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**The role of the macrophage mTOR-S6K axis
effectors –S6K1 and S6K2– in NASH, NASH-HCC
and liver fibrosis**

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1 Abbreviations

#	Number
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ALP	Alkaline phosphatase
ALT	Alanine aminotransaminase
AOC	Area of the curve
Arg1	Arginase 1
α SMA	Alpha smooth-muscle actin
AST	Aspartate aminotransferase
BAT	Brown adipose tissue
BMDMs	Bone-marrow derived macrophages
CCl ₄	Carbon tetrachloride
CD	Cluster of differentiation
CDAHFD	Choline-deficient, L-amino acid defined high-fat diet
CK19	Cytokeratin 19
Clec10a	C-Type Lectin Domain Containing 10A
Col1a1	Collagen type I, alpha 1
Col4a1	Collagen type IV, alpha 1
ColIV	Collagen type IV
Cre	Cre-recombinase
Csf1r	Colony stimulating factor 1 receptor
Ctrl	Control
DAPI	4',6-diamidino-2-phenylindole
dL	Deciliter
eMRI	Echo-magnetic resonance imaging
eWAT	Epididymal white adipose tissue
fl	flox
FOV	Field of view
h	Hours
H&E	Hematoxylin and eosin
HCC	Hepatocellular carcinoma
HNF4a	Hepatocyte Nuclear Factor 4 Alpha
HSC	Hepatic stellate cells
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin
IL-4	Interleukin 4
ipGTT	Intraperitoneal glucose tolerance test
ipITT	Intraperitoneal insulin tolerance test
iWAT	Inguinal white adipose tissue
KC	Kupffer cells
KO	Knockout
L	Liter
LPS	Lipopolysaccharide
Lysm	Lysozyme
MCSF	Macrophage colony stimulating factor 1
mg	Milligram
MHCII	Major histocompatibility complex class II

mL	Mililiter
Mmp	Matrix metalloproteinases
MoMFs	Monocyte-derived infiltrating macrophages
Mrc1	Mannose Receptor C-Type 1
n.s	Not significant
NAFL	Non alcoholic fatty liver
NAFLD	Non alcoholic fatty liver disease
NAS	Non alcoholic fatty liver disease activity score
NASH	Non alcoholic steatohepatitis
ND	Normal diet (chow standard mouse diet)
No.	Number
Nos2	Nitric Oxide Synthase 2
NT	Non-tumor
p-4EBP1	Phosphorylated eukaryotic translation initiation factor 4E-binding protein 1
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline solution
PCR	Polymerase chain reaction
Pdgfa	Platelet-derived growth factor subunit A
pS6	Phosphorylated ribosomal protein S6
ROI	Region of interest
S6K1	Ribosomal S6 kinase 1
S6K2	Ribosomal S6 kinase 2
T	Tumor
TBIL	Total bilirubin
TCHOL	Total cholesterol
Tgf- β	Transforming growth factor beta
WD	Western diet
WT	Wild type
μ	Micro
μ g	Microgram
μ L	Microliter

2 Summary

Excess nutrients can be temporarily stored in the liver in the form of fatty acids and glycogen; however, a chronic positive energy state induces pathological storage of fatty acids in hepatocytes, driving inflammation and leading to non-alcoholic steatohepatitis (NASH).

Macrophages are pivotal for the liver homeostasis, they have distinct roles in NASH, and its complications like NASH-HCC and liver fibrosis. Bridging nutrient sensing and macrophages, the mammalian target of rapamycin (mTOR) is involved in macrophage polarization, but mTOR-inhibition in macrophages has shown conflicting evidence for the treatment of NASH. Additionally, the role of downstream mTOR-effectors S6K1 and S6K2 in the macrophage compartment in NASH-associated carcinogenesis is unknown.

Therefore, to investigate the role of macrophage mTOR-S6K effectors –S6K1 and S6K2– I studied the activation status of myeloid mTOR-S6K axis in human and mice healthy and NASH livers where I found it to be increased relative to the pathology severity.

Further, I established two independent transgenic mouse lines with deletion of S6K1 and S6K2 in mature macrophages and monocytes. I then generated bone-marrow derived macrophages from these mice to explore the role of the S6 kinases in macrophage polarization, where I found no causal link between S6K1/S6K2 and macrophage polarization.

Furthermore, to explore the role of both macrophage kinases *in vivo*, I employed chronic inflammation models of NASH and NASH-HCC either in the context of metabolic syndrome (Western diet feeding) or in the context of profound liver fibrosis (Choline-deficient L-amino acid-defined diet feeding). These experiments showed no causal link between macrophage S6K1/S6K2 in obesity, metabolic syndrome (i.e. glucose/insulin tolerance, obesity/body composition) and liver pathology beyond the progression of liver fibrosis.

Thus, to understand the role of macrophage S6K1/S6K2 in liver fibrosis, I used a hepatotoxic model of carbon tetrachloride (CCl₄) to induce liver fibrosis in mice with macrophages lacking the S6 kinases in myeloid cells. My data demonstrated that the macrophage S6 kinases play a subdued role in liver fibrosis.

Thereby, my work demonstrated that the myeloid mTOR-S6K axis is activated in human and mouse NASH. While not influencing NASH-progression or NASH-HCC, macrophage S6K1/S6K2 loss dampened liver fibrosis in NASH and CCl₄-induced liver injury. My data contributes to resolving conflicting evidence surrounding the role of the myeloid mTOR-S6K axis in obesity, NASH, NASH-HCC and liver fibrosis.

3 Zusammenfassung

Überschüssige Nährstoffe können in Form von Fettsäuren und Glykogen vorübergehend in der Leber gespeichert werden; Ein chronisch positiver Energiezustand induziert jedoch eine pathologische Speicherung von Fettsäuren in Hepatozyten, was zu Entzündungen führt und zu nichtalkoholischer Steatohepatitis (NASH) führt.

Makrophagen sind für die Leberhomöostase von zentraler Bedeutung und spielen eine besondere Rolle bei NASH und seinen Komplikationen wie NASH-HCC und Leberfibrose. Das Säugetierziel von Rapamycin (mTOR) verbindet Nährstofferkennung und Makrophagen und ist an der Makrophagenpolarisierung beteiligt. Die mTOR-Hemmung in Makrophagen hat jedoch widersprüchliche Beweise für die Behandlung von NASH erbracht. Darüber hinaus ist die Rolle der nachgeschalteten mTOR-Effektoren S6K1 und S6K2 im Makrophagenkompartiment bei der NASH-assoziierten Karzinogenese unbekannt.

Um die Rolle der Makrophagen-mTOR-S6K-Effektoren – S6K1 und S6K2 – zu untersuchen, habe ich daher den Aktivierungsstatus der Makrophagen-mTOR-S6K-Achse in gesunden Menschen und Mäusen sowie in NASH-Lebern untersucht, wo ich festgestellt habe, dass er im Verhältnis zum Schweregrad der Pathologie erhöht ist.

Darüber hinaus habe ich zwei unabhängige transgene Mousselinien mit Deletion von S6K1 und S6K2 in reifen Makrophagen und Monozyten etabliert. Anschließend habe ich aus diesen Mäusen aus dem Knochenmark stammende Makrophagen erzeugt, um die Rolle der S6-Kinasen bei der Makrophagenpolarisierung zu untersuchen. Dabei konnte ich keinen kausalen Zusammenhang zwischen S6K1/S6K2 und der Makrophagenpolarisierung feststellen.

Um die Rolle beider Makrophagenkinasen in vivo zu untersuchen, habe ich außerdem chronische Entzündungsmodelle von NASH und NASH-HCC verwendet, entweder im Zusammenhang mit dem metabolischen Syndrom (westliche Ernährung) oder im Zusammenhang mit schwerer Leberfibrose (Cholinmangel-L- Aminosäuren-definierte Diätfütterung). Diese Experimente zeigten keinen kausalen Zusammenhang zwischen den Makrophagen S6K1/S6K2 bei Fettleibigkeit, metabolischem Syndrom (d. h. Glukose-/Insulintoleranz, Fettleibigkeit/Körperzusammensetzung) und Leberpathologie über das Fortschreiten der Leberfibrose hinaus.

Um die Rolle der Makrophagen S6K1/S6K2 bei der Leberfibrose zu verstehen, habe ich ein hepatotoxisches Modell von Tetrachlorkohlenstoff (CCl₄) verwendet, um Leberfibrose bei Mäusen mit Makrophagen zu induzieren, denen die S6-Kinasen in myeloischen Zellen

fehlen. Meine Daten zeigten, dass die Makrophagen-S6-Kinasen eine untergeordnete Rolle bei der Leberfibrose spielen.

Dabei zeigte meine Arbeit, dass die mTOR-S6K-Achse des Makrophagen in NASH von Mensch und Maus aktiviert ist. Der Verlust von Makrophagen S6K1/S6K2 hatte zwar keinen Einfluss auf die NASH-Progression oder NASH-HCC, dämpfte jedoch die Leberfibrose bei NASH- und CCl₄-induzierter Leberschädigung. Meine Daten tragen dazu bei, widersprüchliche Erkenntnisse über die Rolle der Makrophagen-mTOR-S6K-Achse bei Fettleibigkeit, NASH, NASH-HCC und Leberfibrose aufzuklären.

4 Preface

Commended as a highly regenerative organ, the liver has been praised as such since at least the Greek mythology tales of Prometheus and Tityus, both of which were punished by Zeus by having their livers eaten daily by eagles and vultures, only to have their livers regenerate overnight so they could be punished again and again¹. It is not clear whether the Greek had evidence of liver regeneration at the time¹. Today we know the liver is indeed highly regenerative, especially upon acute insults like partial hepatectomy or chemical injuries, however, upon chronic challenges the effectiveness of liver regeneration is challenged, allowing fibrosis and neoplasms to occur as well as loss of function². In the modern world, an important chronic challenge for the liver is caused by the obesity epidemic and metabolic syndrome-related fatty liver disease and its complications^{3,4}. This will be explored in my present doctoral thesis, with a special focus on the consequences of a chronic positive energy balance on the liver microenvironment, the homeostasis of hepatocytes and the role of nutrient sensing by myeloid cells in liver homeostasis.

5 Introduction

5.1 The liver

Often referred to as “the laboratory” of the body, the liver is situated in the abdominal cavity behind the lower ribs, and it is a major regulator of nutrient homeostasis and the largest gland and internal organ in the human body⁵. The liver is compared to a laboratory mostly because of its main functions in metabolizing drugs and toxins, digestion, whole body metabolism and nutrient homeostasis^{6,7}. In line with its major functions, the liver is the fourth highest energy consuming organ at rest (i.e. 12-14 hours fasting humans) only behind the heart, kidneys and the brain⁸. Moreover, the liver is a highly vascularized organ that receives dual blood supply, that is, oxygen rich blood from the hepatic artery and nutrient rich blood from the portal vein ascending from the gastrointestinal tract (GI) with freshly absorbed nutrients⁵. Around one third of the blood received by the liver comes from the portal vein (~1000 mL/min at rest), with the remaining coming from the hepatic artery (~500 mL/min at rest)⁵.

Anatomically, the human liver is composed by two primary lobes termed as the right (the largest), and left main lobes, additionally, an inferior quadrate lobe and a posterior caudate lobe complete the organ⁵⁻⁷. Being that the present thesis deals with the study of the liver using mouse models, it is important to consider the liver anatomy of mice. Thus, the liver from mice is composed by four lobes termed the medial, left (largest) lateral, right and caudate lobes⁶ (Figure 1 and Figure 2). In both species, the liver plays an important role for digestion as it generates, stores, releases and eventually recycles nutrients and bile⁵⁻⁷. Aiding digestion of lipids, bile is doused from the gallbladder into the small intestine through the common bile

duct to emulsify fat, facilitating its breakdown and absorption^{6,7}. Regardless of the minor anatomic differences in the liver between humans and mice, there are no major liver physiological differences between the two species⁶. Consequently, mice models of preclinical research are useful for studying human liver diseases as long as the limitations of each model are acknowledged and respected⁹⁻¹¹. After all, as eloquently put by the famous statistician George Box “All models are wrong, but some are useful”.

Zooming in to the microscopic level, the liver parenchyma is also very similar between mice and humans; it is composed of hepatocytes, bile canaliculi and sinusoids organized in polygonal functional units known as hepatic lobules^{5,7}. Peripheral to these lobules, portal triads are formed by arterioles, venules and a bile duct^{5,7}. These vascular entities bring oxygen (hepatic artery) and nutrient rich (portal vein) blood to the lobules, as well as take away bile through the bile duct^{5,7,12}. Following the blood flow from the periphery, oxygen and nutrients move towards the central vein of each hepatic lobule through liver sinusoids^{5,12}. Said sinusoids are thin microvascular fenestrated projections that combine blood from the hepatic artery and portal vein in the periphery and carry it towards the central vein at the core of each liver lobule^{5,7,12}. Important for immunosurveillance, liver resident macrophages –Kupffer cells (KCs)– sit in the sinusoids surveilling for invading bacteria and microparticulates that may enter through the gastrointestinal tract and travel through the portal vein^{5,7,12}; KCs will be described in more detail in a different section.

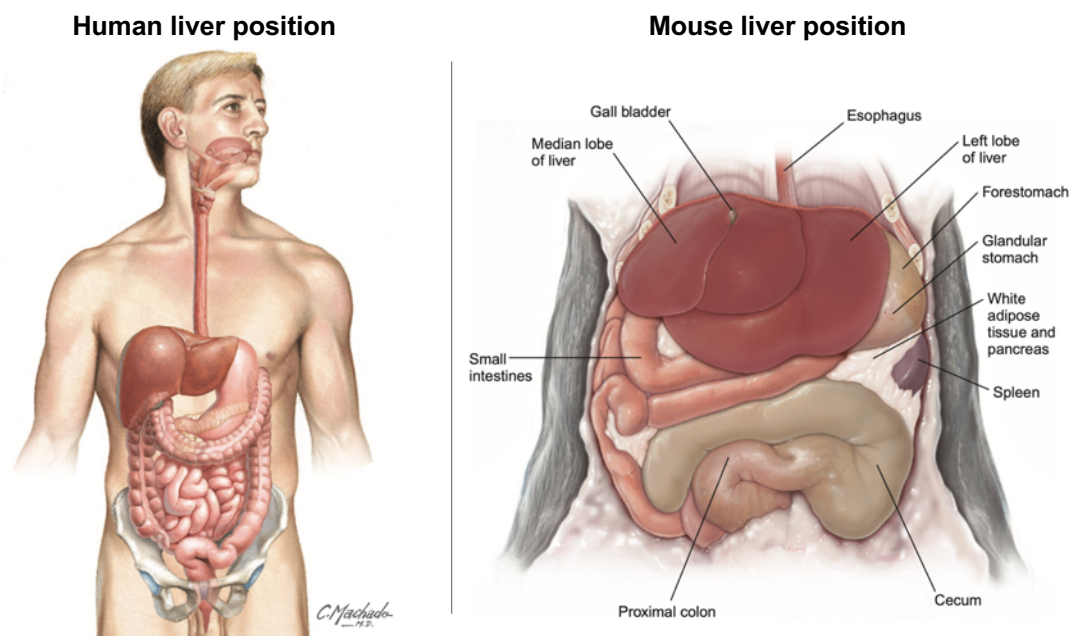


Figure 1. Liver location in humans and mice. The liver of mice and human reside below the ribs in the abdominal cavity. The human liver extends from the flank until right after the medial line of the abdomen. Conversely, in mice the liver occupies most of the abdominal front space. In both cases, the liver is neighbored by the stomach, spleen, pancreas and intestines. Adapted from Rogers et al., 2012⁶.

Although the liver sinusoids are the closest blood carrying microvasculature before reaching the hepatocytes, the latter are not in direct contact with the blood stream. Rather, through a perisinusoidal space, best known as the space of Disse¹³, which separates the sinusoids from hepatocytes and contains plasma (e.g. nutrients, electrolytes and dissolved gasses) that arrives through fenestrations on the sinusoids, excluding the cellular components of blood (i.e. red and white blood cells)¹² (Figure 3). To access the plasma components, hepatocytes reach their basolateral microvilli into the space of Disse, where they uptake and exchange biomolecules^{7,12}. Additionally, the space of Disse is home to hepatic stellate cells (HSC), whose main function is the storage of vitamin A as well as extracellular matrix production for tissue repair thus, in a pathological context, HSC play a key role in liver fibrosis^{5,12}.

Bile is secreted from each hepatocyte not towards the space of Disse and sinusoids, but rather through their apical membrane into bile canaliculi that travels connected to each hepatocyte^{7,12}. The bile canaliculi move bile towards the bile ducts at the portal triads and form a left and right hepatic duct; they eventually unite to form the common hepatic duct^{5,12}. Notably, ~95% of the bile is reabsorbed in the GI and make their way back to hepatocytes for recycling; the remaining 5% is excreted in the feces and represents the main excretory path for cholesterol (a major component of bile), bilirubin and some drugs⁵.

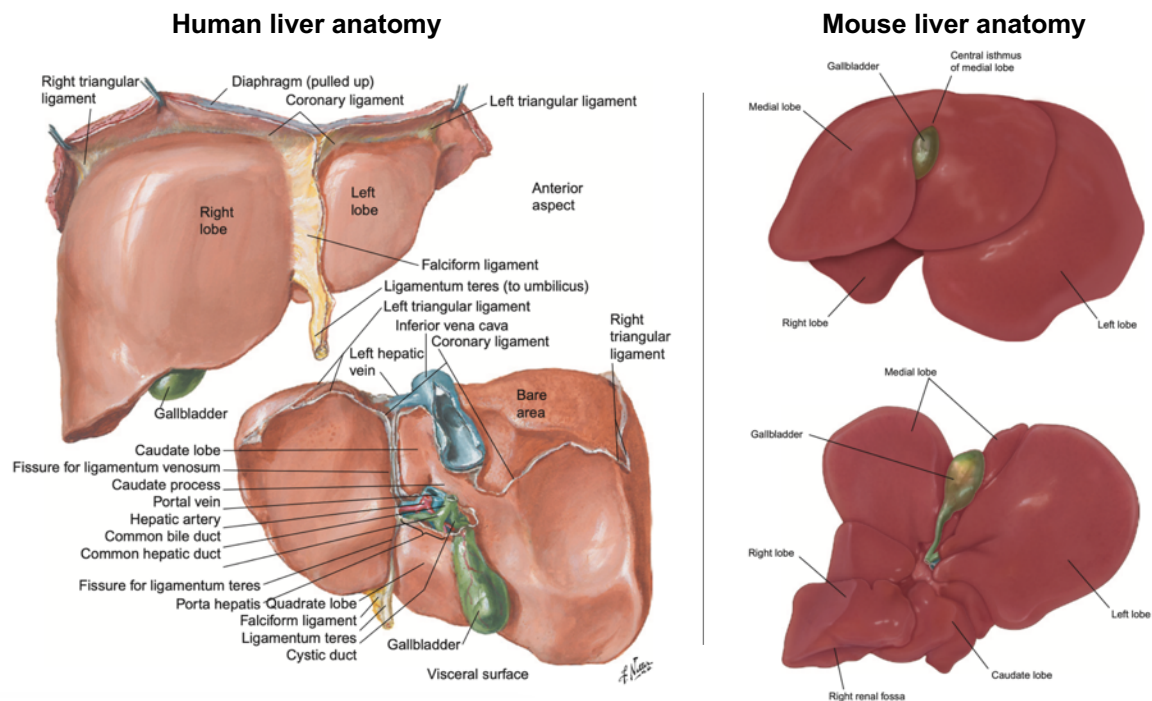


Figure 2. Human and mouse liver anatomy. The human liver is composed by the right (biggest), left, caudate and quadrate lobes. The mouse liver is composed by the medial lobe (biggest), left, right, and caudate lobes. Both livers have a gallbladder that stores bile. Adapted from Rogers et al., 2016⁶.

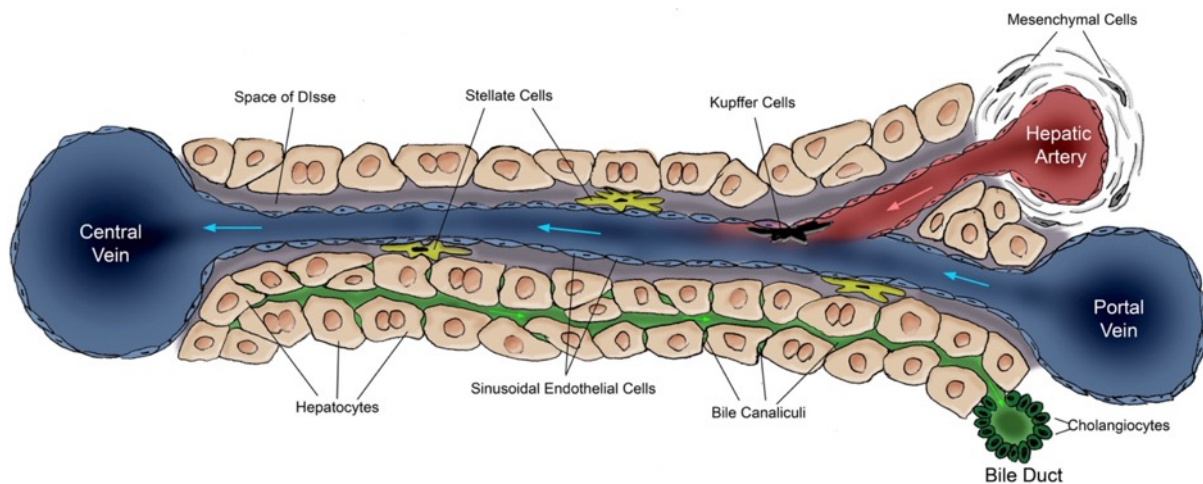


Figure 3. Micro anatomy of the liver lobules. The lobules are the functional unit of the liver. They are conformed by a portal triad (right end) where the hepatic artery, portal vein and a bile duct are in proximity. The hepatic artery and portal vein converge on the liver sinusoid where the combined blood flows towards the central vein at the center of the lobule. The sinusoids are guarded by liver resident macrophages –Kupffer cells–. Other immune cells (not shown) also transit through the sinusoids. The perisinusoidal space of Disse separates the hepatocytes from the sinusoids and is home of hepatic stellate cells. Bile from the apical membrane of hepatocytes flows from the middle of the lobe towards the portal triads through bile canaliculi, eventually flowing into the bile ducts. Adapted from Fanti et al., 2017¹⁴.

5.1.1 The immune system of the liver

The tissues and organs in contact with the exterior world are a first line of defense against foreign invaders such as bacteria, viruses and toxins. Helping with this task, the inner side of organs like the skin, the lungs and the entire gastrointestinal tract, are surveilled and defended by innate and specialized immune cells. Although the liver is not in direct contact with the outside, three quarters of its blood supply comes from the portal vein carrying blood ascending from the GI, where microorganisms and toxins may reach the bloodstream evading immune surveillance, and ultimately reach the liver^{15,16}. Therefore, the liver is a non-obvious, yet important line of defense against the outside world.

As portal blood reaches deep into the liver through the liver sinusoids, a first line of defense is met at this level in the form of phagocytosing KCs patrolling the sinusoids. From there, KC can project extensions of their membrane to reach into the space of Disse, hepatic stellate cells (HSC) and hepatocytes for exchanging biomolecules^{17,18} (Figure 3). The main role of KCs in immunity is that of phagocytosing antigenic molecules and dying red blood cells, antigen presentation and expression of costimulatory molecules (i.e. cytokines and chemokines) to modulate other immune cells^{17,18}. Notably, the iron from dying red blood cells is passed by KC-membrane extensions through the space of Disse to deliver it directly to hepatocytes for storage and recycling^{18,19}. Additionally, KCs are important mediators of tissue homeostasis

and liver regeneration. For example, upon hepatotoxic insults (e.g. aflatoxins²⁰, per- and polyfluoroalkyl substances (PFAS)^{21,22} and carbon tetrachloride (CCl₄)²³) KC mediate the immediate “cleanup” of dead cells while producing costimulatory signals that result in monocyte infiltration and activation¹⁵. Moreover, KC are key mediators of fibrosis resolution through the degradation of extracellular matrix and immune regulation^{15,18,24}. Additionally, KC are highly plastic and readily differentiate or are replenished by infiltrating monocytes¹⁵.

One layer deeper, in the space of Disse, HSC (Figure 3) are normally quiescent cells that store vitamin A as explained before, however, upon activation primarily by TGF- β (produced by macrophages), they transdifferentiate into myofibroblasts and secrete extracellular matrix, leading to its accumulation and liver fibrosis²⁵. Thus, HSC they are critical for liver fibrosis, and are thought to have antigen presenting functions, although this is still debated²⁶.

The liver is also surveilled by immune cells traditionally thought to be rather liver infiltrating; these include neutrophils, monocytes, monocyte derived macrophages (MoMFs), T-cells, B-cells, natural killer cells (NK), and platelets^{16,17,27,28}, all of which reside in the liver sinusoids in the healthy liver but which can infiltrate the parenchyma during inflammatory situations²⁷. Like in the periphery, these immune cells play important roles in innate and acquired immunity in the liver, whereby in the context of an immune surveillance function, they react against antigenic stimuli^{17,29}. However, given the continuous exposure of the liver to bacterial products and toxins arriving through the portal vein, immune cells in the liver are immunotolerant under normal conditions^{17,29,30}. Whenever this immunotolerant balance is subdued by chronic inflammatory insults (e.g. steatosis, fibrosis, superfluous LPS exposure, etc.), autoimmunity can arise and fuel the deterioration of the liver parenchyma rather than solving the pressing insult^{29,31,32}. Thus, diverse immune cells patrol and defend the liver homeostasis against infection and damage with many additional immune cell types and subtypes beyond the scope of this thesis, but all play a role in liver homeostasis (Figure 4).

