receptor coactivator-1/2
SREBP	Sterol regulatory element-binding protein
STAM	Streptozotocin-induced NASH mouse model
STAT	Signal transducer and activator of transcription
SULT2A1	Sulfotransferase family 2A member 1
T1Dm	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAZ	Tazafazzin
TCA	tricarboxylic acid
TCR	T-cell receptor
TGF- β	Transforming growth factor β
TGR5	Takeda G protein-coupled receptor 5
TGs	Triglycerides
Th	T helper
TIE	Angiopoietin receptor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TP53	Tumour protein p53
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
Treg	Regulatory T-cell
Tris	Tris(hydroxymethyl)amino methane
Tropi	Tropifexor
T- β -MCA	Tauro-Beta-Muricholic acid
UDPGA	Uridin 5'-diphosphoglucuronic acid
UGT	UDP-glucuronosyltransferase
UGT2B4	UDP Glucuronosyltransferase Family 2 Member B4
UPLC	Ultra-performance liquid chromatography
USF-1	Upstream stimulatory factor 1
VCAM-1	Vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
VSIG4	V-set and Ig domain-containing 4
WD	Western diet
Wnt	Wingless-related integration site
YAP	Yes-associated protein
β -MCA	Beta-Muricholic acid
μ L	Microliter
μ m	Micrometre
μ M	Micro molar

2. Summary

Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers, is the sixth most frequent cancer and represents the third cause of global cancer-related mortality [1]. The known aetiologies of HCC could be of viral (Hepatitis B, Hepatitis C infection) and non-viral (non-alcoholic fatty liver disease (NAFLD) and alcoholic steatohepatitis (ASH)) nature, although NAFLD is becoming the predominant cause of chronic liver disease in western countries. NAFLD, a condition presenting as the hepatic manifestation of the metabolic syndrome, is favoured by diet-induced obesity and insulin resistance [2]. NAFLD is characterized by progressive degree of severity, inducing Non-alcoholic steatohepatitis (NASH), its most detrimental stage that precedes liver cirrhosis and HCC onset [3, 4]. Currently, no approved therapy is available for NASH, and many studies ongoing worldwide are focused in the effort to develop new treatments.

In the last two decades the essential role of bile acids in regulating metabolism has been uncovered and driven an increased amount of research to find suitable molecules for targeting the main bile acid receptor, FXR [5]. This led to the development of several FXR agonist, many of which are currently under clinical trials testing. Among them, Tropicifexor, a highly potent synthetic agonist, demonstrated safety and tolerability during clinical trials, both alone and in combination with other compounds, and will likely proceed to phase III testing in the near future [6, 7]. However, no research has to date evaluated the effects of sustained treatment with Tropicifexor nor other FXR agonist, in the context of late NASH and during NASH-to-HCC transition. To study this question, I employed different dietary models of murine NASH and tested several FXR agonists including Tropicifexor in both short-and long-term therapeutic fashion. I confirmed the extraordinary efficacy of Tropicifexor in reversing NASH by driving important improvements in obesity, glucose and lipid metabolism, effectively reducing bile acids and leading to a clearance of hepatic steatosis in the short-term experiments, showing also relevant modulations on lymphoid-and myeloid-induced inflammation. Nevertheless, some concerning effects related to hepatomegaly induction stimulated by elevated hepatocyte proliferation and activation of a pro-carcinogenic signature, balanced by activation of cellular senescence and polyploidy, sparked my interest for further investigation after prolonged administration of Tropicifexor. The long-term experiments faithfully recapitulated the efficient metabolic improvements induced in the liver by Tropicifexor, successfully reversing severe NASH and improving fibrosis, but at the expenses of increased amount of hepatic lesions. Additional evaluation led to the identification of pronounced levels of Endoplasmic Reticulum

(ER) stress, likely caused by specific hepatic retention of hydrophobic bile acids species, favoured especially by a low dose of Tropifexor. In addition, a comparison of a high and low dose of Tropifexor revealed essential pharmacokinetic differences that importantly impact on the diversity of liver damage induced and on the biliary stress stimulation driving regenerative changes. Furthermore, long-term administration of Tropifexor presented dual effects on both lymphoid and myeloid immunity, showing contradicting effects in the inflammation modulation. Additional studies are definitely required to dissect in detail the molecular mechanisms at the base of the observed phenotype. Yet, the present study clearly indicated that a prolonged administration of Tropifexor in late NASH in proximity of HCC transition could rather synergize the signals that drive malignant transformation and increase tumour incidence in spite of NASH improvement.

3. Zusammenfassung

Das hepatozelluläre Karzinom (HCC) macht 90 % der primären Leberkrebserkrankungen aus, ist die sechsthäufigste Krebsart und stellt die dritthäufigste Ursache für die weltweite krebsbedingte Mortalität dar [1]. Die bekannten Ursachen von HCC können viraler (Hepatitis-B-, Hepatitis-C-Infektion) und nicht-viraler Natur (nichtalkoholische Fettlebererkrankung (NAFLD) und alkoholische Steatohepatitis (ASH)) sein, obwohl NAFLD zur Hauptursache für chronische Lebererkrankungen wird Krankheit in westlichen Ländern. Die nichtalkoholische Fettlebererkrankung (NAFLD), ist eine Erkrankung, die als hepatische Manifestation des metabolischen Syndroms auftritt und durch ernährungsbedingte Fettleibigkeit und Insulinresistenz begünstigt wird [2]. NAFLD zeichnet sich durch einen fortschreitenden Schweregrad aus, der die nichtalkoholische Steatohepatitis (NASH) auslöst, ihr schädlichstes Stadium, das der Leberzirrhose und dem Beginn des HCC vorausgeht [3, 4]. Derzeit ist keine zugelassene Therapie für NASH verfügbar, und viele weltweit laufende Studien konzentrieren sich auf die Entwicklung neuer Behandlungsmethoden.

In den letzten zwei Jahrzehnten wurde die wesentliche Rolle von Gallensäuren bei der Regulierung des Stoffwechsels aufgedeckt und zu einer verstärkten Forschung geführt, um geeignete Moleküle zu finden, die auf den Hauptgallensäurerezeptor FXR abzielen [5]. Dies führte zur Entwicklung mehrerer FXR-Agonisten, von denen viele derzeit in klinischen Studien getestet werden. Unter ihnen hat Tropifexor, ein hochwirksamer synthetischer Agonist, in klinischen Studien sowohl allein als auch in Kombination mit anderen Verbindungen Sicherheit und Verträglichkeit nachgewiesen und wird wahrscheinlich in naher Zukunft in die Phase-III-Tests übergehen [6, 7]. Bisher wurden jedoch noch keine Untersuchungen zu den Auswirkungen einer dauerhaften Behandlung mit Tropifexor oder einem anderen FXR-Agonisten im Zusammenhang mit spätem NASH und während des Übergangs von NASH zu HCC durchgeführt. Um dies zu untersuchen, habe ich verschiedene Ernährungsmodelle für murines NASH verwendet und mehrere FXR-Agonisten, darunter Tropifexor, sowohl kurz- als auch langfristig als Therapie getestet. Ich habe die außerordentliche Wirksamkeit von Tropifexor bei der Umkehrung von NASH bestätigt, indem es wichtige Verbesserungen bei Fettleibigkeit, Glukose- und Lipidstoffwechsel herbeiführte, Gallensäuren effektiv reduzierte und in den Kurzzeitexperimenten zu einer Beseitigung der Lebersteatose führte, wobei auch relevante Modulationen auf Lymphoid- und Myeloidgewebe gezeigt wurden -induzierte Entzündung. Dennoch weckten einige besorgniserregende Effekte im Zusammenhang mit der Hepatomegalie-Induktion, die durch eine erhöhte Hepatozytenproliferation und die Aktivierung

einer prokanzerogenen Signatur, ausgeglichen durch die Aktivierung von Zellalterung und Polyploidie, stimuliert wird, meinem Interesse für weitere Untersuchungen nach längerer Verabreichung von Tropifexor. Die Langzeitexperimente stellten getreu die durch Tropifexor in der Leber induzierte effiziente Stoffwechselrettung dar, wodurch schweres NASH erfolgreich rückgängig gemacht und die Fibröse verbessert wurde, allerdings auf Kosten einer erhöhten Anzahl von Leberläsionen. Zusätzliche Untersuchungen führten zur Identifizierung ausgeprägter Belastungen des endoplasmatischen Retikulums (ER), die wahrscheinlich durch eine spezifische hepatische Retention hydrophober Gallensäurespezies verursacht werden, die insbesondere durch eine niedrige Tropifexor-Dosis begünstigt wird. Darüber hinaus ergab ein Vergleich einer hohen und einer niedrigen Dosis von Tropifexor wesentliche pharmakokinetische Unterschiede, die sich erheblich auf die Vielfalt der induzierten Leberschäden und auf die biliäre Stressstimulation auswirken, die regenerative Veränderungen vorantreibt. Darüber hinaus hatte die Langzeitverabreichung von Tropifexor doppelte Auswirkungen auf die lymphatische und myeloische Immunität und zeigte widersprüchliche Wirkungen bei der Entzündungsmodulation. Es sind definitiv weitere Studien erforderlich, um die molekularen Mechanismen, die dem beobachteten Phänotyp zugrunde liegen, im Detail zu analysieren. Die vorliegende Studie zeigte jedoch deutlich, dass eine längere Verabreichung von Tropifexor im späten NASH-Bereich zum HCC-Übergang die Signale, die die maligne Transformation vorantreiben, eher verstärken³ und die Tumorzinzidenz trotz der NASH-Verbesserung erhöhen könnte.

4. Introduction

4.1. Liver, gut-liver axis and metabolism

4.1.1. Liver anatomy and physiology

The liver is a major organ of our organism located in the upper abdomen and connected to the digestive system. It has a lobular structure composed of different cell types, like hepatocytes, cholangiocytes, stellate cells, Kupffer cells (KCs), and liver sinusoidal endothelial cells. Hepatocytes perform most liver functions, while cholangiocytes line the bile ducts. Hepatic stellate cells (HSCs) store vitamin A and contribute to collagen deposition during injury. KCs have roles in inflammation. Liver sinusoidal endothelial cells (LSECs) facilitate protein and particle exchange [8-10]

The liver's lobules have a pyramidal shape and are made of hepatocyte strings separated by sinusoids. Sinusoids are blood vessels derived from the hepatic artery and portal vein, allowing nutrient and metabolite exchange with hepatocytes. HSCs and KCs are present in the sinusoids. The interlobular spaces at the meeting points of lobules form the portal space, containing blood vessels, bile ducts, lymphatic vessels, and nerves. Bile capillaries, lined by cholangiocytes, flow towards the portal spaces. Bile is then transported through hepatic ducts to the duodenum via the common bile duct [11, 12].

The liver is mainly an exocrine gland involved in the secretion of bile, but carries out also important endocrine functions, by releasing products of its metabolism into the blood flow. It can regulate the concentration of single plasmatic constituents through altering the circulation of metabolites in the blood stream. The liver is also the endpoint of convergence of basic nutritional substrates absorbed in the intestine, as for the catabolic products of tissues, such as glucose, amino acids and fatty acids (FAs). These are thereby stored, or oxidized and subjected to reactions of interconversion in order to be further secreted based on specific necessities [11].

The liver possesses a great metabolic flexibility, as able to adapting its enzymatic activity in relation to the variation of different substrates. It can provide a constant source of glucose, regulates the amino acids metabolism, is involved in the synthesis of urea, bile acids (BAs), ketone bodies, most of the plasmatic proteins, and regulates the lipids transport. It is also the major organ responsible of the clearance of substances that are not excreted by the

kidneys. In addition, it transforms the by-products of the metabolism into other substances that could somehow be re-cycled or in further waste products sent to excretion via urine or faeces [8, 9].

Lastly, the liver is importantly implicated in the metabolism and removal of active substances like hormones, drugs, or toxins in general. The variety of biochemical processes that take place in the liver ensure that the alterations in its functional state, as a result of the different physiological and pathological state of the body, are mainly subject to quantitative variation and rate of the several enzymatic reactions [13].

The liver is in close connection with the gut as most of the metabolites absorbed in the intestine are directed to the liver through the portal circulation, therefore it is important to mention that alteration in one of these two organs massively affects the overall homeostasis of the whole gastrointestinal tract, with obvious systemic repercussions. That is why the study of the gut-liver axis, both for the normal physiology and for all the pathological alterations affecting the hepatobiliary and intestinal complexes are of high importance for the scientific and medical communities [14, 15].

4.1.2. Glucose metabolism

Glucose metabolism in the liver is a complex process that plays a crucial role in preserving normal blood glucose levels and providing energy for various physiological functions. Approximately 80% of the liver consists of hepatocytes, the primary cell type responsible for glucose metabolism.

Glucose enters hepatocytes through a glucose transporter known as GLUT2. Deletion of GLUT2 specifically in hepatocytes blocks glucose uptake. GLUT2 also facilitates glucose release from the liver, but its deletion does not impair glucose production during fasting, suggesting the contribution of alternative transporters or mechanisms for glucose release [16]. Inside hepatocytes, glucose is converted to glucose 6-phosphate (G6P) by the enzyme glucokinase. This process reduces intracellular glucose levels and enhances glucose uptake. G6P cannot exit hepatocytes, so it remains within them. In the fed state, G6P serves as a precursor for glycogen synthesis and can be metabolized to generate pyruvate through glycolysis. Pyruvate is further oxidized in the mitochondria, producing ATP via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Additionally, pyruvate can be used for FAs synthesis through lipogenesis. The metabolism of G6P through the pentose

phosphate pathway generates nicotinamide adenine dinucleotide phosphate (NADPH), which is essential for lipogenesis and the synthesis of other biologically active molecules. In the fasted state, G6P is transported to the endoplasmic reticulum (ER) and dephosphorylated by an enzyme called glucose-6-phosphatase (G6Pase) to release glucose [17-19].

During the fed state, glucose is transported into hepatocytes via GLUT2 and undergoes phosphorylation by glucokinase (GCK). The phosphorylated glucose is then utilized by glycogen synthase, an enzyme responsible for glycogen synthesis. Conversely, during the fasted state, glycogen phosphorylase (GP), an enzyme involved in glycogenolysis, breaks down glycogen to produce G6P, which in addition to its role as a precursor for glycogen synthesis, acts as an allosteric inhibitor of GP and an allosteric activator of glycogen synthase (GYS1), thereby influencing liver glycogen levels. Post-translational modifications further modulate the activity of glycogen synthase and glycogen phosphorylase. GYS1 is primarily inhibited by glycogen synthase kinase 3 (GSK-3)-mediated phosphorylation, while phosphorylation of GP enhances its activity. Both enzymes can be dephosphorylated by protein phosphatase 1 (PP1) [20, 21]. In the fed state, insulin, released by pancreatic β cells to contrast elevated blood glucose, amino acids, and FAs, plays a crucial role. Insulin activates Protein kinase B (Akt), which phosphorylates and inactivates GSK-3, leading to increased glycogen synthesis. Insulin also promotes the acetylation of GP, facilitating its dephosphorylation and inhibition by PP1, thereby suppressing glycogenolysis. Insulin stimulates the expression of GCK, an enzyme that indirectly enhances hepatocyte glucose uptake by phosphorylating glucose and generating G6P. G6P, in turn, stimulates glycogen synthesis and inhibits glycogenolysis [20].

bisphosphatase (FBPase). F6P is eventually converted to G6P and glucose after transportation into the endoplasmic reticulum and dephosphorylation by G6Pase. Glycerol, transported into hepatocytes through aquaporin-9, is phosphorylated by glycerol kinase to generate glycerate-3 phosphate, a gluconeogenic precursor [25]. Amino acids are converted to α -ketoacids through deamination reactions and further metabolized into TCA cycle intermediates, which can serve as gluconeogenic precursors [26, 27].

Regulation of gluconeogenesis is a complex process involving multiple factors. Transcription factors and coactivators, such as FBP1, CREB, FOXO1, C/EBP α/β , p300/CBP, CRTC2, PGC-1 α , SRC-1, and SRC-2, play crucial roles in the transcriptional regulation of gluconeogenic genes [28-34]. Metabolic states and the circadian clock also exert regulatory control over gluconeogenesis through pathways involving SIRT, AMPK, mTORC1, LKB1, ERK/RSK, and cryptochrome proteins. Insulin signalling via the insulin receptor substrate (IRS) and Akt pathway influences the activity of gluconeogenic transcription factors, including FOXO1, and regulates gluconeogenesis [35-40]

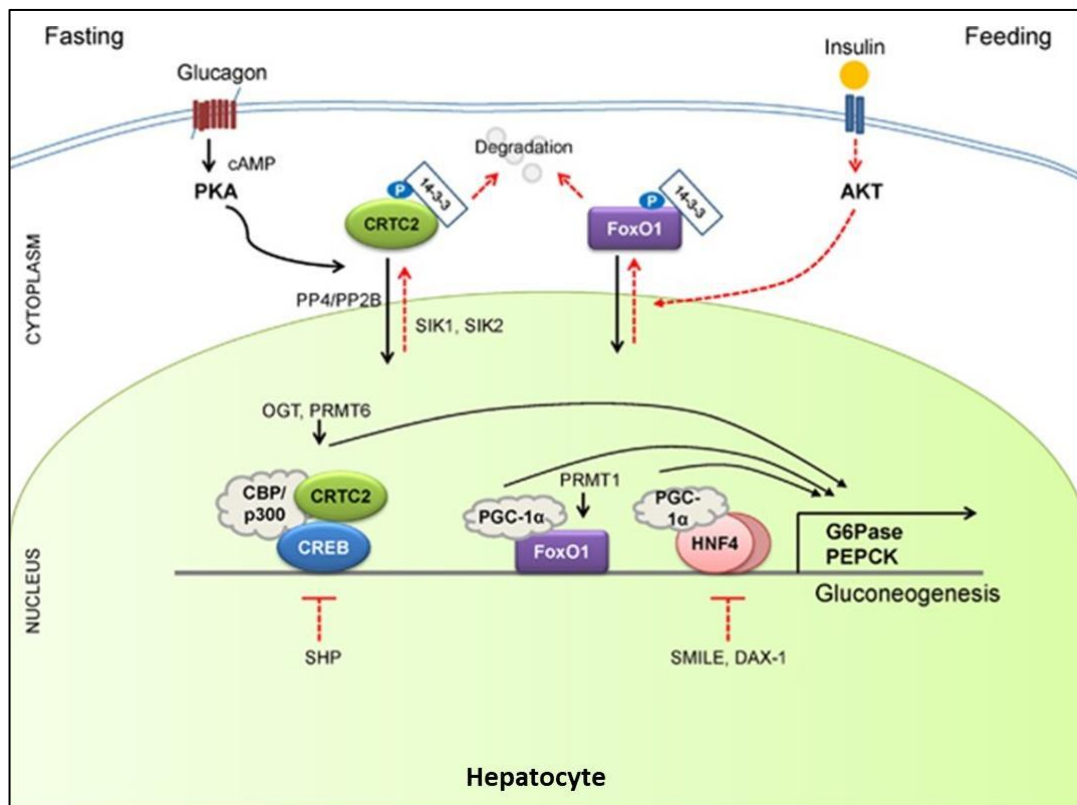


Figure 2. Hepatic gluconeogenesis transcriptional regulation under fasting and feeding conditions. Adapted from Han *et. al.* 2016 [19].

4.1.3. Lipid metabolism

Along with utilizing glucose as its primary fuel, the liver has the capacity to convert glucose into FAs. FAs can be obtained by hepatocytes from the bloodstream, released by adipose tissue, or absorbed from digested food in the gastrointestinal tract. These FAs are crucial for various metabolic processes, including the synthesis of triglycerides (TGs), cholesterol esters, phospholipids, and energy production through oxidation [18, 41].

Following a meal, dietary fats are broken down inside the small intestine and absorbed by enterocytes. These lipids are then converted into TGs and secreted into the lymphatic system as chylomicrons. Chylomicrons travel through the bloodstream to the liver, where lipolysis mediated by lipoprotein lipase (LPL) releases non-esterified fatty acids (NEFAs). Hepatocytes primarily take up NEFAs through CD36, fatty acid transport protein 2 (FATP2), FATP4 and FATP5. The expression of these transporters is regulated by receptors and transcription factors such as Pregnane X receptor (PXR) and Aryl hydrocarbon receptor (AhR). Fatty acid binding proteins (FABPs), particularly L-FABP, are involved in intracellular FAs transport. Long-chain acyl-CoA synthetases (ACSLs) activate FAs, and their expression is regulated by various factors [42-48]. Liver plays a pivotal role in converting carbohydrates into FAs. FAs are then packaged into very low-density lipoprotein (VLDL) particles and transported to adipose tissue and other extrahepatic tissues through the bloodstream [41].

Glucose undergoes glycolysis, leading to the formation of pyruvate. Pyruvate is further metabolized in the mitochondria, generating acetyl-CoA. Acetyl-CoA combines with oxaloacetate to form citrate, which is exported to the cytoplasm and subsequently cleaved into acetyl-CoA and oxaloacetate. Malonyl-CoA, derived from acetyl-CoA, acts as a precursor for FAs synthesis. Fatty acid synthase (Fas) utilizes malonyl-CoA and NADPH to synthesize palmitic acid, a 16-carbon fatty acid. Enzymes like acetyl-CoA carboxylase (ACC) regulate this process. Transcription factors such as ChREBP and SREBP modulate the expression of genes involved in *de novo* lipogenesis [49-52].

The availability of lipogenic substrates, such as glucose and NADPH, governs *de novo* lipogenesis (DNL). Glucose acts as a carbon source, while glycolysis and the pentose phosphate pathway generate NADPH, which is essential for lipogenesis [52]. Lipogenesis is regulated by an interplay of transcription factors and co-regulators. Key regulators include ChREBP, SREBP, LXR, FXR, PPAR γ , and PPAR δ . These factors not only activate genes involved in lipogenesis but also regulate genes related to lipid uptake, trafficking, and storage [53-68].

Insulin plays a critical role in stimulating hepatic lipogenesis during the fed state. The PI3K/Akt pathway suppresses gluconeogenesis and promotes lipogenesis. Insulin activates mTORC1, which further activates SREBP-1, facilitating lipogenesis. Akt also stimulates SREBP-1 activation. Other factors, including USF-1, SIRT1, and glucagon, modulate lipogenesis by regulating the activity of transcription factors and coactivators involved in this process [69-71]. Metabolic states, the circadian clock, and ER stress also influence hepatic lipogenesis. SIRT1 and AMPK, activated during fasting, inhibit lipogenic processes. ER stress promotes hepatic steatosis, while circadian clock genes regulate lipid metabolism. Factors like FGF21, glucagon, and FGF15/19 also play a role in regulating lipogenesis [31, 72-77].

During fasting, FA β oxidation is upregulated in the liver, providing energy for hepatocytes and generating ketone bodies. PPAR α serves as the master regulator of FA β oxidation, while factors such as SIRT3, SIRT6, and FGF21 contribute to its regulation [78-84]. Disruptions in hepatic lipid metabolism can lead to various metabolic disorders, including non-alcoholic fatty liver disease (NAFLD), where excess fat accumulates in the liver, and dyslipidaemia, characterized by abnormal lipid levels in the blood [4, 85]. I will describe NAFLD in detail in a dedicated chapter.

4.1.4. Liver immunosurveillance

The liver is a vital organ enriched with a diverse population of immune cells, both innate and adaptive, which play a critical role in maintaining immune tolerance and regulating inflammatory responses during liver disease. Within the liver, various types of T-lymphocytes are present, including regulatory T-cells (Tregs), cytotoxic T-lymphocytes (CTLs), and different subsets of helper T-cells (Th1, Th2, Th17, and Th22) [86-89]. Additionally, B-lymphocytes and plasma cells can also be found in the liver. Tregs are particularly important in establishing hepatic tolerance and preventing excessive immune responses. They express specific immunoregulatory markers such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), CD39, and lymphocyte-activation gene 3 (LAG-3) [89]. Tregs secrete interleukin-10 (IL-10) [90], a key anti-inflammatory cytokine that helps to maintain a tolerogenic hepatic microenvironment. IL-10 not only has direct anti-inflammatory effects [91] but also interacts with various immune and non-immune cell [92]. Hepatic Tregs work in collaboration with KCs to create a local immunosuppressive niche. This microenvironment prevents the onset of CTLs responses [93]. KCs, in addition to their phagocytic functions, produce immunosuppressive molecules such as prostaglandin E2 (PGE2) and IL-10 [94-97]. They also express V-set and Ig

domain-containing 4 (VSIG4), which is involved in maintaining the immunotolerance in T and natural killer T (NKT) cells [97]. Furthermore, IL-10 up-regulates the expression of programmed death ligand 1 (PD-L1) on KCs, which provides an inhibitory signal to T-cells and promotes the generation of Tregs, further enhancing the IL-10-rich environment in the liver [96, 98]. The contribution of macrophage colony-stimulating factor (M-CSF) and hepatocyte growth factor (HGF) in the hepatic environment facilitates the development of regulatory dendritic cells (DCs) that produce IL-10 and contribute to the anti-inflammatory milieu [99, 100].

Various innate immune cells reside in the liver, including KCs, natural killer (NK) cells, invariant natural killer T (iNKT) cells, mucosal-associated invariant T (MAIT) cells [101, 102], DCs, $\gamma\delta$ -T-cells, and innate lymphoid cells (ILCs) [103-105]. Despite being educated in a tolerogenic environment, these cells actively participate in clearing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) encountered in the liver [106-109]. Human liver-resident NK cells (lrNK) exhibit an immature phenotype with low levels of pro-inflammatory cytokines and cytotoxic mediators, aligning with the tolerogenic liver environment [110]. However, lrNK cells can respond to increased interferon-alpha (IFN- α) by up-regulating molecules that induce hepatocyte and T-cell death, like TRAIL, Fas-L and NKG2D [111, 112].

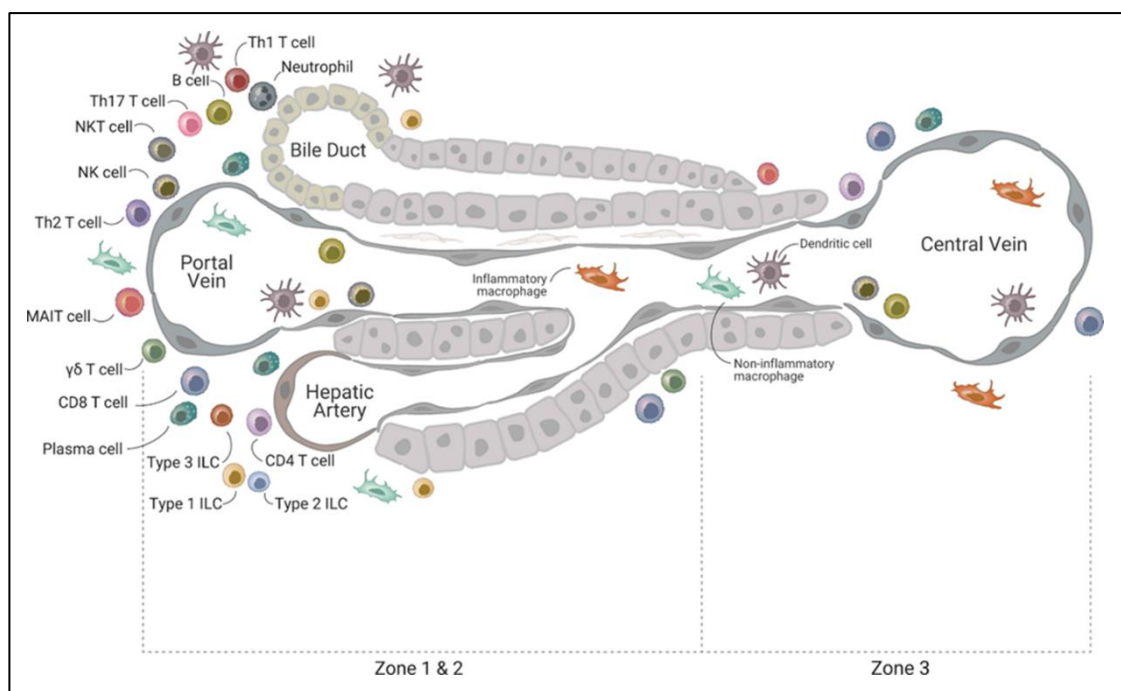


Figure 3. The immune niche of the liver. From Cheng *et. al.* 2021 [89].

The liver's immune response is not solely driven by immune cells but also involves interactions between parenchymal and non-parenchymal cells. Hepatocytes, cholangiocytes, LSECs and HSCs all contribute to the immunological response in the liver. These cells respond to DAMPs and PAMPs by secreting immunomodulatory molecules such as IL-10, and TGF- β , HGF and HSC-derived retinoic acid (RA) [104, 113-123]

The liver maintains immune tolerance through the promotion of a regulatory environment. However, under certain circumstances, such as exposure to toxins or pathogens, liver inflammation can occur when tolerance is disrupted. Inflammatory responses involve the secretion of factors by liver-resident cells to recruit immune cells from circulation [89]. Both parenchymal and non-parenchymal cells can act as antigen-presenting cells (APCs) by expressing immunological receptors, contributing to an immunogenic state, like scavenger receptors, Toll-like receptors (TLRs), major histocompatibility complex (MHC) class I and II, among others [104, 113, 114, 116, 119-121, 123-125].

The mechanisms triggering inflammation can vary depending on the threat. Injured hepatocytes release high mobility group protein B1 (HMGB1) and interleukin 33 (IL-33), which are important for neutrophil chemotaxis and activation [126]. Neutrophils, in turn, secrete cytotoxic substances and inflammatory cytokines, exacerbating local inflammation. They also up-regulate adhesion molecules, like ICAM-1 and VCAM-1, and promote chemokine release [127-129]. Extracellular DNA and ATP released by necrotic cells activate specific receptors such as TLR9, and inflammatory complexes like NLRP3 inflammasome in KCs, thereby driving a pro-inflammatory response [130, 131]. IL-12 and IL-18 facilitate the stimulation of NK cells, which produce interferon-gamma (IFN- γ) and perforin in response to tumour-transformed or virally infected cells [132, 133]. Hepatic innate lymphoid cells 3 (ILC3s) secrete IL-17A/F and IL-22, which play a critical role in contrasting hepatic viral infections by priming T-cells and promoting the secretion of antimicrobial peptides [134, 135]. In a pro-inflammatory liver environment, the suppression of PD-L1 can shift T-cells toward a Th1 response [136]. While the liver is tolerant to small amounts of lipopolysaccharide (LPS), high levels can activate macrophages and HSCs, driving the up-regulation of adhesion molecules, pro-inflammatory cytokines, and chemokines [137, 138].