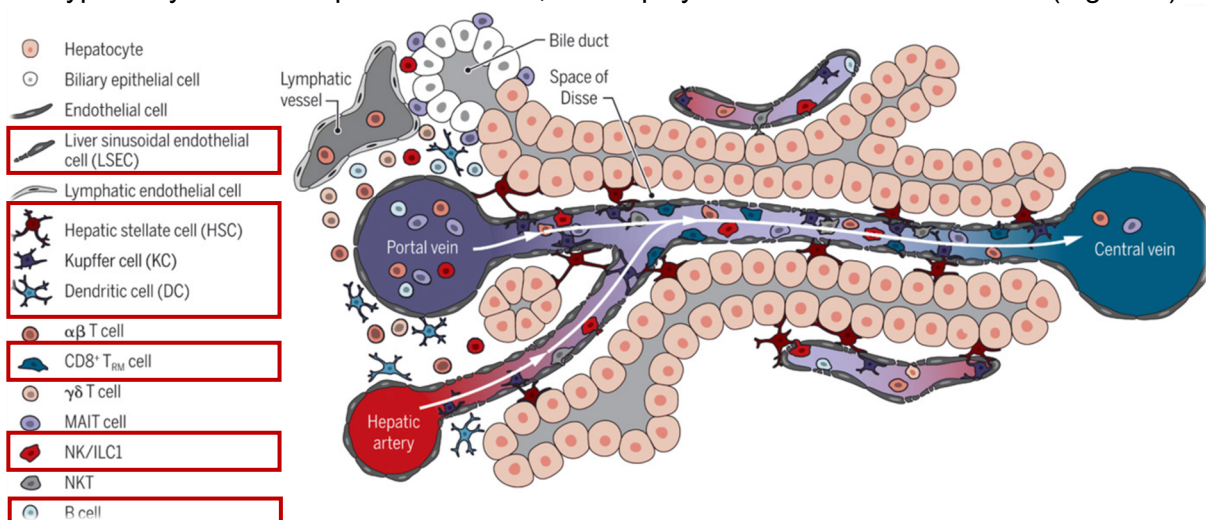


Figure 4. Liver immune cells, several types and subtypes. Many types and subtypes of immune cells contribute to maintaining liver homeostasis by defending against invading bacteria, viruses and toxic biomolecules (e.g. LPS) reaching through the portal vein. Red boxes enclose the cell types discussed. Adapted from Ficht & Iannacone 2020²⁷.

Impaired liver immunity has consequences including modifying therapeutic outcomes in the context of pathology. One example being the impaired response to immune checkpoint inhibition observed in hepatocellular carcinoma associated with non-alcoholic steatohepatitis³¹. There, CD8⁺PD-1⁺ T-cells are non-responsive to immunotherapy by anti-programmed cell death protein 1 (anti-PD-1), and paradoxically, anti-PD-1 therapy accelerates hepatocarcinogenesis³¹. This example presents important limitations that might not otherwise be expected in the clinical treatment of patients with hepatic diseases, and which may well result in harm, rather than benefit for patients. It is therefore imperative to continue studying the hepatic immune landscape in different disease contexts, from where one can inform the clinicians as to whether a therapy might be better for one patient with a specific history or markers, rather than an otherwise “standard-of-care” or “gold-standard” therapy. Moreover, such findings inform the design of clinical trials to increase the rigor and output of their findings.

5.2 The burden of excessive body weight and obesity

Body weight and height are simple measurements used to calculate the body mass index (BMI) of adults³³. The index is calculated by dividing the body weight in kilograms by the height in meters squared^{33,34}. The index is used to roughly categorize adults as underweight (<18.5 kg/m²), normal (18.5 – 24.9 kg/m²), overweight (BMI ≥25 kg/m²) or obese (BMI ≥30 kg/m²) and relates to risks of developing diseases of malnourishment or overnutrition^{35,36}. Notably, the cutoff values can be adjusted for specific ethnicities to consider their specific health risks (e.g. South Asians normal BMI is restricted to 22.9 kg/m² instead of 24.9)³⁷. However, as useful as the BMI can be to suggest health risks, it has limitations since it does not distinguish body composition^{38,39}, whereby two individuals with the same body weight and height would report the same BMI (e.g. overweight), but one could have excessive visceral body fat associated with several health risks, whereas the second could present with increased musculature or subcutaneous fat, which is rather healthier compared to excessive visceral body fat.

The World Health Organization (WHO) collects data from member countries to track the global prevalence of body weight related risks. Notably, in the last decades, the prevalence of overweight and obesity have risen across the world to reach epidemic proportions⁴⁰. It is estimated that in 2016, up to 39% of adults in the world were overweight, while 13% were obese⁴⁰ with similar numbers reported in 2020⁴¹. Additionally, also in 2020, 39 million children under 5 years of age were overweight or obese⁴⁰. Furthermore, it is forecasted that as

developing nations adopt the so-called western diet and lifestyle, more than 50% of the adults will be overweight or obese by 2035, with obesity alone affecting up to 24% of the adult population⁴¹. In 2016, the WHO in collaboration with the organization –Our World in Data– published the map of overweight and obesity prevalence in adults (Figure 5).

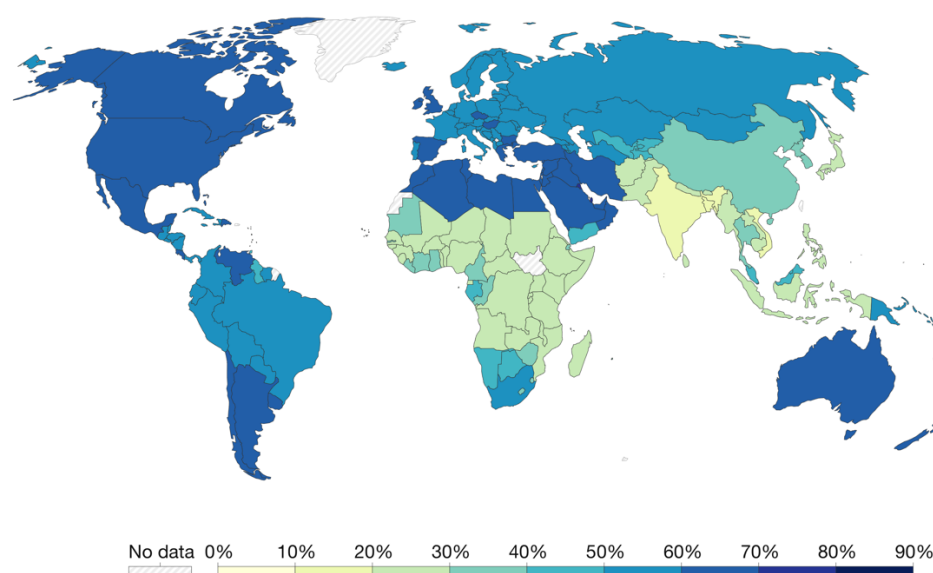


Figure 5. Share of overweight and obese adults in the world (2016 data). All adults with a BMI ≥ 25 Kg/m² are displayed in this map of overweight and obesity in the world. North and South America, the Middle East and Australia had the highest prevalence of overweight and obesity in 2016. Adapted from Our World in Data⁴².

Although obesity is usually seen as a clear-cut excess body weight problem, the reality is that it represents complex insults to the whole body. Obesity has multifaceted consequences on health ranging from the mechanical pressure the excess weight exerts on articulations and bone health⁴³, the increased output demand and deterioration of the cardiovascular system it elicits^{44,45}, to the molecular perturbations associated with obesity, like insulin resistance and increased oxidative stress⁴⁶⁻⁴⁸. Thus, obesity is a major risk factor for the development of diseases like dyslipidemia, cardiovascular disease, type 2 diabetes, fatty liver diseases, metabolic syndrome and several types of cancer such as liver, breast and gastrointestinal cancers⁴⁹⁻⁵¹. In sum, the impact of obesity and its associated diseases on public health and the economy is expected to reduce the gross domestic product of the world by 3% by 2035, an impact similar to that of the COVID pandemic in 2020⁴¹.

Efforts to control the obesity epidemic have tackled both prevention and therapy. Preventive approaches have mostly pivoted around education, whereby dissemination of nutrition and lifestyle information, the public has been made aware of the problems presented by obesity, as well as informed on the importance of a varied, well-balanced diet, food portion

control, exercise requirements, and other lifestyle components⁴⁰. Further, some countries have implemented public health incentives like supplemental taxation of carbohydrate-rich beverages, or mandates of standardized nutritional information on food product labels^{40,52,53}. In line, the WHO has published an “acceleration plan to stop obesity” full of recommendations to prevent and manage obesity by its member states⁵⁴. The plan aims to stop the growth of childhood obesity (younger than 5 years of age) before 2025, while reducing the growth of adolescent and adult obesity⁵⁴. Nevertheless, although the plan tackles several factors like education, creation of public spaces for exercising, implementation of taxes on sugar and fat-rich foods, recommendations for food manufacturers and food labelling, etc.⁵⁴ the plan does not implement any novel recommendations. Thus, it remains to be seen whether the plan has a significant impact beyond the same or similar recommendations that preceded.

Obesity treatment options include personalized nutritional and lifestyle interventions with 15% - 30% reduction of energy intake along increased physical activity, bariatric surgeries and procedures (e.g. gastric bypass, gastric banding and intragastric balloon), as well as several drugs (e.g. bupropion-naltrexone, orlistat, liraglutide and semaglutide)^{51,55,56}. In that regard, semaglutide is becoming a breakthrough in the treatment of obesity by inducing body weight loss of 15% when combined with lifestyle intervention⁵⁷. Nevertheless, there are no long-term assessments so far and its impact on fatty liver and related pathologies is currently debated⁵⁸. However, to date, the most effective long-term obesity treatment is bariatric surgery as it has the strongest impact on reducing mortality risks associated with obesity and in average, the longest lasting bodyweight control along a significant reduction of the healthcare costs compared with non-surgery control patients⁵⁹⁻⁶². Nevertheless, its use is restricted to patients with a BMI ≥ 40 or between 35 and 39.9 kg/m², if the treatment of a comorbidity like diabetes would benefit from weight loss⁵¹. Additionally, it carries common surgical risks, as well as long term intervention-specific risks (e.g. micronutrient malnutrition) that should not be ignored⁶³.

Taken together, although health workers currently have several tools for treating patients with overweight and obesity, applying these tools on the scale required by the current obesity pandemic is challenging. Thus, we must further investigate the molecular mechanisms at the helm of obesity, with the hope that we might find targets amenable to pharmaceutical treatment. Though, finding a singular or a discrete number of obesity-controlling targets might be wishful-thinking, since after all, our evolutionary path has finetuned the human body for rewarding energy intake, while minimizing our energy expenditure⁶⁴⁻⁶⁹. Thus, leaving the lucky of us with access to a surplus of food, in a battle to minimize our energy intake while maximizing our energy use. A battle lost every day by more than a third of the adult population^{40,41}.

5.3 The metabolic syndrome

The metabolic syndrome, formerly –syndrome X–⁴⁶, is a group of associated conditions, namely excess weight, impaired blood glucose and lipid homeostasis as well as hypertension⁷⁰⁻⁷³. Currently the metabolic syndrome is clinically diagnosed mostly in overweight or obese patients with concomitant at least three of the following criteria: 1) a large waistline (≥ 88 cm in women and ≥ 102 cm in men), 2) high blood pressure, 3) hyperglycemia, 4) hypertriglyceridemia, or low HDL cholesterol⁷⁰⁻⁷³. Table 1 contains reference values for each condition. Notably, the diagnostic criteria has been refined throughout the years, mostly by tightening the maximum cutoff values or limits of different markers before diagnosis⁷¹⁻⁷³.

Table 1. Current metabolic syndrome diagnostic criteria.

Condition	Criteria references
Central obesity	BMI ≥ 30 kg/m ² and/or waist circumference ≥ 88 cm in European* women ≥ 102 cm in European men
Prediabetes or diabetes	≥ 100 mg/dL fasting glucose or diabetes diagnosis
Dyslipidemia	Triglycerides ≥ 150 mg/dL or on anti- triglycerides treatment HDL-C < 40 mg/dL in men HDL-C < 50 mg/dL in women or on HDL-C treatment
High blood pressure	Systolic pressure ≥ 130 mm Hg and /or diastolic pressure ≥ 85 mm Hg Or on anti-hypertensive treatment

Diagnostic criteria according to the International Diabetes Federation (IDF) 2005⁷³. Central obesity plus two additional conditions are required for the diagnosis of metabolic syndrome⁷³. *There are ethnic-specific cutoff points⁷³. BMI: body mass index. HDL-C: high density lipoprotein cholesterol.

It is estimated that 1 in 3 adults have metabolic syndrome in the US^{70,74}. Meanwhile, although the precise worldwide prevalence of the syndrome is not available, it is estimated that the metabolic syndrome is three times more common than diabetes, thus, around one quarter of the world population is expected to suffer metabolic syndrome⁷⁴. Supporting its impact on public health and overall burden, the metabolic syndrome is associated with significantly increased risks for cardiovascular disease, as well as conversion to type 2 diabetes and dyslipidemia in otherwise undiagnosed patients^{72,73}. Taken together, it can be argued that alongside the increasing prevalence of obesity, the metabolic syndrome will likely also continue to grow in prevalence worldwide, worsening the burden to the health systems and economy.

Preventing efforts are exercised mostly in the form of widespread education presenting metabolic syndrome and its consequences⁷⁵⁻⁷⁷, as well as the risks of excess weight and obesity, like the accelerated plan to stop obesity by 2035 by the WHO previously mentioned⁵⁴. On the therapeutic side, treatment of the metabolic syndrome has the main goal of reducing the risk of atherosclerotic disease (i.e. cardiovascular risks)⁷².

The treatment of metabolic syndrome is spearheaded by management of dyslipidemia, hypertension and glucose homeostasis⁷². These goals are tackled by changes in modifiable risks factors, for which diet and exercise are prescribed⁷². Additionally, pharmacological therapies can be used by prioritizing the treatment of comorbidities in accordance with applicable clinical guidelines for each disease⁷². Additionally, when applicable, smoking cessation is also encouraged⁷². Thus, individual treatment schemes are tailored for each patient, adding pressure to the health systems.

An elusive “single pill” or general treatment scheme for metabolic syndrome would alleviate pressure on health professionals, but this is unlikely to happen as not all patients present with the same comorbidities, and similarly, the treatment options for each accompanying condition can have different outcomes in each person^{72,78}. Furthermore, as already mentioned, lifestyle interventions are limited by poor patient compliance to changes in lifestyle components (e.g. diet and exercise) on the long term⁷⁹⁻⁸⁴. Taken together, tackling metabolic syndrome at its current pandemic scale represents a great challenge for health systems that is expected to grow in prevalence. Thus, much effort is placed in understanding druggable molecular targets that may one day allow for either preventive or therapeutic modification of metabolic syndrome-associated maladies or its main risk factor –excess weight and obesity– at its core.

5.4 Metabolic syndrome-associated liver diseases

At its core, obesity results from a prolonged positive energy balance^{40,85,86}. In response to a positive energy balance signaled by insulin, the liver and skeletal muscle have the capacity to store carbohydrates in the form of glycogen, a large hydrophilic molecule that branches together glucose molecules for storage⁸⁷. Glycogen synthesis is promoted by insulin signaling an overall positive energy balance, whereas its degradation is controlled by glucagon and epinephrine when glucose is needed⁸⁷. Once the glycogen storage limit is reached (~15 g/Kg body weight⁸⁸), hepatocytes synthesize fatty acids from excess glucose through the *de-novo* lipogenesis pathway^{88,89}. These fatty acids can be transiently stored inside hepatocytes but should ultimately be released to circulation as very-low density lipoproteins (VLDL) and stored in the adipose tissue^{90,91}.

Upon chronic positive balance during the metabolic syndrome, tissues are insulin insensitive and thus, the insulin insensitive adipose tissue continues to release free fatty acids (also known as non-esterified fatty acids) and glycerol into circulation which are taken up by hepatocytes, esterified and stored as triglycerides^{91,92}. Unlike the adipose tissue, it can be said that the liver is not a lipid storage tissue, thus, prolonged storage of lipids in the liver represents a hepatic insult^{90,93}. The insult originates in part from excess free fatty acids saturating mitochondrial β -oxidation in the liver and thus, their storage inside hepatocytes leads to increased reactive oxygen species (ROS) and lysosomal dysfunction through the expression of inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α)⁹³⁻⁹⁶.

Additional insult comes from insulin resistance and increased ROS that generate endoplasmic reticulum (ER) stress that further fuels insulin resistance, inflammatory signals and ultimately results in hepatocyte death^{97,98}. Thus, the hepatic insult posed by the metabolic syndrome represents a vicious cycle of insulin resistance and hepatocyte damage that further encourages liver steatosis and inflammation (Figure 6).

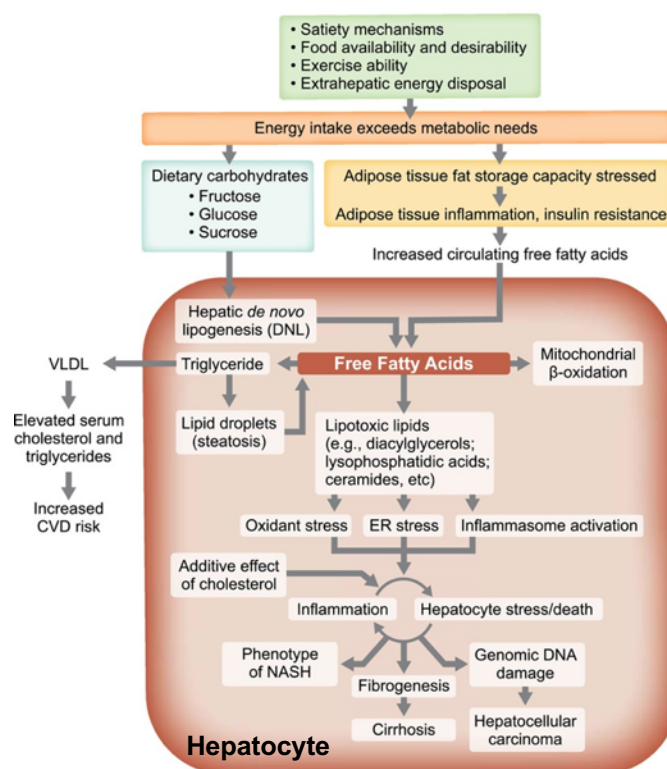


Figure 6. Metabolic syndrome associated drivers of hepatocyte damage. Excess nutrients and energy substrates from food, and insulin resistant adipose tissue result in excess free fatty acids reaching hepatocytes. The mechanisms of mobilization out from hepatocytes (β -oxidation and triglyceride/lipoprotein synthesis) are impaired and saturated, leading to endoplasmic reticulum and oxidative stress. These in turn drive inflammation, DNA damage and hepatocyte death, thereby driving liver inflammation and fibrogenesis. VLDL: very low density lipoprotein. CVD: cardiovascular. ER: endoplasmic reticulum. NASH: non-alcoholic steatohepatitis. DNA: deoxyribonucleic acid. Adapted from Rinella et al., 2023³.

5.4.1 Non-alcoholic fatty liver disease

In the absence of alcoholism, significant liver steatosis results in non-alcoholic fatty liver disease (NAFLD), recently renamed as (metabolic dysfunction-associated fatty liver disease (MAFLD))⁴ –in this doctoral thesis the former name will be used to avoid confusion–, similarly, the American Association for the Study of Liver Diseases (AASLD) practice guidance from 2023 did not yet change its nomenclature either³. To that end, NAFLD is diagnosed in the clinic when $\geq 5\%$ of hepatocytes show macrovesicular steatosis in the absence of alternative explanations such as the use of certain medications, starvation, or significant alcohol consumption (i.e. <20 g/day in women and <30 g/day in men)³. In line, the European clinical guidance guideline requires the quantification of liver fat content by either proton magnetic resonance spectroscopy (showing $\geq 5.6\%$ liver fat), or by imaging magnetic resonance (MRI)⁹⁹. Additionally moderate inflammation can also be present³. Although not specifically mentioned as a diagnostic tool in the latest AASLD-practice guidance, the NADLF-Activity Score (NAS) can be used by pathologists as a reproducible criterion for the characterization of the histological components of NAFLD^{3,100}.

The NAS is calculated by a pathologist assessing the relative degree of steatosis, hepatocyte ballooning and inflammation present in a standardized liver needle biopsy using the following criteria: steatosis (0-3 points), inflammation (0-3 points) and hepatocyte ballooning (0-2 points) with a maximum sum of 8 points being possible¹⁰⁰. The unweighted sum of points is then interpreted according to the criteria in table 2, whereby the liver biopsy is described as either non-non-alcoholic steatohepatitis (NASH), borderline or NASH¹⁰⁰. Additionally, the indication language used in this thesis appears in the third column.

Table 2. NAFLD-Activity Score interpretation.

Total NAS points	Indication	Indication in this thesis
0-2	Non-NASH	Healthy
3-4	Borderline	NAFLD
≥ 5	NASH	NASH

First two columns from the left according to Kleiner et al. 2005¹⁰⁰. NAFLD: non-alcoholic fatty liver disease. NAS: NAFLD-Activity Score. NASH: non-alcoholic steatohepatitis.

To date, the most widely accepted figures regarding the prevalence of NAFLD in adults is that of Younossi and colleagues from 2016, where they showed that up to 25% of the adult population in the world have NAFLD¹⁰¹. In line, the latest AASLD-practice guidance sets the prevalence between at 25%-30% in the general population³. However, it is expected that given its lack of symptoms and the extensive prevalence of its main risk factor –obesity–, the real prevalence of NAFLD might be much higher than so far reported¹⁰².

The treatment of NAFLD is based in lifestyle changes (i.e. healthy diet and regular exercise) with a weight loss goal of at least 3%-5% to improve liver steatosis³. It is recommended to treat patients with a multidisciplinary clinical team formed by primary care/endocrinology, gastroenterology/hepatology and nutrition/lifestyle experts to addressing primarily cardiovascular risks factors. Additionally, in the treatment of comorbidities, the use of liver-beneficial drugs (i.e. weight loss drugs and antidiabetics) like tirzepatide and gliflozins is encouraged as they tend to ameliorate liver histology and serum enzymes³.

Dietary intervention should be based on the reduction not only of energy intake, but also the reduction of saturated fats and refined carbohydrates as well as increased exercise³. Nevertheless, the limitations of lifestyle interventions due to long-term compliance have been addressed before in this work. Notably, the consumption of 3 or more cups of coffee per day is recommended as this has shown beneficial effects on liver health on several epidemiological studies³. Other than this, the treatment of NAFLD is through the control of its comorbidities according to their specific clinical guidelines. Although NAFLD is in principle benign and reversible, if untreated, it can progress with inflammation and liver fibrosis^{3,95,99,103} (Figure 7) which will be addressed in the coming sections.

5.4.2 Non-alcoholic steatohepatitis

The inflammatory progression of NAFLD leads to non-alcoholic steatohepatitis (NASH), also recently renamed under the umbrella concept of MAFLD, where the main difference between NAFLD and NASH is thus given as a grade of disease activity and stage of fibrosis⁴. Nevertheless, like with NAFLD above, the old nomenclature will be used in doctoral thesis to avoid confusion. Thus, like NAFLD, NASH is clinically diagnosed by the presence of liver steatosis ($\geq 5\%$), hepatocyte ballooning (indicative of cellular damage), inflammation (as accumulation of inflammatory foci) with or without fibrosis^{3,4,99,100} (Figure 7).

Thus, in patients with high risk of cardiovascular disease and liver fibrosis, the eventual NASH diagnosis can be made with a liver needle biopsy assessed for the NAS by a certified pathologist, where patients with a NAS sum ≥ 5 points are given a histology-confirmed NASH diagnosis^{99,100,103,104} or otherwise as indicated in table 2. However, the European clinical guidance guideline does not allow the use of NAS as a diagnostic tool, but rather as an evaluation of disease severity⁹⁹.

The prevalence of NASH has not been systematically recorded until recently. Nevertheless, it is estimated that 5% of the world population has NASH, with a higher prevalence (up to 37%) in patients with type 2 diabetes¹⁰⁵. Meanwhile overweight and obese patients have a NASH prevalence of 33%¹⁰⁶. In contrast, other studies set the prevalence of

NASH at 14% in a population screened for asymptomatic colon cancer¹⁰⁷. Further evidence of the current burden of NASH is that in the US from 2002 to 2019, NASH was the number 1 cause of liver transplantation in women and the second reason for inclusion in the transplant waiting list other than liver cancer¹⁰⁸. Therefore, the prevalence of NASH is mostly discussed in the context of its risk factors like obesity and NASH, rather than in the general population. Importantly, like NAFLD, NASH is not part of current health screening efforts^{71,99,103,104}, thus, it can be expected that the real number of adults with undiagnosed NASH is much higher.

The majority of patients screened for NASH arrive to primary care (i.e. house doctor / general practitioner) with incidental discovery of liver steatosis in imaging assays for unrelated conditions, or with elevated liver enzymes in serum^{99,103,104}. In any case, before invasive diagnostic tools are used, the latest AASLD Practice Guidance recommends first to assess the patients in primary care for risk factors using tools like the fibrosis-4-calculator (FIB-4) to rule out significant fibrosis⁹⁹. Briefly the FIB-4 considers age of the patient, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and platelet count to predict liver fibrosis without invasive procedures¹⁰⁹. The goal is to separate high-risk patients from lower risk patients. Lower risk patients should be treated with lifestyle intervention and treatment of comorbidities like mentioned above for the treatment of NAFLD⁹⁹. Briefly, lifestyle changes, namely diet and exercise are prescribed. In contrast, high risk patients should be treated by gastroenterologist and or hepatologists alongside nutrition and psychology experts whenever possible to improve the long-term outcome of lifestyle interventions⁹⁹. Nevertheless, the treatment options are the same, only depth of diagnostic tools is increased to include invasive or costly techniques like the liver biopsy or proton magnetic resonance spectroscopy in high-risk patients³.

Regardless of the severity of NASH, there is currently not a single drug approved for the treatment of NAFLD or NASH^{110,111}. Thus, the pharmacological treatment is similar to NAFLD, limited to the treatment of any present comorbidities using lifestyle interventions aiming for weight loss ($\geq 10\%$ is needed for histologically relevant changes) and anti-diabetics whenever possible^{99,103,104}. All things considered, the current lack of specific NASH treatments warrants the continued research efforts to understand the pathophysiology of the disease, biomarker discovery therapeutic discoveries. In that regard, resmetirom, a liver specific thyroid-hormone receptor- β (THR- β) recently showed promising results in phase III clinical trials (preliminary not yet peer-reviewed data) where it was well tolerated for 52 weeks, showed improved liver steatosis, ameliorated serum liver enzymes and lipid profile as well as improved liver fibrosis^{112,113}. Importantly, liver fibrosis is a large risk factor for disease worsening and carcinogenesis in NAFLD and NASH²⁵. Resmetirom is not yet approved for the treatment of NAFLD/NASH but according to a press release, it has gained approval as a “breakthrough

therapy” by the American Food and Drug Administration (FDA), which allows it an accelerated process for final approval¹¹⁴.

Moreover, current efforts in drug developments are following double or triple target combinatorial treatments that aim to modify several disease driving mechanisms at once, namely those targeting weight loss and glucose homeostasis (glucagon-like peptide 1 receptor agonist), those targeting ER-stress, lipotoxicity and mitochondrial dysfunction (THR- β and fibroblast growth factor (FGF) 19/21 agonists) as well as those targeting inflammation, cholesterol toxicity, and fibrosis (farnesoid X receptor and peroxisome proliferator-activated receptor (PPAR) agonists)^{115,116}. In any case, there is great urgency to find and approve effective NAFLD/NASH therapies given their role as risk factors for the development of hepatocellular carcinoma posed by NAFLD/NASH and their association with increased cardiovascular-related deaths^{99,103,104,117}.

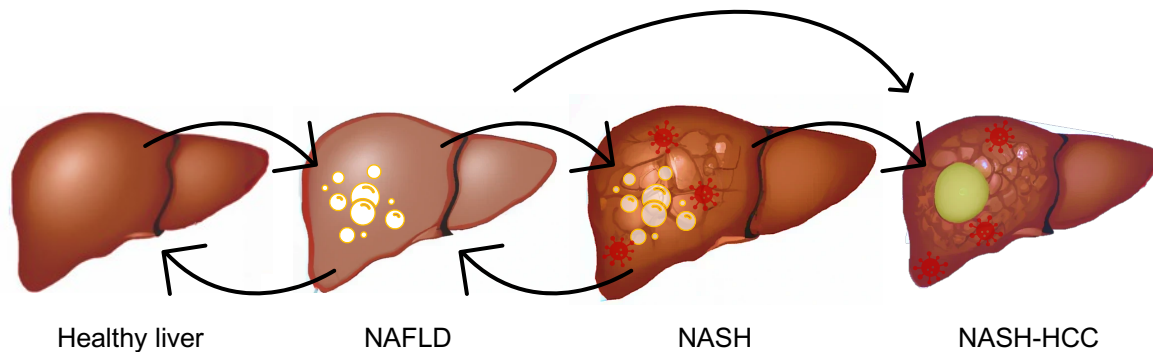


Figure 7. Non-alcoholic fatty liver disease progression. Accumulation of lipids in the liver leads to non-alcoholic fatty liver disease (NAFLD). Inflammation of the fatty liver leads to non-alcoholic steatohepatitis (NASH), which can present with fibrosis. Continued metabolic insult can lead to hepatocellular carcinoma (HCC) which can also arise directly from NAFLD without significant inflammation. NAFLD and NASH can regress in severity. Base liver illustrations created with DALL-E 2 (prompt used in the methods section).

5.4.3 NASH-hepatocellular carcinoma progression (NASH-HCC)

In 2020, liver cancer was the sixth most diagnosed cancer and the third leading cause of cancer deaths in the world¹¹⁸. Hepatocellular carcinoma (HCC) formerly “hepatoma”, arise from low grade dysplastic nodules that become high-grade dysplastic nodules and eventually early or progressed HCC¹¹⁹. HCC is the most common form of liver cancer, accounting for ~90% of all cases¹²⁰. Importantly, NAFLD and especially NASH are major risk factors for the development of HCC^{30,117,120-122} (Figure 7). Although currently most HCC cases have a viral etiology (viral hepatitis B and C), vaccinations and modern current therapies are blunting the prevalence of viral-HCC. In contrast, metabolic diseases like type 2 diabetes, metabolic syndrome and NAFLD/NASH account for a growing incidence of metabolic-associated HCC^{102,117,120,121}. In line, chronic necroinflammation of the liver, like that posed by NASH favors the accumulation of mutations which together with an impaired immune microenvironment and liver regeneration, allow the accumulation of transformed cells that give rise to dysplastic nodules that eventually form HCC⁹⁴.

Broad prevention of HCC is two-fold: viral etiologies are tackled through antivirals in patients with hepatitis B or C virus infection and vaccination for hepatitis B virus, whereas the nonviral etiologies have mostly preliminary observational foundations. These range from the dose-dependent consumption of coffee being protective against HCC¹²³, to aspirin/antiplatelet therapy^{28,124}, statins¹²⁵ and metformin¹²⁶ showing preventive effects¹¹⁷. Beyond these, avoidance of already mentioned risks factors, namely, obesity, type 2 diabetes, metabolic syndrome, NAFLD/NASH and liver fibrosis would be encouraged. Special emphasis should be placed in the diagnosis and management of chronic liver diseases (e.g. NASH and cirrhosis) to avoid progression to HCC¹²⁷.

Diagnosis of HCC must be done through noninvasive imaging since no serum markers are accurate for diagnosis¹¹⁷. Thus, HCC diagnosis is through liver imaging with MRI or CT^{117,127}. Additionally, tumor biopsies are recommended (and the gold standard diagnostic tool) for the precision oncology they can offer^{117,127}. Furthermore, the tumor extent should be assessed by CT or MRI, particularly for tumors bigger than 2 cm, which warrant imaging exploration for lung methastasis¹¹⁷.

In the absence of cirrhosis, the treatment of HCC is based on surgical resection of tumors^{117,120,127}. However, surgical resection is limited and contraindicated by several factors, both anatomical (e.g. the amount of liver that should be removed) and physiological (e.g. overall health and likelihood of decompensation and death), thus not all tumors are resectable^{117,121,127}. Additional therapies are systemic chemotherapies, different forms of radiation therapy, chemoembolization, thermal ablation of tumors, liver transplantation and

immunotherapy^{117,121,127}. Each treatment modality is associated with different outcomes that are linked to the extent of tumor size, number as well as overall health. An example of treatment effectivity is that 40% of people diagnosed with primary liver cancer in England survive for at least one year, whereas only 15% survive for at least 5 years after diagnosis¹²⁸.

New HCC treatment modalities are extending overall survival (OS) and progression free survival (PFS). In line, a recent therapeutic breakthrough came from combination immunotherapy targeting the programmed death ligand 1 receptor (anti-PD-L1) and the vascular endothelial growth factor 1 (anti-VEGF) that resulted in increased OS and PFS in patients with irresectable HCC, compared with the previous standard of care for this subset of patients – sorafenib¹²⁹. In response, the FDA approved the use of anti-PD-L1 and anti-VEGF for the treatment of unresectable locally advanced or metastatic HCC with no prior systemic treatment, which has already reached the American clinical guideline for the treatment of HCC^{117,130}. Nevertheless, although the breakthrough is already significant in OS and PFS, the five-year follow-up will dictate a more complete picture of the efficacy of this treatment. This is important, given the expected prevalence growth of metabolic-related HCC in the next decades. Therefore, it is imperative to accelerate research on more effective and/or lower cost HCC therapies, as well as more effective preventive approaches.

5.5 Macrophages in NASH and NASH-HCC

As previously indicated, NASH and NASH-HCC arise in part due to chronic liver inflammation. Macrophages, both liver resident (i.e. KC) and infiltrating (MoMFs) play an important role in modulating liver homeostasis, inflammation and fibrosis^{18,131-133}. However, not all macrophages are the same. In that regard, upon chronic hepatic insults, liver resident KC self-renewal is not sufficient to maintain the pool of KC in the liver¹³⁴. In turn, KC are activated and release cytokines and chemokines that recruit infiltrating monocytes from circulation that become the so called –monocyte derived macrophages– (MoMFs)^{15,134}. These macrophages eventually acquire a phenotype similar to KC but are associated with an inflammatory phenotype that leads to HSC activation and subsequent liver fibrosis^{15,24,132} (Figure 8).

Many distinct macrophage populations have been identified in the liver according to the expression of different markers and the expression of inflammatory and anti-inflammatory genes and cytokines¹³⁵. Thus, there are abundant publications relating distinct macrophage populations with specific outcomes in NAFLD/NASH and NASH-HCC^{15,25,132,136}. Nevertheless, their interpretation should consider the specific conditions in which macrophages were studied, since after all, macrophages are highly plastic and can readily switch between inflammatory and anti-inflammatory phenotypes throughout the progression of the disease¹³⁷.

There are growing efforts to therapeutically target liver macrophages as modifiers of liver pathology²⁵. Major macrophage-direct and indirect targeting examples include anti platelet therapy (preventing their activation of liver macrophages²⁸), inhibition of C-C motif chemokine receptors 2/5 (CCR2/5; preventing macrophage-HSC activation), and inhibition of toll-like receptor 4 (TLR4; dampening danger recognition by macrophages sensing LPS and free fatty acids) to name some²⁵. Nevertheless, the specific role of the chronic positive energy balance in liver macrophages in NAFLD and NASH and NASH-HCC remains unknown, and its modulation might offer a therapeutic window for preventing or treating liver inflammatory diseases.

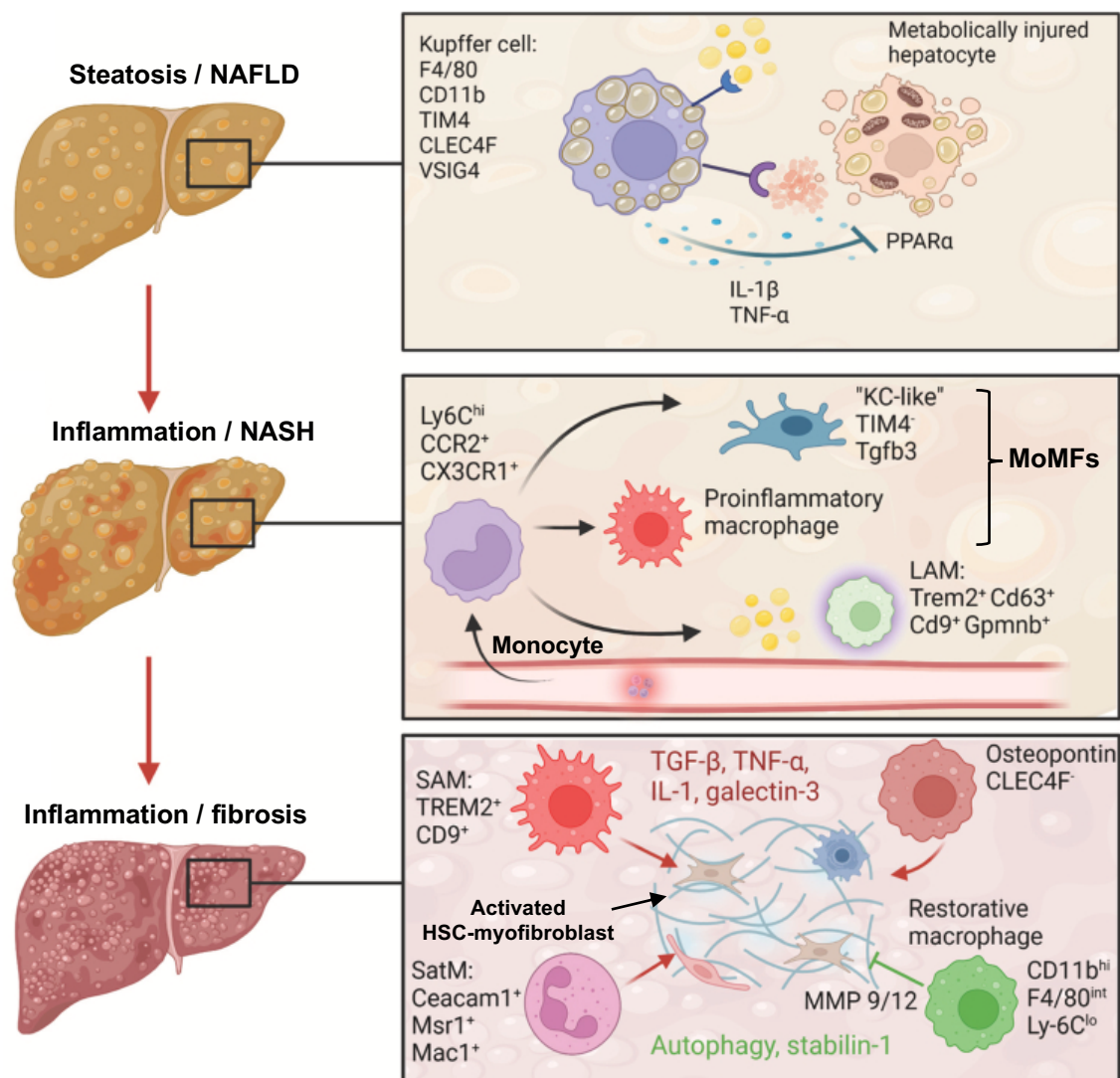


Figure 8. Diverse liver macrophages and functions. Top. Liver resident Kupffer cells are activated by damaged and dying hepatocytes in NAFLD, releasing inflammatory signals that attract infiltrating monocytes. **Middle.** Infiltrating monocytes differentiate into "KC-like" monocyte derived macrophages with proinflammatory phenotype. **Bottom.** In NASH, the different macrophage populations drive pro- and anti-fibrotic cues. Adapted from Vonderlin et al., 2023¹⁵.

5.6 Nutrient sensing, a matter of mTOR-S6K signaling.

As revealed at the beginning of this thesis, the role of nutrient sensing by liver macrophages is the focus of this work. Thus, it is important to delve into nutrient sensing. In that regard, all living cells need to actively build, degrade and recycle biomolecules and nutrients (e.g. DNA, RNA, protein, lipids and carbohydrates) as part of their fight for homeostasis –to live–, to grow and to proliferate¹³⁸. These anabolic (constructive) and catabolic (degrading) processes require mechanisms of nutrient sensing, in a way that signals of positive nutrient and energy balance allow or lead to anabolic processes, whereas signals of nutrient and energy scarcity lead to the activation of catabolic processes^{138,139}. Thus, in eukaryotic cells, anabolic processes are mostly regulated by the mechanistic (formerly mammalian) target of rapamycin (mTOR), whereas catabolic processes are regulated mostly by 5' adenosine monophosphate-activated protein kinase (AMPK)^{138,139}.

mTOR is a serine/threonine protein kinase from the phosphatidylinositol 3-kinases (PI3K)-related kinase (PIKK) family¹³⁸. It forms the catalytic subunit of two major protein complexes: the mTOR complex 1 (mTORC1) and complex 2 (mTORC2)¹³⁸. Both protein complexes share components and are formed as summarized in table 3.

Table 3. mTOR complexes components and function overview.

Complex	Components	Function overview
mTORC1	mTOR	Catalytic subunit
	Raptor	Facilitates substrate recruitment to mTORC1 by binding to the TOS-motif on several mTORC1 substrates. Is required for the correct subcellular localization of mTORC1
	mLST8	Associates with mTOR to stabilize the catalytic domain
	PRAS40	Inhibitory subunit
	DEPTOR	Inhibitory subunit
mTORC2	mTOR	Catalytic subunit
	Rictor	Believe to be functionally analogous to Raptor
	mLST8	Associates with mTOR to stabilize the catalytic domain
	DEPTOR	Inhibitory subunit
	mSin1	Regulatory subunit
	Protor1/2	Regulatory subunit

Raptor: regulatory protein associated with mTOR. mLST8: mammalian lethal with Sec13 protein 8. PRAS40: proline rich Akt substrate of 40 kDa. DEPTOR: DEP-domain containing mTOR interacting protein. Rictor: rapamycin insensitive companion of mTOR. mSin1: mammalian stress-activated protein kinase-interacting protein 1. TOS: TOR-signaling. Summarized from Saxton & Sabatini 2018¹³⁸.

The precise signaling cascades of mTOR are broad, complicated and not the focus of this thesis. Thus, they will be discussed briefly. Central to mTOR signaling, mTORC1 is activated by positive energy and nutrient balance after feeding to promote growth and energy storage in the form of glycogen and lipids. In contrast, mTORC1 is inhibited in the fasting state

to conserve cellular resources. Activating signals include growth factors like the insulin/insulin growth factor 1 (IGF-1), Wtn-pathways and TNF- α ¹³⁸. These growth factors act by ultimately inhibiting the tuberous sclerosis complex (TSC1/2) that is itself a negative regulator mTORC1, thus, inhibition of TSC1/2 by growth factors enhance mTORC1 activity¹³⁸.

mTORC2 is an effector of insulin/PI3K-signalling through the mTORC2 subunit – mSin1– which contains a phosphoinositide-binding PH domain that allows insulin to regulate mTORC2¹³⁸. Briefly, in the absence of insulin, mSin1 inhibits the catalytic activity of mTORC2. This inhibition is reversed when PI3K-generated PIP₃ binds at the plasma membrane¹³⁸. Additionally, mSin1 is also phosphorylated by Akt to promote the activation of mTORC2 which further phosphorylates Akt in a feedback loop¹³⁸. Furthermore, mTORC1 can inhibit mTORC2 through a negative feedback loop between mTORC1 and insulin/PI3K. There, mTORC1 phosphorylates the insulin/PI3K inhibitor (upstream of Akt and mTORC2) –Grb10–. Additionally, S6K1, downstream from mTORC1, can induce the degradation of the insulin receptor substrate-1 (IRS1) by phosphorylated it, thereby creating a feedback regulation mTORC2 by mTORC1¹³⁸.

Amino acids inside lysosomes and in the cytosol are sensed by mTORC1 through several amino acid “sensors” like GATOR1 that upon amino acid abundance phosphorylate and activate mTORC1¹³⁸. Similarly, energy, oxygen and DNA damage regulate mTORC1 through low ATP, hypoxia and DNA damage signals are ultimately relayed to mTORC1 through the activation of TSC and AMPK, both of which inhibit mTORC1¹³⁸.

Further downstream in the mTOR signaling cascade, both mTORC1 and mTORC2 are involved in the regulation of anabolic process. Briefly, mTORC1 integrates the anabolic stimuli to regulate mRNA translation, protein synthesis and turnover, glucose metabolism, lipid synthesis and nucleotide synthesis¹³⁸. Conversely, mTORC2 regulates proliferation and survival (Figure 9) by phosphorylating several AGC (PKA, PKG, PKC) family of protein kinases involved in cytoskeleton remodeling and cells migration¹³⁸. mTORC2 additionally controls insulin stimuli upstream of mTORC1 by phosphorylating and activating Akt so it can signal with insulin and PI3K, thereby promoting cell survival, proliferation growth¹³⁸.

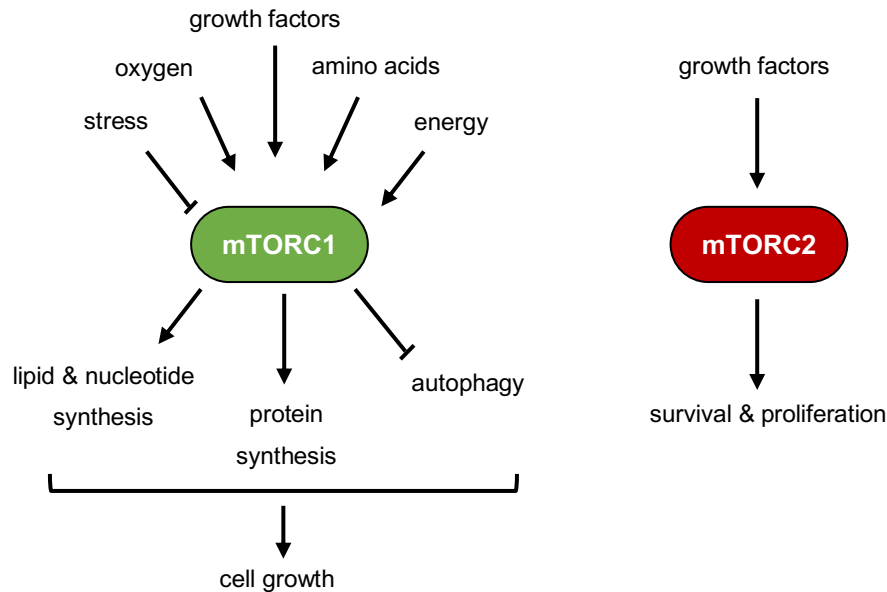


Figure 9. mTORC1 and mTORC2 signal integration. Both mTOR complexes integrate signals of nutrient and energy abundance, as well as growth signals to activate anabolic pathways leading to cell growth, survival, and proliferation. Adapted from Saxton & Sabatini 2017¹³⁸.

Taken together, it is difficult to determine which components warrant continued research, after all, each constituent plays a role in the signaling cascade. Nevertheless, it is clear that S6K1 is a key effector of mTOR signaling. In that regard, S6K2 is closely related to S6K1 and share many characteristics, functions and targets^{140,141}. Although some differences have been reported between both kinases, so far, no major differences that would impede their simultaneous study have come to light^{141,142}.

In humans, S6K1 and S6K2 are ubiquitously expressed, and although they share most targets, some specific targets have been reported^{141,142}. For example, mice lacking S6K2, S6K1 has shown compensatory mechanisms where S6K1^{-/-} mice are small whereas S6K2^{-/-} mice are bigger compared with control mice¹⁴³, so their study might benefit from manipulations targeting both kinases simultaneously to unearth their effect or dispensability for the hypothesis tested. Perhaps such experiments can be followed by independent manipulations of each kinase to weigh their contributions to an asserted effect. In that regard, the functional status of the mTOR-S6K pathway, accounting for both S6K1 and S6K2, can be assessed by examining the phosphorylation of the 40S ribosomal protein S6 (pS6) where pS6 is indicative of mTOR activation^{143,144}.

5.7 mTOR inhibition as a modulator of metabolic diseases.

Rapamycin, the mTOR inhibitor that led to its discovery¹⁴⁵⁻¹⁴⁷, induces an arrest of anabolic cell processes regardless of growth factors and abundant nutrients being available¹⁴⁸. In turn, catabolic processes (i.e. autophagy) increase and break down reserve energy substrates¹³⁸. Therefore, simply put, inhibition of mTOR could be used to manipulate the cells to consume stored fatty acids and thereby treat obesity. In that regard, mTOR inhibition leads to increased longevity in yeast, *c. elegans* worms and mice, which recapitulates the effects of calorie restriction^{149,150}. mTOR inhibition additionally improves age-related liver steatosis¹⁵⁰.

However, although mTOR inhibition has positive effects, it also has significant undesirable effects. For instance, inhibition of mTOR disrupts lipid homeostasis, induces glucose intolerance, insulin resistance, cataracts, liver damage and is immunosuppressive^{138,149-151}. Due to these important side effects, mTOR inhibitors like rapamycin and analogues are clinically relegated to use as immunosuppressants after organ transplant and antiproliferative therapy for some tumors^{152,153}.