Following an inflammatory response, the liver activates self-rescue mechanisms to contain tissue damage. These mechanisms include the phagocytosis of apoptotic bodies, autophagy, and an increased frequency of intrahepatic Tregs [139-146]. Liver-resident

macrophages contribute to this anti-inflammatory response by upregulating PD-L1, reducing costimulatory molecules, and promoting the maturation of Tregs [147].

Liver-resident macrophages can undergo self-renewal within the human liver when derived from yolk sac and fetal liver monocytes [148-152]. In certain contexts, circulating monocytes can replace liver-resident macrophages [152]. In the later stages of fibrosis resolution, monocyte-derived macrophages can switch from a pro-fibrotic phenotype to a scar-resolving phenotype, characterized by elevated levels of matrix metalloproteinases that clear extracellular matrix [153, 154]. The immune microenvironment is essential in orchestrating tissue repair or fibrosis [155-157]. Environmental cues, such as cytokines like IL-4 or IL-17, can influence macrophage polarization and determine whether the immune response is pro-regenerative or pro-fibrotic [156]. Factors like IL-6, Wnt signalling, HGF, EGF, TGF- β , and nitric oxide synthase have a role in hepatic regeneration [158-163]. Chronic inflammation can impair the liver's ability to restore homeostasis and repair tissue, leading to end-stage liver diseases. The liver's tolerogenic environment depends on complex interactions between parenchymal and non-parenchymal liver-resident cells, resulting in the promotion of immunoregulatory immune cell phenotypes and the expression of immunomodulatory molecules. When inflammation is not controlled, it can lead to chronic inflammation, fibrosis, and scarring, causing irreversible damage and increasing the likelihood of disease and cancer development [89, 164].

4.1.5. The intestine and the gut-liver axis

The intestine, also known as gut, is a multifunctional organ that serves as a gateway for nutrient absorption, houses a diverse microbial community, and plays a vital role in maintaining digestive health and immune function. The intestine consists in a long, tubular structure that extends from the stomach to the anus, divided into two main parts: the small intestine and the large intestine [165].

The small intestine is the longest canal of the gastrointestinal tract (on average 7m long in humans). It follows the stomach and precedes the colon until the ileocecal valve. The small intestine is located in the abdominal cavity and divided into duodenum, located just below the stomach and in close proximity with liver's gallbladder and pancreas, and the intestine sitting under the colon and anchored to the abdominal wall by the mesentery. This last tract, long around 6,5 meters, is composed by jejunum and ileum [166].

Small intestine receives chyme coming from the stomach by the duodenal ampulla, filled with glands with alkaline secretions to contrast the gastric acidity. Inside the duodenum, the pancreatic ducts release their secretions from the exocrine pancreas, and by the *ductus coledocus*, the bile pours out, coming from the gallbladder. This mixture, together with other enteric secretions, contributes to ultimate the digestion of chyme, thanks to the emulsifying properties of bile and the lithic capacity of lipases, amylases and proteases of pancreatic or enteric source. The overall inner surface of the small intestine extends to 100 m², achieved by repeated folding of the internal wall forming circular folds, called Kerkring valves [167]. The mucosa is also lifted in extroversions, the intestinal villi, of lamellar shape in the duodenum and gradually narrower forming a fine carpet on the inner ileum surface. The epithelial mucosa, is formed by three cell types: the enterocytes, the most abundant, characterized by a brush border made of microvilli that promote increased absorption surface, Goblet cells, mucin secreting glands and cells belonging to the gastro enterohepatic endocrine system, responsible for secreting hormones, such as gastrin, serotonin, secretin and pancreozymin, also known as cholecystokinin [168].

This complex structural, chemical and enzymatic synergism allows an extremely efficient absorption capacity of all the molecules coming from digestion into the blood and lymphatic circulation. Diffused endocrine cells, together with the submucosal and myenteric nervous plexus, ensure, through the peristaltic movements, a complex coordination between the progression of the content and the secretory and absorbent activity. At the same time, small masses of lymphoid tissue, in the form of aggregated (Peyer's patches), or isolated lymph nodes, are scattered on the *lamina propria* and provide the adequate immunosurveillance, limiting the growth of bacteria present in food and balancing the microbial populations that are physiological part of the intestinal flora [166].

The colon, also known as the large intestine, is a vital element of the digestive system. It is a long, muscular tube situated at the end of the gastrointestinal tract. The colon is divided into several sections, including the ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Its primary role is to ensure water and electrolytes absorption from the undigested food material, which helps in the consolidation of waste into faeces [167]. Furthermore, the colon harbours a diverse population of beneficial bacteria that aid in the fermentation of indigestible carbohydrates, producing essential vitamins and maintaining a balanced gut microbiome. The colon's muscular walls contract in a coordinated manner to propel faecal matter toward the rectum and eventually facilitate its elimination from the

organism through bowel movements [169]. Any disruption in the normal functioning of the colon, such as inflammation or structural abnormalities, can give rise to digestive disorders such as constipation, diarrhoea, or inflammatory bowel disease [170, 171]. Regular screenings, such as colonoscopies, are essential for monitoring colon health, as they allow for the early detection and prevention of conditions like colorectal cancer [172].

The intestine is not only implicated in the digestive process but also has a critical involvement within the immune system. It acts as a barrier, preventing the entry of harmful substances and pathogens into the bloodstream, while also facilitating the uptake of essential nutrients. The immune system in the intestine is highly specialized and in close coordination with the gut microbiota, helping to maintain a delicate balance between tolerance and defence against potential threats [173].

The bidirectional relationship between the liver, the gastrointestinal tract, and its microbiota is referred to as the gut-liver axis. While they operate independently, the key components including diet, microbiota, and intestinal mucosa are interconnected and linked to the host through bile and portal blood [174]. BAs, secreted by the hepatocytes, play a regulatory role in shaping the composition of the microbiota and maintaining intestinal barrier function, while gut-derived products influence BAs synthesis as well as glucose and lipid metabolism in the liver. Disruption of the gut-liver axis has been implicated in the onset of various chronic liver diseases, including cirrhosis [175]. Common elements of a dysfunctional gut-liver axis include an imbalanced intestinal microbiota, compromised gut barrier, increased permeability, and alterations in BAs levels. These changes contribute to reduced intestinal bile acid receptors signalling, impairing the synthesis of protective mucous and antimicrobial peptides, and compromising the integrity of the gut-vascular barrier [176]. The extent of gut-liver axis disruption varies depending on the underlying cause and stage of hepatic disease, where severe cirrhosis exhibits more pronounced barrier damage. Consequently, the functional consequences of a disrupted gut-liver axis involve repeated exposure of liver innate immune cells to bacterial products and metabolites derived from the gut, leading to liver inflammation and contributing to liver malignancies development [177-179].

Advancements in understanding the gut-liver axis have spurred the development of diagnostic, prognostic, and therapeutic tools based on the microbiota for liver disease management. Researchers are actively exploring the identification of specific microorganisms that can serve as indicators of liver damage and disease progression in conditions like alcoholic liver disease (ALD) and NAFLD, as well as predict hospitalization, bacterial infection, and liver-related

complications in cirrhosis [180]. Comprehensive microbiome analysis through multi-omics profiling holds promise in unravelling the mechanistic aspects and identifying distinct microbiota phenotypes associated with ALD and NAFLD [181-183]. Such analyses, when applied to serum, could facilitate diagnostic testing. Modulating bile acid signalling to restore the intestinal barrier homeostasis and regulate the gut-liver axis is a potential therapeutic approach [184]. This involves the use of Farnesoid X Receptor (FXR) agonists to modulate intestinal FXR signalling, thereby improving the synthesis of antibacterial proteins and reversing gut-vascular barrier damage [185]. I will dedicate a separate chapter to describe in detail the FXR signalling and the use of FXR agonists with major focus on NASH. Another therapeutic avenue worth exploring is the modification of intestinal contents through targeted faecal microbial transplantation or the administration of specific probiotics isolated from human faeces [186-188]. The growing recognition of the gut microbiota's contribution in the pathogenesis and progression of liver diseases provides a wide range of opportunities for the engineering of microbiome-based tools for the effective diagnosis and treatment of hepatic disorders [174].

4.1.6. Cholesterol and bile acids metabolism

Cholesterol is the most abundant sterol in mammals, with a plethora of functions and regulated by complex metabolic machineries. It is present in every cell of the organism and is critical for building the cell membrane, for vitamin D production to the generation of several hormones and for the creation of lipoproteins necessary to distribute lipids in various tissues through the bloodstream [189].

Cholesterol biosynthesis starts in the ER with acetyl-CoA, which forms β -Hydroxy β -methylglutaryl-CoA (HMG-CoA). HMG-CoA is converted to mevalonate, a precursor for sterols and isoprenoids. Mevalonate undergoes several reactions to produce geranyl pyrophosphate (GPP) and then farnesyl pyrophosphate (FPP). FPP can join with isoprenyl pyrophosphate to form geranylgeranyl pyrophosphate (GGPP). Squalene is formed from the condensation of two FPP molecules and is then oxidized to 2, 3-epoxysqualene. Lanosterol is produced from 2, 3-epoxysqualene and undergoes further transformations to become cholesterol [190, 191]. Additionally to biosynthesis, cells can acquire cholesterol from blood and diet. Cholesterol in circulation is present in different forms of lipoproteins, such as VLDLs and LDLs. LDL uptake is carried out by the LDL receptor (LDLR) through endocytosis, while dietary cholesterol is absorbed by NPC1L1 in the small intestine. Internalized cholesterol is

hydrolysed to free cholesterol and transported to various organelles, including the plasma membrane and the ER. NPC1L1 expression is also present in the liver and helps reabsorb biliary cholesterol excreted by ABCG5/ABCG8 [190, 191]. About 95% of the cholesterol present in the bile is in a non-esterified form and in a stable and constant concentration, thus independent from variation in cholesterolaemia and exogenous intake [192].

Normally cholesterol is maintained soluble in bile by micelles mixed with BAs and lecithin in adequate concentrations. Through bile secretion, the liver carries out a fundamental action in the micellar aggregation of lipids within the small intestine, which is at the base of the absorption of long chain FAs [193].

The bile is composed by water (97%), inorganic electrolytes (mostly sodium, potassium, calcium, magnesium, chlorides, and bicarbonates), cholesterol, phospholipids, organic anions, bile salts, and bilirubin. The BAs are products of conjugation directly secreted from the hepatocyte into the bile, in which their concentration is up to a thousand fold higher than in the blood [194]. The BAs are sodic salts of the glycocholic, glycochenodeoxycholic, taurocholic and taurochenodeoxycholic acids (GCA, GCDCA, TCA, TCDCA) and they derive from the conjugation of glycine and taurine with the cholic and chenodeoxycholic acids (CA and CDCA), respectively. While in humans we can find the majority of the BAs conjugated to glycine, in rodents the predominant form are taurine-conjugated. The primary BAs are the main products of the metabolic degradation and clearance of cholesterol, and they are formed exclusively in the hepatocytes [195]. BAs are not only essential for the uptake of dietary lipids and lipophilic vitamins, but also carry out both important endocrine and paracrine functions [196]. They can activate many receptors, most importantly the main bile acid receptor FXR [197, 198]. Importantly, FXR has a plethora of function that affect cholesterol, glucose and BAs metabolism [199]. In the following chapters, these functions will be properly described.

Other BAs receptors are the G-protein coupled receptors S1PR2 (sphingosine-1 phosphate receptor 2) and TGR5 (Takeda G protein-coupled receptor 5, also known as Gbpar 1). BAs can also activate the adhesion receptor α -5- β -1 integrin, among others [200-203].

The BAs synthesis comprehends two pathways, the classical way and the alternative one. Through the classical synthetic pathway, cholesterol is converted into 7 α -hydroxycholesterol by the rate-limiting enzyme CYP7A1, and after a series of additional enzymatic conversions ends with the production of the primary BAs, such as cholic acid (mediated by CYP8B1) and chenodeoxycholic acid. The alternative synthetic pathway takes place when the enzyme CYP27A1 carries out a hydroxylation reaction on cholesterol at carbon

27, generating thereby 27-hydroxycholesterol, and a successive hydroxylation by CYP7B1 mediates the conversion into CDCA. In absence of pathological disturbance on the main pathway, only a 10% of the whole BAs pool synthesis takes place through the alternative pathway [204].

Of note, in rodent livers, CYP2C70 hydroxylates CDCA on the carbon in position 6 to produce Tauro- β - and β -muricholic acids (T- β -MCA, β -MCA) and, which are additional major BAs in addition to CA and CDCA [205]. Deconjugation, dehydroxylation, and epimerization are microbial biotransformation processes that lead to the production of additional secondary BAs, including β -MCA, MDCA (murideoxycholic acid), HCA (hyocholic acid), and HDCA (hyodeoxycholic acid) [205]. In comparison to human BAs, rodents have a considerably greater proportion of hydrophilic BAs (muricholic BAs and their taurine conjugates), which results in reduced hepatocyte toxicity [206]. The conjugated BAs, excreted by the liver through the bile, are almost entirely reabsorbed in an active process within the ileum, and return to the liver through the hepatic portal vein [194, 207].

The intestinal bacteria de-conjugate and modify the BAs, forming the secondary BAs. The intervention of bacteria on CA generates deoxycholic acid (DCA), which is hydro soluble and thus easily re-absorbed and directed back into the circulating hepatic BAs pool. On the contrary, bacteria transform CDCA into the insoluble lithocholic acid (LCA), which is excreted through the faeces. The enterohepatic circulation of these molecules regulates the synthesis of hepatic cholesterol and BAs [204, 207]. Cholesterol can also inhibits its own synthesis by limiting the conversion of HMG-CoA into Mevalonate, and promote the production of BAs. In turn, BAs can further inhibit the generation of cholesterol and block their own synthesis [204, 207].

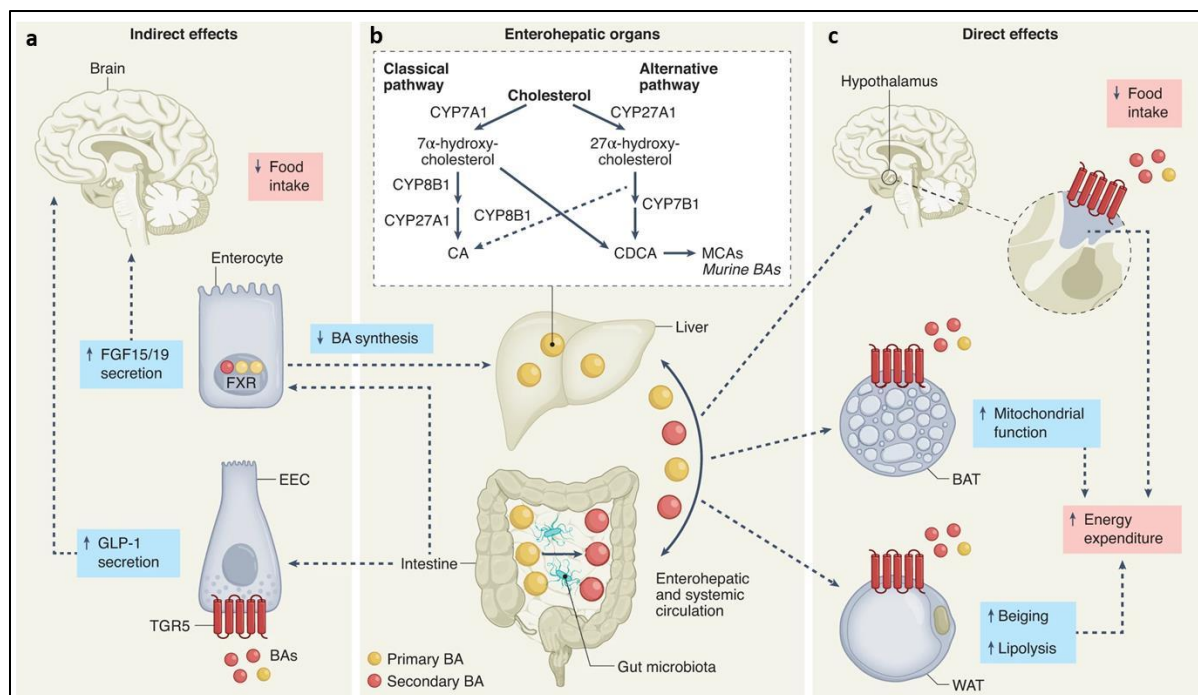


Figure 4. (a) Indirect effects of BAs on food intake. (b) Overview of BAs synthesis and circulation within enterohepatic organs. (c) Direct effects of BAs on food intake and energy expenditure. Adapted from Perino *et. al.* 2022 [208].

The secreted BAs mix with bilirubin glucuronides, secreted by KCs cells to form bile. Bilirubin, the most abundant pigment in bile, derives to a major extent from haemoglobin catabolism, as result of red blood cells destruction. The microsomal heme oxygenase opens up the cyclic tetra-pyrrole porphyrin ring of heme, which allows the further removal of iron and globin, forming biliverdin. The heme oxygenase has a mixed function, and requires molecular oxygen and NADPH and uses the P450 cytochrome as terminal oxidase. The final products of its enzymatic activity are CO, iron and biliverdin. The latter, due to a NADPH-dependent reduction by the bilirubin-reductase, is converted into bilirubin [209].

Bilirubin synthesis takes place within the hepatocytes, spleen's reticuloendothelial cells, lymph nodes and bone marrow. From there, the non-conjugated bilirubin moves into the blood stream and then into the hepatocytes, where, in the cytosol, 2 protein acceptors, Z and Y (ligandin), are able to bind it. Inside the smooth ER of the hepatocytes, takes place the conjugation of bilirubin with two molecules of uridin 5'-diphosphoglucuronic acid (UDPGA) by the UDP-glucuronosyltransferase (UGT), forming thereby the water-soluble conjugated bilirubin, which is excreted through the bile [209, 210].

The elimination process is unidirectional and is the limiting passage of the entire transfer of bilirubin from blood to the bile. Once in the intestine, redox reactions convert the most of

the conjugated bilirubin into urobilinogen, further oxidized to urobilin and excreted through the faeces [210]. Some bacterial beta-glucuronidase can de-conjugate again small amounts of bilirubin, which unite to the small fractions of unconjugated bilirubin coming from the serum (bound to albumin) or the liver, and are part of the enterohepatic circulation [210]. The flux and the composition of bile are determined by the functional activity of the bile ducts and canaliculi. The BAs secretion is an active and energy consuming process, whereas the transport of water and electrolytes through the canaliculi is for the most part a passive process of diffusion and ultrafiltration, even though some inorganic ions seem to be actively secreted [211].

The secretion of bile by hepatocytes is continuous, but the outflow of bile from the gallbladder into the intestine is intermittent and stimulated by the presence of chymus in the duodenum. The emptying of the gallbladder is induced by a nervous stimulus, where the duodenum mucosa secretes cholecystokinin. Other neuro-hormones such as secretin, gastrin, histamine and glucagon can also influence the biliary secretion [212, 213].

The maintenance of the BAs homeostasis is critical to prevent dysregulations, especially in case of excess BAs accumulation, also known as cholestasis. When BAs are too elevated and their release into the intestine is dampened, they can exert toxic and disruptive effects on the liver parenchyma and intrahepatic bile ducts. BA overload is often associated to metabolic disorders such as obesity, Type 2 diabetes mellitus (T2DM), primary biliary cholangitis (PBC) and NAFLD [195, 214].

4.2. Liver disease, NAFLD and HCC

4.2.1. Chronic liver disease

Globally, chronic liver disease (CLD) cause 2 million deaths every year, not to mention the remarkable amount of high burden disability and its enormous weight on healthcare. It is however speculated that the mortality for CLD is underestimated and the real numbers are even higher [215]. The main complications of CLD are cirrhosis and liver cancer, and account for 3.5% of all deaths globally [216]. The conditions that most commonly result in CLD and cirrhosis are chronic infections with hepatitis B and C viruses (HBV, HCV), ALD and NAFLD [215].

According to information from 2017, CLD was diagnosed in 1.5 billion people, in 60% of the cases for NAFLD, in 29% for HBV, in 9% for HCV and in 2% of the cases for ALD [217]. The global burden of CLD is remarkable, and despite vaccination campaigns and anti-viral treatments for hepatitis B and C have improved the trends, and reduced the incidence for CLD in certain regions of the world, other conditions like metabolic syndrome and NAFLD are on the rise [218].

4.2.2. Obesity and NAFLD

In the modern society, the quality of life has seen a dramatic increase in the last century. Thanks to this, it was possible to almost eliminate or dramatically reduce mortality due to famine and malnutrition. However, our society has now to deal with the opposite issue. Indeed, an obesity pandemic is spreading in the western world, and has started to expand also into the developing countries in the last decades, with 2 billion people worldwide estimated to be overweight or obese [219, 220].

While overweight condition is diagnosed based on the body mass index (BMI, calculated as kg/m^2) in the range between 25 and 29.9, obesity is defined with a BMI above or equal to 30 [221]. However, this index alone is now considered of limited value, and should be associated to other important factors, such as body fat, fat distribution, and relevant considerations regarding genetic, metabolic, physiological and psychological predisposition, which can vary importantly between individuals, socio-economic classes and ethnic groups [222]. Although some socio-economic and cultural factors or even genetics may promote (or in certain cases prevent) obesity in certain populations, the major contribution remains the energy imbalance provoked by over nutrition. This can certainly be favoured by the large availability of hyper-caloric-processed food, rich in simple sugars and fat, but also by the absence of physical activity and increased sedentary lifestyle [219, 220].

Obesity can per se increase the risk to develop a number of comorbidities, which are linked to increased mortality, including T2DM and specifically insulin resistance (IR), cardiovascular diseases, metabolic syndrome, chronic kidney disease, hyperlipidaemia, hypertension, obstructive sleep apnoea, depression, osteoarthritis, some types of cancer and NAFLD [219].

NAFLD is a condition favoured by obesity and often associated to the metabolic syndrome, many times even assumed as its hepatic manifestation. NAFLD is a general term

that includes a broad spectrum of liver damage severity, and could be divided into two predominant stages. One stage is defined with hepatic fat accumulation in absence of hepatocellular injury, called non-alcoholic fatty liver (NAFL), which can be benign and easily reversible. The other stage consists in the presence of hepatic damage, which can impair the liver function, and, based on the degree of fibrosis, present a lower likelihood of resolution, called non-alcoholic steatohepatitis (NASH). The simple fatty liver is generally associated to hepatic steatosis below 5% in absence of other features, whereas NASH consists in more than 5% of hepatic steatosis, in addition to hepatocellular injury (cellular ballooning), inflammation, with or without fibrosis [218]. NAFLD is considered alone a major public health issue because its incidence in the global population is estimated between 20-25% and is expected to raise further. According to computer models based on the most recent predictions for obesity, and diabetes and NAFLD, between 2015 and 2030, it is expected to assist to a raise by 21% and 63% for the respective prevalence of NAFLD and NASH. The incidence of decompensated cirrhosis, HCC, and fatalities associated to NAFLD will also respectively increase by 168%, 137%, and 178% [223, 224]. The major driver of NAFLD is the enlargement of adipose depots and the accumulation of ectopic fat caused by over nutrition [225].

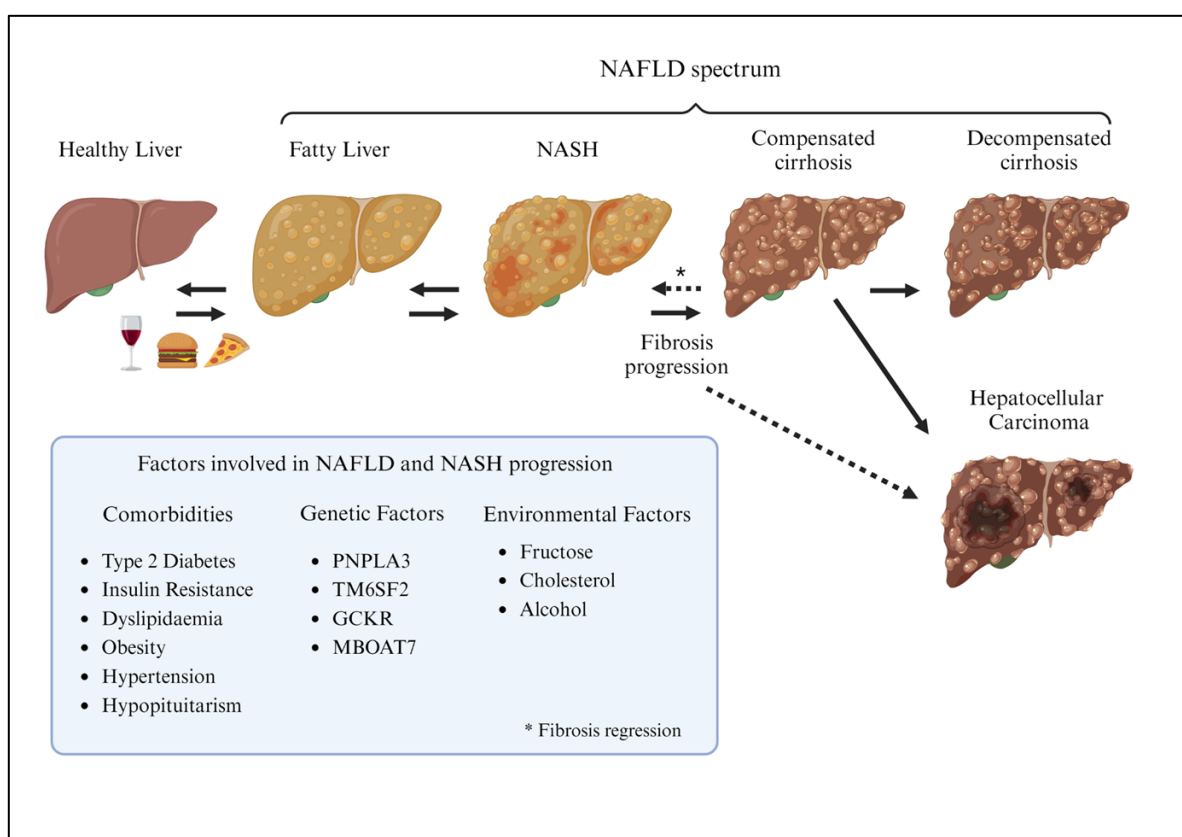


Figure 5. Illustration of NAFLD spectrum and summary of promoting factors. Inspired from Powell *et al.* 2021 [4]. Created with BioRender.com.

The “two hits” hypothesis has been for almost two decades the most accepted theory regarding the pathogenesis of NAFLD. It considered the first hit insulin resistance, driven by the hepatic FA accumulation, and the second hit inflammation, comprehending mitochondria dysfunction, oxidative stress and increased exposure to gut –derived endotoxins [226]. This theory has been revised, as the hits are now considered multiple and keeps into account the contribution of adipose tissue-secreted-hormones, gut microbiota and genetic and epigenetic factors [227, 228]

Being the input and the output of FAs in a critical balance, when the exogenous FAs uptake (through diet and tissue lipolysis) and the endogenous FAs synthesis (from DNL) are quantitatively higher of the FAs release (by FAs oxidation, lipolysis and VLDL-TG complexes secretion), the FAs are stored as hepatic TGs [229, 230]. These lead macrophages to infiltrate the visceral adipose tissue and induce a pro-inflammatory state that favours the onset of insulin resistance. A combination of inappropriate lipolysis in the context of insulin resistance and *de novo* lipogenesis results in a saturation of the liver metabolic capacity and drives the generation of lipotoxic lipids that increase oxidative stress and ER stress, activate the inflammasome and induce cellular apoptosis. The cellular death further promotes the inflammatory status that induces regeneration processes, activating hepatic stellate cells and driving fibrogenesis [3, 4, 218].

Several studies reported that an imbalance between pro-inflammatory and anti-inflammatory adipokines might exacerbate hepatic pathology and promote its progression [231, 232]. For example, adiponectin can reduce DNL and increase beta-oxidation, irisin can improve glucose homeostasis and decrease insulin resistance, and ghrelin can decrease lipid accumulation, inflammation, oxidative stress and apoptotic signals [233-238]. Conversely, adipokines as leptin, resistin and visfatin, can drive insulin resistance and bring a negative impact on steatosis and fibrosis progression [239-244]. The response of adipokines to pharmacological agents with directed effects on liver histopathology may make these molecules attractive therapeutic markers or biomarkers for predicting the severity and progression of NAFLD.

Due to the inappropriate dietary regimens that favour obesity, metabolic syndrome and NAFLD, it is common to find dysbiosis and increased intestinal permeability. The gut microbiome plays a significant role in the development of altered hepatic responses in NASH. It affects hepatic BAs metabolism and contributes to increased gut permeability, allowing the translocation of signals from the gut to the liver [245]. This activation of the gut-liver axis can

amplify inflammatory, pro-fibrogenic, and pro-carcinogenic pathways, promoting NASH and HCC. The intestinal microbiome is one of the factors driving liver steatosis by increasing energy harvest, monosaccharide absorption, and abnormal acetate production in the gut. The dysbiotic leaky gut allows the passage of harmful molecules into the liver, activating immune cells and triggering pro-inflammatory and fibrotic processes. Bacteria themselves can cause the disruption of gut permeability, and the migration of intestinal inflammatory cells to the liver could contribute to bacterial translocation. Specific bacterial species, such as *Proteobacteria*, *Enterobacteriaceae*, *Escherichia*, and *Bacteroides*, have been correlated with NAFLD and NASH in humans. Treatment with non-absorbable antibiotics has shown promise in improving liver function in patients with NASH, further suggesting the involvement of the gut microbiome in disease progression [174, 245].