Aiming to circumvent the undesirable side effects of broad mTOR inhibition, in the last years, several tissue specific and downstream mTOR modulators have been investigated with the premise of circumventing the undesirable side effects of upstream mTOR inhibition¹⁵⁴⁻¹⁵⁸. Particularly, mTOR effectors S6K1 and S6K2 have been investigated. For instance, in the context of positive energy balance, the global lack of mTOR effector –S6K1– in C57BL7/J mice was protective against diet-induced obesity (DIO) and additionally improved glucose homeostasis without evidence of hepatomegaly¹⁵⁹. Additionally, under normal energy settings (i.e. normal diet feeding), mice lacking S6K1 were long-lived compared with controls¹⁵⁶. Similarly, prophylactic adeno-associated virus (AAV)-mediated knockdown of S6K1/S6K2 in the liver recapitulated the beneficial effects of the S6K1 global knockouts by protecting mice from high fat diet (HFD)-induced insulin resistance and liver steatosis¹⁶⁰. The latter recapitulates the longevity-prolonging effects of systemic mTOR inhibition and or calorie restriction previously discussed, thereby exemplifying how downstream mTOR agents could be specifically modulated to recapitulate the beneficial effects of broad mTOR inhibition.

5.7.1 mTOR-S6K signaling by macrophages in the liver, a NAFLD/NASH focus.

Macrophages, both liver resident KC and infiltrating MoMFs, are important players in NASH as they mediate liver homeostasis and liver fibrogenesis by activating hepatic stellate cells into myofibroblasts¹³¹. Thus, modulators of liver macrophages hold therapeutic potential in NASH^{16,116}.

The central role of macrophages in the pathogenesis of NAFLD, NASH and NASH-HCC have been discussed. Additionally, the liver-damage driving chronic positive energy balance of obesity and insulin resistance has been covered in this work. Moreover, nutrient sensing by mTOR-S6K signaling cascade has also been introduced and has been shown to modulate macrophage polarization^{161,162}. Integrating all concepts, in NAFLD, mTOR is activated throughout the liver parenchyma and non-parenchymal cells (NPC)¹⁶³. Nevertheless, its role in liver NPC during NAFLD/NASH is unknown. Thus, we will next explore the role of the mTOR-S6K pathway specifically in liver macrophages during NAFLD and NASH.

In the liver, manipulation of mTOR signaling in macrophages has reported conflicting evidence. For instance, mice fed a high-fat diet (HFD) and harboring macrophage-specific mTOR inhibition by deletion of *Raptor*, were protected from HFD-induced insulin resistance and liver inflammation, and further, their macrophages showed reduced expression of inflammatory genes compared with those from control mice¹⁶⁴. Conversely, in a similar experiment but employing a Western diet (WD), mice with macrophage-specific *Raptor* deletion showed increased liver damage markers (ALT and AST), accompanied by increased liver inflammation and worse glucose homeostasis than wildtype control mice¹⁶⁵. Nevertheless, the limitations of the HFD used in the first experiment should be considered as HFD-feeding merely induces fatty liver without significant inflammation, whereas WD-feeding induces NASH and liver fibrosis⁹. Thus, direct comparison of both experiments is not possible. Therefore, the role of myeloid mTOR-S6K signaling in NASH and eventual NASH-HCC remains unclear.

6 Hypothesis and aims.

Myeloid cells are key mediators of liver homeostasis. In NAFLD and NASH, liver macrophages are exposed, alongside the liver parenchyma, to a chronic positive energy and nutrient balance. Nutrient sensing by mTOR signaling controls anabolic processes in response to a positive energy balance and has been shown to be increased in the NAFLD liver parenchyma and non-parenchymal cells, including myeloid cells. Hepatocyte and global mTOR inhibition have shown to be protective from excess nutrient insults. However, systemic mTOR inhibition has undesirable side effects that limit its clinical use. In turn, myeloid-specific mTOR inhibition has shown conflicting evidence regarding its therapeutic usefulness in NAFLD/NASH.

Consequently, in the frame of this doctoral thesis, to clarify whether nutrient sensing by macrophages play an important role in the pathogenesis of NAFLD/NASH and NASH-HCC, I have formulated the following aims.

- 1) Determine the status of mTOR-S6K signaling in liver myeloid during NAFLD/NASH, both in humans and mice.
- 2) Establish mouse lines with myeloid-specific deletion of the mTOR effectors –S6K1 and S6K2–.
- 3) Clarify whether S6K1/S6K2 play a role in macrophage polarization *in vitro*.
- 4) Determine whether liver myeloid signaling through S6K1/S6K2 has a causal link with obesity, glucose or insulin homeostasis in the context of obesity.
- 5) Elucidate whether liver myeloid signaling through S6K1/S6K2 has a causal link with NASH.
- 6) Clarify whether liver myeloid signaling through S6K1/S6K2 has a causal link with NASH-HCC.
- 7) Resolve whether liver myeloid signaling through S6K1/S6K2 has a causal link with liver fibrosis.

7 Materials and methods

7.1 Animal studies

All animal experiments were conducted in accordance with German Law with the approval of the Regierungspräsidium Karlsruhe Presidium under the licenses: G70-18 or G139-19. Animals without experimental interventions were used under the DKFZ332 general permit. Furthermore, most mouse experiments benefited from daily health controls by the personnel of the Center for Preclinical Research (ZPF) of the DKFZ.

C57BL/6J mice were purchased from Janvier Labs, France. Homozygous floxed S6K1^{fl/fl}/S6K2^{fl/fl} mice¹⁵⁷ –a kind gift from Prof. Dr. Dominic Withers from the Imperial College London– were crossed with Csf1r-Cre or LysM-Cre mice to generate Csf1rS6K1/S6K2 and LysmS6K1/S6K2 mice, respectively. Cre-negative littermates were used as controls for each cohort. Animals were allowed to habituate to our animal facility for at least 1 week before being fed any special diet. All mice were housed in green line individually ventilated cages (Techniplast) on a 12-hour light/dark cycle, between 21 - 23 °C, and under specified-pathogen free conditions in the Center for Preclinical Research (ZPF) from the DKFZ.

7.1.1 Special diets

All mice had *ad libitum* access to water and food. Starting between eight and twelve weeks of age, mice received either a standard chow diet (Provimi Kliba, Switzerland) or a Western-Diet (WD, Research Diets; D16022301i: 40% kcal fat, 20% kcal fructose and 2% cholesterol) for 30 weeks to induce NASH or 12 months to induce NASH-HCC. A Choline-Deficient L-Amino acid-defined High-Fat diet (CDAHFD, Research Diets; A06071302i: 60% kcal fat, L-amino acid-defined, 0.1% choline) was fed for 30 weeks to induce NASH-HCC. The body weight of the mice was controlled on a weekly basis by me. Additionally, the wellbeing of the animals was controlled daily by the caretaker team of the Preclinical Research Center (ZPF) of the DKFZ.

7.1.2 Carbon tetrachloride (CCl₄)-induced liver fibrosis

Acute liver fibrosis was induced with 0.5mL/kg CCl₄ or equivalent volume of corn oil injected intraperitoneal twice-per-week for 6 weeks. Treatments were performed in collaboration with Dr. Suchira Gallage from the DKFZ. Animals were culled 5-days after the last injection to reduce the toxicity risks of CCl₄ (highly volatile) during dissection. Animals in this experiment were also housed in green line cages but with an additional isolating disposable insert (Techniplast) to prevent contaminating the cages.

The insert, embedding and enrichment was exchanged once per week, or earlier if necessary. The body weight of the mice was controlled weekly and the wellbeing of the animals was monitored daily by the caretaker team of the ZPF. For safety, cage handling was done in the cytostatic laminar flow hood, wearing extensive personal protective coat, FFP3 face mask, and thick puncture and chemical resistant gloves over normal gloves. Similarly, CCl₄ aliquots for each injection were prepared observing strict safety measures in the cytostatic-handling central laboratory of the DKFZ.

7.1.3 Organ harvest

At the end of each experiment, mice were euthanized humanly and pain free during the day by time-controlled CO₂ exposure. Blood was collected by cardiac puncture and the harvested organs were weighed before flash freezing or paraformaldehyde fixation. Macroscopic liver tumors (≥ 1 mm) were counted and measured with a ruler immediately after liver harvest. Similarly, all livers were photographed over a graded paper immediately after dissecting them from the mouse and before processing them further.

Several metabolically and immunologically relevant tissues and organs were harvested during dissection from each mouse. Namely: blood, liver, spleen, pancreas, quadriceps, epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), and brown adipose tissue (BAT). Due to the considerable number of mice in each cohort, and the fact that many organs were harvested, processed, measured, and stored, most mouse dissections were carried out by me together with a group of collaborating scientists. Namely: Dr. Suchira Gallage, Dr. Jan Kosla, and Dr. Adnan Ali from the DKFZ. Moreover, during the COVID-lockdown in 2020, Dr. Pierluigi Ramadori, Sandra Prokosh, Ulrike Rothermel, Danijela Heide and Florian Müller (minimal emergency crew) dissected several mice included in the NASH cohorts.

7.2 Mouse bone marrow derived macrophages isolation and culture

I isolated the bone marrow from the femur and tibia of each mouse under sterile conditions. Thus, after dissecting the tissues from the tibia and femur inside the laminar flow hood, the hypophysis of each bone (one at a time) was cut with sterile scissors on a right angle. Then using a 10 mL sterile syringe with a 25G-needle and loaded with 10 mL ice-cold PBS+ (PBS, 2% heath-inactivated FBS), the needle was introduced into the bone marrow cavity and the PBS+ was flushed atop a 70 μ m cell strainer. The procedure was repeated with the remaining bones from each mouse to increase the biological yield. The cell strainer was flushed with 10 mL ice cold DMEM-F12 medium (11320033-Gibco). The cell isolate was washed once by centrifugation (1200 rpm, 5 min, at 4 °C), thereafter I resuspended the cells in BMDM-medium

(DMEM-F12, 1% heat -inactivated FBS, 1% Penycilin/Streptomycin 15140122 -Gibco) and seeded them in three 10 cm sterile Petri dishes (i.e. non cell-culture dishes).

The cells were allowed to attach for three days under standard-culture conditions (37 °C, 5% CO₂). On day four, 10 mL of fresh medium were added back in. On day five, 5 mL of medium and detached cells were aspirated and 10 mL replenished. On day six 10 mL were aspirated and 10 mL replenished. On day 7, cells were split and seeded 5e⁵ (RNA) or 7.5e⁵ (protein) in 6 cm tissue culture dishes (628160, Greiner) in 6 mL media. Cells were allowed to attach overnight.

On day 8, I stimulated the cells for 6 hours with 100 ng/mL LPS (L2630, Sigma), 20 ng/mL IL-4 (404-ML-010, R&D-Systems), or an equivalent volume PBS as control added directly on top of the medium and immediately agitating the plates by hand to avoid the effect of culture media change. Finally, I washed the cells twice with 3 mL ice-cold PBS before harvest with TRizol or 1x- radioimmunoprecipitation (RIPA) buffer with 1x protease and phosphatase inhibitors (04693116001 and 4906845001, Roche) for downstream analysis.

7.3 Mouse metabolic phenotyping

Intraperitoneal glucose tolerance test (ipGTT) was assayed in overnight-fasted mice following 28 weeks of WD-feeding and in collaboration with Dr. Suchira Gallage and or Sandra Prokosch. Thus, I measured the basal glucose concentrations with a handheld glucose analyzer (Accu-Chek Aviva, Roche) shortly before the intraperitoneal administration of D-Glucose (1 g/kg body weight). Thereafter, I repeatedly measured blood glucose after 15, 30, 60, 90 and 120 min.

Intraperitoneal insulin tolerance test (ipITT) was performed in 5-hour fasted mice after 29 weeks of WD-feeding and in collaboration with Dr. Gallage and Mrs. Prokosch as well. Thus, basal glucose concentrations were measured with a handheld glucose analyzer shortly before the intraperitoneal administration of human insulin (0.75 U/kg body weight). Thereafter glucose clearance from circulation was monitored by measuring blood glucose 15, 30, 60, 90 and 120 min after insulin administration.

Body composition was analyzed by me using an EchoMRI machine (EchoMRI) after 27 weeks of WD-feeding. A primary accumulation of 1T4 was used and body weight was measured immediately prior to EchoMRI to calculate the fat and lean mass percentages. Briefly, one mouse at a time, they were fixed inside the sampling tube of the machine in a fetal position and held immobile in place by the plunger. The tube with the mouse was inserted into the already prepared machine and the analysis was immediately started to minimize the stress on the animals. Results were extracted as a CSV file and analyzed using Microsoft Excel.

7.4 Serum parameters

At the indicated times, blood was isolated from mice in collaboration with Mrs. Prokosch and Mr. Müller. Briefly, living mice were held by their scruff on their neck and the v. facialis was punctured with a blood lancet to draw blood. Blood was collected in a gel serum tube (41.1378.005, Sarstedt) and kept at 4 °C until further processing.

Thereafter, to separate the serum, I centrifuged the blood (10,000 rpm, 10 min, at 4 °C), aliquoted the serum into a labelled Eppendorf tube and store at -80 °C until needed. For analysis, 10 µL of serum were used per test to measure liver enzymes alanine aminotransferase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP), bilirubin and total cholesterol (TCHOL) with a DRY-CHEM 500i analyzer (Fujifilm, Japan) following the manufacturers instructions on room temperature analysis chips. Serum was diluted 1:2 to repeat measurements outside the limit of detection of the machine.

7.5 Liver triglyceride and hydroxyproline measurements

Liver triglycerides were measured by me in collaboration with M.Sc. Tania Araujo Ramos using a colorimetric kit (MAK266, Merck) following the instructions from the manufacturer. Shortly, 100 mg of liver were homogenized in 5% Nonidet-P40 substitute (A1694.0250, AppliChem) using zirconium beads in a tissue homogenizer (Precellys, France). Thereafter, the homogenates were slowly heated to 100 °C for 5 minutes and allowed to temperate between rounds. The homogenate was cleared by centrifugation at top-speed at room temperature, diluted in ultra-pure water 1:10 for NASH livers or 1:5 for healthy livers (from ND-fed mice). I then performed lipase digestion according to the manufacturer instructions before measuring absorbance at 562 nm. The concentration of liver triglycerides was calculated from the standard curve and expressed per mg of liver.

7.6 Protein extraction and immunoblotting

After washing the cells culture dishes with ice cold PBS, proteins were extracted by scraping with ice-cold 1x RIPA buffer (150 µL for a 6 cm cell culture dish with cells) (#9806, Cell Signaling) containing 1x-protease inhibitor and phosphatase inhibitor (11697498001 and P4906837001, Roche). The lysates were incubated on ice for 1 hour with occasional shaking (vortex) for 10 seconds to ensure cell lysis. Cells debris was separated from the supernatant by centrifugation (10,000 rpm, 15 min, at 4 °C), carefully avoiding the debris pellet at the bottom and any fat layer floating at the top of the tube. Centrifugation was repeated one more time to ensure no debris was present in the protein samples. Thereafter, protein aliquots were stored at -80 °C until used.

Proteins were quantified using a bicinchoninic acid assay (BCA) kit (23225, Thermo) for immunoblotting. Thereafter, I prepared aliquots containing 10 µg per sample, diluted in RIPA buffer with 1x Laemmli buffer (J61337.AC, Thermo) containing 2-mercaptoethanol as reducing agent.

Proteins were resolved by size in SDS-PAGE, using 4-15% precast polyacrylamide gel (4561086, Bio Rad) with 80 to 140 constant V until the markers reached the bottom of the gel. Proteins were then transferred to nitrocellulose membranes using a wet transferring method and using 1x transfer buffer (35040, Thermo) with 200 mA of constant current for 60 minutes. I then blocked the membranes by incubating them with 5% skim-milk in TBST for 1 hour at room temperature. Thereafter I incubated the membranes shaking overnight at 4 °C with primary antibodies (Table 4) followed by 2-hours of HRP-linked secondary antibodies at room temperature (#7074, #7076 or #7077, Cell Signaling).

For imaging, I used the BioRad ChemiDoc machine (Bio Rad). Briefly, excess buffer was blotted by capillary forces with a clean paper towel from the membrane. I then placed the membrane on the ChemiDoc tray for imaging and added 200 µL of 1x enhanced chemiluminescent (ECL) substrate (34094, Thermo). Thereafter, I acquired a sequence of images with adequate exposure of luminescence. Care was taken to ensure an abundance of image acquisitions. Moreover, immediately after image acquisition, each image file was labeled with the details of the experiment.

Protein densitometric measurements were done using the gel tool in Fiji. Briefly, a box encompassing each band, the same box size for all band, was drawn over the first band. Thereafter, the gel analysis tool in Fiji was used to duplicate and center a box over each band of the gel. The tool produces densitometric peaks that are segmented at the base (avoiding secondary peaks and maintaining a uniform segmentation criteria), then the area of each peak is quantified using the analysis tool. The process is repeated for each band on the membrane and the results are exported to Microsoft Excel for calculations. There, the density of each protein band is normalized by the housekeeping protein and comparisons are made for plotting relative density compared to control (PBS). For presentation purposes, I adjusted the brightness and contrast equally in all images and homogeneously adjusted the image size for all bands.

Table 4. Immunoblotting antibodies.

Antigen	Source/clone	Dilution	Provider
S6K1	Rabbit, 49D7	1:1000	Cell Signaling
S6K2	Rabbit, 14130	1:500	Cell Signaling
4EBP1	Rabbit, 53H11	1:1000	Cell Signaling
p-4EBP1(T37/46)	Rabbit, 236B4	1:750	Cell Signaling
pS6 (S240/244)	Rabbit, D68F8	1:10,000	Cell Signaling
RPS6	Rabbit, 5G10	1:2500	Cell Signaling
Vinculin	Mouse, V9131	1:5000	Sigma-Aldrich

7.7 RNA extraction and gene expression analysis by qPCR

I extracted bulk RNA from 80-100 mg of liver or 7.5e5 cells by homogenization in 0.5 – 1 mL TRIzol (10296010, Invitrogen). Tissues were homogenized in a Precellys tissue homogenizer (Bertin technologies) and aided by 5-10 homogenization beads (1.0 mm zirconium beads) at 5000 rpm, for 3 minutes at 4 – 12 °C or until fully homogenized. RNA was purified using RNeasyMini Kit (74106, Qiagen) following the instructions from the manufacturer and eluting in 50 µL RNase-free water. RNA quality and quantity were measured in technical duplicates using a NanoDrop 2000 spectrophotometer (Thermo).

cDNA was prepared using the QuantiTect Rev. transcription kit (205313, Qiagen) using equal mass of mRNA as starting material (500 – 1000 ng) following the instructions from the manufacturer. Briefly, working on ice, mRNA was diluted with RNase-free and mixed with 2 µL 7x gDNA wipeout buffer from the kit and incubated at 42 °C for 2 min for DNA digestion. Thereafter, the samples were placed back on ice and a master mix containing Quantiscript RT buffer, RT primer mix and Quantiscript reverse transcriptase from the kit was added and mixed. Reverse transcription took place at 42 °C for 25 min. The reverse transcriptase was inactivated by incubating the cDNA products at 95 °C for 3 min. The final cDNA was further diluted 1/10 with RNase free water and stored at -80 °C until used.

Thereafter, gene expression was analyzed by quantitative real-time PCR, (QuantStudio 5, Life Technologies) in duplicates using the standard melting curve analysis. The qPCR master mixes were prepared with Fast Start SYBR Green Master Rox (4913914001, Roche) according to the instructions of the manufacturer and using specific primers (Table 5). The expression data was extracted to Microsoft Excel and analyzed using the $\Delta\Delta\text{CT}$ method, correcting each sample to its own expression of the housekeeping gene *Rps14*.

Table 5. Gene expression primers.

Gene	Forward	Reverse
<i>Arg1</i>	GGAATCTGCATGGGCAACCTGTGT	AGGGTCTACGTCTCGCAAGCCA
<i>Clec10a</i>	GCTGTTGGTGGTCGTCTCCGTG	AGCTGTCAGCCCTGGAGTCCAG
<i>Col1a1</i>	GAGAGGTGAACAAGGTCCCG	AAACCTCTCTCGCCTCTTGC
<i>Col4a1</i>	CTGGCACAAAAGGGACGAG	ACGTGGCCGAGAATTTACC
<i>Il-1β</i>	TCCCGTGGACCTTCCAGGATGA	GGGAACGTCACACACCAGCAGG
<i>Il6</i>	CTCTGGAGCCCACCAAGAAC	GTCACCAGCATCAGTCCCAA
<i>Mmp1a</i>	CCTTGATGAGACGTGGACCAA	ATGTGGTGTGTTGCACCTGT
<i>Mmp2</i>	AACGGTCGGAATACAGCAG	CCACCCATGGTAAACAAGGC
<i>Mmp3</i>	GGCCTGGAACAGTCTTGGC	TGTCCATCGTTCATCATCGTCA
<i>Mmp10</i>	GAGCCACTAGCCATCCTGG	CTGAGCAAGATCCATGCTTGG
<i>Mrc1</i>	GCCCACACCTGCTCCACAAGAC	TGCAAATGCTCGCGCGTTGTC
<i>Nos2</i>	TCCTGGACATTACGACCCCT	CTCTGAGGGCTGACACAAGG
<i>Pdgfa</i>	GTGCGACCTCCAACCCTGA	GGCTCATCTCACCTCACATCT
<i>Rps14</i>	GACCAAGACCCCTGGACCT	CCCCTTTTCTTCGAGTGCTA
<i>Tgf-β</i>	GGAGCAACATGTGGAATC	CAGCAGCCGGTTACCAAG

7.8 DNA extraction and genotyping

DNA was extracted in collaboration with Anna-Lena Eck and Tim Machauer from mouse ear clippings in 500 μ L NaOH 0.06M at 100 °C for 10 min. Thereafter the digest was allowed to cool and was then neutralized with 125 μ L 1M TRIS pH 8. Thereafter, 1 μ L of DNA extract was used for PCR with the DreamTaq (EP1712, Thermo) following the instructions from the manufacturer and appropriate thermo-cycling conditions before resolving the PCR product in 2% agarose gels containing 10 μ L ethidium bromide. Electrophoresis was performed at 120 constant V for ~1 hour or until the molecular weight marker ladder reached the bottom of the gel. The following primers were used for PCR: S6K1 flox -TCCACCCACCAGTAAAGAGC, CCTCAGTCTCCTGAGTGTTAAGG-. S6K2 flox - GCA-CACCAGGGGCTACATAATG, GCACTGCATACAATCTTGGCCTAG, and CACAACGGG-TTCTGTAGTCC. Lysmcre GCA-TTGCAGACTAGCTAAAGGCAG, GTCGGCCAGGCTG-ACTCCATAG and CCCAGA-AATGCCAGATTACG. Csf1rcr -CAGGGCCTTCTCCAC-ACCAGC, and CTGGCTGTG-AAGACCATC.

7.9 Immunohistochemistry

I fixed the tissues in 4% buffered paraformaldehyde (P087.3, Roth) for 48-hours, thereafter the tissues were dehydrated, embedded, and processed in collaboration with the coworkers of the histology lab: Danijela Heide, Jenny Hetzer, and Tim Machauer and with occasional aid from Anna-Lena Eck and Florian Müller from the DKFZ.

Antigens and hematoxylin & eosin (3801582E & 3801601E, Leica) were detected in 2-µm tissue slices whereas Sirius-red (17540, Morphisto) staining was performed on 7-µm slices. In brief, cuts were deparaffinized and rehydrated, antigens were retrieved with Bond citrate solution (AR9961, Leica), Bond EDTA solution (AR9640, Leica) or Bond proteolytic enzyme kit (AR9551, Leica). Thereafter, the slides were incubated with primary antibodies (Table 6) in Bond primary antibody diluent (AR9352, Leica) followed by secondary antibodies (Leica) and stained with the Bond Polymer Refine Detection Kit (DS9800, Leica).

The slides were scanned in collaboration with Jenny Hetzer and Anna-Lena Eck with a whole-slide scanner (Aperio AT2, Leica) at 20x. Thereafter I annotated and analyzed the slides using Aperio ImageScope (ver. 12.4.0.5043, Leica), Fiji¹⁶⁶ (ver. 1.52e) or QuPath¹⁶⁷ (ver. 0.3.0 and 0.4.3). All histological analysis were performed in 6-12 fields of view (FOV; 10x magnification) per mouse (~10 mm² tissue). Positive areas were calculated by color deconvolution with Fiji using the same thresholds for each stain throughout all images. Cell aggregates were manually counted with a stable criterion. Individual cells were counted using watershed cell segmentation in QuPath with appropriate settings. All analysis were manually checked for accuracy. Mice NAFLD-Activity Score (NAS) was determined by two collaborating trained pathologists (Dr. Alphonse Charbel and Prof. Dr Stephanie Rössler from the Universitätsklinikum Heidelberg).

Table 6. Antibodies for immunohistochemistry and double immunofluorescence.

Antigen	Source/clone	Dilution	Provider
aSMA	Mouse, A2547	1:5000	Sigma
CD8	Rat, 4SM15	1:200	Invitrogen
CD68	Rabbit, 125212	1:200	Abcam
CHOP	Rabbit, D46F1	1:100	Cell Signaling
CK19	Rat, TROMA-III	1:500	DSHB, deposited by R. Kemler
Collagen IV	Rabbit, CL50451AP-1	1:100	Cedarlane
F4/80	Rat, BM8	1:250	Linaris
HNF4A	Rabbit, 181604	1:2000	Abcam
Ki67	Rabbit, SP6	1:100	Fisher Scientific
MHCII	Rat, M5/114.15.2	1:500	Novus Biologicals

7.10 Immunofluorescence

In collaboration with the coworkers of the histology lab, double immunofluorescence was assayed in 2-µm thick FFPE liver sections in collaboration with the coworkers of the histology lab. The tissue slides were deparaffinized and rehydrated, thereafter incubated with antibodies targeting CD68, F4/80 or pS6^(S240/244) (Table 6), AKOYA Biosciences Opal Fluorophore kit (Opal 520, FP1487001KT and Opal 620, FP1495001KT), and counter-stained with AKOYA 10x Spectral DAPI (FP1490) following the manufacturer's instructions.