Studies analysing paired liver biopsy samples provide valuable data on disease progression rates. Fibrosis, a key prognostic marker for liver-related outcomes and mortality, can develop in NAFLD and NASH. Fibrosis progression tends to be faster in individuals with NASH, likely due to necro-inflammation, the inflammatory cascade involved after non-apoptotic death, determining the release of several molecules that affect importantly the surrounding tissue [246-249]. A meta-analysis of NAFLD studies using paired liver biopsies revealed an average worsening of fibrosis by one stage over 7.1 years for NASH patients and 14.3 years for NAFL patients. Pronounced fibrosis (stage 3 and 4) is particularly significant, as it correlates with higher all-cause mortality and liver-related mortality [250].

The development of NASH is strongly correlated with significant fibrosis, but this relationship weakens at the cirrhotic stage [251]. Most people with cryptogenic cirrhosis (cirrhosis of unknown cause) and metabolic comorbidities are likely to present burned-out NASH, with aggressive fibrosis but reduction of hepatic fat, if not even complete fat loss [252]. It is frequent for individuals with NAFLD to remain undiagnosed for many years, even after cirrhosis has developed. Diagnosis often occurs when patients show evidence of portal hypertension or develop liver-related complications. The progression from compensated cirrhosis (therefore asymptomatic) to decompensated disease with complications of portal hypertension or liver failure occurs at a rate of approximately 3-4% per year. Cirrhosis is also the major significant risk factor for developing HCC, with an increased incidence of NASH in cirrhotic patients [4, 253].

The relationship between NAFLD and all-cause mortality is still not fully understood. Some studies suggest a slight increase in the mortality risk compared to the general population,

while others find no association [254-258]. NAFLD is a diverse condition with varying rates of disease progression and outcomes. Most patients have stable or slowly progressing liver disease that does not lead to cirrhosis or liver-related death. However, a small percentage of individuals develops advanced fibrosis and is at risk of complications like end-stage liver disease and liver cancer. Recognizing the different disease progression patterns and contributing factors is crucial for guiding patient care [4].

NAFLD has become the second driving cause of end-stage liver disease and the second most common cause of primary liver cancer among adults in the United States awaiting liver transplant [259, 260]. In Europe, NAFLD accounts for 8.4% of annual transplantations, and individuals with NAFLD have a higher proportion of HCC compared to those without NAFLD [261]. The increasing prevalence of severe hepatic disease and liver-related outcomes caused by NAFLD is a concerning trend.

Although progressive liver disease poses a risk, cardiovascular disease is the leading cause of death in NAFLD patients, followed by extrahepatic malignancies such as colorectal or breast cancer [262]. These causes of death are probably associated with shared cardio metabolic risk factors between NAFLD and cardiovascular disease [263]. The extent to which NAFLD directly contributes to cardiovascular disease development is however still unclear. NAFLD is associated with an increased risk of incident cancers, particularly those involving uterus, liver and gastrointestinal tract. The mechanisms driving this association may be related to visceral adiposity and chronic low-grade inflammation, although further research is needed to determine the exact cause [264]. Patients with NAFLD, especially those with clinically significant fibrosis, also face a higher risk of COVID-19 infection complications. This risk may be independent of metabolic comorbidities, although diabetes and obesity are established risk factors [265].

As of now, NAFLD is still devoid of approved therapies. The major effort in contrasting the onset and the progression of this condition remains a change of lifestyle, mainly oriented to a healthy diet regimen and physical exercise. Individuals with severe obesity may undergo bariatric surgery in absence of successful body weight reduction after diet and exercise alone. Patients that can successfully improve their obesity are generally treated for the various comorbidities: Thiazolidinedione, GLP-1 analogue or SGLT2 inhibitor for T2DM and IR, statin for dyslipidaemia, ARB or ACE inhibitor for hypertension, vitamin E and pioglitazone for improving liver function [266]. It is always important to remember that obesity alone is a risk factor for cardiovascular diseases, diabetes, hypertension and cancer, even in absence of

NAFLD [267, 268]. Therefore, every effort to rescue obesity is per se an all-round remedy for a number of related comorbidities. The problem lies, as we know, in the extremely challenging effort in changing one's lifestyle and habits. Indeed, the drop-out rate of patients enrolled in anti-obesity nutritional programs is very high, and is probably related to the known addictive effects of junk food, which some studies compared as dangerous as drugs of abuse [269]. Furthermore, the western society has made some efforts in raising awareness about the risks related to obesity and unhealthy diets, both by employing health campaigns and by introducing severe restrictions and limitations for food companies regarding amounts of sugars and fat that can be added to their products. However, the lack of regulations in developing countries, together with poor knowledge of their populations, still allow food companies to use aggressive advertisement and to include more fats and sugars in their products in such countries [270].

4.2.3. NASH-induced HCC

HCC accounts for 90% of primary liver cancers, represents the sixth most common cancer and the third cause of cancer-related mortality worldwide. As before mentioned, HCC is often the final result of chronic liver disease and cirrhosis, induced by chronic viral hepatitis infections, aflatoxin, ALD or NAFLD - if the patients do not perish before due to liver failure-comorbidities or related complications [1].

Although improved sanitation, early screening and therapies have dramatically reduced overall the mortality of multiple cancer types, the one for liver cancer has nevertheless increased. It is indeed estimated that by 2025, one million individuals each year will develop this kind of tumour [2].

HCC is often diagnosed late as its development is in most cases asymptomatic and it is diagnosed through MRI only during screening for other pathologies or when the patients display abnormal serum markers for liver damage associated to some form of abdominal pain [2].

Contrary to HBV and HCV infection, where 80-90% of HCC develops on established cirrhosis, in 30-40% of the NASH-related HCC occurs in patients with severe fibrosis but without cirrhosis. This trend underlines the complexity of the metabolic factors and extrahepatic drivers of cancer associated to the metabolic syndrome [3, 271].

NASH is multifactorial disease presenting a complex interplay of several dysregulated pathways contributing to injury and subsequent fibrosis, making difficult to date to identify

crucial therapeutic vulnerabilities, also due to the lack of a temporal hierarchy of causality. A big effort has been made in distinguishing patients with NASH from those with simple steatosis, as 80% of patients with NAFLD do not display NASH, and the main difference lies in the fibrosis onset [272]. An important driver of injury in NASH is lipotoxicity, resulting from increased uptake of the liver of FAs and exosome from peripheral fat, together with increased insulin resistance and inflammation from abnormal adipose tissue. Hepatocytes subject to lipotoxicity develop ER stress, oxidative stress, activate the inflammasome and apoptotic responses. The resulting hepatocellular injury sets the stage for pre-malignant events, such as DNA damage and mutations in the master metabolic genes, like FOXO1, CIDEA and GPAM, known to confer protection to the hepatocytes from lipotoxicity but at the expenses of enhancing the risk of malignant transformation [3, 4, 271, 273]. Gut-derived endotoxins, such as LPS, play a significant role in the development of NAFLD/NASH and HCC by promoting invasive potential and inducing the epithelial-mesenchymal transition (EMT), thanks to the activation of JNK and MAPK via TLR4 [274].

To counteract the cellular death occurring in NASH, several developmental pathways such as YAP-TAZ, Notch and Hedgehog are reactivated in hepatocytes, fuelling further proliferation, inflammation and risk for cancer development [4].

Adipokines, including adiponectin and leptin, are inflammatory factors with known correlations with HCC development. Adiponectin has chemo- and hepatoprotective effects, while leptin is involved in obesity-related tumorigenesis [275, 276].

Hepatokines, cytokines released by the liver, are important in the development of NAFLD/NASH and HCC. Adipokines from adipose tissues contribute to FFA uptake and energy source for NAFLD, while additional factors like oxidative stress and mitochondrial dysfunction are needed for the progression to NASH and development of HCC [277-279]. Free non-esterified cholesterol and its oxidized derivatives, along with increased TNF levels, contribute to the transition from NASH to HCC, and hepatokines secreted from the liver play an important function in this process [280-282].

The stage of hepatic fibrosis becomes determinant of the clinical outcomes as it represents the key factor driving the progression of NASH to cirrhosis and liver failure, and creating the favourable environment for HCC development. The major contributor to fibrosis is the activation or trans-differentiation of HSCs into proliferating myofibroblasts, driving a progressive accumulation of extracellular matrix and therefore tissue scarring [3, 283].

Experimental models of NASH have identified increased levels of vascular endothelial growth factor (VEGF), implication of TIE1 and TIE2 angiopoietin receptors and dysregulated LSECs, suggesting that also angiogenesis plays a role in NASH and NASH-to-HCC transition [127, 148, 159, 272].

The immune system is also importantly involved both in NAFLD and in HCC. NASH is characterized by lobular inflammatory responses that are assumed as the leading causes in driving the progression to fibrosis, cirrhosis or HCC. There is a complex interplay of both innate and adaptive immunity driving inflammation in NASH. Studies observed that the recruitment of resident KCs and various leukocytes releasing cytokines, chemokines, eicosanoids, nitric oxide and reactive oxygen species all converge to increased inflammatory responses in NASH. An important function in NASH is played by Th1 and Th17 CD4⁺ cells, but more in general the majority of T-cells have an anti-tumorigenic effect, as depletion of CD8⁺ T or CD4⁺ T-cells promotes tumour growth in mice [3, 4, 127, 159, 273].

The immune system also affect the response to immunotherapies in the context of NASH and NASH-related HCC development. Adaptive and innate immune cells, such as CD4⁺ and metabolically activated CD8⁺ T-cells, platelets, dendritic cells, and neutrophils, influence the transition from fatty liver to steatohepatitis and affect the efficacy of immunotherapy [273, 284, 285]. Different NASH-inducing diets can alter immune cell function and drive the NASH-HCC transition. Macrophages and T-cells in NALFD exhibit impaired function, and specific macrophage subsets have been associated with non-response or response to immune-checkpoint inhibition in HCC patients. Impaired T-cell fitness in NAFLD can hinder the response to immune-checkpoint inhibition, but metformin treatment and neutrophil reprogramming show promise in overcoming this obstacle [286]. Further studies are needed to understand the complex interactions between immune cells, metabolism, and the NAFLD-specific tumour microenvironment. In NASH-derived HCC, CD8⁺PD1⁺ T-cells acquire auto-aggressive phenotype, becoming dysfunctional and even pro-tumorigenic, potentially contributing to non-responsiveness to immunotherapy [273, 285]. Studies documented similar phenotypes in human NASH-derived HCC, suggesting that CD8⁺PD1⁺ T-cells could be used as predictors of treatment success or resistance [284, 285, 287]. Studying the immune microenvironment composition can guide the engineering of more effective therapies.

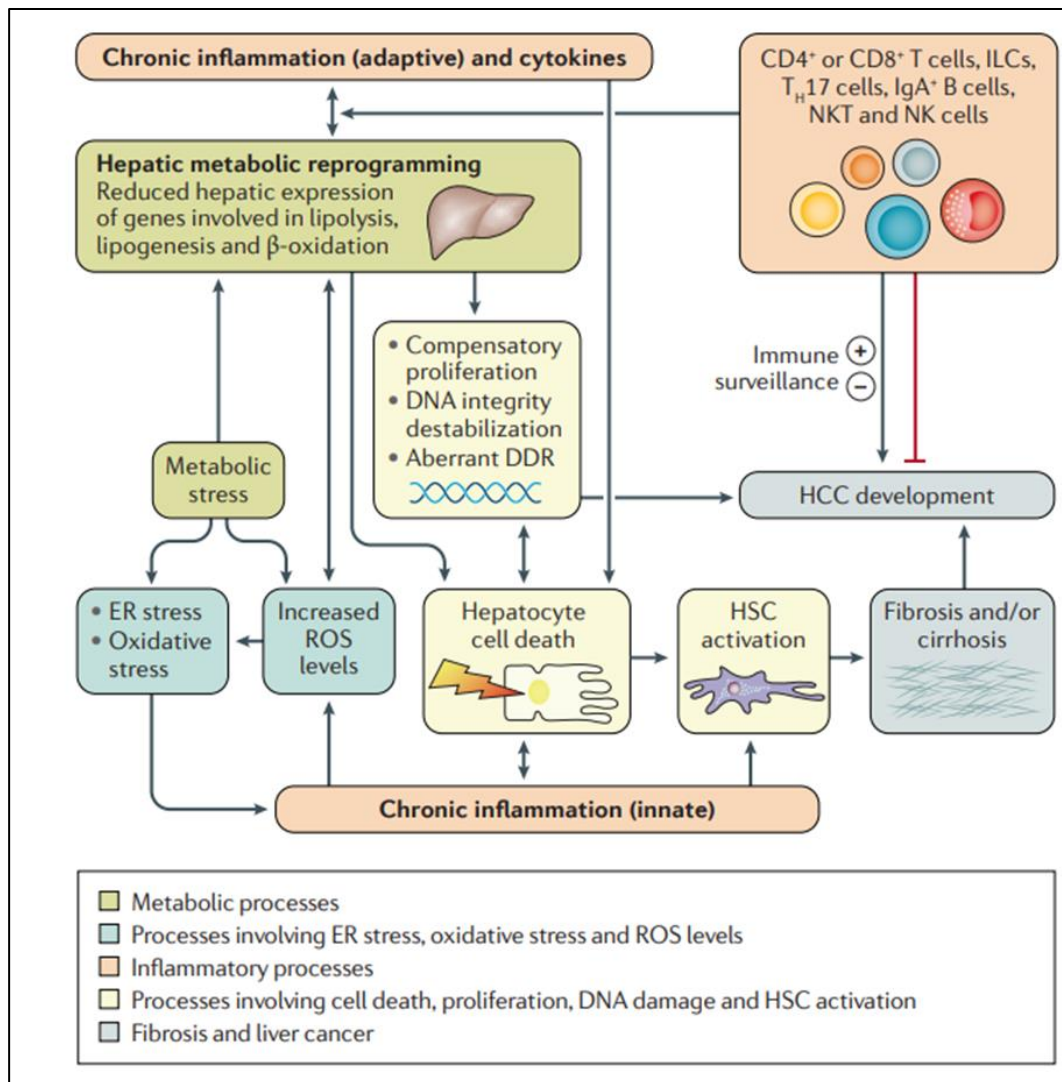


Figure 6. Influence of chronic inflammation induced by innate and adaptive immunity on hepatic metabolic reprogramming and tumorigenesis. From Anstee *et. al.* 2019 [3].

The main treatment strategies for early and intermediate stage HCC consist in resection, transplantation or loco regional therapies [271]. Depending on the tumour stage, the mutational landscape and other factors, approximately half of the patients tend to be eligible for supplemental systemic treatments over the course of the disease, which include several combinational therapies (like dual atezolizumab-bevacizumab or durvalumab-tremelimumab treatment) and single-agent tyrosine kinase inhibitors (like sorafenib, lenvatinib, regorafenib and cabozantinib) or monoclonal antibodies (ramucirumab) [271]. Checkpoint inhibitor therapy is currently under evaluation, but apparently for NASH-related HCC its efficacy alone is questionable and requires further studies, with combined approach [285].

Among the key relevant factors that could influence the outcome of the therapy for HCC, its mutational landscape is one of the most studied. The major molecular traits identified

to date in NASH-HCC consist in ACVR2A and TP53 mutations, and enrichment in the proliferative class S1-WNT-TGFB have been observed. A recent study has identified a *de novo* mutational signature (called MutSig-NASH-HCC), present in 25% of NASH-HCC patients, characterized by a higher frequency of C>T and C>A transitions [2, 3, 288]. In comparison to patients presenting with HCC from other aetiologies, patients with NASH-derived HCC display more hepatic DNA damage induced by oxidative stress. Lastly, there is a strong involvement of epigenetic events in abrogating the transcription of genes involved in bile and FAs metabolism and upregulating proliferative pathways in NASH-HCC, where experimental models of epigenetic reprogramming have shown promising effects in reversing carcinogenesis [195].

HCC is a highly lethal cancer, and its treatment is even more challenging due to the peculiar molecular and metabolic landscape that can assume when originating from NASH. Therefore, it is imperative to identify novel mechanisms and molecular targets that could lead to developing compounds eligible for clinical trials validation. To this end, investigating pathways and testing novel molecules using preclinical mouse models constitutes the most efficient and valuable tool to date for cancer research.

4.2.4. Mouse models of NASH

During the last decades, the scientific community has developed several different approaches in the effort to recapitulate NASH in the animal, generally with but not limited to mice and rats models. The main and probably most faithful approach consists in dietary model, where the mice are fed *ad libitum* with diets, which exist in a large variety of commercially available formulations, and can result in different flavours of NASH based on the specific combination nutrients, their abundance, or lack thereof. Diets that are very popular nowadays are rich in fat, carbohydrates (especially rich in sugars such sucrose, glucose or fructose, which can also be supplemented into the drinking water) and cholesterol [289]. These diets are often called Western Diets, for a formulation generally resembling the classical fast food and fizzy drinks nutritional profile. Some diets may be also choline deficient, and when matched to a high fat content (e.g. choline-deficient high-fat diet; CDHFD), they drive the mouse to an accelerated development of obesity and hepatic steatosis [273]. Choline is indeed an essential nutrient for the production of phosphatidylcholine, one of the building units of Very Low Density Lipoprotein (VLDL), which are involved in extra-hepatic transfer of lipids and cholesterol. In addition, choline is also pivotal for a proper functioning of the fat beta-oxidation reactions.

Therefore, a lack of choline incapacitates the liver to mobilize and metabolize any fat that is absorbed and ultimately this leads to hepatic steatosis development. Choline is also important for those pathways involved in the anti-oxidative response, and its deficiency leads to an accumulation of reactive oxygen species (ROS), driving the aggravation of the pathology [289]. Ultimately, also accelerated models for NASH exist, and combined diet poor in choline and in methionine in so called methionine-choline deficient diet (MCD), or a diet without choline, a restricted amount of methionine and L-defined amino acids (CDAHFD) [290, 291]. CDAHFD drives severe NASH in 6 months and liver cancer after 8 months with full penetrance. However, these models are not particularly translational due to lack of obesity (CDAHFD is considered a model of lean NASH), and induction of cachexia in the case of MCD [289, 292].

Other mouse models of NASH include the use of genetically manipulated mice strains, which spontaneously develop steatosis and liver damage overtime, and the progression is often accelerated by matching the genetic knockouts with a special diet feeding, like those above mentioned. Examples of these models include the MUP-uPA mice, fed HFD, probably one of the best approach to study ER stress [282], the DIAMOND, which drives consistent NASH and could be employed in treatment studies [293], or the STAM, which drives a very particular form of lean NASH starting from induction of T1DM [294].

Alternatively, it is possible also to induce a liver damage with the use of toxins, like DEN or CCl₄, which were found able to accelerate fibrosis and HCC development in the mice fed also HFD or WD [289, 295, 296].

Of note, the genetic and combinatorial models often do not allow a physiological progression of NAFLD, whereas the dietary models maintain a higher translational value. In the last few years, the scientific community is indeed debating on which model could faithfully phenocopy the human NASH in the mouse [289, 297]. To this day, modified versions of the AMLN dietary model (such as GAN or the Western Diet) that include the use of non-trans-fat up to 40%, matched with high fructose and cholesterol content, seem to best recapitulate the key features of NASH. They can induce metabolic syndrome, including obesity, T2DM with insulin resistance and portal hypertension, with development of severe hepatic steatosis within 4 months of diet feeding in C57BL/6J mice and full-blown NASH with fibrosis after 6-8 months. These models work well to study also NASH-to-HCC transition, as they induce hepatic lesions after 12 to 15 months, up to 80% [284, 298-300].

It is also important to mention that the basic inbred mouse strain of choice has particular relevance on the final liver disease outcome, as, for example, already important differences across genetic and metabolic predisposition could be found between BL6/J and BL6/N [289].

Preclinical models of NASH have become a fundamental pillar of drug studies prior clinical trials for drug studies and development. To date the most promising therapeutic strategy in clinical development for NASH treatment consists in the modulation of FXR signalling with FXR agonists. In the next chapters, I will introduce the basics of FXR signalling and its relevance in NASH, which made it a valuable target worth further investigation.

4.3. Modulation of FXR as a therapeutic target for NASH

4.3.1. FXR signalling

The farnesoid X receptor (FXR) belongs to the nuclear receptor (NR) superfamily, and designated as NR1H4. Two genes, namely *Fxr α* and *Fxr β* , have been identified for encoding for FXR. While *Fxr α* is conserved in humans and encodes for four isoforms (FXR α 1, FXR α 2, FXR α 3 and FXR α 4), *Fxr β* is considered a pseudogene [301, 302].

The N-terminal ligand-independent transcriptional activation AF1 domain, the DNA-binding domain (DBD), a hinge region, and the C-terminal ligand-binding domain (LBD) containing a transcriptional activation function domain 2 are all modular domains which constitute the typical NR structure of the FXR protein (AF2) [301, 302]. The length of the AF1 region and the sequence of the hinge region change amongst the four FXR isoforms. Agonists that bind to the pocket created by LBD increase its binding to FXR response regions in downstream target genes, which in turn stimulates transcriptional activation. FXR is most abundantly expressed by the enterohepatic system [303, 304]. Similar amounts of FXR1/2 and FXR3/4 are expressed in the liver, but FXR3/4 isoforms are mostly expressed in the gut. According to its expression profile, FXR primarily elicits its activities, which places it at the centre of the pathophysiological developments in the enterohepatic system [302, 305].

Since certain BAs were identified to be this receptor's endogenous ligands, FXR is also known as the bile acid receptor (BAR) [197, 198, 306]. As a BA sensor, FXR is essential for regulating the synthesis, transport, and metabolism of BAs, which helps to maintain their homeostasis [65]. Both the intra-hepatic FXR-small heterodimer partner (SHP) pathway and

the extra-hepatic FXR-FGF19/15-FGFR4- β -klotho pathway are major mechanisms by which FXR inhibits BA production [307]. Both pathways indeed converge to the suppression of the hepatic cholesterol 7 α -hydroxylase (CYP7A1), a rate-limiting enzyme involved in the classical pathway for BAs synthesis. Furthermore, FXR is also able to induce BAs secretion via bile salt export pump (BSEP) and multidrug related protein 2 (MRP2) activation, and to downregulate the BAs absorption via apical sodium-dependent bile acid transporter (ASBT) and sodium taurocholate co-transporting polypeptide (NTCP)-mediated suppression. FXR can also control BAs conjugation and detoxification through direct action on bile acid CoA synthase (BACS), BA-CoA: amino acid N-acetyltransferase (BAAT), UGT2B4, and SULT2A1. Experiments on FXR null mice showed dramatic accumulation of BAs, while treatments with FXR agonists decreased BAs concentration, demonstrating how essential FXR is in maintaining the BAs below hepatotoxic level, making this receptor a valuable target for therapy of cholestatic liver disease, like primary biliary cirrhosis and intrahepatic cholestasis of pregnancy [305, 308].

Given the accumulating data on the effects of FXR activation on cholesterol metabolism, there is growing interest on this BAR also in the therapeutic landscape for many metabolic diseases, from atherosclerosis to NAFLD. For this reason, I delved into my PhD project investigating the effects of FXR agonism in NASH using different compounds. Many molecules are to date available for this purpose, and will be summarized in the next chapter.

4.3.2. FXR modulators

After several studies identified FXR as the main receptor of BAs and the strong impact that exerts on metabolism, many different compounds have been synthesized to better characterize its intestinal and hepatic activity, in the attempt to deliver a valid therapeutic target and ligand to the clinic to treat metabolic and BA-related disorders. BAs are known for being FXR ligands, and primary BAs like CA and CDCA, as well as the secondary BAs DCA and LCA exert FXR agonists function [305, 306].

The main synthetic steroidal FXR agonists have been created from modifications of CDCA, the most potent endogenous activator (EC₅₀ 50 μ M). One popular derivative was obtained from and introduction of an ethyl group at 6 position of CDCA, generating thereby 6 α -ethyl-chenodeoxycholic acid, more popular with the name Obeticholic acid (OCA) [309]. Patented by Intercept Pharmaceuticals, OCA has a potency 500-fold higher than CDCA, with an EC₅₀ of 99nM [310-312]. Some analogues of OCA also exists, and seem to be even more

potent. Other endogenous steroidal compounds have affinity for FXR and their agonistic activity have been demonstrated, such as 5 α -androstan-3 α -ol-17-one (androsterone), 5 β -androstan-3 α -ol-17-one (etiocholanolone) and MFA-1 [17-(4-hydroxybenzoyl) androsta-3,5-diene-3-carboxylic acid] [305, 313, 314].

Nonsteroidal compounds can also be FXR agonists, like GW4064, the first of its kind, patented in 1998 and published in 2000. GW4064 exerts a full agonist activity without off-target effects on other NRs up to a 1 μ M concentration [315, 316]. However, the clinical application of GW4064 are limited, due to poor bioavailability and potential hepatobiliary toxicity [317, 318].

A decade ago, Phenex Pharmaceuticals have developed and patented Px-102 (PX20606) and its eutomer Px-104 (GS-9674), as analogues of GW4064 with similar affinity for FXR but improved aqueous solubility and membrane permeability [319-321].

GlaxoSmithKline scientists also worked on a less toxic alternative to GW4064, and generated FXR agonists like GSK8062 and GSK2324 [317, 318]. Of recent development is ST-1892, an effective FXR agonist with low cellular toxicity and good solubility, based on the fragmentation of GW4064 [322]. Tropicifexor (LJN452) is another non-steroidal small molecule, which Novartis Pharmaceuticals developed and patented in 2017. Tropicifexor, known for its highly potency and selective activity, with an EC₅₀ of 0,2nM, is characterized by decent solubility and improved pharmacokinetics in rats, like low clearance and long terminal half-life [323, 324]. Given its proven safety and tolerability, Tropicifexor worked its way to clinical trials for PBC and NASH.

Another compound worth of mention is Fexaramine, developed by combinatorial chemistry with 100-fold increased affinity compared to CDCA, but still distinct from GW4064 or any other steroidal FXR ligand in terms of structure [313, 325, 326]. Due to its activity, predominantly intestinal, some newly synthesized derivatives are currently preferred to Fexaramine [305].

Some BA species, such as CDCA, DCA, and LCA, activate FXR, while less hydrophobic ones act as FXR antagonists. T- β -MCA and G-MCA are FXR antagonists, and T- β -MCA was found to downregulate ileal FGF19/15 and SHP expression, activated by TCA. These BAs show potential therapeutic benefits against obesity and metabolic syndrome, and G-MCA is resistant to gut bacterial bile salt hydrolase, making it more stable within the intestine than T- β -MCA [327, 328]. In addition, some bile alcohols, intermediates in the BA synthetic

pathway, can have FXR-ligand properties, and as 5 β variants such as 5 β -cyprino and 5 β -bufol normally act as FXR agonists, their 5 α -counterparts work as antagonists [329].

FXR partial agonists are ligands that activate FXR with lower efficacy than a full agonist. Some BA species and derivatives act as FXR partial agonists. CDCA analogues with a substituted distal carboxylic acid group behave as partial agonists, and NCDCN is a partial agonist that is 3-fold more potent than its carbamoylated derivative [309]. LY2562175 and UPF-838 are well-known partial agonists, with LY2562175 being applicable to dyslipidaemia and atherosclerosis treatment [330]. Ivermectin is documented as partial agonist that has less capability to recruit coactivators than a typical full agonist does [331]. Thiazolidinediones, including troglitazone, and epigallocatechin-3-gallate act as partial agonists that can act as competitive antagonists with full agonists for receptor occupancy [332].

Important compounds are also those called selective bile acid receptor modulators (SBARMs), drugs that activate specific downstream target genes of the FXR receptor while avoiding potential side effects [305]. This can be achieved by designing compounds that interact with the receptor's "back door" or S2 pocket, altering the conformation of the binding cleft for co-regulators without affecting the conformation of H12 or H3 [333]. SBARMs have potential therapeutic benefits for various enterohepatic diseases, particularly in lipid metabolism, and can be derived from compounds such as guggulsterone, polyunsaturated FAs, xanthohumol, tetrahydrotetramethylmethylnaphthalenyl (-propenyl) benzoic acid (TTNPB), and mometasone furoate (MF) [315, 333-338].

In addition, FGF19 and FGF21, being hormones that play a role in regulating metabolism, also shown to have potential therapeutic applications for metabolic disorders, including obesity, type 2 diabetes, and NASH. For this reason, FGF-based clinical analogues or mimetic are being investigated to tackle fatty liver and fibrosis in NASH, and definitive answers on their development prospects are expected in the next few years. FGF21 analogues revealed promising efficacy in preclinical and clinical studies, but more research is needed to fully understand their therapeutic potential and safety. The most relevant FGF21 analogues to date are LillyLY2405319 and Akero's AKR-001. NGM282, an FGF19 analogue developed by NGM, is the only asset in clinical development for NASH, demonstrating effective reduction of liver fat content in patients [339].

4.3.3. FXR modulation in NAFLD

In recent years, therapeutic agents able to modulate FXR signalling gained particular interest in the field of NAFLD. Indeed, it became apparent that FXR plays also an important role in regulating lipid homeostasis, as studies on FXR-null mice described elevated serum glucose and circulating FFAs, as well as severe fatty liver, suggesting that FXR can influence cholesterol catabolism and transport as well lipoprotein metabolism [340]. FXR activation drives the activation of the ATP-binding cassette subfamily G member 5/8 (ABCG5/G8), an important mediator of intestinal and biliary secretion of cholesterol [341-344]. The two major regulators of LPL are Apo lipoprotein C-II (Apo C-II) and Apo C-III, respectively activator and inhibitor, and are under control of FXR, which induces Apo C-II and suppresses Apo C-III [345, 346]. FXR activation also reduces the hepatic triglycerides levels, by upregulating carboxylesterase 1 (CES1) and PPAR α and by suppressing sterol regulatory element-binding protein-1c (SREBP-1c) [347-350]. Importantly, previous studies reported that FXR and PPAR α are key regulators of the fasted and fed states in the liver, with opposing metabolic regulatory functions in glucose and lipid metabolism, and an essential function in coordinating autophagy and liver secretome [348, 351].