The slides were imaged in collaboration with Danijela Heide with a NanoZoomer-S60 (Hamamatsu) scanner at 20x. For analysis of double positive cells, CD68⁺ or F4/80⁺ cells were segmented as objects in QuPath and classified according to their expression of pS6^(S240/244). Cell segmentation and positive/negative thresholds were adjusted and controlled manually to ensure accuracy.

Sequential-multiplex immunofluorescence was assayed in collaboration with Dr. Alix Bruneau from the Charité Universitätsmedizin Berlin on 2-µm thick FFPE liver sections after deparaffination and rehydration, the unspecific binding was blocked by incubation in 2% normal goat serum (31872, Thermo) for 1 hour at room temperature. Thereafter, 3 antigens were simultaneously assayed with primary antibodies diluted in 1% BSA PBS overnight in the fridge (Table 7). This was followed by incubation in secondary antibodies which include: #4408S, 4410S, 4412S, 4413S and 4417S (Cell Signaling) for 1 hour at room temperature before nuclear staining in PBS with 2 µg/mL DAPI (D9542, Sigma) for 5 min at room temperature.

The slides were washed first with PBS and then deionized water, then and mounted with VectaMount AQ aqueous mounting medium (Vector BioLabs) and a region of interest (~17 mm²) imaged on an Observer.Z1 (Zeiss) microscope at 20x. The slide was dismounted, striped and re-probed using primary antibodies (Table 7). The single channel images were mounted as a concatenated hyperstack in Fiji. The scans from several staining cycles were aligned with the DAPI channel as guide using the FIJI HyperStackReg V5.6 plugin with the affine setting. Thereafter, each channel was assigned a color with the hyperstack tool in Fiji for visualization proposes.

For analysis, Iba1⁺ cells were segmented by watershed cell segmentation based on the Iba1 channel instead of the nuclear channel to reduce computing time and more accurately segment the irregular shapes of myeloid cells. Thereafter, the expression of Clec4f, F4/80 and pS6^(S240/244) was measured using QuPath.

Using sequential single measurement classifiers with accurate settings representative of all slides, the cells were categorized as liver-resident Kupffer cells (KC; Iba1⁺, Clec4f⁺ and F4/80⁺), monocyte-derived macrophages (MoMFs; Iba1⁺, F4/80⁺ and Clec4f⁺) or monocytes (Iba1⁺, Clec4f⁺ and F4/80⁺). The same segmented cells were additionally classified as positive or negative according to their expression of pS6(S20/244). The total number of Iba1+ segmented cells was used to calculate the percentage represented by each population or presented as a natural number. Cell segmentation and positive/negative settings were manually adjusted for accuracy and standardized per batch of slides stained on the same day.

Table 7. Antibodies for multiplex-immunofluorescence.

Antigen	Source/clone	Dilution	Provider
Iba1	Rabbit, 100369-764	1:1000	VWR
Clec4f	Rat, 370901	1:1000	R&D Systems
F4/80	Rat, BM8	1:250	Linaris
pS6 ^(S240/244)	Rabbit, D68F8	1:5,000	Cell Signaling

7.11 Statistical analyses and data visualization

Data are expressed as mean $\pm$ standard error of mean (SEM) unless otherwise stated. Statistical analyses and base data visualization were performed in Graph Pad Prism 9 and Affinity Designer (ver. 1.10.5.1342), respectively. The liver figures in Figure 7 were generated with DALL E using the prompt: “Medical illustration style. A series of 5 livers aligned from left to right. The first liver looks normal. The second liver has fat droplets inside. The third liver has more fat droplets than the second and several pointy cells inside. The fourth liver is slightly deformed as if a net had been pressed on its surface. The fifth liver looks like the third but has a big tumor growing from it.” on October 10.10.2023. Thereafter the figures were segmented, arranged and enhanced in PowerPoint for presentation.

Statistical significance for comparison of three groups was calculated using one-way analysis of variance with Tukey’s multiple comparison test. Statistical significance for four groups with two variables (diet and genotype) was calculated using two-way analysis of variance with Tukey’s multiple comparison test. Statistical significance for the glucose/insulin tolerance test was carried out by analysis of area of the curve for each group in Graph Pad Prism 9. Statistical significance for the tumor incidence was carried out using the Fischer’s exact test. Statistical significance for the correlation plot was carried out using linear regression. A p-value of < 0.05 was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

8 Results

8.1 Myeloid mTOR-S6K signaling is upregulated in NAFLD and NASH

Studying the role of myeloid mTOR-S6K signaling in NASH required a measure of whether the signaling pathway is indeed active in the pathological setting and to which extent. Thus, I, in collaboration with Dr. P. Ramadori from the German Cancer Research Center (DKFZ) gained access to formalin-fixed paraffin-embedded (FFPE) liver tissues from obese patients undergoing bariatric surgery in the surgical department of PD. Dr. Susanne Roth in Heidelberg. Notably, although all samples came from obese patients, upon NAS-examination by a pathologist (an external collaborator of PD. Dr. Roth and Dr. Ramadori), the livers had heterogenous histology compatible with a NAS-based diagnosis of healthy liver, NAFLD and NASH. Thereby allowing me to study the activation status of mTOR-S6K in livers with different grades of pathology.

Thereafter, I, in collaboration with the coworkers of the histology lab (Danijela Heide, Jenny Hetzer and Tim Machauer from the DKFZ), performed double immunofluorescence staining of CD68⁺ human macrophages alongside the mTOR-S6K functional readout – pS6(S240/S244)⁺– (pS6; from here onwards) on the liver FFPE tissues (Figure 10 A). Analysis of the co-occurrence of CD68 and pS6 revealed significantly increased mTOR-S6K activation in NAFLD and NASH livers, compared with the healthy counterparts (Figure 10 B-C). Moreover, myeloid mTOR-S6K activity positively correlated with the severity of NASH as measured by the pathologist assessed NAS (Figure 10 D). These findings confirmed that in humans, myeloid mTOR-S6K signaling is significantly increased in human NAFLD and NASH.

After confirming increased myeloid mTOR-S6K signaling during NAFLD/NASH in humans, I next sought to investigate whether the effect was reproduced in the mice as this would allow the study of the phenomenon in mouse model-systems of liver pathology. Thus, I employed a well characterized model of Western diet (WD) induced NASH⁹ alongside normal chow diet (ND)-feeding for healthy control livers (Figure 10 E). Thus, using double immunofluorescence, I in collaboration with the coworkers of the histology lab examined the healthy and NASH livers of mice for the co-occurrence of mouse macrophages (F4/80⁺) and pS6. Corroborating the human situation, I found significantly higher myeloid mTOR-S6K activity in NASH livers, compared with the healthy control livers (Figure 10 F-G). Thus, like in humans, macrophages have increased mTOR-S6K signaling in mouse NASH, thereby supporting the study of this signaling in mice and the continuation of my research.

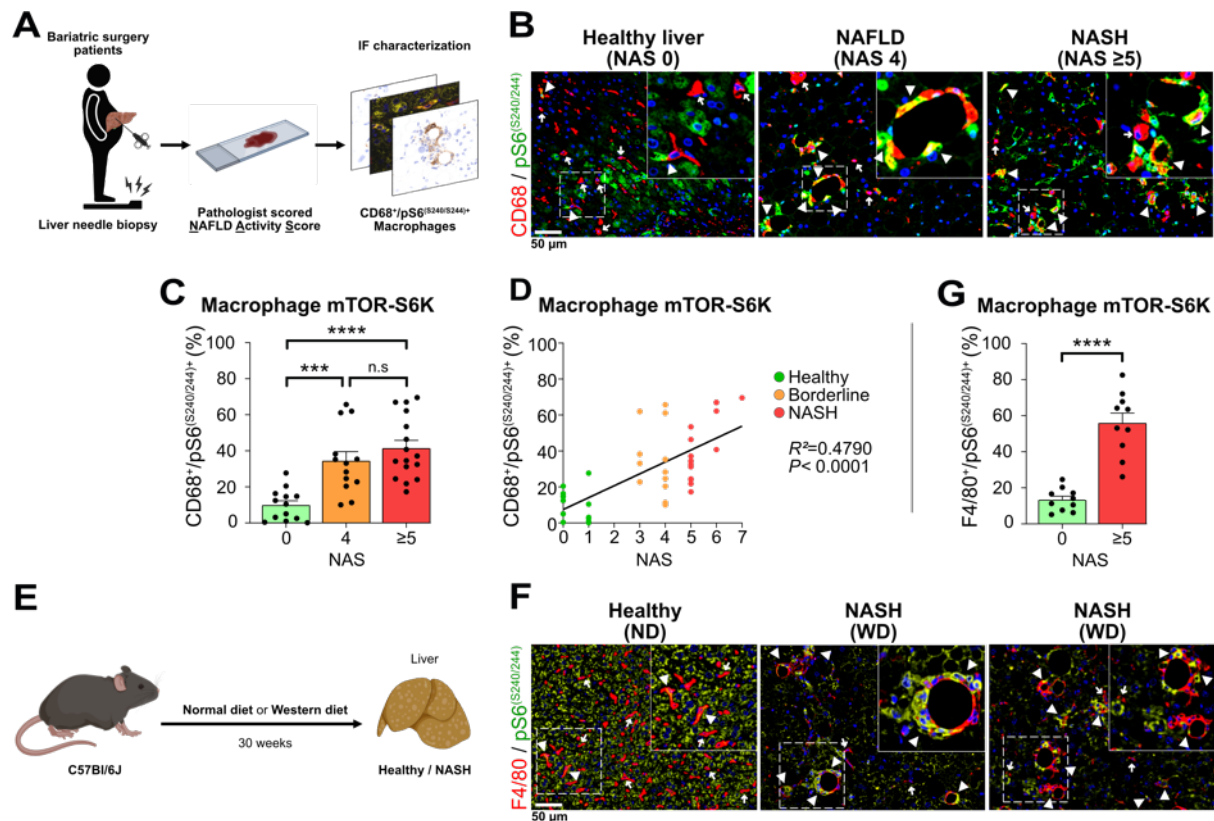


Figure 10. Human and mouse liver myeloid cells display upregulated mTOR-S6K axis in NAFLD and NASH. **A.** Experimental scheme. Human liver biopsies collected from bariatric surgery patients stratified by their NAFLD-Activity Score (NAS) were examined by immunofluorescence. **B.** Representative liver double immunofluorescence of macrophages (CD68⁺) and pS6(S240/244)⁺. Scale, 50 μ m. White triangle, double positive macrophages. White arrow, single positive macrophages. Dotted square, zoomed area. **C.** Quantification percentage of double positive macrophages per sample. **D.** Correlation plot CD68⁺/pS6(S240/244)⁺ macrophages and NAS. CD68⁺/pS6(S240/244)⁺ co-occurrence was increased in human livers with NAFLD and NASH compared with healthy livers and positively correlated with disease severity. **E.** Experimental scheme. C57BL/6J mice received control normal diet or Western diet for 30 weeks to induce NASH. **F.** Representative liver double immunofluorescence of mouse macrophages (F4/80⁺) and pS6(S240/244)⁺. Scale, 50 μ m. White triangle, double positive macrophages. White arrow, single positive macrophages. Dotted square, zoomed area. **G.** Quantification percentage of double positive macrophages per sample. F4/80⁺/pS6(S240/244)⁺ co-occurrence was increased in mouse livers with NASH compared with healthy livers. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using one-way analysis of variance with Tukey's multiple comparison test. *** P < 0.001, **** P < 0.0001. n.s: non-significant.

8.1.1 mTOR-S6K signaling is upregulated in polarized macrophages

Macrophages have crucial roles in the pathophysiology of NASH and NASH-HCC^{18,131-133}. Importantly, their functions are governed by their activation and polarization status which is partly controlled by mTOR signaling^{161,162}. My previous experiment uncovered that liver macrophages have upregulated mTOR-S6K signaling in NAFLD and NASH. Nevertheless, the specific role of myeloid S6K1 and S6K2 in macrophage activation remains unknown.

Therefore, to investigate whether mTOR-S6K signaling is modulated in macrophage polarization, I cultured bone marrow derived macrophages (BMDMs) from C57BL/6J mice which were polarized into classical “M1” pro-inflammatory and “M2” anti-inflammatory phenotypes with lipopolysaccharide (LPS) and interleukin 4 (IL-4), respectively (Figure 11 A). Immunoblots from these BMDMs revealed significantly enriched pS6 (i.e. mTOR-S6K signaling) in M1-polarized macrophages, and to a lower extent M2-polarized macrophages (Figure 11 B-C). This indicates that polarized macrophages have increased mTOR-S6K signaling. Nevertheless, whether the mTOR-effectors, S6K1 and S6K2 mediate macrophage polarization remains unclear.

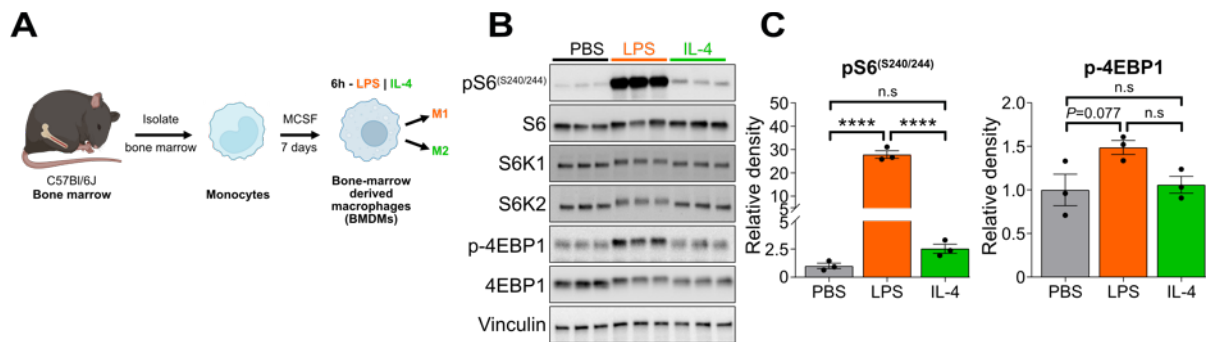


Figure 11. mTOR-S6K axis is upregulated in polarized macrophages. **A.** Experimental scheme. Bone marrow from C57BL/6J mice was isolated to generate bone marrow derived macrophages (BMDMs). BMDMs were treated for 6 hours with LPS, IL4 or PBS as control. **B-C.** Representative immunoblots from basal and polarized BMDMs and densitometric quantification of pS6(S240/244) and p-4EBP1. Polarized macrophages display increased mTOR-S6K signaling targets. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using one-way analysis of variance with Tukey’s multiple comparison test. **** $P < 0.0001$. n.s: non-significant.

8.2 Generation of myeloid-specific S6K1 and S6K2 knockout mice

After confirming that in human and mouse NAFLD and NASH, liver macrophages have increased mTOR-S6K signaling, and to enable my preclinical studies of the mTOR effectors –S6K1 and S6K2–, I, in collaboration with Dr. Suchira Gallage from the DKFZ generated two mousselines with macrophage-specific deletion (knockout: KO) of the *Rps6k1* and *Rps6k2* genes. Therefore, I crossed S6K1^{fl/fl} and S6K2^{fl/fl} double-floxed mice (a kind gift from Prof. Dr. Dominik J. Withers from the Imperial College London) with mice expressing a Cre-recombinase driven either by Colony Stimulating Factor Receptor 1 (Csf1rS6K1/S6K2) or Lysozyme (LysmS6K1/S6K2) (Figure 12 A-B). These mousselines would allow further investigation on the role of the S6 kinases in all monocytes -to capture monocyte derived macrophages– and mature macrophages namely –Kupffer cells–, respectively.

Thereafter, I genotyped the tissues to confirm the presence or absence of the floxed exons and each Cre-recombinase (Figure 12 A-B). Furthermore, I functionally confirmed the deletion of S6K1/S6K2 by generating bone marrow derived macrophages (BMDMs) from these mice. BMDMs immunoblots from both *Csf1r*S6K1/S6K2 and *Lysm*S6K1/S6K2 revealed loss of S6K1, S6K2 and consequently pS6 albeit with some residual pS6 in *Lysm*S6K1/S6K2 BMDMs, likely originating from undifferentiated monocytes on the *ex vivo* setting (Figure 12 C & E).

Finally, to confirm the deletion of S6K1/S6K2 in the liver, I, in collaboration with the coworkers of the histology lab, analyzed the expression of pS6 in F4/80⁺ macrophages in the livers of *Csf1r*S6K1/S6K2 and *Lysm*S6K1/S6K2 mice though double immunofluorescence. This revealed a depletion of pS6⁺ macrophages in KO mice, while the livers from Cre-negative littermate control mice showed basal levels of F4/80⁺/pS6⁺ cells (Figure 12 D & F). Importantly, this confirmed the deletion of S6K1/S6K2 *in vivo*, validating the use of these mice for subsequent experiments.

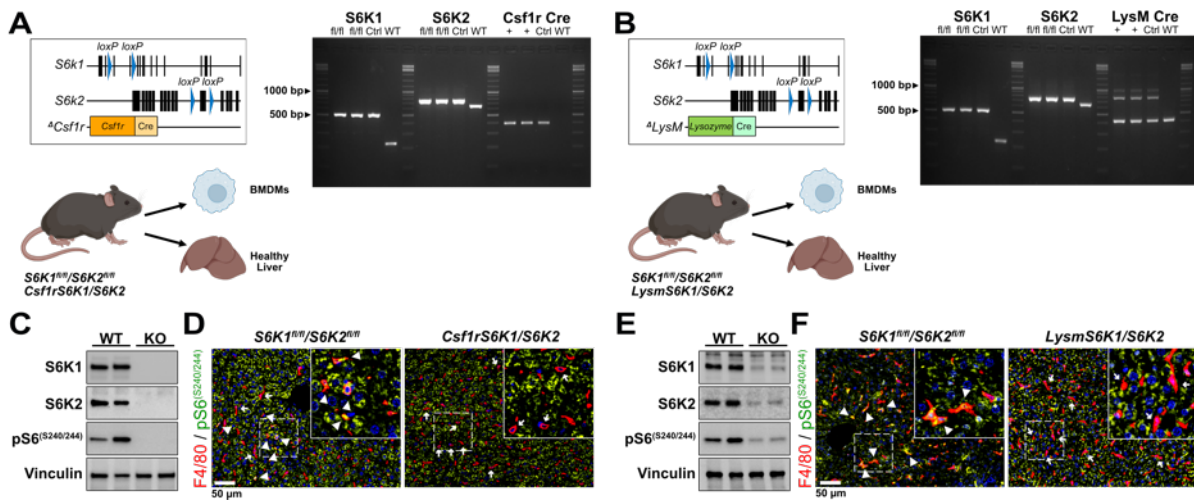


Figure 12. Generation of myeloid-specific S6K1/S6K2 knockout mouse lines. A-B. Mice genetic schemes. Homozygous-floxed *S6k1* and *S6k2* (S6K1^{fl/fl}/S6K2^{fl/fl}) alleles are excised by a Cre-recombinase expressed under the control of *Csf1r* (liver resident and infiltrating macrophages; *Csf1r*S6K1/S6K2) or *Lysozyme* (liver resident macrophages; *Lysm*S6K1/S6K2), respectively. Representative genotyping gel. The floxed alleles (fl/fl) are larger than the wildtype (WT) alleles. Ctrl, positive control. Livers and BMDMs from each mouse line and Cre-negative littermates were characterized. C & E. Representative immunoblots from *Csf1r*S6K1/S6K2 and *Lysm*S6K1/S6K2 and Cre-negative BMDMs. Cre+ macrophages lack S6K1 and S6K2 expression and downstream pS6(S240/244) signal. D & F. Representative healthy liver double immunofluorescence of mouse macrophages (F4/80⁺) and pS6(S240/244)⁺. Scale, 50 μm. White triangle, double positive macrophages. White arrow, single positive macrophages. Dotted square, zoomed area. Cre-positive mice showed no double positive macrophages, whereas Cre-negative littermates had double positive macrophages (F4/80⁺/pS6⁺).

8.3 S6K1 and S6K2 are dispensable for macrophage polarization

Previous experiments showed that polarized macrophages have increased mTOR-S6K signaling (Figure 11). Nevertheless, it is unclear whether the mTOR effectors S6K1/S6K2 have a causal role in macrophage polarization, or if increased mTOR-S6K signaling is rather a passenger phenomenon. Moreover, I established and validated two mousselines with myeloid-specific S6K1/S6K2 deletion which could be used to investigate the role of macrophage S6 kinases for uncovering potential therapeutic targets in NAFLD/NASH or other inflammatory conditions where macrophages might play a role.

Thus, to investigate whether S6K1/S6K2 play a functional role in macrophage polarization, like before, I derived and polarized BMDMs from *Csf1r*S6K1/S6K2, *Lysm*S6K1/S6K2 and littermate control mice (Figure 13 A). In line with the treatments (i.e. LPS and IL-4), M1-associated genes (*Nos1*, *IL-6* and *IL-1 β*) were upregulated only in the LPS treated macrophages, whereas M2-associated genes (*Arg1*, *Mrc1* and *Clec10a*) were upregulated only in IL-4 treated macrophages. Nevertheless, the lack of S6K1/S6K2 did not significantly alter gene expression (Figure 13 B-E). Therefore, mTOR-S6K signaling mediated through S6K1/S6K2 does not have a major role in macrophage polarization in this setting.

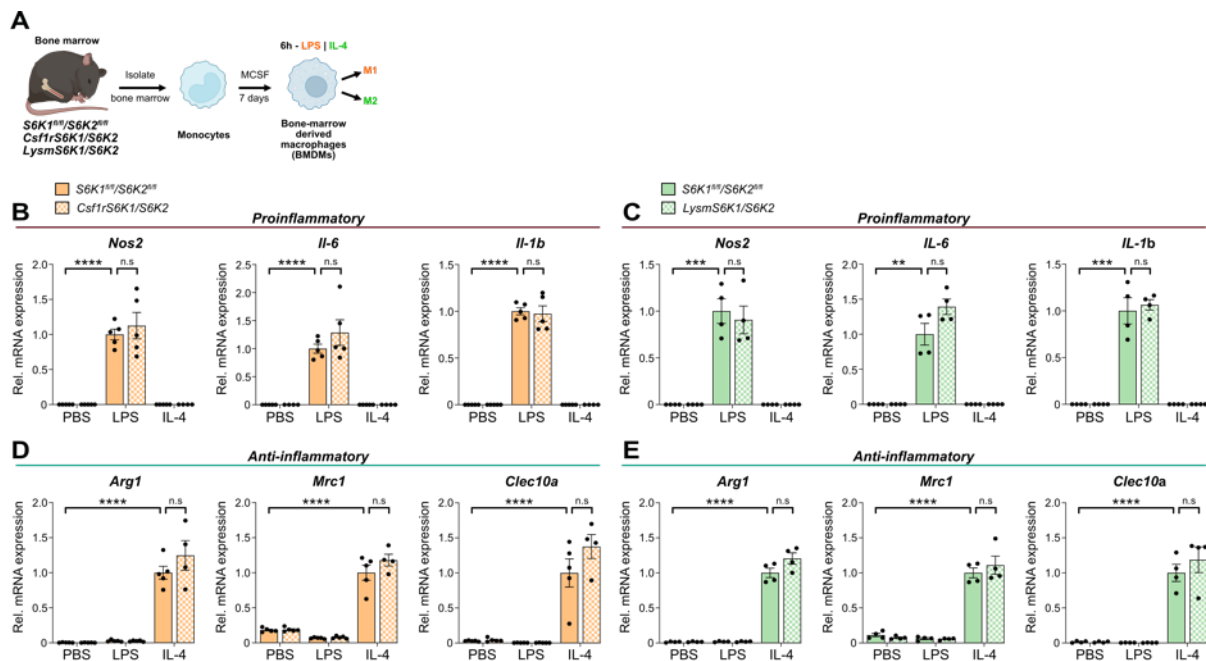


Figure 13. S6K1 and S6K2 are dispensable for macrophage polarization ex vivo. A. Experimental scheme. Bone marrow derived macrophages from *Csf1r*S6K1/S6K2 and *Lysm*S6K1/S6K2 mice were polarized with LPS, IL4 or PBS as control. **B-E.** Relative gene expression of the indicated pro- and anti-inflammatory genes in polarized BMDMs. Loss of S6K1/S6K2 did not modulate the expression of classical polarization-associated genes. Gene expression relative to the expression of the housekeeping gene *Rps14*. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance

with Tukey's multiple comparison test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. n.s: non-significant.

8.4 Myeloid S6K1 and S6K2 are dispensable for diet-induced obesity, glucose and insulin homeostasis

Earlier analyses showed that liver macrophages have increased mTOR-S6K signaling in human and mouse NAFLD and NASH (Figure 10). Using BMDMs from mice with macrophage-specific deletion of S6K1/S6K2 I investigated the causal link between the S6 kinases and macrophage polarization. Nevertheless, my data suggested that in macrophages, S6K1/S6K2 are not necessary for classical polarization *ex vivo* (Figure 13). However, macrophages are highly plastic, which gives rise to many macrophage populations and polarization *in vivo*. Therefore, I still had to elucidate the role of myeloid S6K1/S6K2 in the context of a chronic positive energy balance-induced liver damage as this could have potential roles in whole body metabolism and glucose homeostasis.

Thus, to investigate the role of myeloid S6K1/S6K2 in the context of obesity-mediated liver damage, I utilized the WD model of NASH to induce obesity and related metabolic insults *in vivo*. This experiment would allow me to include variables not present in the acute *ex vivo* settings of BMDM experiments. Therefore, I fed Csf1rS6K1/S6K2, LysmS6K1/S6K2 and control mice with WD (40% kcal fat, 20% kcal fructose and 2% cholesterol) for 30 weeks to induce NASH. Meanwhile control mice received ND (Figure 14 A).

Compared with ND-fed mice, those fed WD significantly gained body weight increasing with time of feeding and peaking between 24 - 30 weeks but without differences attributable to their genotype (Figure 14 B & D). In line, WD-feeding induced a marked shift in body composition after 29 weeks, where fat mass increased while lean mass remained comparable between WD and ND-fed mice (measured by EchoMRI) (Figure 14 C & E). Additionally, the ND-fed Csf1rS6K1/S6K2 mice showed reduced lean mass compared with Cre-negative controls. However, normalization of lean mass relative to body weight revealed a relatively lower lean mass in WD-fed mice but without differences between genotypes (Figure 14 F-G). Similarly, the mass of the main fat depots, namely the epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT) and brown adipose tissue (BAT) was increased by WD-feeding but without changes related to the lack of myeloid S6K1/S6K2 (Figure 14 H-I). Taken together, the data shows that myeloid S6K1/S6K2 signaling is dispensable for diet induced obesity.

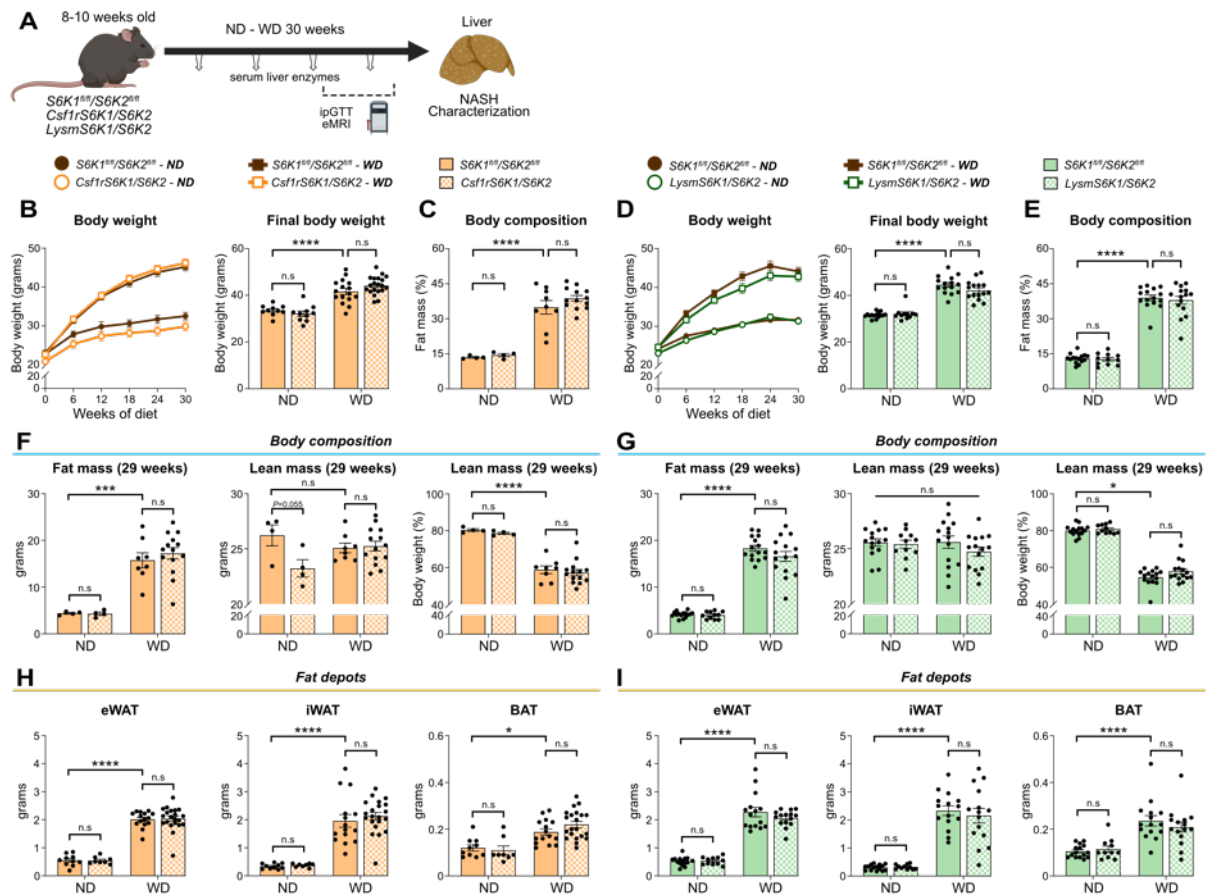


Figure 14. Myeloid S6K1 and S6K2 are dispensable for WD-induced obesity and gain in adipose mass. **A.** Experimental scheme. 8-10 week old Csf1rS6K1/S6K2 (left side of the figure), LysmS6K1/S6K2 (right side of the figure) and Cre-negative littermate mice received normal diet (ND) or Western diet (WD) for 30 weeks. Serum was collected every 8 weeks. Intraperitoneal insulin and glucose tolerance test (ipITT and ipGTT) and body composition analysis (echoMRI; eMRI) were assayed. **B & D.** Body weight development over 30 weeks. **C & E & F-G** Body composition measurements. WD-feeding induced body weight and adipose tissue mass increase. WD-fed mice had elevated fat mass compared with ND mice. **H-I.** Fat depots (i.e. epididymal, inguinal and brown adipose tissue) weight after 30 weeks of the indicated feedings. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. n.s: non-significant.

Furthermore, analyses of glucose and insulin homeostasis in these mice revealed WD-induced higher fasting glucose after 4 and 6 hours of fasting (Figure 15 B-E), as well as impaired glucose clearance in glucose and insulin tolerance tests (Figure 15 F-I). Nevertheless, no differences were mediated by the genotype. Thus, in the context of obesity, myeloid mTOR-S6K signaling through S6K1/S6K2 does not play a role in glucose homeostasis, indicating that macrophage S6 kinases do not represent a therapeutic target for obesity or glucose homeostasis.

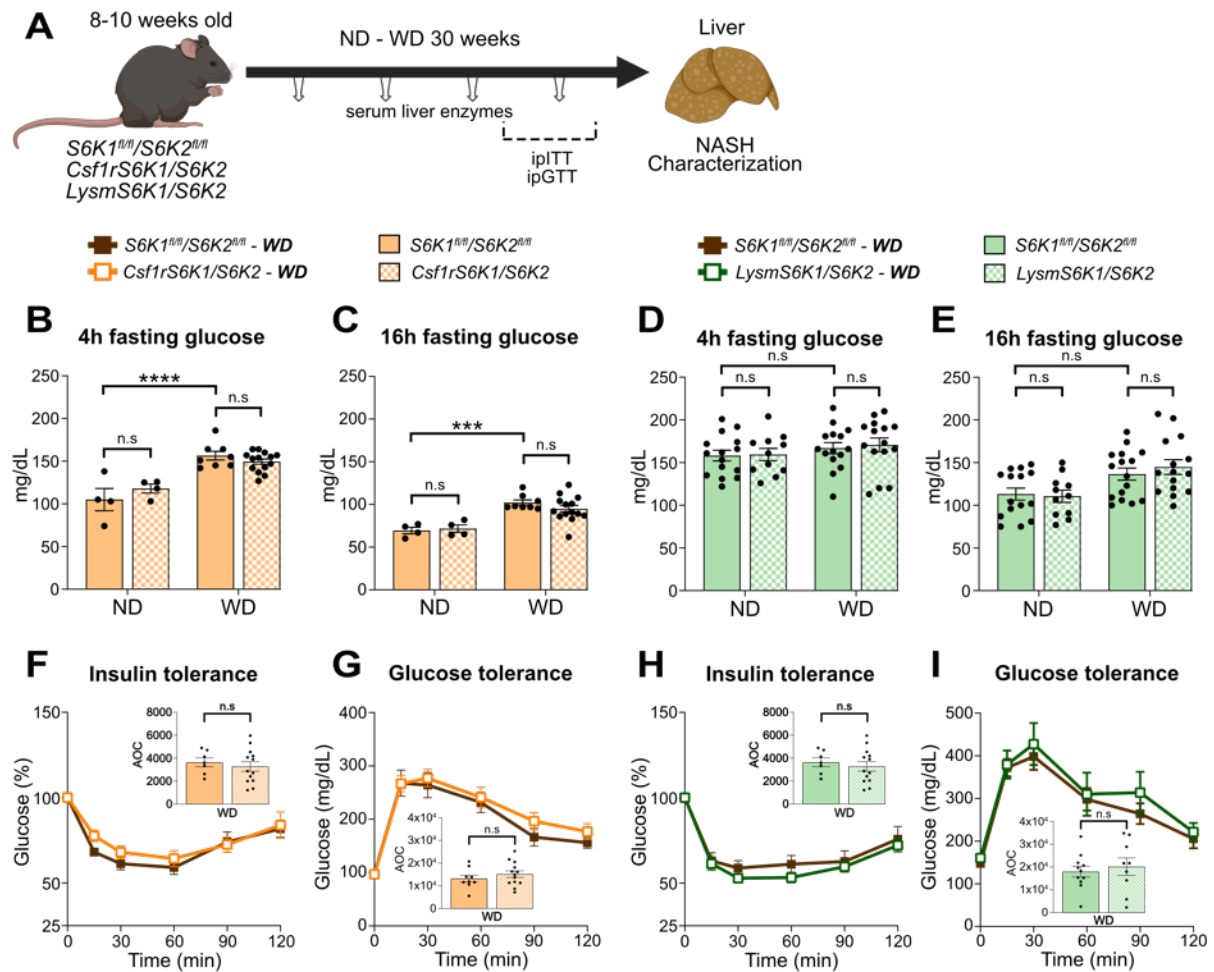


Figure 15. Myeloid S6K1 and S6K2 are dispensable for glucose and insulin homeostasis. **A.** Experimental scheme. 8-10 week old *Csf1rS6K1/S6K2* (left side of the figure), *LysmS6K1/S6K2* (right side of the figure) and Cre-negative littermate mice received normal diet (ND) or Western diet (WD) for 30 weeks. Intraperitoneal insulin and glucose tolerance tests (ipITT, ipGTT, respectively) were assayed. **B-E.** Serum glucose after 4 and 6 hours of fasting. WD-feeding induced higher basal glucose compared with ND-feeding. **F-I.** Serum glucose clearance curves from ipITT and ipGTT and area of the curve (AOC). Loss of myeloid S6K1/S6K2 did not affect glucose nor insulin homeostasis. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test and area of the curve analysis *** $P < 0.001$, **** $P < 0.0001$. n.s: non-significant.

8.5 Myeloid S6K1 and S6K2 are dispensable for NASH

Although liver macrophages have significantly upregulated mTOR-S6K activity in NASH, and polarized BMDMs also showed increased mTOR-S6K activity, the lack of both kinases does not prevent nor impact macrophage polarization. Moreover, in WD-fed mice, macrophage-specific loss of the S6 kinases did not impact obesity nor glucose homeostasis. However, the role of both kinases in liver pathophysiology remains unknown. Conceivably, it is at this level that macrophages might have a more extensive impact.

Therefore, to understand whether myeloid S6K1/S6K2 signaling play a role in liver damage during NASH pathogenesis, I analyzed (time series) serum markers of liver damage (alanine aminotransferase - ALT, aspartate aminotransaminase - AST and alkaline phosphatase - ALP) from *Csf1r*S6K1/S6K2, *Lysm*S6K1/S6K2 and Cre-negative littermate controls mice fed WD for 30 weeks. As expected from the model⁹, ALT, AST and ALP increased in serum with time (Figure 16 A-F), thereby indicating cumulative liver damage. Moreover, serum total cholesterol (TCHOL) also increased with time of WD-feeding (Figure 16 G-H) likely as a result of impaired cholesterol export and metabolism. Nevertheless, these serum markers increased with WD-feeding time in both KO and control mice, indicating that macrophage S6K signaling does not play an important role in mediating liver damage.

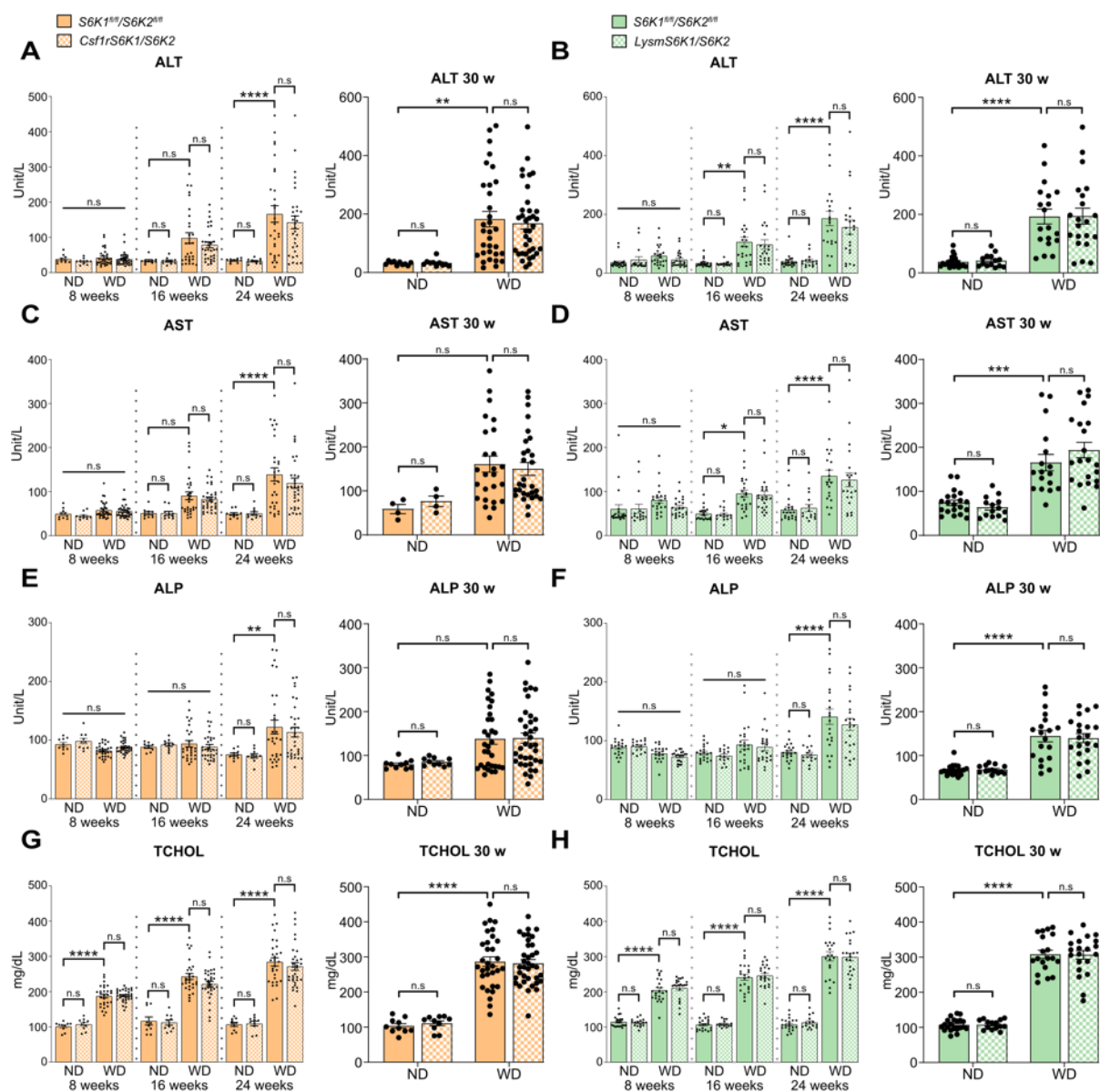


Figure 16. Myeloid S6K1 and S6K2 are dispensable for WD-induced liver damage. *Csf1r*S6K1/S6K2, *Lysm*S6K1/S6K2 and Cre-negative littermates were fed Western diet (WD) or normal diet (ND) for 30 weeks. Serum was collected every 8 weeks. **A-H.** Serum alanine

aminotransferase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP) and total cholesterol (TCHOL) over time of WD-feeding. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. n.s: non-significant.

Additionally, 30 weeks WD-feeding induced hepatomegaly which was macroscopically evident and confirmed by their liver to body weight ratio, however this was similar between KO and control mice (Figure 17 A-B), suggesting that myeloid S6K1/S6K2 are not necessary for the pathogenesis of NASH at the macroscopic level. Nevertheless, the role of macrophage S6 kinases in NASH histological features remained to be elucidated.

In turn, to elucidate whether macrophage S6 kinases may modulate NASH histopathology, I, in collaboration with the coworkers of the histology lab, executed histological analysis of the livers described. Firstly, hematoxylin & eosin (HE) staining revealed abundant macro- and micro-steatosis as well as inflammatory foci on the livers of WD-fed mice, whereas ND-fed livers appeared healthy (Figure 17 C-D). Given that histology analysis can be prone to bias, I, in collaboration with M.Sc. Tania Araujo from the DKFZ, quantified liver triglycerides from flash frozen liver aliquots. Additionally, I enlisted a collaborating pathologist (Dr. Alphonse Charbel from the Universitätsklinikum Heidelberg) to assess the NAS from these livers. Both, the steatosis component from NAS and liver triglycerides quantification corroborated the pronounced liver steatosis (Figure 17 C-F). Additional to steatosis, the NAS analysis revealed that WD-feeding but not ND-feeding, induced significant inflammation as well as hepatocyte ballooning compatible with a NASH diagnosis for most mice (Figure 17 C-E & G). In summary, these data showed that in diet-induced obesity, myeloid S6K1/S6K2 signaling of nutrient sensing is dispensable for the diagnostic histopathological signs of NASH.

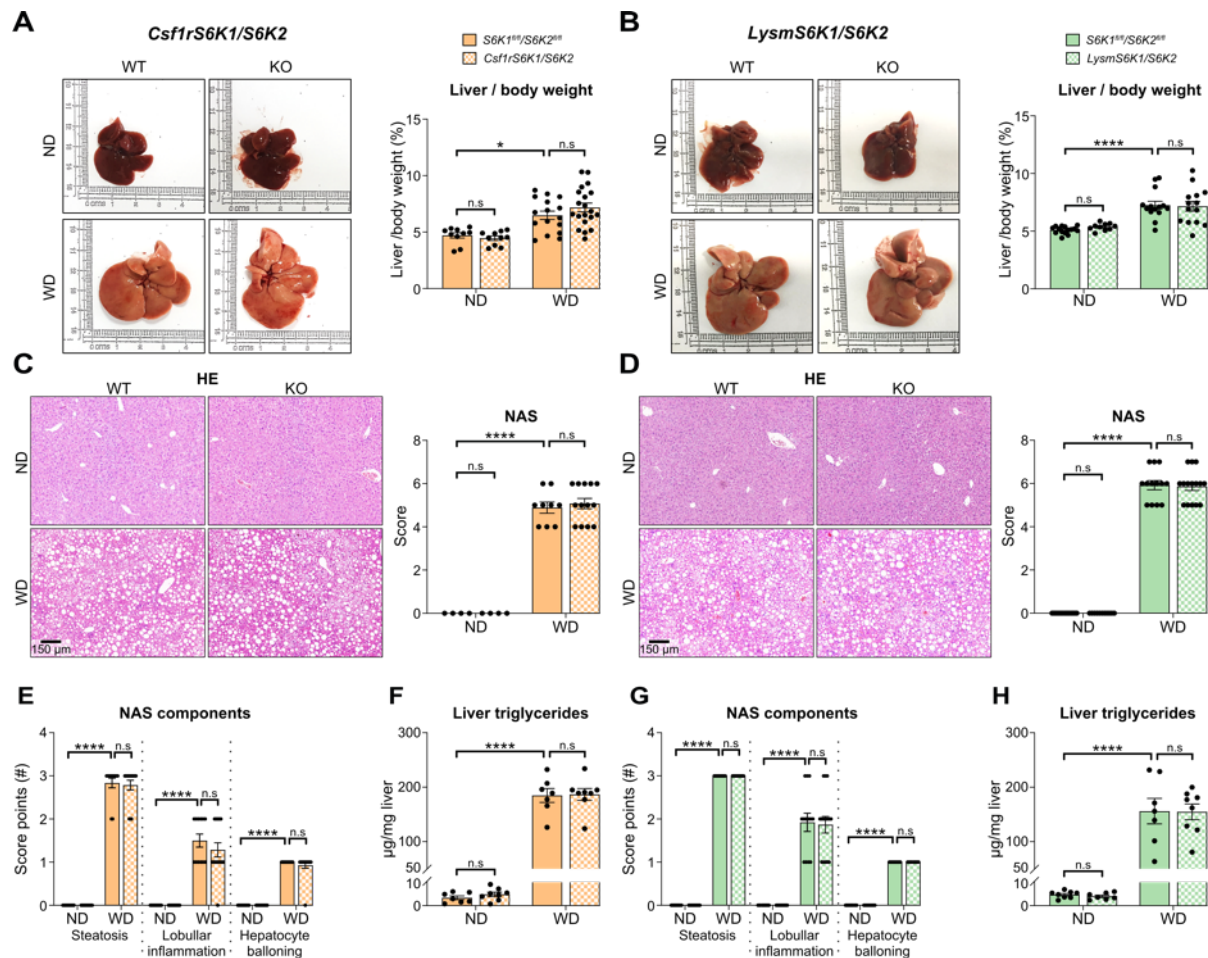


Figure 17. Myeloid S6K1 and S6K2 are dispensable for WD-induced NASH development. **A & B.** Representative liver pictures and liver to body weight ratio from *Csf1rS6K1/S6K2* and *LysmS6K1/S6K2* mice after 30 weeks of normal diet (ND) and Western diet (WD) feeding. **C & D.** Representative immunohistochemistry images for liver hematoxylin-eosin (HE) and pathologist-assessed NAFLD-Activity Score (NAS). **E-H.** NAS components scores and liver triglycerides quantifications. WD-feeding induced hepatomegaly and NASH histological characteristics in control mice as well as in the absence of myeloid S6K1/S6K2. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test * P < 0.05, **** P < 0.0001. n.s: non-significant.

8.5.1 Myeloid S6K1 and S6K2 are dispensable for liver inflammation in NASH

Previous analyses from obesity-related NASH mouse livers revealed that myeloid S6K1/S6K2 as dispensable for the histopathological signs of NASH, namely liver steatosis, inflammation and hepatocyte damage i.e. ballooning degeneration; however, the role of the S6 kinases in macrophages in the quality or type of liver inflammation remained unknown.

In an effort to understand whether myeloid S6K1/S6K2 were related to changes in the liver inflammatory milieu, I, in collaboration with the coworkers of the histology lab, conducted additional immunohistochemical (IHC) analysis. These analyses showed that WD-feeding induced significant liver immune infiltration by antigen presenting cells (MHCII⁺), macrophages (F4/80⁺) and T-cells (CD-8⁺). However, this was similar between

Csf1rS6K1/S6K2 and LysmS6K1/S6K2 mice (Figure 18 A-F). Apart from increased inflammatory cells, both MHCII⁺ and F4/80⁺ cells formed aggregates or hepatic crown-like structures, which in agreement with the NAS of these livers, are associated to advanced inflammatory stages in NASH^{168,169} (Figure 18 A-D).