When considered collectively, these studies indicate that FXR activation decreases plasma and hepatic lipid levels via inhibiting hepatic lipogenesis and secretion as well as enhancing lipoprotein clearance [347, 352, 353]. However, HDL-C levels are concurrently reduced following FXR agonism as a result of its stimulation of scavenger receptor and inhibition of Apo A-I (SR-BI) type I B1 [354]. Additionally to being activated, decreased FXR expression in the liver was shown to control hepatic steatosis in aged mice, indicating the expression levels of FXR also control how lipids are metabolized [355]. FXR overexpression or activation can indeed lower liver and serum TG levels in circumstances like obesity and NASH [305].

Since FXR deficient mice presented with high serum glucose and major impairment to glucose and insulin tolerance, it was concluded that FXR is also essential in maintaining glucose homeostasis [340]. Upon activation FXR operates, directly or indirectly, a negative control on multiple genes involved in glycolysis and gluconeogenesis, such as ChREBP and GLP-1 secretion, or PEPCK and G6Pase, respectively. FXR can also improve insulin sensitivity, suggesting again that this receptor can be a promising therapeutic target for obesity and T2DM [356, 357].

Energy homeostasis seems to be as well under control of FXR, as its intestinal activation drives accumulation of T-LCA, an activator of G protein-coupled bile acid receptor-1 (Gpbar-1, or TGR5) and stimulates the secretion of GLP-1, FGF15 and FGF21, thus promoting white adipose tissue browning and thermogenesis[325, 326, 358].

Interestingly, studies reported that also intestinal FXR antagonism promotes energy expenditure and body weight reduction, through reducing the intestinal ceramides biosynthesis, which directly compromises beige fat thermogenic function and improve obesity.[327]. It is likely that this rescuing effects on steatosis were achieved because only intestinal FXR was inhibited, leaving intact the hepatic signalling, and the drug employed did not have any effect on TGR5 signalling, which was modulated using intestinal-biased FXR agonists, such as Fexaramine, obtaining positive effects on liver steatosis, insulin resistance and driving adipose tissue browning [325, 359].

It's also worth to mention that FXR suppresses inflammation, by inhibiting NF-kB upon activation, or promotes it in obesity by dysregulation of acetyl/SUMO switch, and is a suppressor of NLRP3 inflammasome [360-362]. Lastly, some evidence suggests a role of FXR also in controlling the course of viral hepatitis as it's been shown that upon activation can inhibit hepatitis B virus replication [363].

Another important involvement of FXR is in liver fibrosis, preventing hepatic stellate activation via FXR-PPAR γ and FXR-SHP-TIMP (tissue metalloproteinase inhibitor) pathways. FXR activation also leads to improved portal hypertension, where stimulation of eNOS activity promotes the reduction of vascular remodelling and sinusoidal dysfunction [364-368].

Some studies have reported that the liver, capable of full regeneration after injury or partial hepatectomy, requires BAs dependent FXR activation, both from intestinal and hepatic source, to induce compensatory regrowth [369, 370]. Hepatic FXR can in fact control the cell cycle and proliferation genes Cyclin D1 and Forkhead box m1b (Foxm1b), and the intestinal signalling, via FXR-FGF15 (FGF19) controls the size of BA pool, thereby promoting liver regeneration [371-373].

Several FXR modulators, including GW4064, OCA, INT-767, GS-9674, and Fexaramine, showed to improve insulin resistance, protect against weight gain and liver fat accumulation in obese mice [325, 359, 374-376]. FXR agonists such as OCA, WAY-362450, Px-102 and Tropicifexor have been demonstrated to protect against NASH, and some have

progressed to clinical trials [377, 378]. FXR antagonists T- β -MCA and G-MCA were also reported to improve metabolic parameters in obese mice [327, 358].

Eight years ago, interim data from the FLINT trial on a single dose of OCA led to further interest for research into novel FXR agonists [378]. The study demonstrated OCA's potential to improve histopathology and fibrosis. Despite the success and the further corroboration of these results in the phase III REGENERATE trial, the FDA has not approved OCA for NASH due to safety concerns [379]. OCA consistently increases pruritus and decreases HDL-c while increasing LDL-c, posing a potential atherosclerotic risk. These effects may be FXR agonism manifestations since other FXR agonists have demonstrated similar effects. Interestingly, a recent study observed that high dose OCA led to cholesterol accumulation and FXR dependent induction of hepatotoxicity [380]. Another compound that seemed to be promising was Cilofexor, even if unfortunately did not reach the necessary milestones during clinical trials. Tropifexor, due to its specificity for FXR and its potency, seemed to reach so far the expectations of scientific community [5]. Recent reports from phase 2a/b trial observed decreased levels of ALT and hepatic fat fraction up to 48 weeks of treatment, but no similar trends of AST at week 12 and dose-related pruritus as the major adverse effect [6]. Another recent study from Phase 2b TANDEM tested the efficacy of Tropifexor in combination with cenicriviroc compared to respective monotherapies and reported no major synergism of the combined therapy, while Tropifexor presented sustained ALT and reduction of body weight with pruritus again documented in 40% of the patients [7].

The promising therapeutic efficacy of FXR modulators has sparked a large interest in attempting to deliver the best classes of molecules to the clinic, for monotherapy or even combined therapeutic approaches. However, there is still a lack of clarity for the underlying mechanisms of action of many compounds in this field, and further research is required, to not only improve their efficacy, but also to further confirm their safety.

5. Scope of the study

NASH is quickly emerging as a major worldwide health issue, being an often-underdiagnosed risk factor for hepatic chronic inflammation and cancer and being still devoid of approved therapies. Among limited available solutions, the selective targeting of FXR and related bile acid signalling starts to bring some hope in this field. In preclinical models of NASH, many FXR agonists revealed a striking efficacy in improving the liver pathology, and these results reflect the enthusiasm in pursuing their validation in clinical trials. However, to date no study evaluated the targeting of NASH through use of FXR agonists in late stages of the disease, where it could be possible to determine also the impact on liver cancer onset. This is an important although overlooked aspect, since it is not new that FXR activation already drives pro-proliferative signals as is known for inducing transient liver hypertrophy, which has been observed in pregnancy, for upregulating Cyclin D1 and Foxm1b, and due to its control on autophagy might favour tumour survival and progression [349, 381-383].

Several studies reported that FXR activation showed protective effects towards hepatocarcinogenesis, by upregulating for example tumour suppressors NDRG2 or by inhibiting STAT3 through SOCS3 activation [384, 385]. However, it is still unclear if this effects could counteract other questionable interaction in the context of liver regeneration, or if the exogenous activation could effectively deliver a signalling that truly recapitulates the endogenous one [373, 386]. However, an accurate evaluation of the effects of chronic exogenous FXR agonism has never been assessed, especially in the context of NASH-induced HCC. Taking mentioned aspects into account, we can speculate that FXR agonists may exert potential additional synergism with liver microenvironment during NASH, making it more prone to hepatocarcinogenesis and accelerating it.

Having these points in mind, I formulated the following aims:

- Validating mouse models of NASH in our animal facility and define time frame necessary for NASH development
- Administering FXR agonist in the form of diet supplementation
- Evaluating the impact of various FXR agonists on early NASH: assessed body weight, lipid and glucose metabolism, liver damage and NASH severity
- Detecting the impact of FXR signalling in the gut and liver across the different agonists tested

- Identifying metabolic, immune and molecular signatures that could exert potential pro-carcinogenic effects on the liver
- Determining the impact of various FXR agonists in advanced NASH on body weight, lipid and glucose metabolism, liver damage and NASH severity
- Performing immunophenotyping of intrahepatic immune cells and assessed the effects of FXR agonists on inflammation
- Investigating the role of identified in early NASH rescue study signatures in severe NASH progression
- Defining the influence of various FXR agonists on hepatocarcinogenesis by assessing hepatic lesions incidence and characterization

6. Materials and methods

6.1. FXR agonists

Fexaramine was purchased from Advanced ChemBlocks Inc. Tropifexor was purchased from MedChem Express. OCA was purchased eNovation Chemicals LCC and GW4064 was purchased from Ambeed, Inc.

6.2. Animal experiments and treatments

4-6 week old male C57BL6/J mice were purchased from Charles River. The mice were housed in the animal facilities of the German Cancer Research Centre (DKFZ), under specific pathogen-free and centralized conditions (temperature 20-24°C, 45-65% humidity, 12-h light-dark cycle). The experiments were conducted in compliance to German Law and the governmental bodies, and approved by the Regierungspräsidium Karlsruhe (approval Nr. DKFZ332, G7-17, G159-18, G180-19). The mice had *ad libitum* access to food and water when not differently specified. For the dietary experiments, the mice received normal chow diet (ND) or the diets indicated in Table 1.

Table 1: Animal Diets employed

Diet Name	Diet Composition	Source
Normal Diet (caloric matched)	10% kcal fat, 70% kcal carbohydrates, 20% kcal protein	Research Diets D18090606
Choline Deficient High-Fat Diet (CD-HFD)	45% kcal fat, 35% kcal carbohydrates, 20% kcal protein, without choline	Research Diets D05040102
Western Diet (WD)	40% kcal fat (non-trans-fat), 20% kcal fructose, 2% cholesterol	Research Diets D16022301
Normal Diet + Fexaramine	10% kcal fat, 70% kcal carbohydrates, 20% kcal protein, Fexaramine 429 ppm	Research Diets D18090607

Normal Diet + Tropifexor	10% kcal fat, 70% kcal carbohydrates, 20% kcal protein, Tropifexor 2,61 ppm	Research Diets D18090608
Choline Deficient High-Fat Diet (CD-HFD) + Fexaramine	45% kcal fat, 35% kcal carbohydrates, 20% kcal protein, without choline, Fexaramine 500 ppm	Research Diets D18090610
Choline Deficient High-Fat Diet (CD-HFD) + Tropifexor (dose 0,3 mg/kg/day)	45% kcal fat, 35% kcal carbohydrates, 20% kcal protein, without choline, Tropifexor 3,04 ppm	Research Diets D18090611
Choline Deficient High-Fat Diet (CD-HFD) + Tropifexor (dose 0,15 mg/kg/day)	45% kcal fat, 35% kcal carbohydrates, 20% kcal protein, without choline, Tropifexor 1,8 ppm	Research Diets D21020801
Western Diet (WD) + Fexaramine	40% kcal fat (non-trans-fat), 20% kcal fructose, 2% cholesterol, Fexaramine 500ppm	Research Diets D19020502
Western Diet (WD) + Tropifexor (dose 0,3 mg/kg/day)	40% kcal fat (non-trans-fat), 20% kcal fructose, 2% cholesterol, Tropifexor 3 ppm	Research Diets D19020503
Western Diet (WD) + Tropifexor (dose 0,15 mg/kg/day)	40% kcal fat (non-trans-fat), 20% kcal fructose, 2% cholesterol, Tropifexor 1,8 ppm	Research Diets D20021901
Western Diet (WD) + Tropifexor (dose 0,08 mg/kg/day)	40% kcal fat (non-trans-fat), 20% kcal fructose, 2% cholesterol, Tropifexor 0,9 ppm	Research Diets D20052603
Western Diet (WD) + GW4064	40% kcal fat (non-trans-fat), 20% kcal fructose, 2% cholesterol, GW4064 270 ppm	Research Diets D20072904
Western Diet (WD) + Obeticholic Acid (OCA)	40% kcal fat (non-trans-fat), 20% kcal fructose, 2% cholesterol, OCA 180,3 ppm	Research Diets D20072905

For the short term experiments, 4-6 weeks old male C57BL/6J mice, were fed either a normal standard diet (ND), a choline-deficient high-fat diet (CD-HFD) or a Western Diet (WD) for 3,5 months and further fed the same diet supplemented with FXR agonists (Fexaramine, Tropifexor, OCA, GW4064) for 5-6 weeks.

For the long term experiments, 4-6 weeks old male C57BL6/J mice were fed ND, CD-HFD or WD for 6 months and further fed the same diet supplemented with FXR agonists (Tropifexor, OCA, GW4064) for 4 to 6 months. The mice were fasted overnight and the last dose of compound was delivered via oral gavage four hours prior dissection. The mice were euthanized by asphyxiation with CO₂. Organs harvested were weighted and either frozen in liquid nitrogen or fixed 48-72 hours in 4% buffered formalin and stored in 70% ethanol prior paraffin embedding.

6.3. Magnetic Resonance Imaging

In collaboration with the Small Animal Imaging co-facility at the DKFZ, the mice undergoing long term experiments were screened periodically for tumour follow-up at the given time points (6 months, 7,5 months, 9 months, 10,5 months).

6.4. Metabolic phenotyping

Metabolic phenotyping was carried out in collaboration with Dr. Suchira Gallage and Dr. Adnan Ali, performing indirect calorimetry on mice in PhenoMaster (TSE systems). Mice were kept in individual housing throughout the analysis. Following two to three days of acclimatisation, measures including food intake, water intake, oxygen consumption, carbon dioxide production, respiratory exchange ratio (RER), and overall activity were assessed five times hourly for at least a week in a row. Later, to eliminate the possibility that body weight could have an impact on the obtained values, analysis of covariance (ANCOVA) was carried out using SigmaPlot (14.0). The values plotted represent the average of three consecutive days.

6.5. Intraperitoneal glucose tolerance test

Mice were fasted overnight, and further i.p. injected a 20% glucose (Sigma) solution (5uL/gr body weight) At the indicated time intervals, small blood drops were collected by puncturing the lateral tail vein and blood glucose was measured using “Accu-check Performa Glucometer”.

6.6. Measurement of serum biomarkers

In collaboration with Sandra Prokosch, blood was collected from the facial vein during the indicated time-points or from the heart during dissections. Serum was isolated and the parameters were measured on a Fuji DRI-CHEM NX500i machine with dedicated test slides for ALT, AST, ALP, bilirubin, total cholesterol, HDL, and triglycerides or alternatively on a Cobas Reader in collaboration with the Institute for Clinical Chemistry and Pathobiochemistry, TUM, Munich

6.7. Flow cytometry analysis

During dissections, the mice were perfused from the heart with cold HBSS, and livers were harvested. Livers were mechanically homogenized and further incubated for 30 to 45 minutes in 37°C with digestion buffer, made with Collagen IV 1:10 (60 U f.c.-Sigma) and DNase I 1:100 (25µg/ml f.c.-Sigma). The livers were then filtered through 100um mesh, washed with RPMI1640 (#11875093) medium and centrifuged for 7min/300g/4°C. Lymphocyte enrichment was performed with a Percoll (Cytiva) gradient. Briefly, the liver pellet was gently re-suspended in 8mL 40% Percoll/HBSS and overlaid on 4mL 80% Percoll/HBSS, following a centrifugation for 25min/1100g/4°C (Acc:1 Dcc:0). The isolated immune cells were twice washed with HBSS and centrifuged 5min/300g/4°C, followed by plating in 96-well plates with a V-shaped bottom.

Splenic lymphocytes were isolated by meshing the spleens through 100um filters, washing and centrifuging. The isolated pellet was re-suspended with 1mL of Red Blood Cell (RBC) lysis buffer (BioLegend) for 5 min RT.

The re-stimulation of T-cells was performed by incubation for 2h, 37°C, 5% CO₂ in RPMI 1640 supplemented with v/v 2% fetal calf serum using 1:500 BioLegend Cell Activation Cocktail (with Brefeldin A) (#423304) and 1:1000 Monensin Solution (1,000X) (#420701).

Live/Dead discrimination was performed by staining the cells with ZombieDyeNIR (BioLegend), in accordance to the manufacturer's instructions. FC blocking was carried out with CD16/CD32 antibody (BioLegend) at 1:300 dilution for 15 minutes on ice. After wash, the cells were stained in 25µL with a master mix of titrated antibodies indicated in Table 2 for 20 min at 4°C.

Table 2: Antibodies for flow cytometry

Target	Fluorochrome	Clone	Cat #
Foxo1	/	C29H4	2880
CD192 (CCR2)	AF647	SA203G11	150603
CD4	AF700	RM4-5	100536
CD86	AF700	GL-1	105023
CD366 (TIM3)	APC	RMT3-23	119706
IFN γ	APC	XMG1.2	505810
Nk1.1	APC	PK136	108710
CD11c	BV421	n418	117343
Donkey anti-rabbit IgG	BV421	poly4064	406410
Ki-67	BV421	sola15	48-5698-80
NK1.1	BV421	pk136	108741
CD45	BV510	30-f11	103138
CD279 (PD-1)	BV605	29f.1a12	135220
Ly6G	BV605	1a8	127639
CD11b	BV650	m1/70	101239
TCR β	BV650	h57-597	742483
CD186 (CXCR6)	BV711	sa051d1	151111
Ly6C	FITC	HK1.4	128006
TCR $\gamma\delta$	FITC	UC7-13D5	107504
CD49b	PerCP-Cy5.5	dx5	108916
CD8a	PerCP-Cy5.5	53-6.7	100734
MHC II (I-A/I-E)	PerCP-Cy5.5	M5/114.15.2	107625
CD274 (PD-L1)	PE	10F.9G2	124307
CD69	PE	H1.2F3	104508
GzmB	PE	NGZB	12-8898-80

mCD1d/α- GalCer	PE	/	YD8002-PE
CD206	PE/Dazzle	C068C2	141731
CD62L	PE/Dazzle	MEL-14	104448
Nkp46/CD335	PE/Dazzle	29A1.4	137630
TNFα	PE/Dazzle	MP6-XT22	506346
CD19	PE-Cy7	6D5	115520
CD44	PE-Cy7	im7	103029
F4/80	PE-Cy7	BM8	123114

The samples were fixed using eBioscience IC fixation (#00-8222-49) or Fix/Perm buffer (Invitrogen) for cell panels requiring intracellular staining. Intermediate washing steps were carried out with eBioscience Perm buffer (#00-8333-56) and finally samples were re-suspended in MACS buffer (PBS with 0.5% BSA and 0.4% EDTA).

Prior acquisition CountBright Absolute Counting Beads (Life technologies) were added to the samples.

Cells acquisition was carried out with BD FACS Fortessa and data were analysed using FlowJo v10.8.

The data are displayed as number of cells, normalized by beads count and grams of liver employed for immune cell isolation of as relative percentage of CD45+ live single cells.

6.8. Histological analysis

Tissue processing and subsequent immunohistochemical staining was carried out in collaboration with Jenny Hetzer, Danijela Heide, Florian Müller and Tim Machauer at the DKFZ, Department of Chronic Inflammation and Cancer (Heidelberg).

2µm sections from FFPE (formalin fixed, paraffin embedded) and cryo-preserved tissues were prepared and stained with Haematoxylin and Eosin or IHC antibodies summarized in Table 3 on a Bond MAX (Leica). For Sudan Red staining, cryo sections (5µm) were cut and stained with Sudan Red (0.25% Sudan IV in ethanolic solution). Slides were scanned with SCN400 slide scanner (Leica), and analysed manually using area based staining ImageScope analysis software v12.4 or with a macro-based analysis by ImageJ. Prof. Thomas Longerich (Institute

of Pathology, University Clinic Heidelberg) performed NAFLD activity scoring (NAS) on murine livers.

Table 3: IHC antibodies

Target	Dilution	Clone	Cat#
CD3	1:250	SP7	ab16669
F4/80	1:50	BM8	123105
Ki-67	1:200	RM-9106-S1	RM-9106-S1
Ly6G	1:600	1A8	551459
p21	1:1000	EPR18021	ab188224
γ H2ax	1:500	polyclonal	NB100-2280
NQO1	1:800	polyclonal	ab34173
CHOP	1:100	D46F1	3177
BIP	1:200	C50B12	5554
Sox9	1:2000	EPR14335	ab185230

6.9. Protein extraction and Immunoblotting

Protein homogenates were obtained from liver by employing Precellys homogenizer (Bertin, France) and processing the samples in dedicated tubes with zirconia beads (Carl Roth) and radio immunoprecipitation assay (RIPA – Cell Signaling Technology) buffer supplemented according to the manufacturer's instructions with complete protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche).

Proteins were extracted from cells by re-suspending the cell pellet with RIPA buffer with inhibitors.

Protein concentrations were determined with BCA Protein assay. Briefly, 5 μ L of protein were mixed with 195 μ L of BCA (Sigma) and 1:50 copper sulphate solution (Merck), incubated at 37°C for 30 minutes and absorbance was measured at 562nm using a Perkin Elmer plate reader and confronted to a BSA (Bovine Serum Albumin) standard curve.

30 to 60 μ g of proteins were separated under reducing conditions (using SDS and 2.5% 2-Mercaptoethanol) in 10%-12% polyacrylamide gels and blotted 2h onto PVDF or Nitrocellulose membrane (Thermofisher). Membranes were blocked 30 min at RT in a 5% BSA TBS-T solution (Tris-Base, Salt, Tween 20) or 5% Milk TBS-T.

Primary antibodies indicated in Table 4 were incubated overnight at 4°C on a roller. Secondary antibody (Table 3) incubation was carried out at RT for 1h. Detection was performed using Supersignal West Pico Plus (#1963095- Thermo), with a Chemidoc Imaging System (Bio-Rad).

Table 4: Antibodies for Western Blot

Target	Cat #	Supplier
FXR	sc-25309	Santa Cruz Biotechnology
CYP7A1	ab65596	abcam
SHP	sc-271511	Santa Cruz Biotechnology
P-p65 (Rel A)	3033	Cell Signaling Technology
P65 (Rel A)	6956	Cell Signaling Technology
Rel B	4922	Cell Signaling Technology
P-p38	9211	Cell Signaling Technology
p38	9212	Cell Signaling Technology
P-p44 (ERK)	4370	Cell Signaling Technology
p44 (ERK)	4695	Cell Signaling Technology
p-JNK	9251	Cell Signaling Technology
JNK	9252	Cell Signaling Technology
p70 (S6K)	9202	Cell Signaling Technology
NQO1	ab34173	abcam
GAPDH	sc-32233	Santa Cruz Biotechnology
BIP	3177	Cell Signaling Technology
CHOP	5554	Cell Signaling Technology
Vinculin	V9131	Sigma-Aldrich
ATF6	TA336753	Origene
P-PERK	3179	Cell Signaling Technology
P-eIf2 α	3597	Cell Signaling Technology
eIf2 α	9722	Cell Signaling Technology
Anti-rabbit IgG, HRP-linked	7074	Cell Signaling Technology
Anti-mouse IgG, HRP-linked	7076	Cell Signaling Technology

6.10. RNA extraction and qPCR

Tissues were processed with the “precellys” homogenizer and RNA was isolated using RNeasyMini Kit (Qiagen) according to manufacturer’s protocol. Quantity and quality of the RNA was measured with Nanodrop analyser (Thermo). 1µg of RNA was employed for further cDNA production using Quantitect Reverse Transcription Kit (Qiagen). 3µL of 1:10 diluted cDNA were mixed to 0.12µL of forward and reverse primer mix (see Table 5) and 6µL Sybr green mix (Roche) for qRT-PCR (amounts for one reaction). The reactions were performed in duplicates in 384 or 96-well plates on a Quant Studio 5 light cycler (Thermo) using $\Delta\Delta CT$ method. Relative quantification was performed by comparing target genes to the housekeeping genes *Rps14* for liver and *Gusb* for ileum.

Table 5: qPCR primer sets

Gene	Forward Primer	Reverse Primer
<i>Abca1</i>	GTTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
<i>Acac</i>	GAGAGGGGTCAAGTCCTTCC	CTGCTGCCGTCATAAGACAA
<i>Acad10</i>	TGGCTGTGGGCTGGGAG	AAATAGGAGCTGACGGGCAC
<i>Acat2</i>	GACCCCGTGGTCATCGTC	CCACAACCTGCCGTCAAGA
<i>Acat3</i>	TGGCCACTTTGACAAGGAGAT	CTGTTGCATTAGCAGTTGTGA
<i>Acly</i>	TTCGTCAAACAGCACTTCC	ATTTGGCTTCTTGGAGGTG
<i>Acot2</i>	CTCGTCTTTCGCTGTCCTGA	CTCAGCGTCGCATTTGTCCG
<i>Acox3</i>	GGGTGATGGTCGGTGACATT	CGGACATCCTTAAAGGGGCT
<i>Acs11</i>	ATCTGGTGGAACGAGGCAAG	TCCTTTGGGGTTGCCTGTAG
<i>Acsm2</i>	GCCAGACAGAAACGGGACTT	ACATGCCGATAGGCCAGATG
<i>Acta2/Asma</i>	CTCTCTTCCAGCCATCTTTCAT	TATAGGTGGTTTCGTGGATGC
<i>Bsep</i>	ACAGAAGCAAAGGGTAGCCATC	GGTAGCCATGTCCAGAAGCAG
<i>Ccl2</i>	GCATCCACGTGTTGGCTCA	CTCCAGCCTACTCATTGGGATCA
<i>Ccl5</i>	AGATCTCTGCAGCTGCCCTCA	GGAGCACTTGCTGCTGGTGTAG
<i>Colla1</i>	GAGAGGTGAACAAGGTCCCG	AAACCTCTCTCGCCTCTTGC
<i>Cpt1</i>	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
<i>Cyp7a1</i>	GCTGTGGTAGTGAGCTGTTGCA	CACAGCCCAGGTATGGAATCA
<i>El</i>	CTATCCCAATGGCGGTGACTTC	CGTGCTCGCATTTACCATC
<i>Fasn</i>	GTTGGCCCCAGAACTCCTGTA	GTCGTCTGCCTCCAGAGC
<i>Fgf15</i>	GAGGACCAAAACGAACGAAATT	ACGTCCTTGATGGCAATCG

<i>Fgf21</i>	GTGTCAAAGCCTCTAGGTTTCT	GGTACACATTGTAACCGTCCTC
<i>Fgfr4</i>	GCCTCCGACAAGGATTTGGCA	GAGTGCAGACACCCAGCAGGT
<i>Fxr</i>	AAAGGGGATGAGCTGTGTGT	AGTCACGCCCTTCGCTGT
<i>Gpat</i>	CTTGCCCGATGTAAACACACC	CTTCCGGCTCATAAGGCTCTC
<i>Gusb</i>	CCGATTATCCAGAGCGAGTATG	CTCAGCGGTGACTGGTTTCG
<i>Hl</i>	CTATGGCTGGAGGAATCTG	TGGCATCATCAGGAGAAAG
<i>Ifng</i>	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
<i>Il10</i>	GCCCAGAAATCAAGGAGCATT	TGCTCCACTGCCTTGCTCTTA
<i>Il1b</i>	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
<i>Light</i>	TCCGCGTGCCTGGAAA	AAGCTCCGAAATAGGACCTGG
<i>Lipe</i>	CTTCCAGTTACCTGCCA	AATCGGCCACCGGTAAAGAG
<i>Lpl</i>	TTTGGCTCCAGAGTTTGACCGC	CGAAGGTCTTGCTGCTGTGGTT
<i>Ltb</i>	TAGTTCTGAGTCTGGATGGG	GATCTTCTCAACCCGACG
<i>Mvd</i>	GAGGGAGACCTCTCCGAAGT	GTCTGCARGCCCACTGTACT
<i>Ppard</i>	GAACAGCCACAGGAGGAGAC	GAGGAAGGGGAGGAATTCTG
<i>Rps14</i>	GACCAAGACCCCTGGACCT	CCCCTTTTCTTCGAGTGCTA
<i>Scd1</i>	CTACAAGCCTGGCCTCCTGC	GGACCCCAGGGAAACCAGGA
<i>Shp</i>	CTCTCTTCCTGCTTGGGTTG	GGTACCAGGGCTCCAAGACT
<i>Sqle</i>	GCAATCTACGCCACGTATTTCT	GGGCCCCGTGGTTTTGT
<i>Tgfb1</i>	ATCCTGTCCAACTAAGGCTCG	ACCTCTTTAGCATAGTAGTCCGC
<i>Tnfa</i>	CGATGGGTTGTACCTTGTC	CGGACTCCGCAAAGTCTAAG

6.11. Bile acids analysis

The analysis on BAs and relative conjugated derivatives were performed in collaboration with Dr. Diran Herebian (University Clinic Düsseldorf) on bile, serum, and liver tissue samples that were frozen upon necropsies. The analysis was carried out by UHPLC-MS/MS. The system consists of an ExionLC (Sciex) or I-Class (Waters) coupled to a QTrap 5500 mass spectrometer (Sciex) or Xevo-TQS (Waters). Electrospray ionization is performed in the negative ionization mode. Chromatographic separation is run on a BEH C18 column (2.1x100 mm, 1.7µm) after solid phase Extraction (SPE) of the extract using C18 cartridges (MN). The mobile phase consists of water containing 0.1% formic acid and 5 mM ammonium acetate (Eluent A) and Acetonitrile (Eluent B). Analytes are separated by a gradient elution. Sample volumes of 1 µL for bile, 10 µL for serum/plasma and 20-60 mg for liver/feces samples are required for analysis.

Detection of the bile acids and their glycine and taurine conjugates is performed in the multiple reaction monitoring (MRM) mode, as described in previous studies [387, 388].

6.12. Ploidy analysis and PHH3 immunostaining

Analysis carried out in collaboration with Dr. Chantal Desdouet and Pierre Cordier, (Proliferation, Stress & Liver Physiopathology department, Cordeliers Research Center, Paris University).

For ploidy analysis, β -catenin and Hoechst immunostaining were performed on 7 μ m sections and stereological image analysis was carried out, following by in situ nuclear ploidy analysis. A customized R software script with TukeyHSD (ANOVA) statistical analysis was employed for evaluating 2n, 4n, and 8n nuclear abundance.

Representative mice whose ploidy profiles were close to the mean ploidy profile of the group were selected to perform the pHH3 immunostaining, carried out with Anti-phospho-Histone H3 (Ser10) antibody (MERCK MILLIPORE #06-570), and using Hoechst 33342 for counterstaining.

Positive nuclei were counted on 10 randomly chosen 200X fields covering all the liver sections present on the slide (around two to three 200X fields per liver lobe). The quantification was performed directly on the microscope to better visualize the morphology of positive nuclei. Under the microscope, the 200X field of view has a diameter of 1.1 mm and spans a tissue area of 0,95 mm² (Field Number (FN) = 22, objective = 20X). Hence, the total area covered by ten 200X fields is 9.5 mm². G2 nuclei were discriminated from M (Mitotic) nuclei based on both the nuclear morphology (envelope, condensation, chromosome distribution) and the pattern of fluorescent staining (foci or diffuse). Early M nuclei (rounded shape with a nuclear envelope still present but a diffuse fluorescent staining) were counted within G2 nuclei since they can be recognized by our nuclear ploidy programme. Positive non-parenchymal cell (NPC) nuclei were not taken into account and discriminated based on their nuclear size and morphology.

Each time a spleen tissue section was present on the slide, splenic lymphoid follicles were checked first for proliferating cells to be sure of the adequate pHH3 staining.

Data are expressed as a density of positive nuclei (number of positive nuclei per surface area). Since cell density might be variable under the various diet conditions due to different degree of

steatosis (hepatocyte hypertrophy and reduced number of hepatocyte per 200X field), results were compared only between groups of similar hepatocyte lipid overload.

6.13. Immunofluorescence, microscopy and Three-dimensional (3D) cell reconstruction of liver macrophages

The analysis was performed in collaboration with Dr. Lukas Amann (Institute of Neuropathology, University Clinic Freiburg). For the 3D reconstruction of liver macrophages, paraffin embedded sections were cut at 20 µm thickness with a microtome (CUT 4062, Slee medical GmbH). Antigens were retrieved by cooking in citrate buffer (pH 6.0) for 45 minutes. The sections were blocked and permeabilized with blocking buffer (PBS containing 5 % normal donkey serum and 0.5 % Triton-X 100) for 1h at room temperature. Primary antibodies were incubated in blocking buffer at a dilution of 1:400 for anti-F4/80 (70076, Cell Signaling Technology) and 1:200 for anti-CLEC4F (AF2784, R&D Systems) for 48h at 4 °C. Secondary antibodies were incubated in blocking buffer at a dilution of 1:500 for donkey anti-rabbit Alexa Fluor® 568 (A10042, Thermo Fisher Scientific) and 1:500 for donkey anti-goat Alexa Fluor® 647 (A21447, Thermo Fisher Scientific) for 48h at 4°C. To quench auto fluorescence, sections were treated for 30 seconds with TrueBlack® Lipofuscin Autofluorescence Quencher (23007, Biotium) according to the manufacturer's instructions. Coverslips were mounted with ProLong™ Diamond Antifade Mountant with DAPI (P36971, Thermo Fisher Scientific).

Images for 3D reconstruction were taken at a resolution of 1024 x 1024 pixels with a step size of 1.04 µm and zoom 3 with a TCS SP8 X (Leica, LAS X 3.5.7.23225) using a 20 x 0.75 NA objective (HC PL APO 20x/0.75 IMM CORR CS2).

The 3D reconstruction of liver macrophages was performed using the IMARIS software (V9.6.0, Oxford Instruments). The F4/80 immunofluorescence signal of each individual image was reconstructed using the surface reconstruction tool. Due to high variability of the signal-to-noise ratio between the different sections, thresholds were determined individually for every images. Following the automated surface reconstruction, the reconstructed cells were manually curated and each surface without nucleus (i.e. without visible DAPI signal) was excluded from the further analysis. The sphericity index of each cell was then calculated using the statistics function in the IMARIS software.

6.14. Measurement of liver triglycerides

The amount of liver triglycerides was determined by pulverising 20 to 50 mg of liver tissue in liquid using a pestle and adding 250µL of 0.9% NaCl. Samples were incubated on a heat block for 10 minutes at RT and 450 rpm. Subsequently the samples were again vortexed and incubated for 30 minutes at 71°C and 450 rpm after addition of 250µL of ethanoic 0.5KOH. After adding 500µL of 0.15M MgSO₄, samples were vortexed and centrifuged for 10 minutes at RT at 13,000g. The collected supernatants were diluted 1:4 and analysed at optical densitometry O.D 505 with GPO-PAP from Roche Diagnostics.

6.15. Compound deposition analysis

The analysis was carried out in collaboration with Dr. Manfred Birkel (ex Phenexpharma) and Dr. Claus Kremoser (WMT). Plasma and liver samples were analysed for compound concentration by Synovo (Tübingen).

6.16. Statistical analysis

For statistical analysis, I carried out one-way ANOVA with Tukey's or Dunnett's multiple comparison, two way ANOVA with Tukey's multiple comparison, or Mixed –effects model (REML) with Dunnett's multiple comparison. Analyses were performed using GraphPad Prism software version 9 (GraphPad software). Data are shown as mean ± SEM, as individual points, or as box and whiskers for FACS data. Statistical significance is indicated as follows: 0.1<p<0.05, *p< 0.05, **p< 0.01, ***p< 0.001 and ****p< 0.0001.

7. Results

7.1. Early signs of NASH are evident in the mice after 3.5 months of CDHFD/WD feeding

The first important step for this project was to determine at which time point it is possible to detect the early development of NASH in the C57BL6/J mouse, and characterize the basic phenotype, in order to follow-up with a therapeutic approach. To this end, I collaborated with Dr. Marta Szydlowska and started an experiment with 5-week-old C57BL6/J male mice, divided into three groups: (i) one fed a standard chow diet (from now on referred as Normal Diet – ND); (ii) fed a choline-deficient high-fat diet (see materials and methods - CDHFD); (iii) one fed a western diet (see materials and methods – WD). I maintained the mice on diet *ad libitum* for three months and half and performed necropsies.

At visual inspection, the mice on CDHFD or WD were bigger and more obese, and indeed gained more body weight (Fig. 7a). I recorded the weights of the tissue collected and observed that the average liver weight of the mice fed WD was significantly higher (Fig. 7b). Additionally, the weight of both subcutaneous and abdominal fat, measured respectively as inguinal White Adipose Tissue (iWAT) and epididymal White Adipose Tissue (eWAT), were significantly increased in mice fed CDHFD or WD (Fig. 7c-d). I further observed a slight enlargement of the Brown Adipose Tissue (BAT) in the groups fed CDHFD or WD, but without a significant increase (Fig. 7e). In addition, I performed histological and immunohistochemical analyses of the collected livers. I confronted the H&E sections in the various groups, observing a mild degree of hepatic steatosis in the CDHFD and WD groups (Fig. 7f). I collaborated with Prof. Thomas Longerich, and, after pathological evaluation of the liver sections, I could determine a significant increase of NAFLD activity score (NAS) in the livers of the mice fed CDHFD and WD, consistent with early NASH, normally diagnosed with a score of three or above (Fig. 7g). Furthermore, I observed a trend of increased numbers of CD3-positive cells in the livers of mice fed CDHFD and WD, although still not significant (Fig. 7f-h). I could also determine a non-significant increase of F4/80-positive cells in the CDHFD-fed group, and, to a minor extent in the WD group (Fig. 7f-i). Lastly, I observed a net decrease of Ly6G-positive cells in the mice fed CDHFD and WD (Fig. 7f-j). I then measured some biological markers present in the serum. I observed a significant increase in cholesterol, both the total amount and the abundance of the HDL form, in the groups fed CDHFD and WD (Fig. 7k-l), while in contrast the analysis of triglycerides showed no difference between the groups (Fig. 7m). I did not

observe particular alterations in terms of liver damage, measured as Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) (Fig. 7n-o), but I noted a significant reduction in Alkaline Phosphatase (ALP) levels in the WD group, compared to ND (Fig. 7p).

Based on the mentioned data, I could conclude that the mice after 3.5 months of CDHFD and WD feeding developed an early stage of NASH, with major differences observed in body weight, fat deposits and histological signs of hepatic steatosis, with a minor degree of inflammation, as still too early to occur in severe form.

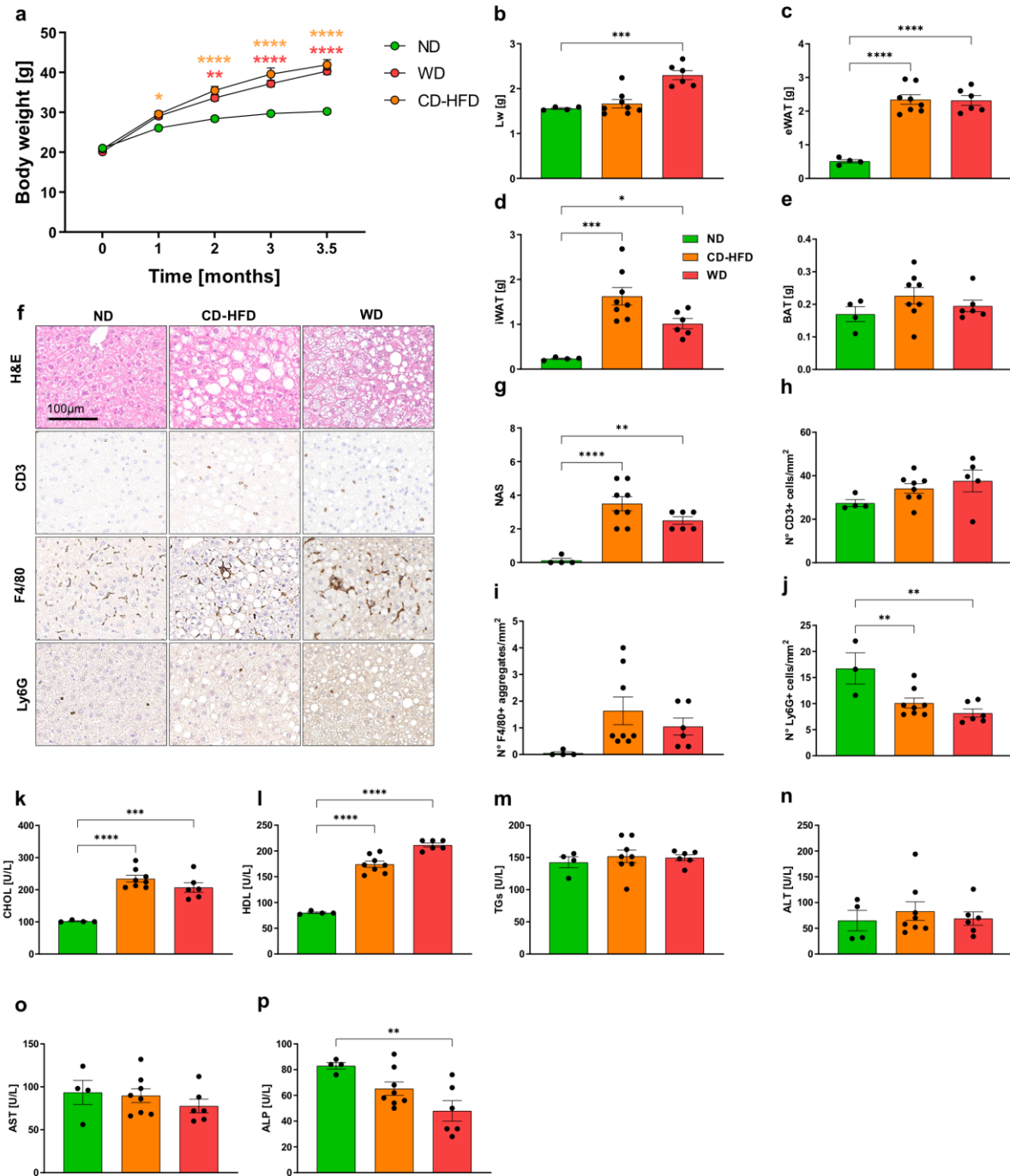


Figure 7. The early NASH signs in C57BL/6J mice after 3.5 months of CDHFD and WD feeding. (a) Body weight development of 3.5 months ND, CDHFD or WD fed mice. Asterisks indicate significance of groups compared to ND fed mice (b) Liver weight of the mouse groups shown in (a) (c) eWAT weight of the mouse groups shown in (a). (d) iWAT weight of the mouse groups shown in (a). (e) BAT weight of the mouse groups shown in (a). (f) H&E, CD3, F4/80 and Ly6G staining with (g) NAS evaluation, (h) CD3 quantification, (i) F4/80 aggregates quantification, (j) Ly6G quantification of the mouse groups shown in (a). Scale bar: 100µm. (k) Serum cholesterol of the mouse groups shown in (a). (l) Serum HDL cholesterol of the mouse groups shown in (a). (m) Serum TGs of the mouse groups shown in (a). (n) Serum ALT of the mouse groups shown in (a). (o) Serum AST of the mouse groups shown in (a). (p) Serum ALP of the mouse groups shown in (a). (ND n= 4 mice, except for j, where n=3; CDHFD n=8 mice; WD n= 6 mice). All data are shown as mean ± SEM. All data were analysed by one way ANOVA

and Dunnett's multiple comparison test, or two way ANOVA and Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

7.2. Tropifexor can reverse NASH in an early therapeutic approach

After establishing a mouse model of early NASH consisting of 3.5 months of CDHFD or WD feeding, I collaborated with Dr. Marta Szydlowska to define a therapeutic experimental plan to test the effects of FXR agonism on early NASH progression. To this end, I designed the initial treatment options using several FXR agonists: Fexaramine (Fex), Tropifexor (Tropi), Obeticholic Acid (OCA) and GW4064, opting for an oral administration by supplementing the compounds to the diets.

In these newly designed study, after feeding the mice with ND, CDHFD and WD for three months and a half as above mentioned, I maintained the mice for additional five to six weeks on the respective diets supplemented with the FXR agonists (Fig. 8a). ND and CDHFD fed mice received Fexaramine or Tropifexor, while WD fed mice received Fexaramine, Tropifexor, OCA or GW4064. After overnight fasting, the mice received the last dose of compound via oral gavage, 4 hours prior to dissection.

One key analysis I performed in collaboration with Dr. Manfred Birkel and Dr. Claus Kremoser, to confirm the bioavailability of the compounds, revealed that Tropifexor, in contrast to Fexaramine, was detectable in plasma and liver in large amounts. It also confirmed that Tropifexor could act on both intestinal and hepatic FXR, while Fexaramine is rather intestine-restricted, due to its limited absorption (Fig. 8b-c).

During the experiment, I observed a progressive reduction of body weight in the mice treated with Tropifexor in the ND fed mice (Fig. 8d). The intra-peritoneal glucose tolerance test (IPGTT) revealed a major reduction of blood glucose levels in the group treated with Tropifexor under ND feeding (Fig. 8e). Histological analysis performed in collaboration with Prof. Thomas Longerich, followed by NAS scoring, revealed that Tropifexor reduced the score even below the ND baseline, while Fexaramine on the contrary induced a slight deposition of lipids, confirmed with Sudan Red staining (Fig. 8f-g-h). In the CDHFD group, I observed that Tropifexor showed a remarkable reduction of body weight, together with improved glucose sensitivity (Fig 8i-j). After histological and immunohistochemical analysis of the liver sections, I observed a mild worsening of hepatic steatosis in the livers after Fexaramine treatment and a complete clearance of lipids after Tropifexor (Fig. 8k).

Interestingly, Fexaramine performed slightly better than Tropifexor in the NAS evaluation (Fig. 8l), although was probably due to reduction of inflammation, since, as mentioned above, the lipid content was overall increased in Fexaramine livers. I carried out the experiment in the same modalities above described also with mice under WD feeding, and I implemented groups treated with other FXR agonists such as OCA and GW4064 in addition to the previously described ones. I noticed that body weight was in part reduced after treatment with each of the FXR agonists, but the group treated with Tropifexor lost more body weight than the others (Fig. 8n). Furthermore, all agonists improved the intrahepatic glucose tolerance to an average extent, and Fexaramine only partially (Fig. 8o). However, Tropifexor, but not the other compounds, reversed the hepatic steatosis, and showed a significantly lower NAS (Fig 8p-q). Lastly, I performed a triglyceride assay on the liver tissue, and I noticed a significant reduction in the groups fed CDHFD and WD after Tropifexor, consistent with the Sudan Red staining in the previous groups (Fig. 8r).

With the current information, I concluded that Tropifexor was the only FXR agonists capable of reversing NASH, driving consistent improvements of body weight, glucose metabolism, hepatic steatosis, and reduced the NAS below the NAFLD threshold. Fexaramine did not show any relevant effect on NASH, OCA and GW4064 presented some mild degree of improvement, especially towards glucose tolerance, but did not respond similarly well in the context of liver steatosis.

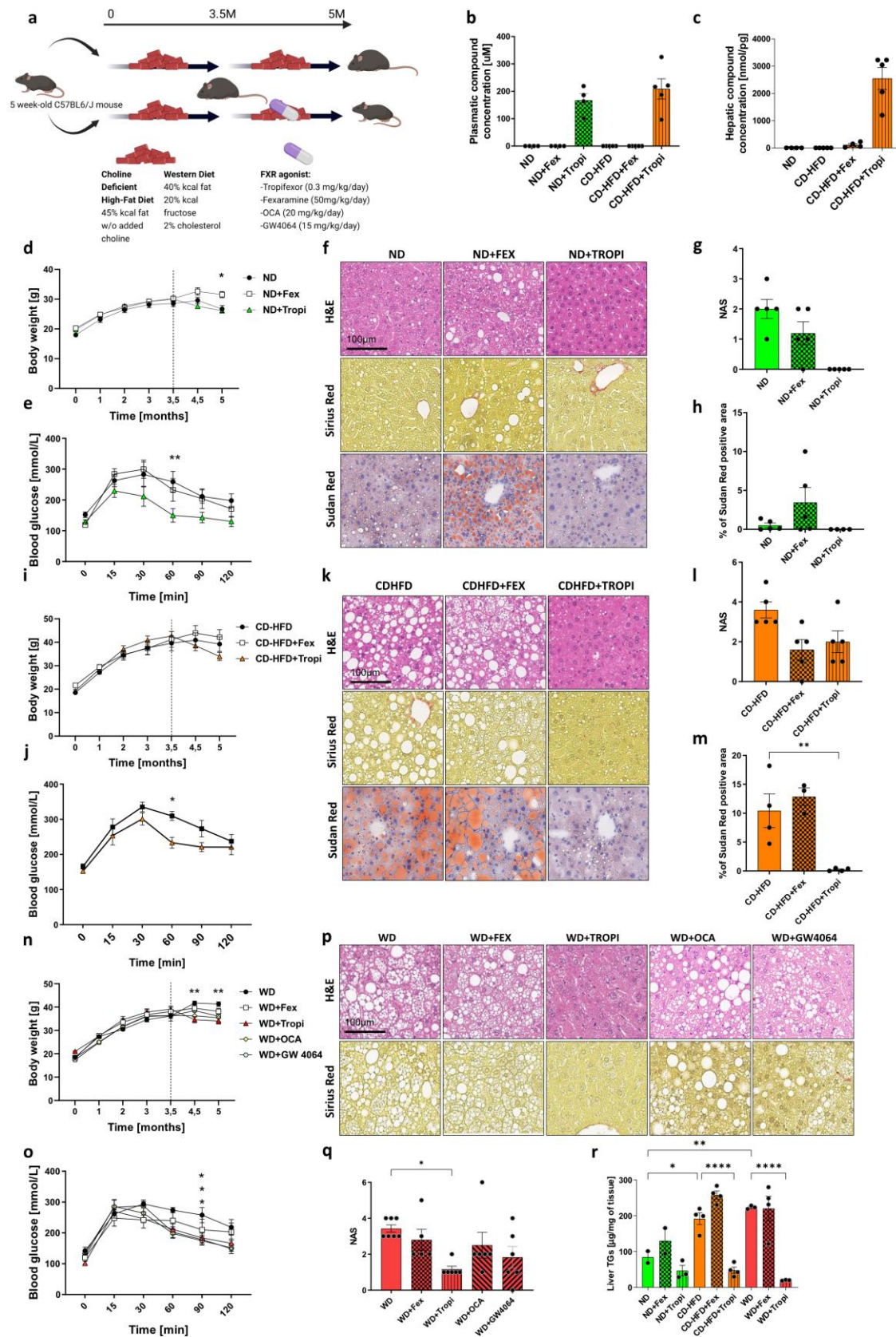


Figure 8. Tropifexor reverses early NASH from mice fed CDHFD and WD after 6 weeks of treatment. (a) Experimental setup of the short-term therapeutic approach with FXR agonists, where mice were initially fed ND, CDHFD or WD for 3.5 months and administered the respective diet supplemented with FXR agonists. (b) Plasmatic concentration of Fexaramine or Tropifexor in the

various mouse groups (c) Hepatic concentration of Fexaramine or Tropifexor in the various mouse groups (from the figures above ND n=4 mice, ND+Fex n=4 mice CDHFD n=5 mice, CDHFD+Fex n=5 mice in b and n=4 mice in c, CDHFD+Tropi n=5 mice). (d) Body weight development of mice administered FXR agonists under ND feeding. Asterisks indicate significance of group ND+Tropi compared to ND fed mice. (e) IPGTT of the mouse groups shown in d. Asterisks indicate significance of group ND+Tropi compared to ND fed mice. (f) H&E, Sirius Red and Sudan Red staining with (g) NAS evaluation, (h) Sudan Red quantification (ND n=5 mice except in d where n=6 mice and in e where n=4 mice, ND+Fex n=5 mice, ND+Tropi n=5 mice except in d where n=6 mice and in h where n=4 mice) of the mouse groups shown in d. Scale bar: 100µm. (i) Body weight development of mice administered FXR agonists under CDHFD feeding. (j) IPGTT of the mouse groups shown in i. Asterisks indicate significance compared to CDHFD fed mice. (k) H&E, Sirius Red and Sudan Red staining with (l) NAS evaluation, (m) Sudan Red quantification of the mouse groups shown in i (CDHFD n=6 mice except in l where n=5 mice and in m where n=4 mice, CDHFD+Fex n=6 mice in i, n=5 mice in l, n=3 mice in m, CDHFD+Tropi n=4 mice except in i where n=6 mice and in l where n=5 mice). Scale bar: 100µm. (n) Body weight development of mice administered FXR agonists under WD feeding. Asterisks indicate significance of group WD+Tropi compared to WD fed mice. (o) IPGTT of the mouse groups shown in d. Asterisks indicate significance of groups WD+Tropi, WD+OCA and WD+GW4064 compared to WD fed mice. (p) H&E and Sirius Red staining with (q) NAS evaluation of the mouse groups shown in p (In all WD groups above n=6 mice except q where WD n=7 mice and WD+Fex n=5 mice). Scale bar: 100µm. (r) Hepatic TGs of the mouse groups shown in d, i and n (ND n=2 mice, ND+Fex n=2 mice, ND+Tropi n=3 mice, CDHFD n=4 mice, CDHFD+Fex n=4 mice, CDHFD+Tropi n=4 mice, WD n=3 mice, WD+Fex n=4 mice, WD+Tropi n=3 mice). All data are shown as mean ± SEM. All data were analysed by one way ANOVA and Tukey's multiple comparison test, or two way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.3. Tropifexor promotes adipose tissue reduction and lowers serum cholesterol through activated gut-liver FXR signalling, without increased energy expenditure

As mentioned previously, Tropifexor induced a remarkable reduction of body weight in the mice. From the tissue collected during necropsies, I noticed that the reduction in body weight reflects mostly the decrease of fat deposits. Indeed, Tropifexor showed significant improvement of eWAT in the WD group and trends of reduction of adipose tissue in all other compartments (Fig. 9a-b-c). This supported the elevated reduction of cholesterol I detected in the serum after Tropifexor, together with a minor improvement of triglycerides (Fig. 9d-e). The measurement of serum markers for liver damage identified that Fexaramine induces ALT higher than the control diet alone, Tropifexor maintained relatively lower values but did not arrest completely the progressive increase, while OCA and GW4064 seemed to perform slightly better (Fig. 9f). Serum AST levels showed on the contrary that OCA and GW4064 could elevate this marker more than the control diet, while Fexaramine or Tropifexor did not induce relevant changes. Interestingly, Tropifexor induced ALP elevation, in particular in the ND and WD fed mice, while Fexaramine induced ALP only in CDHFD (Fig. 9h).

It became reasonable to question whether the effects on body weight and adipose reduction, together with lowering of cholesterol were a consequence of altered food intake or changes in energy expenditure. To answer this question, I repeated the experiment with FXR agonists after early NASH induction. The experimental setup was the same as above described, however, during the last week prior necropsies I performed, in collaboration with Dr. Adnan Ali and Dr. Suchira Gallage, a metabolic phenotypic analysis, by housing the mice individually in a TSE unit. The results showed some differences, but these were exclusively diet dependent, as the food intake did not change between the groups fed the same diet, independently from the compound tested (Fig. 9i). A similar observation was true for the water intake, although Tropifexor induced a slight, non-significant increase of the water intake in the CDHFD group (Fig. 9j). The energy expenditure, calculated as volume of oxygen consumed, was comparable across all groups (Fig. 9k). Similarly, the Respiratory exchange ratio (RER), an index which can indicate whether the metabolism is based on carbohydrates or fat, was showing differences only between ND and CDHFD or WD, as the latter two diets are richer in fat and thus inducing a slightly lower carbohydrate utilisation; however, neither Fexaramine or Tropifexor induced any change in RER (Fig. 9l).

Ruling out changes in energy expenditure and food intake, I performed gene expression analysis of the ileum, which indicated that Tropifexor strongly activated the signalling downstream FXR by elevating *Shp* and *Fgf15* expression. Fexaramine seemed to induce the expression of these genes only partially in the CDHFD group (Fig. 9m). I hypothesized that the activation of the intestinal FXR signalling was critical to bridge FXR induction in the liver and drive the observed effects, denoting the difference between compounds such as Fexaramine, with a low potency and a mild intestinal-restricted efficacy, and Tropifexor, on the other hand highly potent and more bioavailable, allowing a broader effect across the gut-liver axis. Therefore, I analysed the expression of FXR and downstream targets in the liver by qPCR. I could notice that, although the expression of FXR itself did not seem to change, the major downstream target, *Shp* was upregulated, and this culminated in the downregulation of the rate-limiting enzyme for the classical BA synthetic pathway, *Cyp7a1* (Fig. 9n-o). In addition, I observed an increase of *Fgf21*, in particular in the WD group, indicative of activation of a pivotal metabolic target, which likely was responsible to bridge the signalling with the adipose tissue (Fig. 9n-o). In the effort to describe the response of liver metabolism upon Tropifexor administration, I screened several metabolic markers with qPCR. Genes as Acyl-CoA Synthetase Medium Chain Family Member 2 (*AcsM2*), acyl-CoA synthetase long-chain family member 1 (*Acs11*) and *Ppar δ* were downregulated, among others, to denote an abrogation of

FAs oxidation. However, I noticed an upregulation of Squalene epoxidase (*Sqle*) and Mevalonate Diphosphate Decarboxylase (*Mvd*) consistent with a general increase of cholesterol metabolism and lipid droplet formation. Lastly, I reported a downregulation of Stearoyl-CoA Desaturase (*Scd1*), indicative of a remarkable reduction of *de novo* lipogenesis (Fig. 9p). As the primary effect of FXR activation is the downregulation of the BAs synthesis, I collaborated with Dr. Diran Herebian to analyse the total BAs pool in the collected samples. I observed from the analysis of the bile that Tropifexor efficiently reduced the BAs in all diet groups, while Fexaramine did it only in part. However also OCA and GW4064 significantly reduced the BAs (Fig. 9q). The decrease of the BAs content in bile was in general the most prominent, but also in the liver this effect was observed (Fig. 9r). Oddly, the BAs in serum were found slightly higher in Tropifexor, but only on ND and CDHFD groups, whereas the trend was maintained in the WD group, denoting also some diet-dependent responses (Fig. 9s).

With the above described results, I concluded that the strong intestinal FXR signalling activation, and likely the high bioavailability of Tropifexor described in the previous part, was essential for an induction of the hepatic FXR signalling that allowed a proper abrogation of BAs synthesis. This further stimulated a reduced intestinal lipid uptake and the metabolic changes that favoured a loss of a remarkable amount of adipose tissue. Interestingly, these major metabolic effects were not dependent on altered food intake nor energy expenditure, suggesting that the fats stored in the liver (and most likely in the adipose tissue), were rather mobilized not for energy production but exported in lipid droplets, while the production and storage of lipids *de novo* was halted. However, it is important to specify that the reduction of BAs were certainly necessary, but not sufficient to bring to the body weight reduction and steatosis resolution observed, as other compounds importantly reduced BAs production, but without exerting any relevant impact on body weight and hepatic steatosis. Some important considerations on liver damage should also be made, as despite not particularly high ALT or AST levels were detected, the observed elevated ALP could be indicative of cholestasis, which is in net contrast with the lower BAs in bile and liver, therefore it is likely that some degree of stress at the level of bile ducts and cholangiocytes was present.