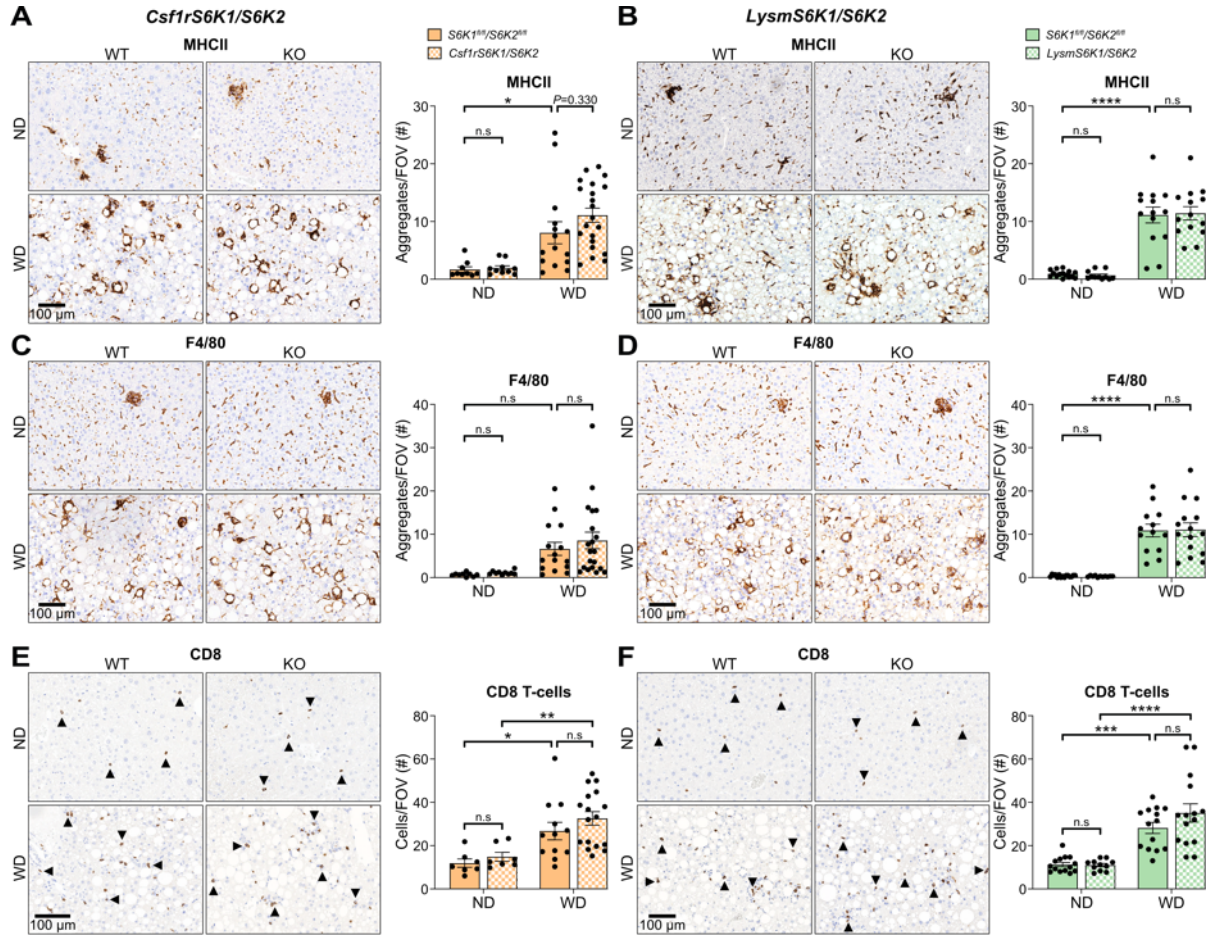


Figure 18. Myeloid S6K1 and S6K2 are dispensable for WD-induced liver inflammation. A-F. Representative liver immunohistochemistry images for antigen presenting cells (MHCII), macrophages (F4/80), CD8 T-cells and quantifications. WD-feeding induced significant liver infiltration of antigen presenting cells, macrophages and CD8 T-cells but the lack of myeloid S6K1/S6K2 had no effect on inflammation. FOV: field of view. Data are expressed as mean ± SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. n.s: non-significant.

Further, to analyze the distinct mouse myeloid populations in the liver, I, in collaboration with Dr. Alix Bruneau from the Charité Universitätsmedizin Berlin, analyzed representative livers by multiplex-immunofluorescence. In accordance with the literature⁹, WD-feeding induced a reduction of liver resident Kupffer cells (Iba1⁺/Clec4f⁺/F4/80⁺) and increased monocyte derived macrophages (MoMFs; Iba1⁺/F4/80⁺/Clec4f⁻) and monocytes (Iba1⁺/F4/80⁻/Clec4f⁻) (Figure 19 A). However, this analysis was conducted with representative

Csf1rS6K1/S6K2 and Cre-negative littermate controls. Nevertheless, no significant changes were attributable to the lack of macrophage S6 kinases. In summary, the multiplex immunofluorescence further suggests that myeloid S6K1 and S6K2 are dispensable to the liver inflammatory milieu in the context of obesity-induced NASH. Nevertheless, this conclusion is limited to the gross analysis of myeloid infiltrates in the liver, as it does not capture the quality or activation status (i.e. polarization) of these cells.

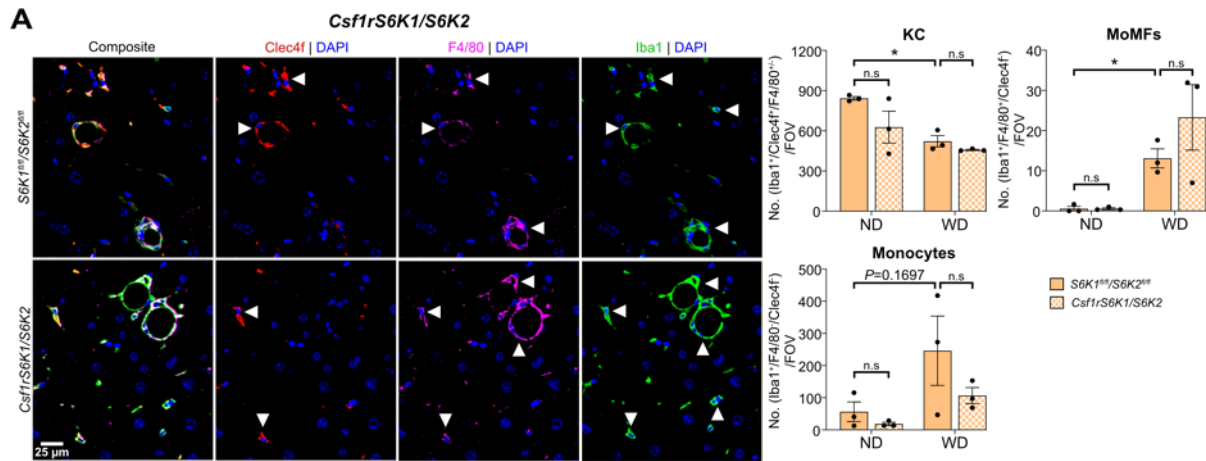


Figure 19. Myeloid S6K1 and S6K2 do not significantly modulate myeloid liver composition.

A. Representative multiplex immunofluorescence liver images after 30 weeks of WD-feeding. Clec4f-red, F4/80-purple, Iba1-green and nuclear staining DAPI-blue and quantifications. Scale bar 25 μ m. White triangles point to cells positive for the indicated antigen. Cells categorized as Kupffer cells (KC; Iba1⁺, Clec4f⁺, F4/80^{+/+}), monocyte derived macrophages (MoMFs; Iba1⁺, F4/80⁺, Clec4f⁻) and monocytes (Iba1⁺/F4/80⁻/Clec4f⁻). WD-feeding reduced KC abundance compared with ND-feeding, while MoMFs and monocytes increased with WD-feeding, nevertheless myeloid S6K1/S6K2 did not significantly modulate cell number in these myeloid populations. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. * $P < 0.05$, n.s: non-significant.

8.5.2 Myeloid S6K1 and S6K2 promote WD-induced liver fibrosis

Liver fibrosis is the main risk factor for liver-related outcomes and mortality³. In line, fibrosis halting or regression is a main outcome in clinical trials for NASH therapeutic candidates¹⁷⁰. Macrophages are key modulators of liver fibrosis as they release cytokines that activate HSC into myofibroblasts that secrete extracellular matrix, thereby driving liver fibrosis^{15,25}. Thus, macrophage modulators have the potential of indirectly modulating liver fibrosis.

My data on the role of myeloid S6K1/S6K2 showed that lack of macrophage S6 kinases did not modulate diet induced liver damage markers, hepatomegaly nor NASH histological features. However, the role of myeloid S6K1/S6K2 in liver fibrosis remained unclear. Thus, I, in collaboration with the coworkers of the histology lab, performed histological analyses of the NASH livers, namely Sirius red staining from *Csf1rS6K1/S6K2*, *LysmS6K1/S6K2* and Cre-negative littermate mice fed WD or ND as control. Notably, WD-feeding significantly induced liver fibrosis compared with ND-feeding, and remarkably, *LysmS6K1/S6K2* mice showed significantly less fibrosis than Cre-negative control mice (Figure 20 A-B). Meanwhile, *Csf1rS6K1/S6K2* also had less liver fibrosis than Cre-negative control mice, however, this did not reach statistical significance as a results of heterogenous fibrosis measurements (Figure 20 A). This suggested that myeloid S6K1/S6K2 have a role in liver fibrosis, however, the mechanism behind was elusive.

To assess whether macrophage S6 kinases played a role in macrophage-mediated activation of HSC, I, in collaboration with the coworkers of the histology lab, analyzed the expression of alpha-smooth muscle actin (α SMA) in the NASH livers. Notably WD-feeding induced significantly more HSC activation than ND-feeding, however, this was not affected by the genotype of mice (Figure 20 C-D). Indicating that HSC were activated in the context of NASH, but their general activation status was not affected by macrophage S6 kinase signaling.

Finally, to understand whether the differences in liver fibrosis mediated by myeloid S6K1/S6K2 derived from fibrogenesis modulation, I, in collaboration with Anna-Lena Eck from the DKFZ, analyzed the expression of fibrogenesis (*Col1a1* and *Col4a1*) and HSC activation-related genes (*Tgf- β* and *Pdgfa*). In line with the α SMA histology analysis, the expression of fibrogenic and HSC-activation genes was significantly upregulated by WD-feeding, however, this was not modulated by the lack of macrophage S6 kinases (Figure 20 E-L).

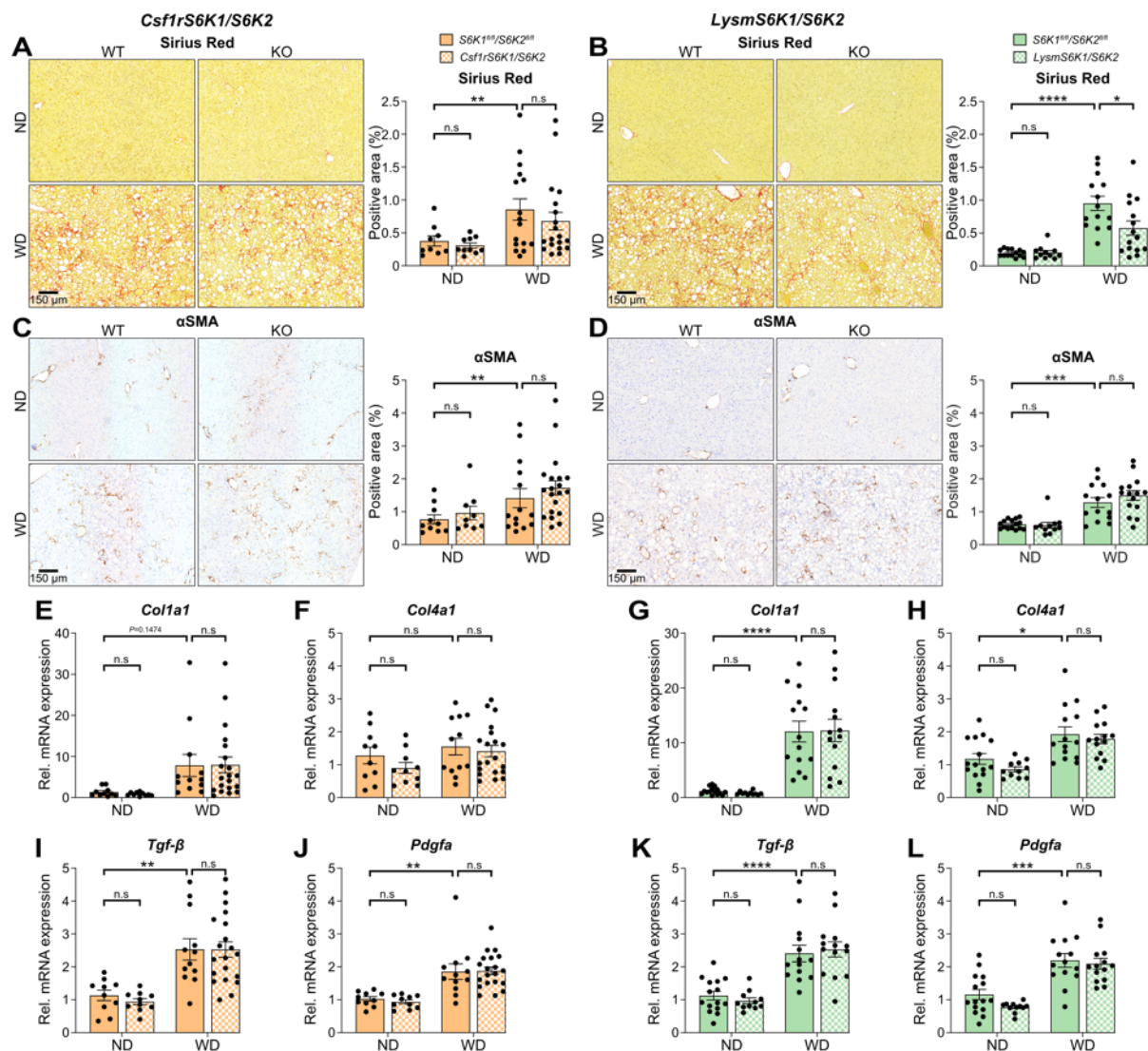


Figure 20. Loss of myeloid S6K1 and S6K2 dampened WD-induced liver fibrosis. **A & B.** Representative liver fibrosis histology (Sirius red) images and quantification. *Csf1rS6K1/S6K2* mice showed reduced fibrosis while *LysmS6K1/S6K2* mice showed mild protection from liver fibrosis. **C & D.** Representative hepatic stellate cells activation (alpha smooth-actin; αSMA) immunohistochemistry images and quantifications. WD-feeding increased αSMA but loss of S6K1/S6K2 did not significantly affect it. **E-L.** Relative liver gene expression of fibrogenesis-associated genes: collagen type I, alpha 1 (*Col1a1*), collagen type IV, alpha 1 (*Col4a1*), transforming growth factor beta (*Tgf-β*) and platelet-derived growth factor subunit A (*Pdgfa*). Gene expression relative to the expression of the housekeeping gene *Rps14*. Data are expressed as mean ± SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. n.s: non-significant.

8.5.3 Csf1r-Cre maintained myeloid S6K1 and S6K2 deletion upon liver macrophage pool replenishment

During the natural history of NASH, liver-resident macrophages –Kupffer cells– are replenished by monocyte derived macrophages –MoMFs–^{24,136}. This represents an important limitation of genetically modified macrophages and their study in liver pathologies¹⁷¹. Different drivers of Cre-recombinase have been described in the literature where they show distinct effectivity on different macrophage populations. Thus, from the inception of this project, two different Cre-drivers were used. Namely the Csf1r-Cre that targets all monocytes early in their differentiation, meaning that it should account for both liver resident and infiltrating macrophages, as well as Lysm-Cre that targets mostly mature macrophages in the liver, spleen and peritoneum¹⁷¹. Importantly, I had previously validated the KO efficiency in F4/80⁺ macrophages in the liver under basal conditions (Figure 12).

Therefore, to control the extent of myeloid S6K1/S6K2 deletion in the context of NASH, where infiltrating macrophages have replenished the liver, I, in collaboration with the coworkers of the histology lab, performed double immunofluorescence. Analysis of NASH and control livers showed extensive abrogation of pS6 in macrophages of Csf1rS6K1/S6K2 mice. However, ~40% of liver macrophages had escaped S6 kinase deletion in LysmS6K1/S6K2 mice fed WD (Figure 21 A-B). Given the baseline confirmation of myeloid S6K1/S6K2 KO driven by Lysm-Cre, this suggested that Lysm-Cre recombinase did not manage to delete the S6 kinases in MoMFs during liver immune infiltration. Thus, to avoid this confounder I decided to only employ Csf1rS6K1/S6K2 mice going forward.

Moreover, to ensure S6 kinase deletion in different liver myeloid cells, I, in collaboration with Dr. Bruneau, performed multiplex-immunofluorescence of representative livers. The analysis showed reduced KC, and increased MoMFs and monocytes attributable to WD-feeding, as well as robust deletion of S6 kinases the same three myeloid populations in Csf1rS6K1/S6K2 mice (Figure 21 C). This further validated the use of Csf1rS6K1/S6K2 mice for chronic liver inflammation experiments to ensure efficient deletion of the S6 kinases in macrophages both at baseline and upon challenge.

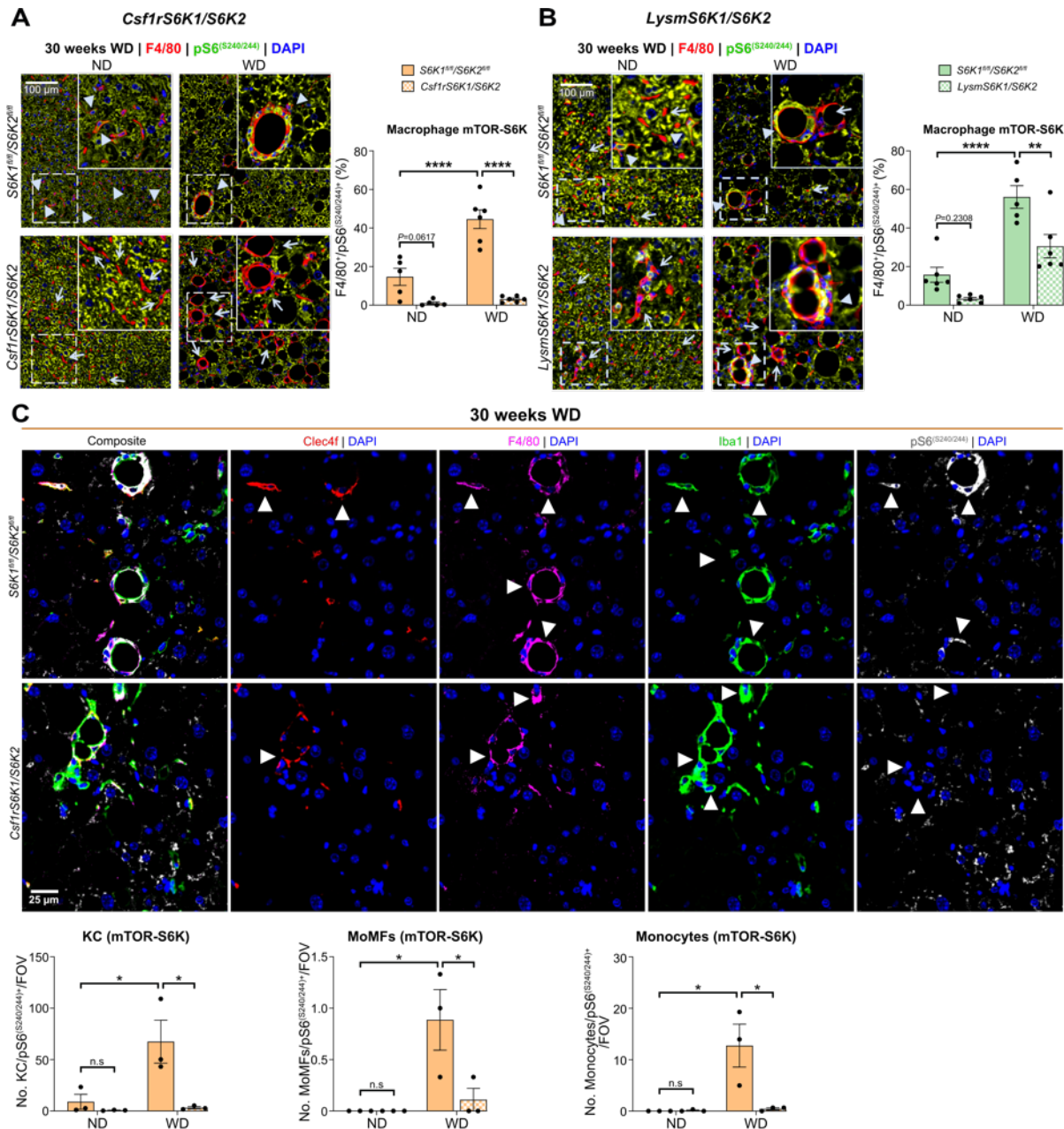


Figure 21. *Csf1r*-Cre targeted diverse myeloid cell compartments in NASH. A-B. Representative liver double immunofluorescence and quantification of macrophages (F4/80-red), pS6(S240/244)-green and nuclear staining DAPI-blue in mouse livers after 30 weeks of WD or ND-feeding. Scale bar 100 μ m. White triangle, double positive macrophages. White arrow, single positive macrophages. Dotted square, zoomed area. *Csf1r*S6K1/S6K2 mice showed significant loss of S6K1/S6K2 (evidenced by lack of pS6(S240/244)) in F4/80⁺ macrophages, whereas *Lysm*S6K1/S6K2 mice had a significant number of deletion-escapees in NASH. **C.** Representative liver multiplex immunofluorescence from representative mice from A. and quantifications. Clec4f-red, F4/80-purple, pS6(S240/244)-white and nuclear staining DAPI-blue. Scale bar 25 μ m. White triangles point to cells positive for the indicated antigen. Cells categorized as Kupffer cells (KC; Iba1⁺, Clec4f⁺, F4/80^{+/+}), monocyte derived macrophages (MoMFs; Iba1⁺, F4/80⁺, Clec4f⁺), monocytes (Iba1⁺/F4/80⁻/Clec4f⁻) and pS6(S240/244)⁺. WD-feeding increased myeloid mTOR-S6K signaling. *Csf1r*S6K1/S6K2 mice show lack of myeloid pS6 in liver KC, MoMFs and monocytes in NASH. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance

with Tukey's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. n.s: non-significant.

8.6 Myeloid S6K1 and S6K2 promote carbon tetrachloride (CCl₄)-induced liver fibrosis

Liver fibrosis and or its advanced setting –cirrhosis– is present in 70% – 90% of primary liver cancers¹²¹. Macrophages are key players in the activation of HSC to produce excess extracellular matrix that constitutes fibrosis^{15,25,136}. Moreover, the previous NASH experiments showed that the lack of myeloid S6K1/S6K2 dampened liver fibrosis in the chronic positive energy balance setting (Figure 20). Nevertheless, fibrogenic-gene expression analysis did not show marked differences attributable to the lack of macrophage S6 kinases. However, both the fibrosis quantification and gene expression analysis were studied in a chronic setting of inflammation, where several alternative variables could account for the observed differences.

Therefore, to validate and explore the role of myeloid S6K1/S6K2 in liver fibrosis, I, in collaboration with Dr. Gallage, employed the CCl₄ model of acute liver fibrosis in Csf1rS6K1/S6K2 and Cre-negative littermate mice. The model is based on 6 week-long biweekly intraperitoneal injections of CCl₄ or oil (vehicle) to induce liver fibrosis (Figure 22 A). At the end of 6.5 weeks, all mice had comparable body and liver weights (Figure 22 B-C). Moreover, a marker of biliary damage, –serum bilirubin– was significantly increased in Cre-negative mice treated with CCl₄ but not in Csf1rS6K1/S6K2 mice (Figure 22 D), with a genotype effect revealed by 2-way ANOVA.

Moreover, histological examination of the livers (done in collaboration with the coworkers of the histology lab) revealed significant bridging fibrosis and activation of HSC evidenced by Sirius red staining and α SMA IHC, both of which were significantly dampened in CCl₄-treated Csf1rS6K1/S6K2 mice compared with controls (Figure 22 F-H). Additionally, mouse monocytes (CD68⁺ cells), but not macrophages (F4/80⁺) or antigen presenting cells (MHCII⁺), were significantly increased in CCl₄-treated mice although without differences between the genotype of mice (Figure 22 I-M). These results supported the claim that myeloid S6K1/S6K2 have a role in liver fibrosis. However, whether the effect is mediated through modulation of fibrogenesis or fibrosis-resolving pathways remains unknown. Moreover, these results additionally confirmed that myeloid S6K1/S6K2 do not have a role in modulating the extensiveness of inflammation in the liver that was previously observed in the NASH experiments.