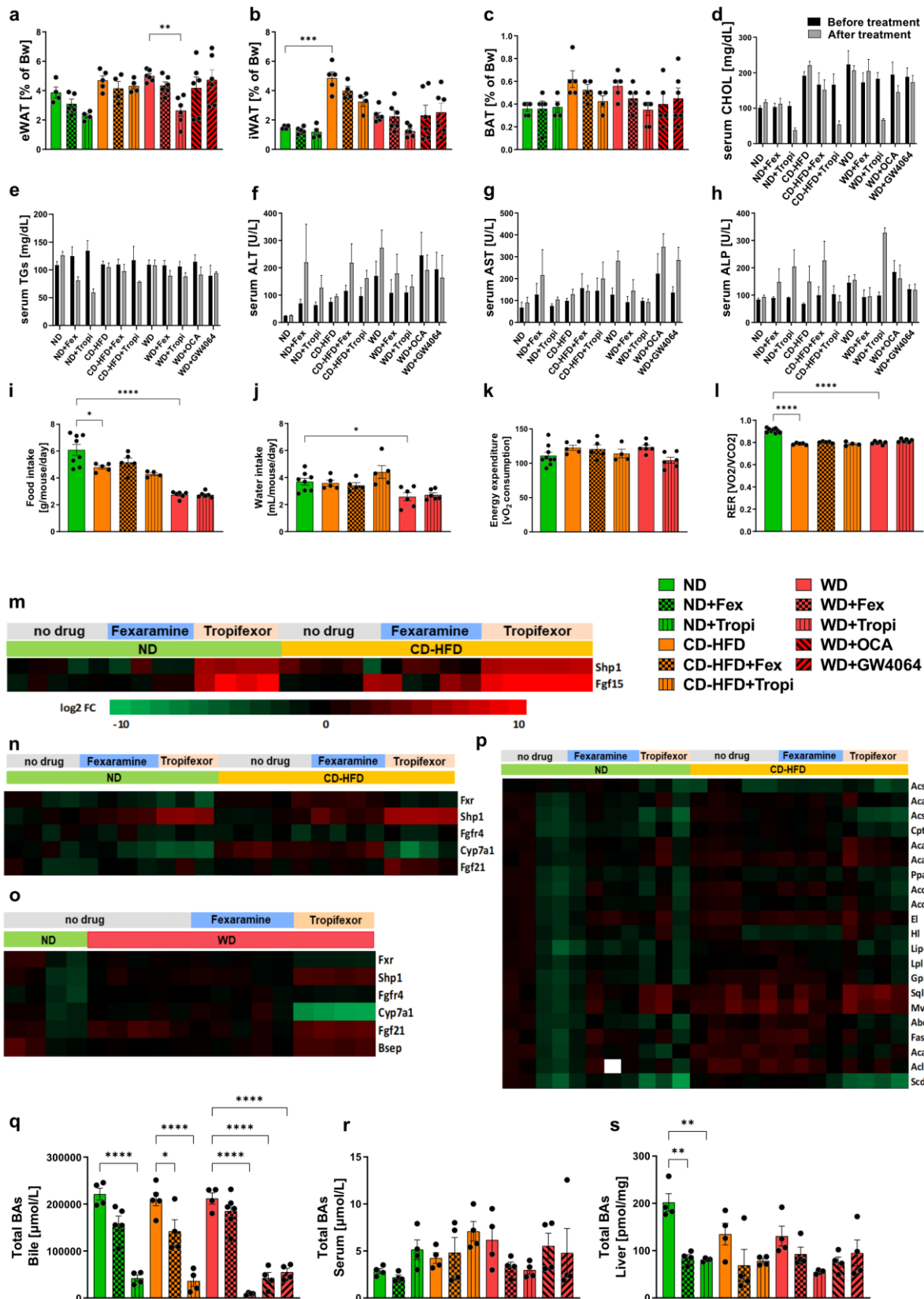


Figure 9. Tropifexor drives adipose tissue reduction and lowers serum cholesterol and triglycerides without affecting energy expenditure, due to intestinal and hepatic FXR signalling activation that leads to bile acids reduction. (a) eWAT to body weight ratio, (b) iWAT to body weight ratio and (c) BAT to body weight ratio of the mice shown in Figure 8. (d) Serum cholesterol, (e) serum TGs, (f) serum ALT, (g) serum AST and (h) serum ALP of the mouse groups shown in Figure 8. (i)

Metabolic phenotyping measuring average of 3 days of food intake, (j) water intake, (k) energy expenditure and (l) RER of mice under ND, CDHFD, CDHFD+Fex, CDHFD+Tropi, WD, and WD+Tropi (n) Heat map of qRT-PCR analysis of *Shp* and *Fgf15* performed on ileum from ND and CDHFD groups shown in Figure 8 (displayed as Log2 FC). (o) Heat map of qRT-PCR analysis of *Fxr* and downstream targets performed on liver from ND and CDHFD groups and (o) WD groups shown in Figure 8 (displayed as Log2 FC). (p) Heat map of qRT-PCR analysis of metabolic genes performed on liver from ND and CDHFD groups shown in Figure 8 (displayed as Log2 FC). (q) Total BAs pool measured on bile, (r) liver tissue and (s) serum from the mouse groups shown in Figure 8. ND n=4 mice, except in d,e,f,g,h, where n=6 mice, in i, where n=8 mice, and in j,k,l where n=9 mice; ND+Fex n=5 mice, except in r,s where n=4 mice, ND+Tropi n=4 mice, except in d,e,f,g,h where n=5 mice, CDHFD n=5 mice, except in r,s where n=4 mice, WD n=6 mice, except in c where n=5 and in q,r,s where n=4 mice, WD+Fex n=6 mice, except in q, where n=4 mice, WD+Tropi n=6 mice, except in q,r,s where n=4 mice, WD+OCA n=6 mice, except in q,r,s where n=4 mice, WD+GW4064 n=6 mice, except in q,r,s where n=4 mice). All data are shown as mean $\pm$ SEM. All data were analysed by one way ANOVA and Tukey's multiple comparison test, or two way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.4. Tropifexor modulates inflammation by altering T-cell infiltration and immune profile

With an interest in characterizing cell infiltration and inflammation after FXR agonism, I analysed the livers after IHC staining for CD3. I could note a slight expansion of lymphocytes after Tropifexor, consistent across all diets (Fig. 10a-b). I performed a more detailed analysis with flow cytometry and observed an increase of CD45 cells after Tropifexor (Fig. 10c); specifically, both CD4⁺ and CD8⁺ T-cells were increased, although none significantly (Fig. 10d). An initial screening of CD4⁺ and CD8⁺ T-cells revealed no peculiar changes in T-cell activation, as CXCR6 levels were comparable; Fexaramine slightly increased PD-1 and TIM3 abundance in both CD4⁺ and CD8⁺ T-cells, although not significantly (Fig 10e-f). Tropifexor drove a minor expansion of CD19⁺ cells, restoring physiological levels of B-lymphocytes from WD-induced reduction (Fig. 10g). Although I did not see alterations in NKT or NK cells abundance, Tropifexor induced a significant increase of NKT cells expressing α -Gal and $\gamma\delta$ -T-cells positive for PD-1, both described for a potential role on anti-tumour effect (Fig 10h) [389-392]. Furthermore, Tropifexor induced a decrease of effector T-cells and increased the amount of naïve T-cells, in both CD4⁺ and CD8⁺ compartments (Fig. 10i-j). As for cytokines expression, I observed a sharp decrease of all the pro-inflammatory markers investigated, such as Granzyme B (GzmB), IFN γ , and TNF α , and a slight depression of proliferation, assessed with Ki-67, in both CD4⁺ and CD8⁺ T-cell compartments in the group WD+ Tropifexor (Fig. 10k-l). I observed the same reduction to a lower extent also in the CXCR6⁺ subset of CD4⁺ and CD8⁺ T-cells, indicating that also the activated cells presented a dampened inflammatory

phenotype. Lastly I assessed the expression of the same markers based on PD-1 expression, and observed the same trends in the Tropifexor group (Fig. 10o-p).

I therefore concluded that Tropifexor drove an immunosuppressive response, which likely was compensated by an increase of circulating naïve T-cells, however not activated and promoting an anti-inflammatory environment.

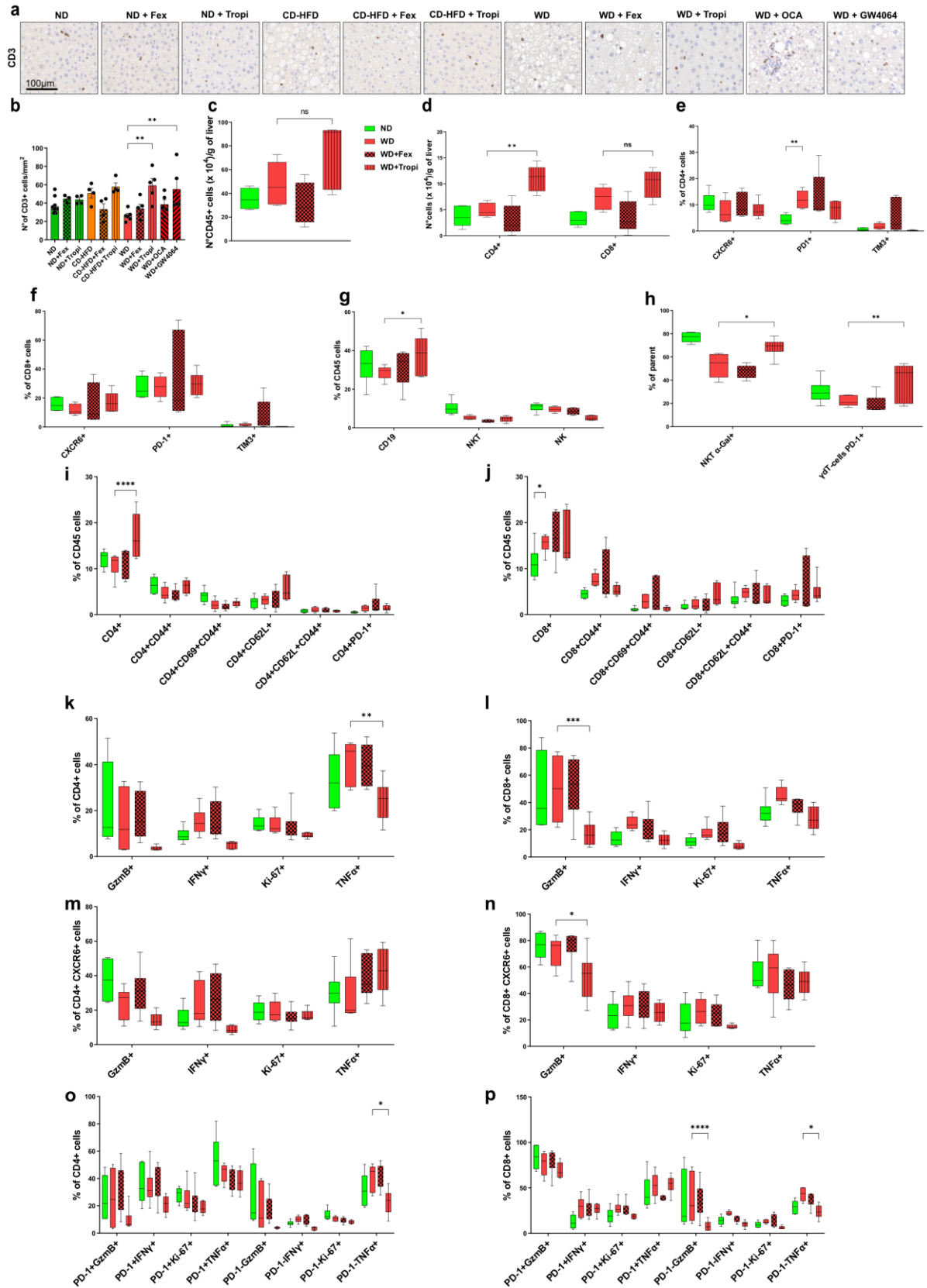


Figure 10. Tropifexor mediates an anti-inflammatory response in the T-cell compartment favouring early NASH resolution. (a) CD3 IHC staining and (b) CD3 quantification of the mouse groups shown in Figure 8 (ND n=11 mice, ND+Fex n=3 mice, ND+Tropi n=4 mice, CDHFD n=4 mice, CDHFD+Fex n=4 mice, CDHFD+Tropi n=4 mice, WD n=6 mice, WD+Fex n=5 mice, WD+Tropi n=5 mice, WD+OCA n=5 mice, WD+GW4064 n=5 mice). Scale bar: 100µm. (c) Flow cytometry analysis

displaying total number of CD45+ cells, (d) total number of CD4+ and CD8+ T-cells, (e) percentage of CD4+ T-cells and (f) percentage of CD8+ T-cells expressing CXCR6, PD-1 and TIM3, (g) percentage of CD45+ cells of B cells (CD19+), NKT cells (Nk1.1+ TCR β +) and NK cells (Nk1.1+ TCR β -), (h) percentage of α -Gal+ NKT cells and PD-1+ $\gamma\delta$ -T-cells, (i) percentage of CD45+ cells of CD4+ T-cells, effector memory cells (CD44+), resident memory cells (CD69+ CD44+), naïve cells (CD62L+), central memory cells (CD62L+ CD44+) and PD-1+ cells within the CD4+ T-cell compartment and (j) CD8+ T-cell compartment of the mouse groups shown in Figure 8. (m) Flow cytometry analysis of cytokines expression within CD4+ CXCR6+ T-cells and (n) CD8+ CXCR6+ T-cells. (o) Expression of cytokines within CD4+ T-cell compartment and (p) CD8+ T-cell compartment, divided for PD-1 positivity or negativity of the mouse groups shown in Figure 8 (all groups n=6 mice/group, except for c, where ND n=5 mice, WD n=5 mice, WD+Fex n=4 mice, WD+Tropi n=5 mice, and d, where n=5/group). Data in b are shown as mean $\pm$ SEM; all other data are displayed in box and whiskers showing Min to Max. All data were analysed by one way ANOVA and Tukey's multiple comparison test, or two way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.5. Tropifexor shapes myeloid immunity by altering Kupffer cells spatial properties and inducing a tolerogenic shift.

As the T-cell infiltration was increased with Tropifexor, but with a marked reduction of pro-inflammatory features, I wanted to determine the effects also in the myeloid immune compartment. I thus analysed the F4/80 IHC staining and noted a remarkable reduction of aggregates together with major alteration in the cell shape (Fig. 11a-b). I analysed further these changes and, in collaboration with Dr Lukas Amann, performed a 3D reconstruction of KC, which was further evaluated to measure volume, sphericity and spatial distribution (Fig. 11c). I could observe a net increase of macrophage sphericity in all the groups treated with Tropifexor in all diets, driving likely an immunosuppressive phenotype and dampening phagocytic capacity (Fig. 11d).

To verify the reshaping of innate immunity I further evaluated through flow cytometry the abundance of myeloid cells and attempted to define the immune activation of macrophages. As already emerged from IHC analysis, I noticed that after Tropifexor all myeloid cells were in vast majority reduced, especially KC, plasmacytoid and classical dendritic cells (pDCs and cDCs) as well as reparative and inflammatory infiltrating monocytes (Fig. 11e).

A characterization of KC identified an increase of CD206 and a slight decrease of MHCII and PD-L1, suggesting a shift towards M2 polarization, which correlates with an anti-inflammatory phenotype (Fig. 11f). I detected no particular changes in the pDC compartment; however, cDCs presented with strongly reduced MHCII and PD-L1 (Fig. 11g). In a similar way, also the infiltrating monocytes presented with a reduction of the same markers after Tropifexor (Fig 11h-i).

With the obtained data, I could conclude that Tropifexor also reshaped myeloid-driven immunity and induced a more tolerogenic environment. KCs were importantly affected by the treatment, and the three-dimensional alterations observed could indicate impairments in phagocytic capacity and aggregates formation, possibly influenced by FXR signalling activation in myeloid cells. Furthermore, macrophages have an important role in orchestrating the immune response in coordination with the T-cells, and a reduction of the markers assessed, like MHCII and PD-L1, normally important in maintaining the T-cell response within specific boundaries, could be a compensation for the lack of cytotoxic response in T-cells observed in the previous chapter.

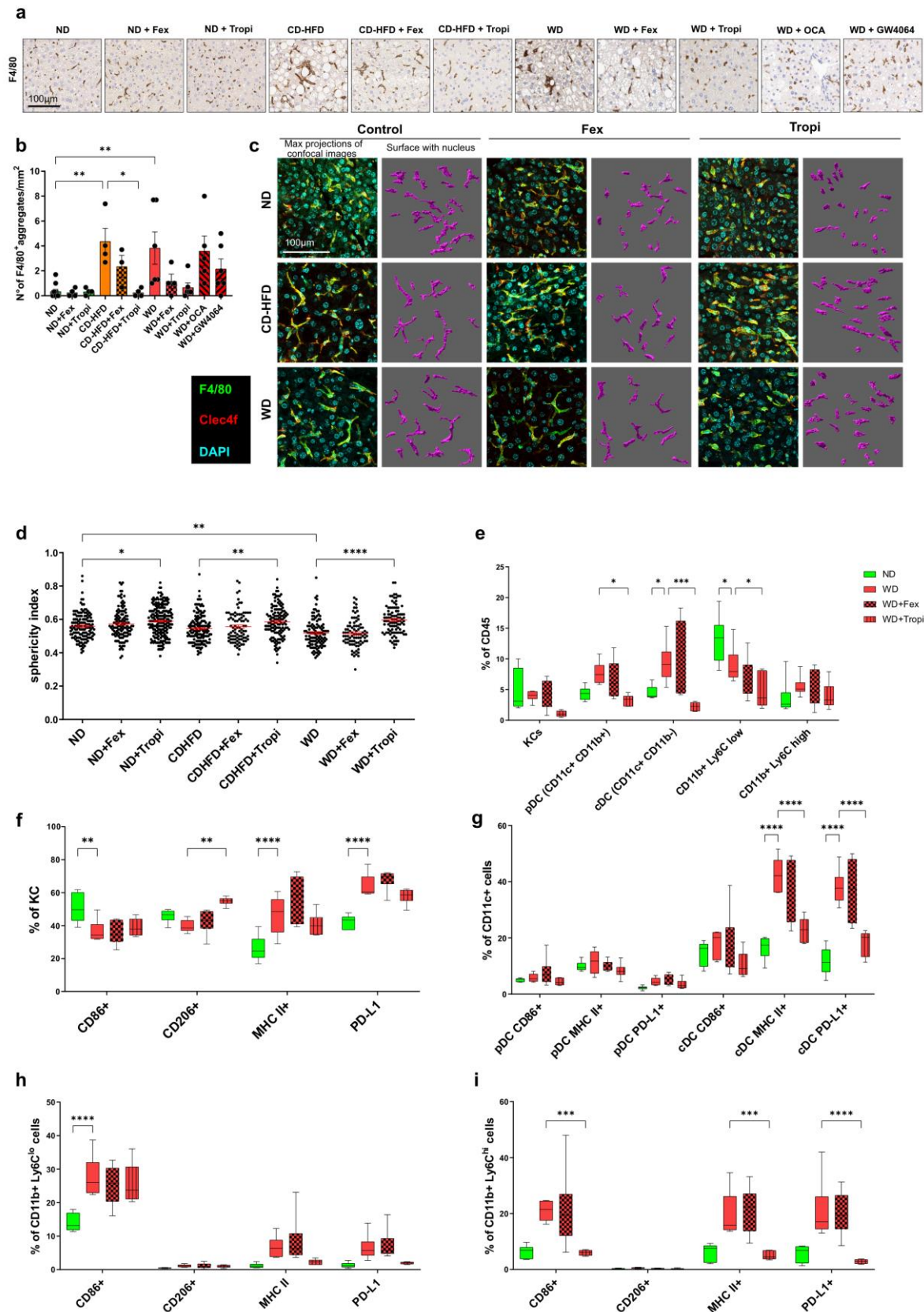


Figure 11. Tropifexor induces morphological changes in the myeloid compartment supporting anti-inflammatory activity. (a) F4/80 IHC staining and (b) F4/80 quantification of aggregates of the mouse groups shown in Figure 8 (ND n=11 mice, ND+Fex n=4 mice, ND+Tropi n=4 mice, CDHFD n=4 mice, CDHFD+Fex n=3 mice, CDHFD+Tropi n=4 mice, WD n=6 mice, WD+Fex n=4 mice, WD+Tropi n=6 mice, WD+OCA n=5 mice, WD+GW4064 n=6 mice). Scale bar: 100µm. (c) 3D

reconstruction of macrophages from analysis on confocal images after immunofluorescence staining with F4/80 (green channel), Clec4f (red channel) and DAPI as counterstaining (blue channel) on liver sections from mouse groups shown in Figure 8. (d) Analysis of sphericity index of cells from the 3D reconstruction shown in e (ND n=137 cells, ND+Fex n=122 cells, ND+Tropi n=208 cells, CDHFD n=147 cells, CDHFD+Fex n=85 cells, CDHFD+Tropi n=133 cells, WD n=115 cells, WD+Fex n=86 cells, WD+Tropi n=98 cells). (e) Flow cytometry analysis showing percentage of CD45⁺ cells of KCs, pDCs, cDCs, CD11b+Ly6C^{lo} and CD11b+Ly6C^{hi} cells, (f) percentage of KCs expressing CD86, CD206, MHCII, PD-L1, (g) percentage of CD11c⁺ cells expressing CD86, MHCII and PD-L1 divided in pDCs (CD11b⁻) and cDCs (CD11b⁺), (h) percentage of CD11b+Ly6C^{lo} cells and (i) CD11b+Ly6C^{hi} cells expressing CD86, CD206, MHCII, PD-L1, from the mouse groups shown in Figure 8 (n=6 mice/group). Data in b and d are shown as mean $\pm$ SEM; all other data are displayed in box and whiskers showing Min to Max. All data were analysed by one way ANOVA and Tukey's multiple comparison test, or two way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.6. Tropifexor's influence on hepatocyte proliferation drives polyploidy and induces pro-carcinogenic pathways, balanced by senescence at the expenses of DNA damage.

After determining positive effects of Tropifexor on NASH, I also reported and characterized relevant concerning effects. The first important observation that required investigation was hepatomegaly, which Tropifexor induced in all diet groups (Fig. 12b). As this enlargement of the liver could have been the result of increase hepatocytes proliferation, I assessed Ki-67 abundance through IHC and could notice a strong induction driven by Tropifexor in all diet groups (Fig 12a-c). As the hepatocytes in the livers treated with Tropifexor presented with increased nuclear size, I suspected that the induction of proliferation was somehow halted, and tested this through p21 staining, confirming that Tropifexor induction of proliferation was counteracted by activation of cell cycle arrest, which also coincides with the induction of senescence (Fig 12a-d). Furthermore, I evaluated the presence of DNA damage by staining the liver sections for γ H2ax. The staining revealed that an important degree of DNA damage was mounting on especially in the WD group, after Tropifexor treatment (Fig 12a-e). In addition, as a previous study reported the induction of antioxidant response after Tropifexor, I wanted to test NQO1, marker belonging to the Nrf2/Keap pathway and found it strongly upregulated in IHC (Fig. 12a).

I suspected that the induction of hepatocytes proliferation with a concomitant induction of cell cycle arrest could result in the presence of polyploidy hepatocytes, already described to be a feature of FXR-dependent proliferative response. To verify this hypothesis, I collaborated with Dr. Chantal Desdouet and Pierre Cordier, and tested the livers from the cohorts previously described for polyploidy. The results indicated in the groups treated with Tropifexor a marked

reduction of the 2n nuclei followed by an expansion of the tetra-and-octaploid nuclear fraction, consistent with polyploidy induction (Fig. 12f-h-i). Furthermore, a staining of phospho-histone H3 (pHH3), a protein phosphorylated during chromatin condensation in mitosis, revealed a significant increment of G2 and M nuclei, especially in the WD group treated with Tropifexor (Fig 12g-j-k).

I further evaluated the signalling downstream FXR with western blot to assess the induction of the molecular markers that govern the BAs metabolism and concomitant factors that could have a role in the proliferation and are known for having a role in cancer. I detected that CYP7a1 was partially decreased, corroborating gene expression data, together with FXR, due to probable feedback inhibition that counterbalanced its exogenous constitutive activation. SHP, major downstream effector of FXR was increased as expected, and lastly, several markers of the NF- κ B and MAPK/ERK and JNK pathways were higher in the groups that received Tropifexor, together with NQO1, as assessed with IHC (Fig 12l). Gene expression analysis revealed a slight downregulation of genes involved in fibrosis, however, contrary to expectations, I found upregulated markers of inflammatory response such as *Tnf- α* , *Ccl2* and *Ccl5* (Fig. 12m).

With the observed findings to this point, I validated reports from other research groups and casted further light on the role and effects of FXR agonism in NASH. The induction of proliferation observed was clearly fuelling the hepatomegaly observed during necropsies, the induction of p21 was an attempt of the hepatocytes to put a limit to the mounting proliferation, impeding the cells to divide and causing an important increase of ploidy in the livers. The induction of polyploidy is not alarming per se, but in the context of NASH could allow a synergism of molecular alteration driving malignant transformation. In addition, the high levels of NQO1 were indicative of a strong anti-oxidant response induced by Tropifexor, and could be increased to contrast the effects that NASH has on oxidative stress, but NQO1 was not particularly elevated in the control WD and CDHFD. Furthermore, the cells were exposed to increased DNA damage, and a concomitant activation of several pathways, well described for their role in cancer, would definitely favour its development if the conditions were maintained for a long time.

I therefore speculated that a constitutive exogenous FXR activation, in particular through Tropifexor administration, could ameliorate NASH and reverse major features that drive its progression, such as hepatic steatosis and fibrosis. However, a long-term administration could harbour a negative impact on cancerous transformation, as well as tumour

survival and progression. To verify this possibility, I further investigated the effects of therapeutic administration of experimental FXR agonists in the context of established NASH and progression of NASH towards development of HCC.

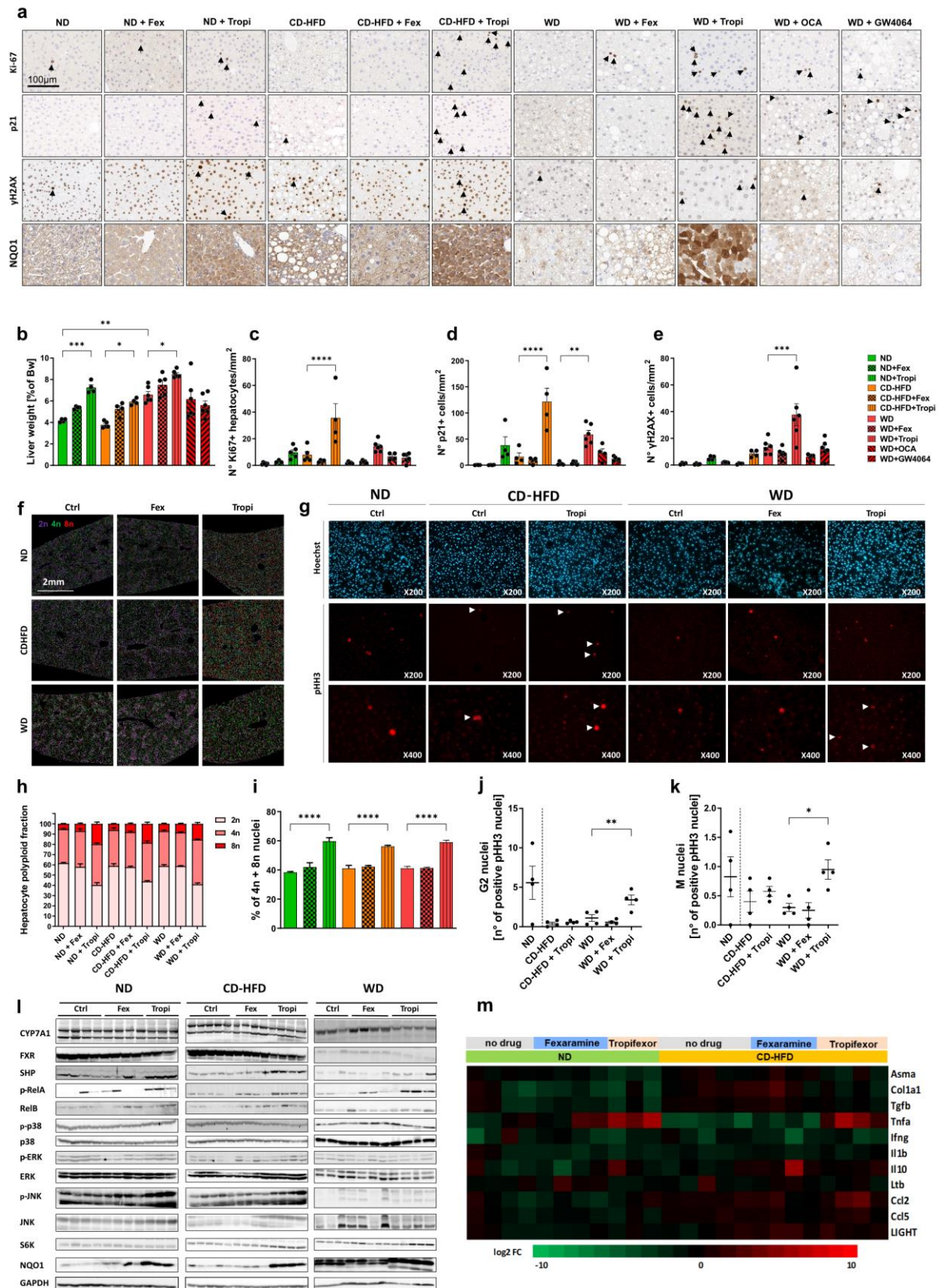


Figure 12. Tropifexor pro-carcinogenic signature drives proliferation and hepatomegaly, contrasted by p21 activation and induction of polyploidy, favouring DNA damage and potentially increased mutational load. (a) IHC staining of Ki-67, p21, γ H2ax, NQO1 of the groups shown in Figure 8. Scale bar: 100 μ m. (b) Liver to body weight ratio of the mouse groups shown in Figure 8. (c) Ki-67 quantification of the staining shown in a. (d) p21 quantification of the staining shown in a (e)

γ H2ax quantification of the staining shown in a. (f) in situ hybridization for ploidy analysis on livers of the mouse groups shown Figure 8. 2n nuclei indicated in purple, 4n nuclei indicated in green, 8n nuclei indicated in red. (g) pHH3 and Hoechst immunostaining on livers of the mouse groups shown in Figure 8 (200x). (h) Percentage of Hepatocyte polyploidy fraction from analysis shown in f (i) Percentage of 4n and 8n nuclei from analysis shown in f. (j) Quantification of G2 nuclei from staining shown in g. (k) Quantification of M nuclei from staining shown in g (in j and k n=4 mice/group). (l) Western blot analysis on liver lysate from the mouse groups shown in Figure 8. (m) qRT-PCR on liver tissue on fibrogenic and inflammatory genes from the mouse groups shown in Figure 8. (ND n=4 mice except in c where n=6 mice, ND+Fex n=4 mice, except in c where n=3 mice, ND+Tropi n=5 mice, except in d and e where n=4 mice, CDHFD n=5 mice, except in d where n=4, CDHFD+Fex n=3 mice, except in c, d and e where n=4 mice, CDHFD+Tropi n=4 mice, WD n=6 mice, except in c and d where n=5 mice, WD+Fex n=6 mice, except in c and d where n=5 mice, WD+Tropi n=6 mice, WD+OCA n=4, WD+GW4064 n=6 mice, except in d where n=5 mice). All data are shown as mean $\pm$ SEM. All data were analysed by one way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.7. Tropifexor significantly improves metabolic features of established NASH but increases incidence of hepatic lesions

As mentioned above, FXR agonists could harbour a great potential for the treatment of NASH. From the short-term experiments I performed, I was able to detect several positive effects leading to the improvement of glucose and lipid metabolism and modulation of inflammation and liver pathology with Tropifexor administration. However, the constant stimulation of hepatocyte proliferation, induction of ploidy and senescence with concomitant DNA damage raised a serious concern in the context of hepatic carcinogenesis that required further evaluation.

To this end, I established a new experimental setup by prolonging the initial feeding duration up to six months and allowing the mice to develop a more severe NASH. After this time, I administered the diets supplemented with the FXR agonists for four to six additional months, and as the previous experiment, the last dose was delivered via oral gavage (Fig. 13a). Given the major interest in Tropifexor and its previously seen positive effects on the FXR signalling and the hepatic pathology, I administered three distinct doses to the mice, 0.08mg/kg/day (low dose), 0.15mg/kg/day (mid dose) and 0.3mg/kg/day (high dose). Of note, I maintained the mice on Tropifexor low dose until twelve months, together with control groups on ND and WD, while I kept all the other groups until ten months. I performed also the experiment with mice on CDHFD and Tropifexor mid dose, to confirm the findings with another dietary model, as well as tested Tropifexor high dose on mice on ND, to have the chance to determine the effects of prolonged FXR agonism in absence of NASH. In parallel, I also administered OCA and GW4064 to the WD fed mice, with the identical dose employed during the short-term experiments.

The initial finding I observed was, as for the short-term experiments, a marked reduction of body weight, which was particularly pronounced during the initial month of treatment, and reached a plateau for the remaining duration of the experiment, with different extent of reduction depending on the diet employed (Fig. 13b). I assessed the effects of the agonists on liver histology and observed a remarkable clearance of hepatic lipids, especially with Tropicifexor high dose (Fig. 13c). The mice that received OCA or GW4064 did not show any improvement of the hepatic steatosis, and I observed no particular impact on liver steatosis on ND with Tropicifexor high dose, anyway absent in the control diet (Fig. 13d). The NAS evaluation I performed in collaboration with Prof. Thomas Longerich revealed a dose-dependent reduction of NASH, which was completely resolved for the mice on Tropicifexor mid and high dose, and partially for the mice on Tropicifexor low dose, but not changing in the case of OCA and GW4064 treatment (Fig. 13e). In addition, quantification of Sirius Red staining showed that Tropicifexor high dose significantly reduced liver fibrosis (Fig. 13f).

As previously stated, body weight was markedly reduced, and the observed results were clearly dose-dependent, with significant improvement also in the CDHFD with Tropicifexor mid dose group (Fig. 13g). The final body weight measured at twelve months in the mice fed WD and Tropicifexor low dose indicated a significant decrease compared to the WD fed control mice, although in this case the mice were still obese and only partially improved (Fig. 13h). As previously assessed, I measured the weight of adipose tissue, and could report once again a highly significant reduction on the groups that received Tropicifexor, observing dose-dependent effects (Fig. 13i). Liver-to-body weight ratio indicated once again the induction of hepatomegaly, with highly significant increase also in the ND group (Fig. 13j).

As expected for the duration of the experiment with WD feeding, the mice developed hepatic lesions (Fig. 13k). Despite the metabolic rescue achieved with Tropicifexor administration, the mice under Tropicifexor mid dose developed more lesions than the mice fed pure WD, the mice under Tropicifexor low dose developed almost twice as many lesions than the control mice on WD at twelve months. Interestingly, the mice with Tropicifexor high dose developed less lesions, but still an amount comparable to the WD control group, despite their NASH phenotype significantly improved. The mice under OCA and GW4064 also developed lesions to a similar extent to WD control mice, but in absence of any improvement of NASH, as expected (Fig. 13l). I collaborated further with Prof. Thomas Longerich and characterized the lesions that were sufficiently large to allow a histological evaluation, and represented them based on HCC, HCC-CCA, dysplastic foci or borderline nodular lesions (Fig. 13m).

With the presented data, I concluded that Tropifexor, despite rescuing metabolically the mice and reversing multiple features of NASH, induced more hepatic lesions. The different effects observed with the varying doses of Tropifexor were quite puzzling, almost ambiguous. Of note, the cohorts were treated with different doses of Tropifexor in separated experiments and for a different duration, therefore it is not possible to draw definitive conclusions from this data. However, some differences could probably depend on a very specific pharmacokinetic and require additional evaluation on the side of changes on metabolism, immunity and impact on liver toxicity and BAs reduction.

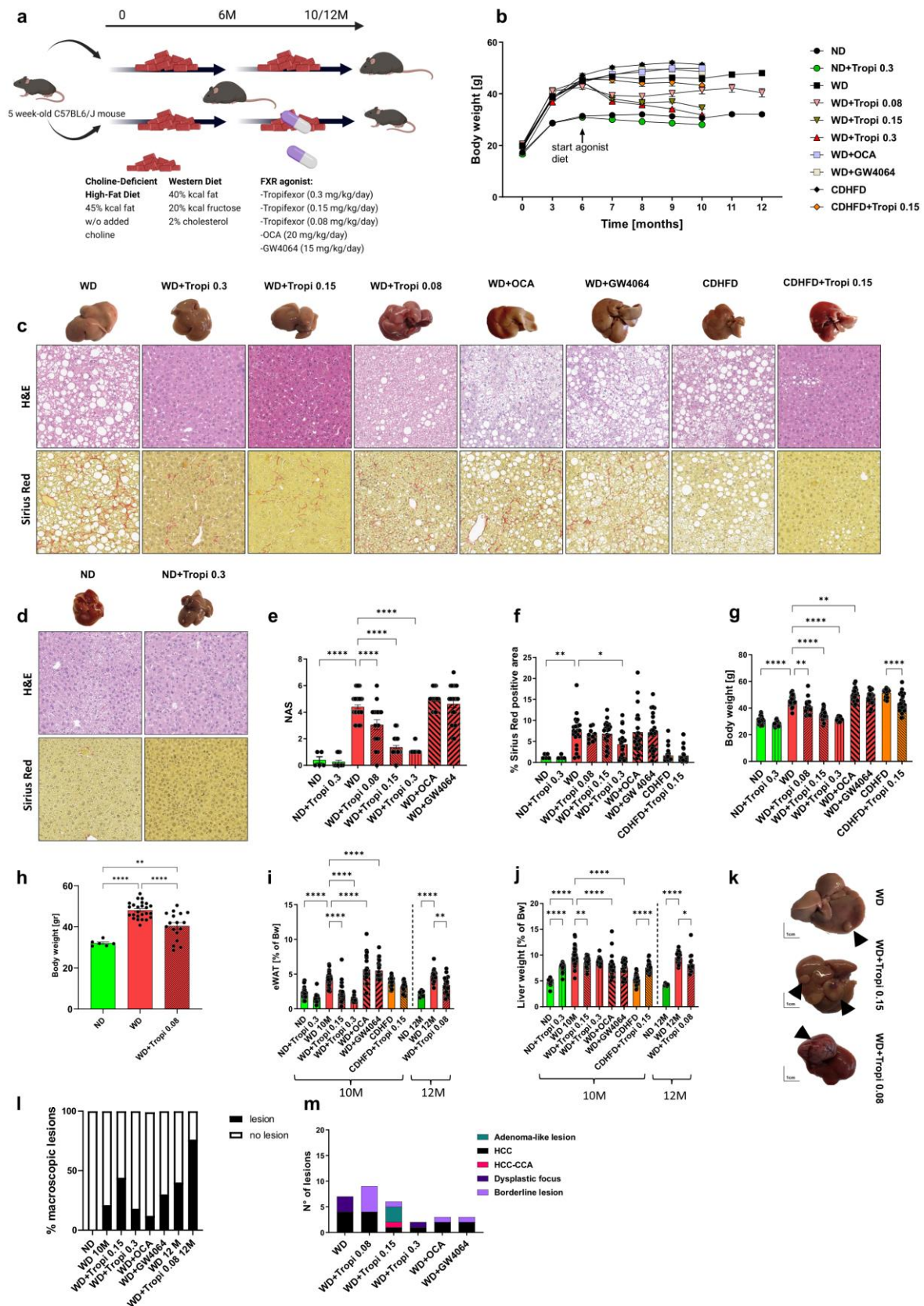


Figure 13. Tropifexor successfully reverses NASH but drives more hepatic lesions (a) Experimental setup of the long-term therapeutic approach with FXR agonists, where mice were initially fed ND, CDHFD or WD for 6 months and administered the respective diet supplemented with FXR agonists for additional 4 to 6 months. (b) Body weight development of different mice cohorts fed ND, CDHFD and WD and treated with different doses of Tropifexor, or OCA or GW4064. Significance not indicated. (c)

and (d) liver macroscopic image, H&E and Sirius Red staining (Scale bar: 100µm), with (e) NAS evaluation and (f) Sirius Red quantification of the mouse groups shown in b. (g) Body weight after 10 months of the mouse groups shown in b. (h) Body weight after 12 months of the mouse cohorts shown in b. (i) eWAT to body weight ratio of the mouse cohorts shown in b. (j) Liver to body weight ratio of the mouse cohorts shown in b. (k) Representative macroscopic picture of livers with tumours under WD, WD+Tropi 0.15 and WD+Tropi 0.08. Scale bar: 1cm (l) Percentage of macroscopic lesions of the mouse cohorts shown in b. (ND n=28 mice, except in b where n=30 mice, in e and f where n=5 mice and i where n=27 mice; ND+Tropi n=26 mice, except in e where n=15 mice, and in f where n=5 mice, WD n=46 mice, except in b where n=49 mice, in e where n=47 mice, and in f where n=22 mice, WD+Tropi 0.15 n=45 mice, except b where n=48 mice, in e where n=46 mice and f where n=23 mice, WD+Tropi 0.3 n=22 mice, except in b where n=24 mice, WD+OCA n=24 mice, except in b where n=25 mice, in e, f and g where n=23 mice, WD+GW4064 n=24 mice, except in b and f where n= 23 mice, CDHFD n=24 mice, CDHFD+Tropi n=25 mice, except in b where n=26 mice, ND12M n=6 mice, WD12M n=25 mice, WD+Tropi 0.08 n=17 mice). (m) Number and phenotyping of lesions from the WD cohorts shown in b. All data are shown as mean $\pm$ SEM. All data were analysed by one way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.8. Tropifexor causes retention of hydrophobic BAs, allowing PERK activation and driving ER stress, inducing pre-neoplastic lesions and regenerative changes, counteracted by polyploidy

In this part, I tried to identify the effects that could be responsible of the increased amount of lesions after Tropifexor. Of note, in the previous section I treated the mice with Tropifexor high dose under ND feeding ad was unable to identify any lesion. However, after an accurate pathological evaluation, in collaboration with Prof. Thomas Longerich, I determined the induction of small cell changes and regenerative response, consistent with pre-neoplastic transformation, in most of the livers treated with Tropifexor high dose under ND feeding. In addition, I observed several dysplastic foci and necrotic areas, quite unusual for livers from ND fed mice (Fig. 14a). I evaluated once again the extent of hepatocyte proliferation through Ki-67 staining, and could identify significantly higher degree of Ki-67-positive hepatocytes in the group Tropifexor low dose, compared to the other two doses (Fig. 14b-c). In addition, contrary to expectations, I observed an even higher amount of Ki-67 in the groups OCA and GW4064 (Fig. 14b-c). After collaborating again with Dr. Chantal Desdouet and Pierre Cordier for evaluating the extent of ploidy induction in the livers, I observed an increase with Tropifexor following a dose dependent trend, strangely in contrast with the data on proliferation above reported (Fig. 14d). In addition, the measurement on nuclear density and nuclear area on tumours revealed a strikingly increase of 4n fraction, also in a dose dependent fashion (Fig. 14f).

I attempted to evaluate several markers through IHC evaluation, and identified a very high elevation of CHOP and BIP, indicative for ER stress induction. The signal was present following a dose-dependent elevation for CHOP, and BIP was increased to a minor extent (Fig. 14g) I further confirmed the induction on CHOP and BIP with western blot, detecting high levels in the group Tropifexor high and mid dose (Fig. 14h).

I wanted then to identify which molecular marker upstream was responsible for the induction of ER stress. I then verified through western blot that ATF6 was not changing in the ND after Tropifexor high dose and in the WD was decreased after Tropifexor high dose. Further evaluating the markers upstream, I detected higher levels of phosphorylated PERK in Tropifexor high dose, both in ND and WD. In line with this I observed high levels of phosphorylated eIf2 α . Interestingly, despite having observed higher levels after Tropifexor in the short-term experiments, I did not notice particularly elevated levels of phosphorylated p65, which presented however high levels of total protein, while p38 showed to be decreased after Tropifexor high dose in WD (Fig. 14i). I evaluated then the serum levels for hepatic damage, and noticed that ALT were reduced by Tropifexor, and just in part elevated by OCA (Fig. 14j). I found AST to significantly change only in OCA and GW4064, and in the ND fed mice, even though, they were also elevated in the ND control mice (Fig. 14k). However, as before observed, I found a remarkable elevation of ALP under Tropifexor, in both ND and WD, but only with a mid and high dose (Fig. 14l).

I was once again interested in evaluating the BAs and, in collaboration with Dr. Diran Herebian, tested the BAs pool in different compartments. I observed as before a marked reduction of BAs in all diet groups with all agonists (Fig. 14m). Not surprisingly, as the main pool was reduced, also the Tauro-Conjugated BAs followed the same trend (Fig. 14n). However, TDCDA, was after Tropifexor low and high dose still in supra-physiologic amounts (Fig. 14o). Similarly, also the BAs in the liver were reduced after FXR agonists (Fig. 14p). Most of the BAs in the liver were Tauro-Conjugated, and presented the exact same profile (Fig. 14q). Also in this case I observed a retention of TCDCA, which due to its toxic effects could have a role in the stress induction (Fig. 14r).

I concluded that ER stress induction, likely through PERK activation, plays a significant role in driving the signals that favoured an increased amount of lesions in the mice treated with Tropifexor under WD. Similarly, mice fed ND developed pre-neoplastic lesions and regenerative changes under the influence of ER stress, the only common denominator consequent to Tropifexor administration across the diets. The administration of Tropifexor led

to a dose-dependent induction of hepatocellular polyploidy both in the tumour-free tissue and in the nodules assessed. The increase of polyploidy in tumours correlates with an advanced stage, higher progression and resistance to apoptosis, thereby worsening the prognosis.

In the following chapter, I attempted to identify more evidence that could explain the difference in lesions incidence between doses of Tropifexor.

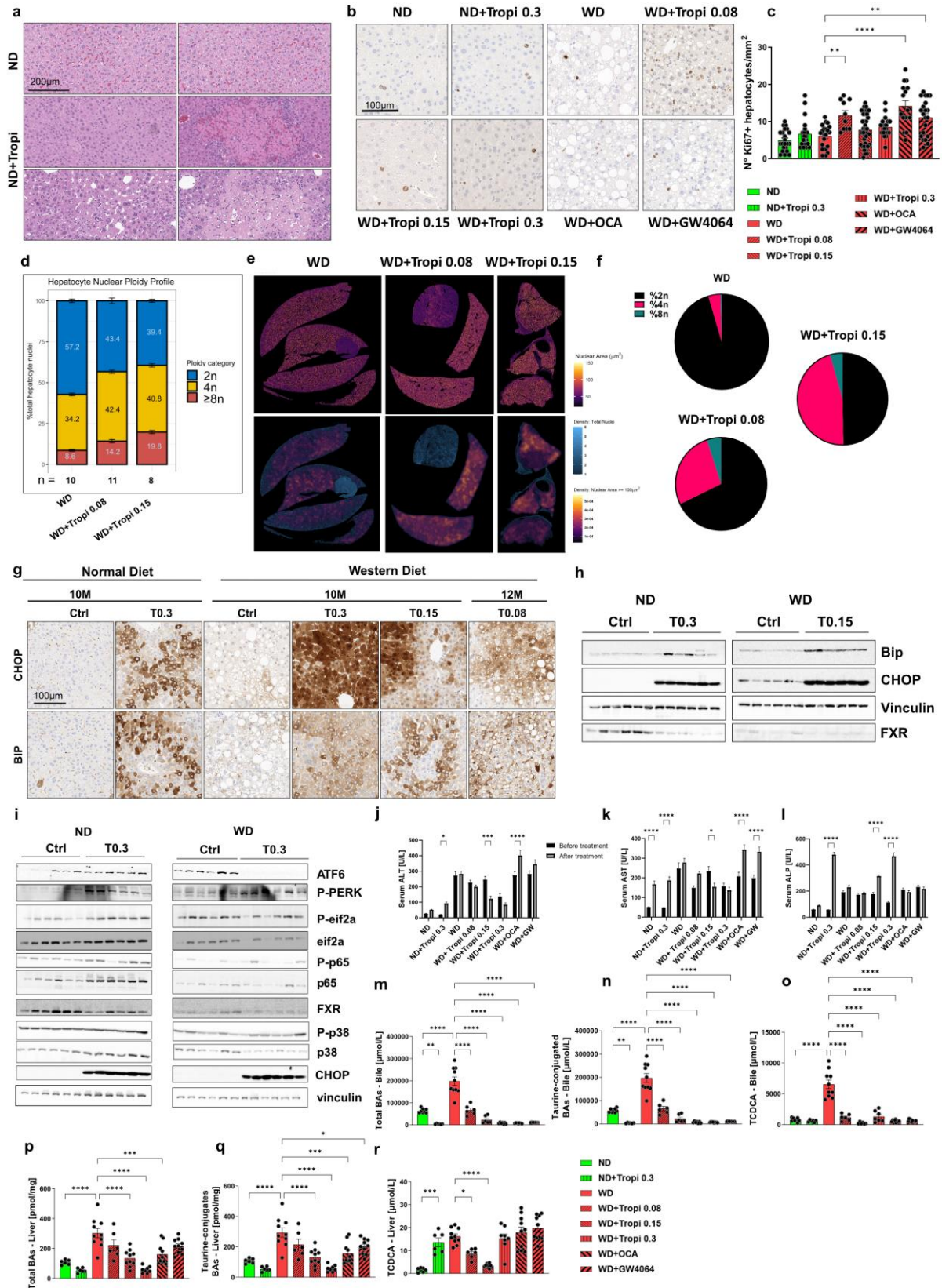


Figure 14. Tropifexor's potentially detrimental reshape of BAs pool activates PERK and drives ER stress, balanced by polyploidy induction, also increased in tumours. (a) Representative H&E staining showing dysplastic foci and necrotic areas of the ND and ND+Tropi groups shown in Figure 13. Scale bar 200µm. (b) Ki-67 staining and (c) Ki-67 quantification of the mouse groups shown in Figure 13 (ND n=22 mice, ND+Tropi n=21 mice, WD n=21 mice, WD+Tropi 0.08 n=10 mice,

WD+Tropi 0.15 n=38 mice, WD+Tropi 0.3 n=19 mice, WD+OCA n=16 mice, WD+GW4064 n=21 mice). Scale bar 100µm. (d) Percentage of 2n, 4n and 8n hepatocytes in tumour free lesions from WD (n= 10 mice), WD+Tropi 0.08 (n=11 mice) and WD+Tropi 0.15 (n=8 mice). (e) Representative pictures of liver tumours from ploidy analysis. (f) Pooled composition of 2n, 4n and 8n in tumour tissue under WD, WD+Tropi 0.08 and WD+Tropi 0.15. (g) CHOP and BIP staining of livers from mouse groups shown in Figure 13. (h) and (i) Western blot analysis from liver lysates of ND and WD cohorts shown in Figure 13. (j) Serum ALT, (k) serum AST and (l) serum ALP comparison before and after the treatment with FXR agonists of the mouse groups shown in Figure 13 (for j, k, l, ND n=27 mice, ND+Tropi n=24 mice, WD n=23 mice, WD+Tropi 0.08 n=15 mice, WD+Tropi 0.15 n=19 mice, WD+Tropi 0.3 n=23 mice, WD+OCA n=20 mice, WD+GW4064 n=21 mice). (m) Total BAs pool, (n) Taurine-conjugated fraction and (o) Taurochenodeoxycholic Acid levels measured in bile from the mouse groups shown in Figure 13. (p) Total BAs pool, (q) Taurine-conjugated fraction and (r) Taurochenodeoxycholic Acid levels measured in liver from the mouse groups shown in Figure 13 (ND n=6 mice, except for m, n, o where n= 8, ND+Tropi n=6 mice, WD n=10 mice, WD+Tropi 0.08 n=6 mice, WD+Tropi 0.15 n=10 mice, except for m,n, o where n= 6, WD+Tropi 0.3 n=8 mice, except for m, n, o where n=7, WD+OCA n=10 mice, except for m,n, o where n= 9, WD+GW4064 n=10 mice). All data are shown as mean ± SEM, except for f, with values plotted as part of a whole. All data were analysed by one way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.9. The dose of Tropifexor determines different effects on liver damage and cholestasis and drives senescence, which balances the induction of stemness and regenerative changes, also favouring the detrimental retention of hydrophobic BAs

As previously described, I observed more hepatic lesions in the mouse cohort that received Tropifexor low dose, than the one that received Tropifexor high dose. Due to different reasons, namely different cohort size and treatment duration, and since I performed the experiments separately, it was not possible to ultimately conclude that the difference in lesion induction was dependent on the dose of Tropifexor administered. To verify this, I performed again the experiment as above described, having in parallel two groups of mice receiving Tropifexor low and high dose respectively for six months. I observed reproducible effects in terms of progressive body weight reduction (Fig. 15a), decrease of visceral and subcutaneous adipose tissue (Fig. 15b-c), and induction of hepatomegaly (Fig. 15d), following dose-dependency trends as before.

Interestingly, I observed again more hepatic lesions in the mice treated with Tropifexor low dose than with a high one (Fig. 15e). It could appear that Tropifexor administration is reducing the amount of lesions rather than increasing it, but considering the smaller size of the control group, this conclusion would probably be biased.

I then proceeded to evaluate serum markers for liver damage, and observed peculiar differences between doses of Tropifexor. In particular, Tropifexor low dose determined a transient increase of ALT and AST, which normalized after nine months (Fig. 15f-g). On the other hand, Tropifexor high dose remarkably induced a progressive increase of ALP and Bilirubin (Fig. 15h-i).

The discrepancy of effects observed with the different doses could be dependent on pharmacokinetic clearance of Tropifexor, which has been reported to change in both humans and rodents based on the dosage. However, as in the case of high dose, high ALP and Bilirubin could indicate signs of cholestasis or biliary stress, and for what concerns the low dose, an elevation of ALT and AST, even temporary, could set the stage for additional liver damage, which in the context of NASH, further favours the ideal microenvironment for cancer initiation.

I previously confirmed that the induction of hepatomegaly was driven by enhanced liver proliferation, which was balanced by activation of p21 driving cell cycle arrest, favouring polyploidy and senescence. I assessed again p21 in IHC and observed a significant increase only in Tropifexor high dose (Fig. 15j-k). I also evaluated Sox9 IHC staining and could not detect significant changes, however, as Tropifexor high dose presented a slight reduction, Tropifexor low dose showed some increase (Fig. 15j-l). This two information are important to determine that a low dose of Tropifexor was not enough to induce a sufficient degree of p21 activation, balancing out the regenerative changes and proliferation driven by FXR signalling activation. Furthermore, Sox9, marker for stemness, with an important role in cancer initiation and associated with several solid tumours, could be partially induced with a low dose of Tropifexor, and in absence of senescence induction further enhance the proliferation observed.

Lastly, as the reshape of BAs pool observed in the previous experiment raised some concerns about a potential retention of hydrophobic BAs, I wanted to assess the differences of the two doses of Tropifexor on BAs in bile and liver. I then, in collaboration with Dr. Diran Herebian, attempted to evaluate the relative quantity of the most abundant Taurine-conjugated BAs, and could observe in both biliary and hepatic compartments a major depletion of TCA, replaced by T- β -MCA as the predominant BA after Tropifexor (Fig. 15m-n). It is important to underline that T- β -MCA is a natural FXR antagonist; thereby its increased relative abundance is likely suppressing the endogenous signalling, which is then sustained exclusively by Tropifexor administration.

In addition, other BAs, such as T- ω -MCA and T- α -MCA, also presented an increased relative abundance, although not significantly (Fig. 15o-p-u-v). Interestingly, I observed again

a significant increase of the relative abundance of TCDCA and TUDCA in both bile and liver compartments after Tropifexor (Fig. 15q-r-w-x). These two BAs have opposite effects on liver toxicity, where TCDCA has been described for being detrimental, whereas TUDCA, on the contrary, has protective effects. It is possible to speculate that the ratio of these two BAs, variable and probably dependent on the dose of Tropifexor and to the response of the individual mouse, could partially be responsible for the outcome on ER stress, regenerative responses and cancer initiation.

These findings showed that Tropifexor induced different levels of damage that lead to increased serum markers with effects specific to the dose utilized. The reshape of the BAs pool was not particularly different between the doses, and the increased relative abundance of some more hydrophobic BAs could be playing a role in driving ER stress and regenerative changes. At this point, the high dose of Tropifexor made a critical difference in activating p21, thus preventing an excessive degree of hepatocyte proliferation, as on the other hand the low dose further promoted the cell expansion and did not decrease Sox9 expression.

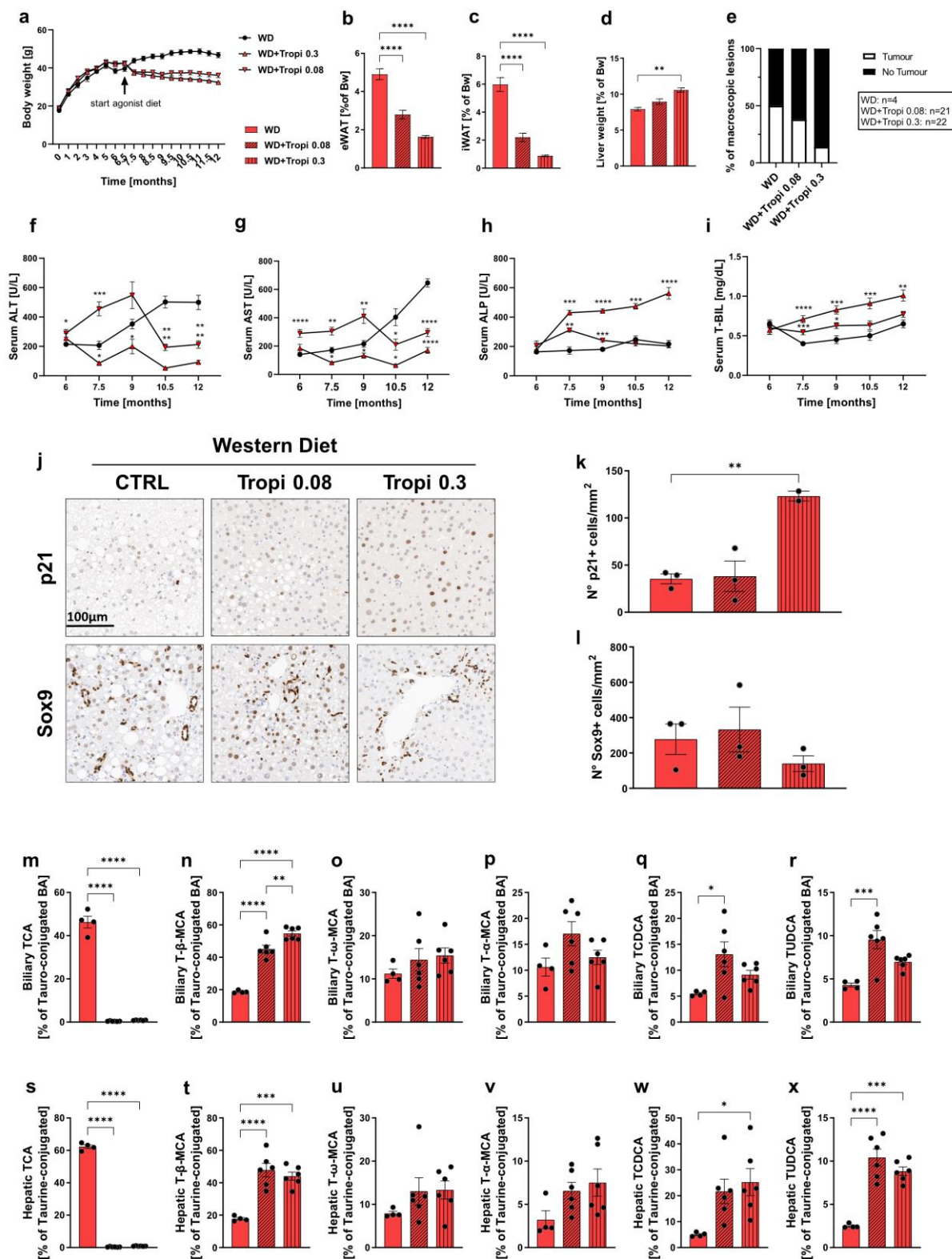


Figure 15. Tropifexor presents different effects on NASH improvement, liver damage, lesions incidence and senescence induction based on the employed dose and confirms to elevate the relative concentration of potentially toxic BAs in the liver (a) Body weight development of mice treated with Tropi 0.08 or Tropi 0.3 under WD feeding for 12 months. Significance not indicated. (b) eWAT to body weight ratio of the mouse groups shown in a. (b) iWAT to body weight ratio of the mice

groups shown in a. (d) Liver to body weight ratio of the mice groups shown in a. (e) macroscopic lesion incidence of the mice groups shown in a (n indicated in figure as the number of mice for each group). (f) serum ALT, (g) serum AST, (h) serum ALP, (i) serum Bilirubin measured over the course of the treatment in the mice from the groups shown in a. (for all the groups above, WD n=4 mice, WD+Tropi 0.08 n=21 mice, WD+Tropi 0.3 n=22 mice) Asterisks indicate significance compared to the group WD. (j) p21 and Sox9 staining on livers with (k) p21 and (l) Sox9 quantification of the mice groups shown in a (WD n=3 mice, WD+Tropi 0.08 n=3 mice, WD+Tropi 0.3 n=3, except for k, when n=2). Scale bar 100µm. (m) percentage of Tauro-conjugated BAs for Taurocholic Acid, (n) Tauro-β-Muricholic Acid, (o) Tauro-ω-Muricholic Acid, (p) Tauro-α-Muricholic Acid, (q) Taurochenodeoxycholic Acid and (r) Tauroursodeoxycholic Acid from bile of the mouse groups shown in a. (s) percentage of Tauro-conjugated BAs for Taurocholic Acid, (t) Tauro-β-Muricholic Acid, (u) Tauro-ω-Muricholic Acid, (v) Tauro-α-Muricholic Acid, (w) Taurochenodeoxycholic Acid and (x) Tauroursodeoxycholic Acid from liver of the mouse groups shown in a. (WD n=4 mice, WD+Tropi 0.08 n=6 mice, WD+Tropi 0.3 n=6 mice) All data are shown as mean ± SEM, except for e with values plotted as single percentages. All data were analysed by one way ANOVA and Tukey's multiple comparison test, or Mixed –effects model (REML) with Dunnett's multiple comparison. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.10. Tropifexor shows dual effects on adaptive immunity and tumour immunosurveillance

Given the important effects observed on adaptive immunity after Tropifexor administration in the short-term experiments, I wanted to characterize the effects of the two doses of Tropifexor also in the long-term experiments, in the attempt to gather further evidence that could explain the difference in outcomes.

I initially evaluated the infiltration of lymphocytes in the liver by CD3 IHC staining, and could note a dose dependent reduction of T cells in the liver, particularly significant after Tropifexor high dose (Fig. 16a-b). This was in net contrast with the result observed in the short-term experiment, where T-cells were increased after Tropifexor. I then further evaluated the immune cells through flow cytometry, and observed a significant decrease of total number of CD45+ cells in Tropifexor low dose, while cells Tropifexor high dose were slightly elevated but not particularly changing compared to WD (Fig. 16 c). When looking at the count of CD4+ T-cells I observed no changes, but CD8+ T-cells, particularly high in WD were significantly reduced in Tropifexor low dose, and Tropifexor high dose to a minor extent (Fig. 16d). I then observed a highly significant increase of CD19+ cells in the Tropifexor high dose group, which explained the apparent discrepancy in the total number of CD45+ cells, while NKT and NK cells did not change (Fig. 16e). I then assessed markers of activation and exhaustion and could note a decrease of CD4+ T-cells expressing PD-1 after Tropifexor high dose (Fig. 16f). To an even higher extent, CD8+ T-cells presented a significant, dose-dependent reduction of CXCR6, PD-1 and TIM3 after Tropifexor (Fig. 16g).

When evaluating the sub-compartments for effector, memory and naïve CD4⁺ T-cells I observed no major differences (Fig. 16i), however, in the CD8⁺ T-cell compartment, there was a major, dose-dependent, highly significant reduction of effector cells (CD44⁺) and resident memory cells (CD44⁺ CD69⁺), together with a reduction of PD-1 expression, as above mentioned (Fig. 16h). Interestingly, CD4⁺ T-cells presented higher Granzyme B and TNF α levels after Tropifexor high dose (Fig. 16j), with similar trends in the CXCR6⁺ CD4⁺ T-cells compartment (Fig. 16k). Similarly, in the CD8⁺ T-cells, I observed elevated levels of Granzyme B and TNF α after Tropifexor, with a decrease of IFN γ and increase of Ki-67, supporting augmented proliferation (Fig. 16l), however, not relative to the CXCR6⁺ CD8⁺ T-cell compartment (Fig. 16m). I then evaluated the cytokine production based on PD-1 expression and could observe that CD4⁺ T-cells PD-1 negative were the major producers of GzmB and TNF α (Fig. 16n). I reported GzmB and TNF α levels following a similar pattern in CD8⁺ PD-1⁻ cells; however, in this case, I observed major changes in the PD-1 positive CD8⁺ T-cell subpopulation, with significant increase of GzmB production after Tropifexor, together with Ki-67 elevation (Fig. 16o). Lastly, despite no major alteration in NKT cells number and no differences of PD-1 levels in $\gamma\delta$ -T-Cells, I observed a significant increase of α -Gal production after Tropifexor high dose (Fig. 16p).

These findings suggest that Tropifexor has a dual effect on the immune system in mice with NASH. At a high dose, Tropifexor has an immunosuppressive effect, reducing the number of CD3⁺ lymphocytes and the expression of PD-1, CXCR6, and TIM3 on CD8⁺ T-cells. This may be due to the fact that Tropifexor activates the transcription factor FoxP3, which is involved in the development of regulatory T-cells. However, Tropifexor also has an immunostimulatory effect, increasing the production of GzmB and TNF α by CD4⁺ and CD8⁺ T-cells, particularly in the PD-1-negative subpopulation. This could be the result of increased NF- κ B expression upon FXR signalling activation. Importantly, α -Gal increase with high dose of Tropifexor plays in favour of better NKT response in tumour immunity, giving a potential explanation of the differences in lesion incidence.

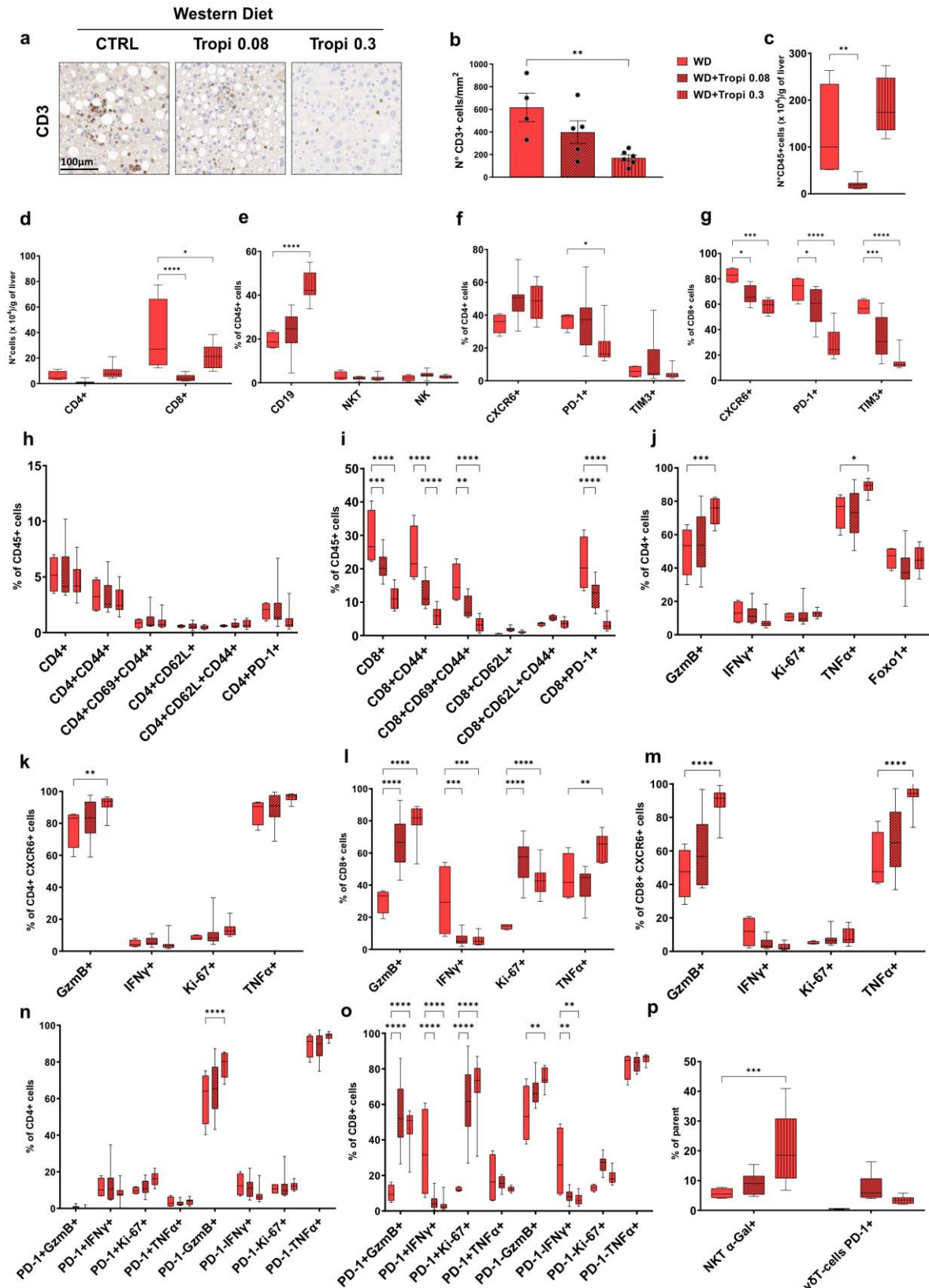


Figure 16 Tropifexor displays mixed effects on T-cell immunity. (a) CD3 IHC staining and (b) CD3 quantification of the mouse groups shown in Figure 15. Scale bar: 100 μ m. (c) Flow cytometry analysis displaying total number of CD45+ cells, (d) total number of CD4+ and CD8+ T-cells, (e) percentage of CD45+ cells of B-cells (CD19+), NKT cells (Nk1.1+ TCR β +) and NK cells (Nk1.1+ TCR β -) (f) percentage of CD4+ T-cells and (g) CD8+ T-cells expressing CXCR6, PD-1 and TIM3, (h) percentage

of CD45⁺ cells of CD4⁺ T-cells, effector memory cells (CD44⁺), resident memory cells (CD69⁺ CD44⁺), naïve cells (CD62L⁺), central memory cells (CD62L⁺ CD44⁺) and PD-1⁺ cells within the CD4⁺ T-cell compartment and (i) CD8⁺ T-cell compartment of the mouse groups shown in Figure 15. (j) Flow cytometry analysis of cytokines expression within CD4⁺ and (k) CD4⁺ CXCR6⁺ T-cells, (l) cytokines expression within CD8⁺ and (m) CD8⁺ CXCR6⁺ T-cells, (n) cytokines expression within CD4⁺ T-cell compartment and (o) CD8⁺ T-cell compartment, divided for PD-1 positivity or negativity, (p) percentage of α -Gal⁺ NKT cells and percentage of PD-1⁺ $\gamma\delta$ -T-cells of the mouse groups shown in Figure 15. WD n=4 mice, WD+Tropi 0.08 n=10 mice (except for a where n=5 mice), WD+Tropi 0.3 n=10 mice (except for a where n=6 mice). Data in b are shown as mean $\pm$ SEM; all other data are displayed in box and whiskers showing Min to Max. All data were analysed by one way ANOVA and Tukey's multiple comparison test, or two way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.11. Tropifexor induces opposite effects on myeloid immunity

In the short-term experiments, I could assess peculiar effects on myeloid cells upon Tropifexor administration, and wanted to verify them also in the long-term experiments. To this end, I started with evaluating F4/80 IHC staining and could detect a significant, dose-dependent decrease of positive cell aggregates (Fig. 17a-b). I proceeded then to characterize the myeloid cell compartment by flow cytometry. Oddly, in contrast to the previous finding, KCs were increased after both doses of Tropifexor. I could observe also increased amounts of cDCs (CD11c⁺ CD11b⁺) after Tropifexor low dose, which were in contrast partially reduced after Tropifexor high dose. I also noted a slight expansion of Cd11b⁺ Ly6C^{lo} cells after Tropifexor high dose, which on the other hand induced a significant reduction of Cd11b⁺ Ly6C^{hi} (Fig. 17c).

I screened KCs for the expression of different markers and did not observe relevant changes in CD86, CD206, MHCII or PD-L1 levels; however, CCR2 was significantly increase with both doses of Tropifexor (Fig. 17d). Even pDCs (CD11c⁺ CD11b⁻) did not change after Tropifexor, I noted a significant decrease of MHCII after Tropifexor low dose, where instead this was higher in the cDCs, together with a significant decrease of CD86 (Fig. 17e). When evaluating Cd11b⁺ Ly6C^{lo} cells, I could observe a significant decrease of MHCII after Tropifexor high dose (Fig. 17f), which was even more significantly decreased in Cd11b⁺ Ly6C^{hi}, together with lower CD86 and PD-L1 levels (Fig. 17g).

These data confirmed again the dual nature of Tropifexor on the immune hepatic microenvironment, with opposing effects, potentially both immunosuppressive and immunostimulatory, on myeloid cells, as it was for T-cells. Interestingly, despite a reduction of F4/80 aggregate, an increase of KCs could denote an attempt to compensate the dampened scavenging and phagocytic activity, while CCR2 increase predisposes for augmented

susceptibility for chemokine-mediated inflammation. At the same time, reduction of MHCII and CD86 in $CD11b^+ Ly6C^{hi}$ could denote a major reduction of the inflammatory response from infiltrating monocytes, which also supports the decreased T-cell infiltration and activation. However, these data also add another layer of complexity to the unclear picture of the ambiguous effects of Tropifexor on hepatic carcinogenesis.

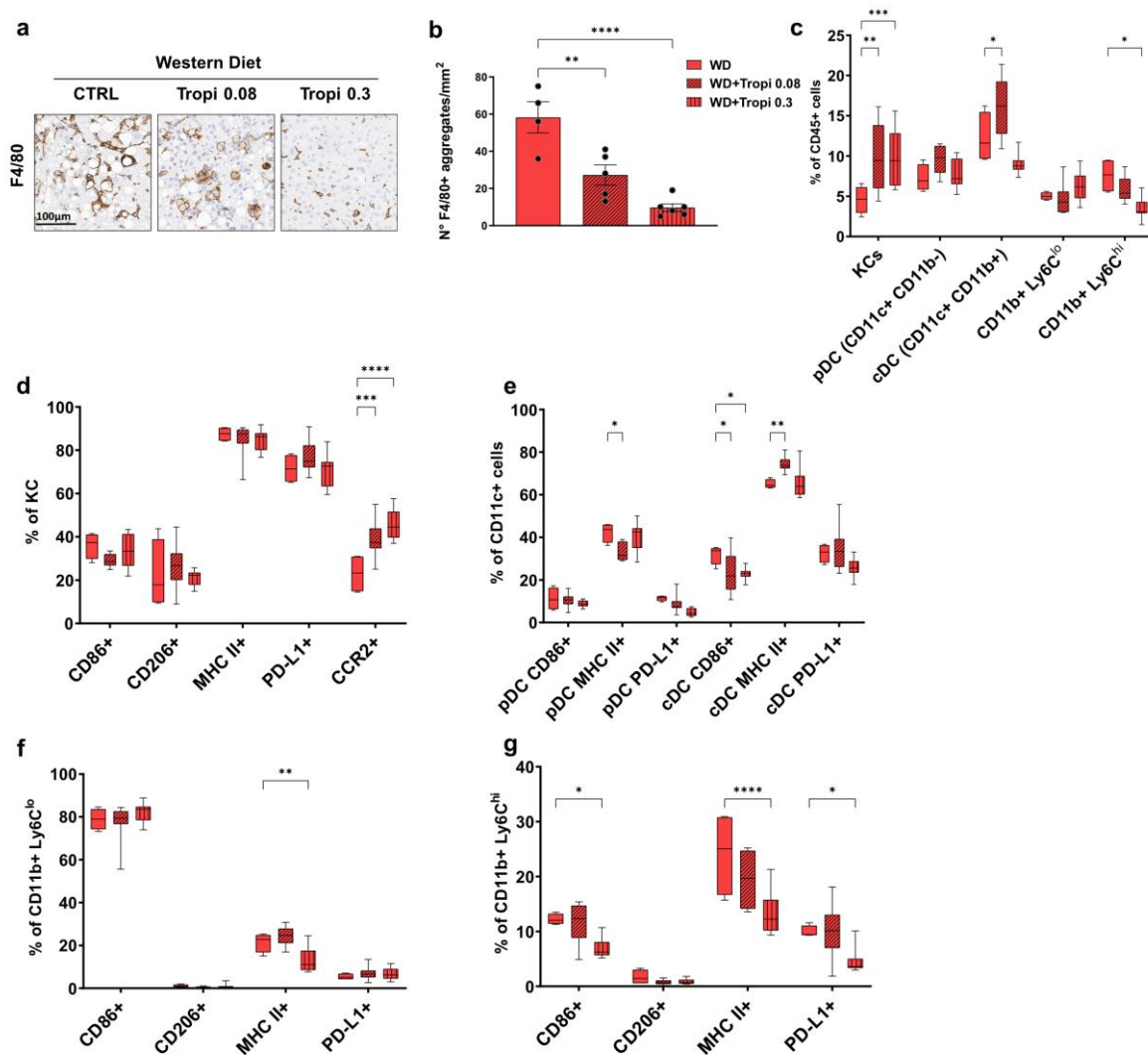


Figure 17. Tropifexor affects macrophages aggregate formation and present dual effects on myeloid immunity. (a) F4/80 IHC staining and (b) F4/80 quantification of aggregates of the mouse groups shown in Figure 15. Scale bar: 100 μ m. (c) Flow cytometry analysis showing percentage of CD45⁺ cells of KCs, pDCs, cDCs, CD11b+Ly6C^{lo} and CD11b+Ly6C^{hi} cells, (d) percentage of KCs expressing CD86, CD206, MHCII, PD-L1, (e) percentage of CD11c⁺ cells expressing CD86, MHCII and PD-L1 divided in pDCs (CD11b⁻) and cDCs (CD11b⁺), (f) percentage of CD11b+Ly6C^{lo} cells and (g) CD11b+Ly6C^{hi} cells expressing CD86, CD206, MHCII, PD-L1, from mouse groups shown in Figure 15. (WD n=4 mice, WD+Tropi 0.08 n=10 mice, except in a where n=5 mice, WD+Tropi 0.3 n=10 mice, except in a where n=6 mice). Data in b are shown as mean $\pm$ SEM; all other data are displayed in box and whiskers showing Min to Max. All data were analysed by one way ANOVA and Tukey's multiple comparison test, or two way ANOVA and Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

8. Discussion

NASH is a complex multifactorial disease that involves a plethora of different systemic components, from nutrition to metabolism, with important roles played by the gut microbiota and the immune system, relevant contribution from genetics and BAs signalling, all converging to the development of the different features that set the stage for cirrhosis and liver cancer. Due to this high complexity, NASH is still devoid of approved therapies, and the urgency that drives the scientific community to shed light on the various mechanisms that favour NASH development in the hope to identify promising molecular targets, it's only aggravated by the predictions that expect this disease to further increase in the population in the future.

In the last two decades, many studies unravelled the essential role of the BAs signalling in metabolism and its capacity to regulate and bridge many functions across the gut-liver axis and beyond. BAs are not only molecules relevant within the frame of proper uptake of fat and lipophilic vitamins from food, or the necessary way to ensure an adequate catabolism of heme through bilirubin clearance, but also hormones, signalling molecules, immunity modulators and much more. Their impact on glucose and lipid metabolism could be the key in strategizing a successful therapeutic approach for NASH and other metabolic disorders. To fully harness the potential of the BAs signalling, researchers have developed and engineered several molecules capable of modulating FXR, the most important bile acid receptor. From early studies where the employed molecules were effective but too toxic for any use beyond research, much progress has been made, bringing to clinical trials several promising candidates, one of which was Tropicifexor. This molecule has demonstrated high safety, tolerability and efficacy, thereby successfully completing phase II clinical trials, and further steps will probably be initiated in the near future to engage phase III. To date, the major element of concern in the clinical use of this drug was pruritus, experienced by a large number of patients, not new as a side effect for FXR agonists and documented during testing of other molecules [6, 7, 378, 379].

However, given the difficulty of NASH patients to engage a full and prolonged lifestyle change, with a healthy diet and regular exercise, in favour of a resolution of obesity and a stabilization of diabetes and liver pathology, it is likely that drug as Tropicifexor, or other FXR agonists, could be employed in a prolonged therapeutic regime lasting years. This sustained exogenous activation of the BAs signalling, in presence of damage to the liver driven by NASH, could potentially synergize and drive side effects that have not yet been explored in patients.

With the study I conducted, utilizing different diets, I was able to establish a highly translational mouse model of early NASH, characterized by obesity, increase fats deposits and hepatic steatosis, in absence of severe hepatic immune cell infiltration driving inflammation and without any degree of fibrosis. This condition was ideal to test different FXR agonists and evaluate the effects of an early therapeutic approach on further NASH progression.

Performed experiments revealed that Tropicifexor, and not the other compounds, was able to efficiently improve obesity, ameliorate glucose metabolism and reverse completely hepatic steatosis, thereby resolving NASH in its early onset. The great efficacy of Tropicifexor was supported by elevated bioavailability, robust activation of intestinal and hepatic FXR signalling and consistent reduction of BAs in all the assessed compartments. It was interesting to determine that the clearance of lipids induced by Tropicifexor was not directing them to energy production, as no difference in energy expenditure nor switch in energetic source was observed. Indeed, it could be actually possible that the lipids were directly exported from liver and adipose tissue to the intestine, leading to faecal excretion. This was further in line with the abrogation of *de novo* lipogenesis and by the important upregulation of cholesterol metabolism, which was necessary to generate lipid droplets and facilitate the export out of the liver. Tropicifexor also showed remarkable effects in creating an anti-inflammatory profile in the liver by reducing the amount of effector T-cells and dampening the expression of inflammatory cytokines. Furthermore, I determined interesting properties of Tropicifexor in regards to the myeloid compartment: as a matter of fact, it remarkably reduced cell aggregates formation and reshaped their spatial conformation and phagocytic activity in support of M2 polarization.

In spite of that, the effects I documented were not exclusively positive. Further assessments of the differences between high and low dose of Tropicifexor determined important repercussions on liver damage, with a transient elevation of ALT and AST with a low dose and progressive increase of ALP and Bilirubin accumulation with a high dose. This discrepancy finds probable explanation in the different pharmacokinetic clearance of Tropicifexor at low and high dose, documented to follow similar patterns in humans and rodents. In particular, studies reported that at a high dose Tropicifexor clearance is carried out through phase II conjugation by the enzyme UGT1A, which is also involved in Bilirubin glucuronidation [393]. It could be possible to speculate that at a high dose Tropicifexor competes with Bilirubin for conjugation and excretion, leading accumulation of either of the two highly detrimental. However, I was able to measure only total Bilirubin in serum, and to verify this hypothesis, it would be necessary to determine the levels of the unconjugated form.

Following my observations, as shown by Ki-67 staining, Tropifexor provoked a proliferative response through FXR activation and mobilization of several downstream effectors, from NF- κ B to MAPK/JNK. The parenchyma counteracted excessive proliferation by increasing p21 expression, but the absence of apoptotic induction brought the cells to a state between polyploidy and senescence, which further exposed the hepatocytes to increased DNA damage. In addition, early signs of ALP elevation induced by Tropifexor denoted the presence of disrupted biliary homeostasis. The reported negative effects of Tropifexor on hepatocytes and biliary cells, together with the pro-inflammatory and potentially pro-carcinogenic microenvironment typical of NASH, could have a detrimental outcome, and therefore required further investigation with a prolonged administration.

As a result of a long-term treatment with FXR agonists, I determined that Tropifexor would be an ideal candidate for reversing not only early, but also more severe, advanced stages of NASH. Indeed, I observed an extraordinary response in terms of body weight reduction, resolution of hepatic steatosis and even improvement of fibrosis. The results showed that the dose of Tropifexor plays an important role in defining the outcome on NASH, with most of the effects being dose-dependent.

Despite impressive beneficial effects of long-term administration of Tropifexor, I documented a higher amount of hepatic lesions in the mice treated with this compound, or in the best case, an incidence comparable to the untreated groups. An interesting finding was the difference in hepatic lesions not following dose-dependent trend but showing rather increased numbers in the mice treated with a low dose of Tropifexor, although, not all of the groups were studied in parallel, which could lead to cohort specific effects.

Next, since NASH itself is a factor having an important role in tumorigenesis, I wanted to evaluate the effects of Tropifexor outside of this environment and tested the compound in mice fed ND. Even if the livers were free of macroscopic nodules, a histological evaluation determined the induction of small-cell changes, which are considered pre-neoplastic, as well as increased amount of dysplastic foci and necrotic areas. Ki-67 elevation was confirming increased amount of hepatocellular proliferation, but were not matching the dose-dependent induction of observed polyploidy. Importantly polyploidy was increased also in the lesions from the groups that received Tropifexor, and this could support tumour survival and progression.

Additional assessments on the effects of different doses of Tropifexor revealed an important activation of p21, as expected, which was not observed at a low dose. Conversely, with a low dose, Sox9 showed some slight elevation, while was reduced at a high dose. This

could allow the hepatocytes to proliferate more and be exposed to cancer initiation signals under a low dose, while at a high dose, the induction of p21 could interfere with these effects, likely not halting but just delaying them, due to increased susceptibility to DNA damage, which was observed to raise already in the short-term treatments.

Apart from disruptions of the cell cycle, I identified ER stress, demonstrated by significant increase of CHOP and BIP, to be the major inducer of the damage driving regenerative changes and starting the chain of events that led to increased lesions incidence after Tropifexor administration. Among the upstream activators of the Unfolded Protein Response (UPR), I detected higher PERK phosphorylation in the liver after Tropifexor, together with increased amounts of eIF2 α phosphorylation.

Another potential piece of the puzzle could be found in the changes in the BAs composition. In fact, while Tropifexor efficiently decreased BAs in all compartments at a high dose, it induced at a low dose a slight elevation of certain BAs classes in some cases, or it failed to adequately reduce some classes in others. TCA, the most abundant bile acid in mice, shows significant reduction after Tropifexor, which could lead to important alterations in the overall composition, changing the relative abundance of some BAs that would be otherwise low. TCDCA, a bile acid documented for toxic effects and roles in cancer, was found to increase its relative abundance in the liver from 5% to up to 30% after Tropifexor administration. This could have an impact on BAs-induced toxicity, although this hypothesis requires further studies.

Lastly, I evaluated the effects of Tropifexor on immune cells after long-term administration. While in the short-term experiments, the impact on both lymphoid and myeloid immunity seemed to be predominantly anti-inflammatory, in the long-term experiments the effects observed were controversial, with Tropifexor presenting a dual effect partially immunosuppressive but also immunostimulatory. Indeed, the reduction of effector and resident memory sub-populations of CD8⁺ T-cells was counteracted by increased amounts of GzmB and TNF α . At the same time, high dose of Tropifexor importantly induced α -Gal⁺ NKT cells, a subclass with documented peculiar anti-tumour effects, which could definitely help in the understanding why high dose of Tropifexor presented with lower lesions despite inducing a potentially pro-tumorigenic environment resulting from increased liver damage and ER stress. The dual role of Tropifexor in liver inflammation consisted in a concomitant reduction of F4/80⁺ cells aggregation and elevation of KCs numbers. Again, a high dose of Tropifexor reduced CD86 and MHCII levels, contributing to a dampened T-cell recruitment, but also increased CCR2, promoting chemokine-mediated inflammation.

Some additional clues from available studies seem to partially support my findings. Indeed, studies on rats showed induction of liver injury after OCA administration in presence of partial hepatectomy or bile duct ligation [394]. Further studies observed reduced cell viability of hepatocytes and induced mitochondrial damage after treating HFD fed mice with OCA and Px-102, and attributed these effects to FXR/SHP pathway activation [395]. One point in common between the studies above mentioned and my work is the presence of oxidative stress, with potential accumulation of ROS. It is also worth mentioning, that in my case, by employing aggressive models of NASH, I provoked the induction of a remarkable amount of liver damage, perpetuating a cycle of hepatocyte death and regeneration. Within this environment a further stimulation of proliferation, supported by anti-apoptotic signals could favour cancer progression, and FXR activation could be responsible [386]. It is known that FXR regulates the apoptosis in a cancer-dependent fashion, for the liver it seems to support anti-apoptotic signals, although this is in contrast with the findings of low expression of FXR in liver cancer [396]. Nevertheless, the tumours that occurred after Tropicifexor treatment are likely to represent the exception rather than the rule, and prolonged systemic administration drives effects hardly comparable with simple endogenous FXR signalling, therefore comparisons with spontaneous NASH-induced hepatocarcinogenesis are difficult to make.

The results from my work could harbour a higher value if confronted with the response induced by Tropicifexor in patients. Yet, as the preclinical studies were just recently conducted, the access to human material from the trials could be complicated and rather cumbersome. In addition, as the preclinical studies did not extend the experimental treatment with Tropicifexor beyond the 48 weeks, it is also possible that in humans it would be required a longer duration of the treatment, possibly several years, before the manifestation of the detrimental effects I observed. This could be complicated, expensive and potentially dangerous to the patients.

Some additional studies on human cells could be the alternative at the moment, but the lack of gut-liver axis signalling and the absence of enterohepatic BAs circulation in simple cell systems strongly limits the amount of information that *in vitro* studies could provide. One valid possibility could be to test FXR agonists responses in separate organoid cultures and attempt to bridge the signalling by exposing one another to the secretions released in the medium after the treatments. However, the establishment of such system would certainly require much time and expertise, far beyond the one I acquired during the frame of my PhD studies.

Overall, with the data in my hands, I can conclude that Tropicifexor effects on reversing the major features of NASH are without doubt promising, showing a degree of efficacy that no

other of the tested compounds could reproduce. Nevertheless, given the shift from the increased risk of carcinogenic signature to the real development of hepatic lesions in spite of a resolution of NASH as I reported, it would be advisable to carry out further studies before advocating for a sustained administration in patients, which potentially could slowly dissipate the positive effects in favour of more detrimental ones.

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Who was trained not to spit in the fan,

Who was told what to do by the man,

Who was broken by trained personnel,

Who was fitted with collar and chain,

Who was given a pat on the back,

Who was breaking away from the pack,

Who was only a stranger at home,

Who was ground down in the end,

Who was found dead on the phone,

Who was dragged down by the stone" (Pink Floyd – Dogs, 1977)

Enrico Focaccia