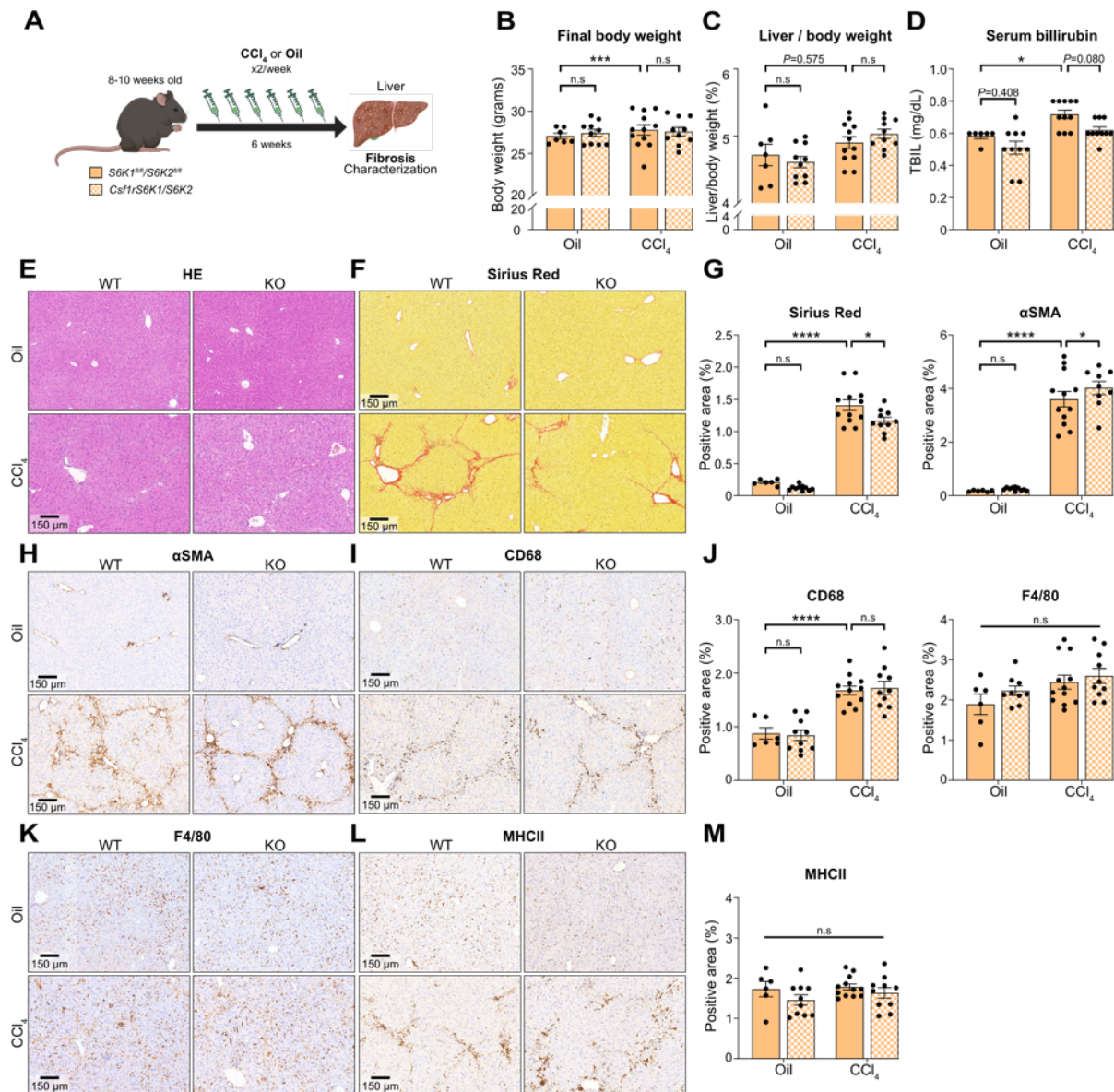


Figure 22. Loss of myeloid S6K1 and S6K2 dampened CCl₄-mediated liver fibrosis. A. Experimental scheme. 8-10-week old, Csf1rS6K1/S6K2 and Cre-negative littermate mice received CCl₄ or corn oil twice per week for 6 weeks to induce liver fibrosis. **B.** Final body weight. **C.** Liver to body weight ratio. **D.** Serum bilirubin 5 days after the last treatment. **E-H.** Representative liver eosyn & hematoxilin (HE) images Fibrosis (Sirius Red), hepatic-stellate cells activation (αSMA) and quantification. **I-M.** Mouse monocytes (CD68), macrophages (F4/80), antigen presenting cells (MHCII) and quantifications. Monocytes were increased by the CCl₄ treatment. Scale bar 150 μm. Data are expressed as mean ± SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. * P < 0.05, *** P < 0.001, **** P < 0.0001. n.s: non-significant.

Liver fibrosis is regulated by fibrogenic and extracellular matrix (ECM)-remodeling or resorptive cues. Therefore, to dissect which aspects of liver fibrosis might be regulated by myeloid S6K1/S6K2, I, in collaboration with Ana-Lena Eck from the DKFZ, explored fibrogenic and ECM-remodeling-associated gene expression in CCl₄-treated and control livers. The analysis revealed significantly increased fibrogenic gene (*Tgf-β*, *Col1a1*, *Col4a1* and *Pdgfa*) expression in the liver from mice treated with CCl₄ compared with control mice (Figure 23 A-D). Moreover, ECM-remodeling genes (*Mmp1a*, *Mmp2*, *Mmp3*, *Mmp10*) were significantly increased by CCl₄ treatment but also significantly dampened in *Csf1rS6K1/S6K2* mice (Figure 23 E-H). Thus, the data indicates that myeloid S6K1/S6K2 are involved in modulating the expression of ECM-remodelers in the context of liver fibrosis. Nevertheless, together with the pro-fibrogenic gene expression and HSC activation observed in the NASH experiments (Figure 20), these results suggest that macrophage S6 kinases do not play a major role in liver fibrogenesis. Nevertheless, macrophage S6 kinases do appear to play a role in ECM-remodeling or liver fibrosis resolution in this acute model of liver fibrosis. Nevertheless, the specific mechanism remains unknown.

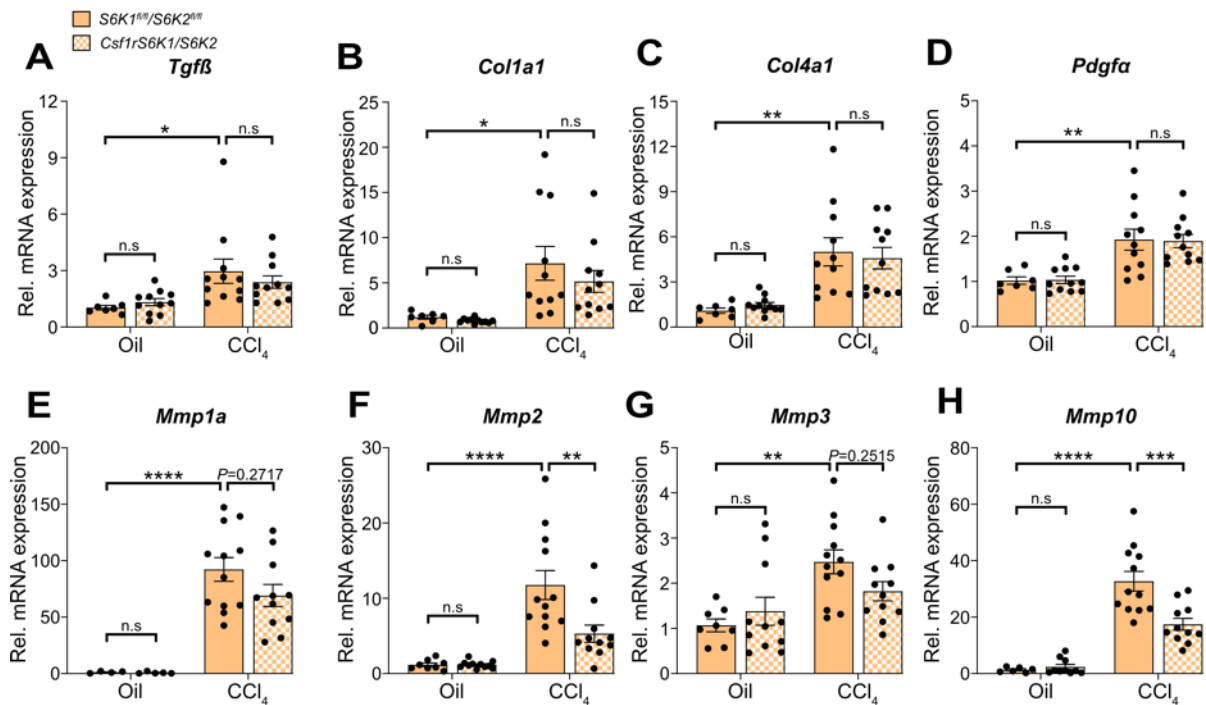


Figure 23. Myeloid S6K1 and S6K2 modulate extracellular matrix remodeling genes in the liver. A-D. Relative liver gene expression of transforming growth factor betha (*Tgf-β*), collagen type I, alpha 1 (*Col1a1*), collagen type IV, alpha 1 (*Col4a1*) and platelet derived growth factor subunit a (*Pdgfa*). E-H. Relative liver gene expression of matrix metalloproteinases 1a1 (*Mmp1a*), 2 (*Mmp2*), 3 (*Mmp3*) and 10 (*Mmp10*). Gene expression relative to the expression of the housekeeping gene *Rps14*. Data are expressed as mean ± SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, n.s: non-significant.

8.7 Myeloid S6K1 and S6K2 are dispensable for the NASH-HCC transition

The severity of liver fibrosis associates with increased cardiovascular and liver cancer risks³. Moreover, tumor-associated macrophages (TAM) have a critical role in liver carcinogenesis¹⁷². In that regard, my previous NASH and CCl₄ experiments showed that myeloid S6K1 and S6K2 play a role in modulating liver fibrosis (Figure 20 & Figure 22). However, whether this modulation of liver fibrosis could lead to changes in NASH-associated carcinogenesis, namely NASH-HCC, remains unknown.

Thus, I employed an obesogenic, spontaneous model of chronic NASH-HCC by feeding Csf1rS6K1/S6K2 and Cre-negative littermates with WD for 12 months (Figure 24 A). In line with the data from the NASH experiment, WD-feeding induced body and liver weight gain, fat mass gain and elevated serum markers of liver damage (ALT, AST and ALP) as well as increased total cholesterol (Figure 24 B-J). Moreover, in line with the literature⁹, 40% - 53% of the mice developed liver tumors. However, tumor incidence, quantity and size were not significantly different between genotypes (Figure 25 B-E). Thus, indicating that macrophage S6 kinases are dispensable for NASH-associated liver carcinogenesis in this spontaneous-obesogenic model of NASH-HCC.

To investigate whether macrophage S6 kinases may be involved in shaping tumor quality, liver fibrosis and the inflammatory milieu of NASH-HCC, I, in collaboration with the coworkers of the histology lab, analyzed tumor quality and liver inflammation markers by histological analysis. However, tumor quality, liver fibrosis (Sirius red), immune infiltration (MHCII⁺ and F4/80⁺ cells) and hepatocyte proliferation (Ki67⁺ hepatocytes) were not different between Csf1rS6K1/S6K2 and control mice (Figure 25 F-I). Thus, these results from histological analysis suggest that myeloid S6K1/S6K2 do not play a role in shaping NASH-associated tumor quality. Moreover, in this chronic setting, the loss of macrophage S6 kinases did not modulate liver fibrosis, suggesting a disease-stage limitation to the role of macrophage S6 kinases in liver fibrosis. Furthermore, like the NASH and CCl₄ experiment suggested, these results further confirmed that macrophage S6 kinases do play a role in mediating the liver inflammatory milieu in the context of obesity.

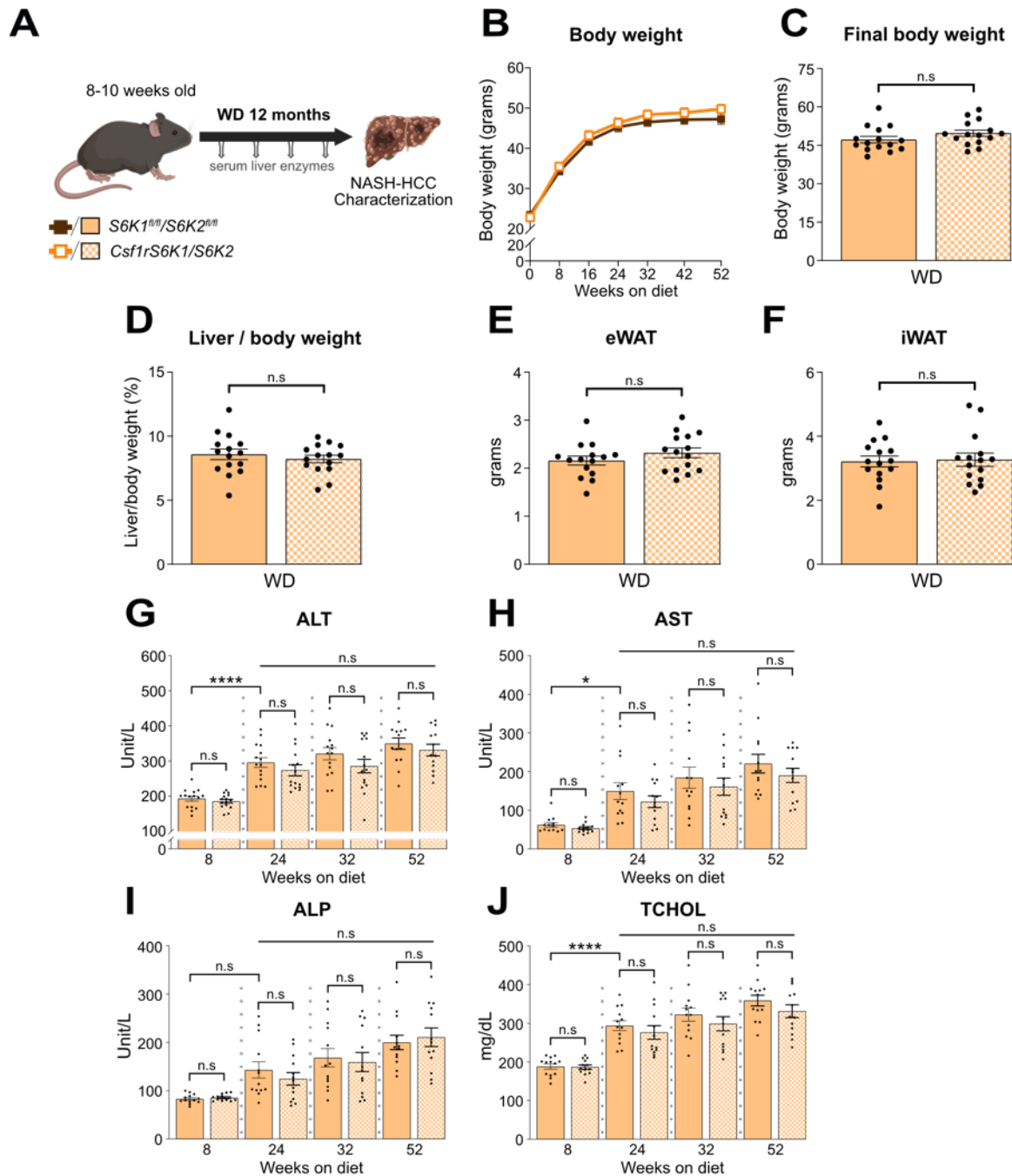


Figure 24. Myeloid S6K1 and S6K2 are dispensable for obesogenic NASH-HCC (1/2). **A.** Experimental scheme. 8-10 week old, CsfrS6K1/S6K2 and Cre-negative littermate mice received western diet (WD) during 12 months to induce (obese) NASH-HCC. **B-C.** Body weight curve and final body weight. **D.** Liver to body weight ratio. **E-F.** epididymal and inguinal white adipose tissue masses (eWAT and iWAT). **G-J.** Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total cholesterol (TCHOL) over time of WD-feeding. WD-feeding induced weight gain, hepatomegaly and liver damage. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. * $P < 0.05$, **** $P < 0.0001$. n.s: non-significant.

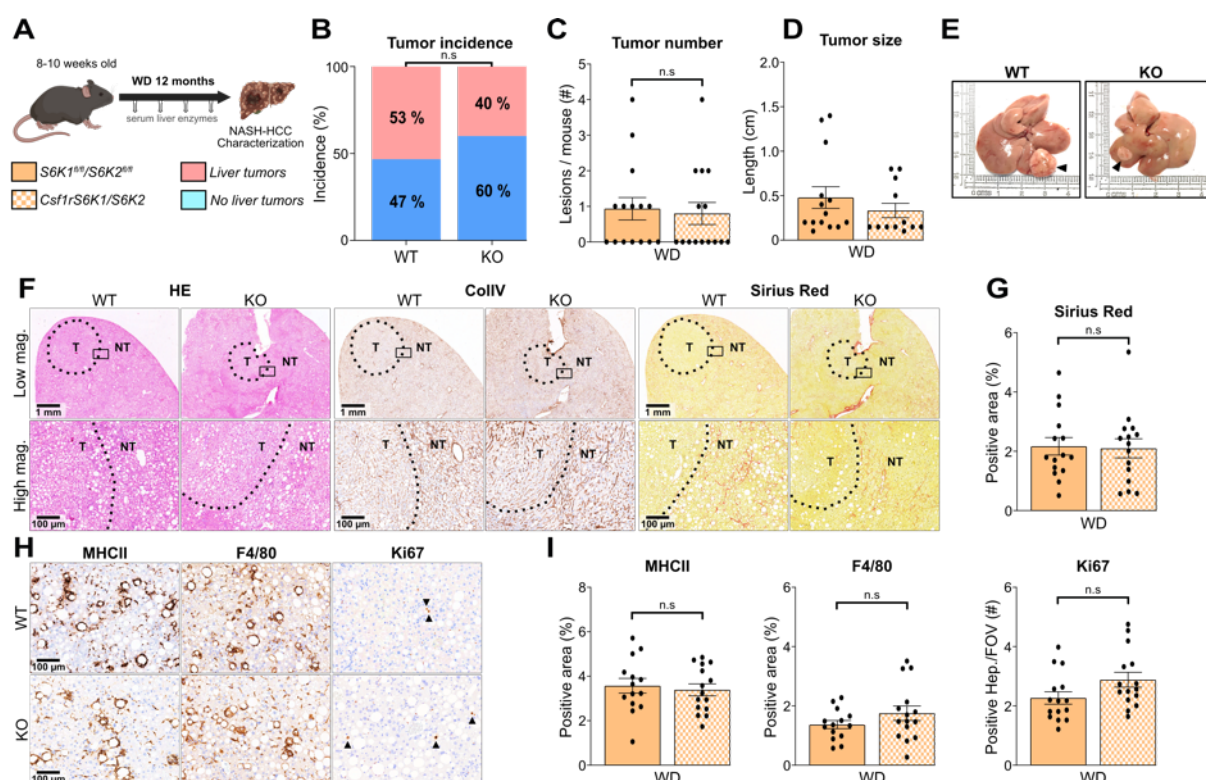


Figure 25. Myeloid S6K1 and S6K2 are dispensable for obesogenic NASH-HCC (2/2). **A.** Experimental scheme. 8-10 week old, CsflrS6K1/S6K2 and Cre-negative littermate mice received western diet (WD) during 12 months to induce (obese) NASH-HCC. **B.** Liver tumor incidence; red-positive, blue-negative. Myeloid S6K1/S6K2 were dispensable for liver tumor incidence. **C & D.** Liver tumor number and size per mouse, respectively. **E.** Representative macroscopic liver pictures. Black arrow-tumor. **F-I.** Representative liver immunohistochemistry and hematoxylin-eosin (HE), collagen type IV (ColIV), liver fibrosis (Sirius Red), antigen presenting cells (MHCII), macrophages (F4/80), proliferating cells (Ki67) and quantifications. FOV: field of view. Scale bars 1 mm and 100 μ m as indicated. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. Tumor incidence statistical significance was calculated using Chi-square test. n.s: non-significant.

Macrophage S6K1 and S6K2 did not play a role in obesogenic NASH-HCC as demonstrated in the previous experiment. Additionally, in that setting, liver fibrosis was no longer modulated by the lack of macrophage S6 kinases like in the NASH and CCl₄ experiments presented before. Thus, to validate these findings in an additional model of NASH-HCC, I employed the highly fibrotic model of NASH-HCC in lean mice. Namely, a model that induces metabolic inflammation, extensive liver fibrosis and rapid (30 weeks) NASH-HCC carcinogenesis⁹. Thus, I fed choline deficient, L-amino acid defined high-fat diet (CDAHFD: 60% kcal fat, L-amino acid-defined, 0.1% choline) to CsflrS6K1/S6K2 and Cre-negative littermates for 30 weeks (Figure 26 A). In contrast to WD, CDAHFD-feeding did not induce significant body weight gain nor loss (Figure 26 B-C). However, it did induce hepatomegaly

(Figure 26 D). Further, in accordance with the lack of change in body weight, fat depots did not grow in mass either (Figure 26 E-F). Moreover, unlike WD-feeding, CDAHFD-feeding induced a peaking elevation of serum liver damage markers (ALT, AST and ALP) already after 8 weeks of feeding, thereafter, the enzymes stabilized at a level consistent with the peaks induced by WD-feeding (150-300 units/L) (Figure 26 G-I). This highlighted the rapid onset of liver damage by the diet. Moreover, total serum cholesterol was not altered by the diet (Figure 26). Furthermore, in line with the literature, all mice developed liver tumors, however, tumor quantity and size were not significantly different between genotypes (Figure 27 B-E). This indicated that macrophage S6 kinases are dispensable for liver carcinogenesis independently of obesity.

Moreover, histological analysis (done in collaboration with the coworkers of the histology lab) showed liver steatosis and profound liver fibrosis that surrounded regenerative nodules (Figure 27 F-G). Moreover, tumors were HNF4a⁺/CK19⁻ suggesting a hepatocyte origin while the biliary marker (CK19) was increased in the non-tumor parenchyma (Figure 27 F & I), suggesting expansion of liver progenitor cells as response to the liver damage. Furthermore, CDAHFD-feeding induced profound inflammation (MHCII⁺ and F4/80⁺ cells) that was similar between Csf1rS6K1/S6K2 and Cre-negative littermates (Figure 27 H-I). Taken together, these data indicate that myeloid S6K1/S6K2 are dispensable for NASH-induced HCC development in two independent diet-induced models of NASH-HCC and independently from obesity. Moreover, they further confirm that macrophage S6 kinases do not play a role in modulating liver inflammation or liver damage and that their role in modulating liver fibrosis is likely limited to earlier stages of hepatopathology.

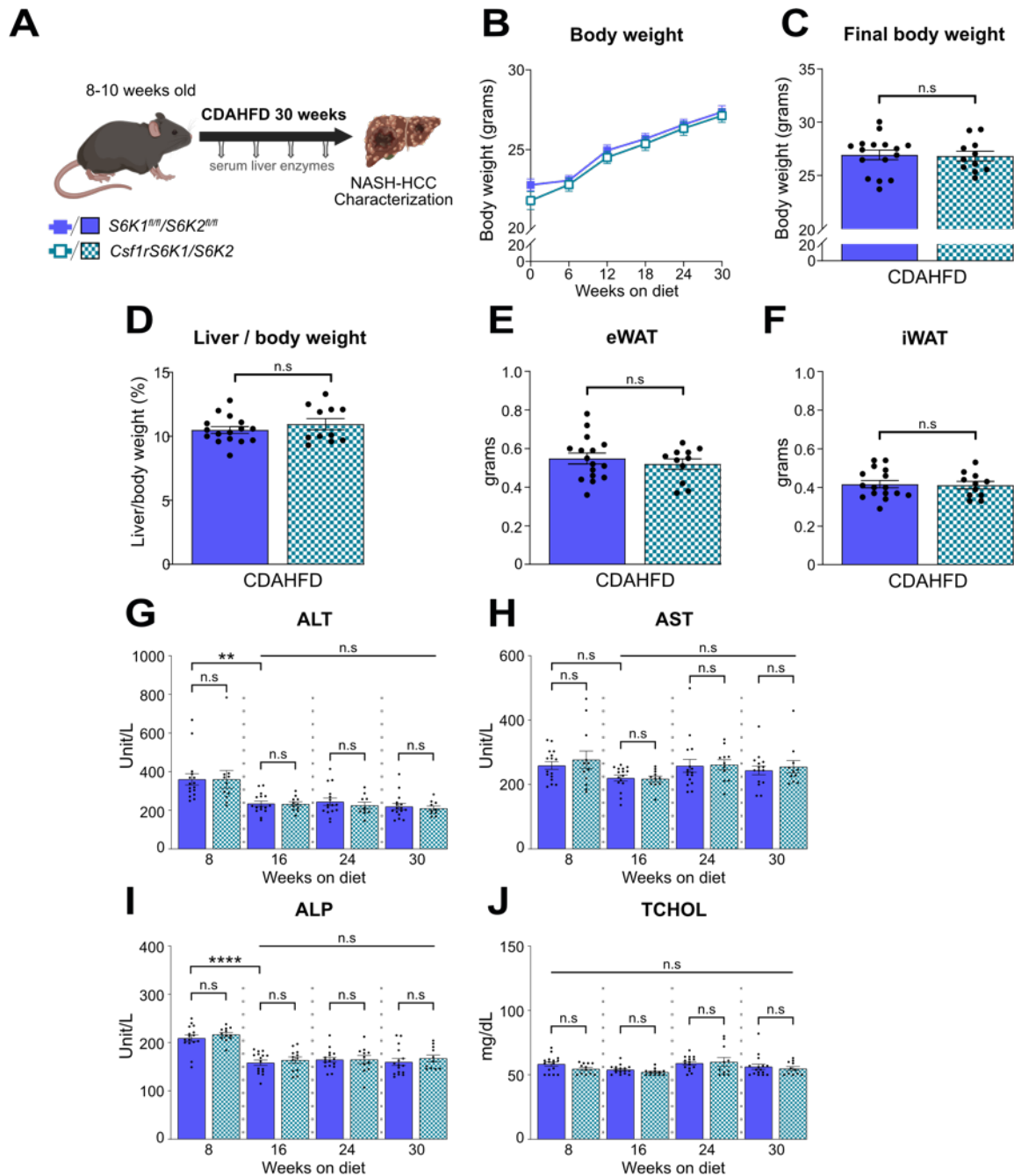


Figure 26. Myeloid S6K1 and S6K2 are dispensable for fibrotic, non-obesogenic NASH-HCC (1/2). **A.** Experimental scheme. 8-10 week old, CsflrS6K1/S6K2 and Cre-negative littermate mice received choline deficient L-amino acid defined high fat diet (CDAHFD) during 30 weeks to induce (lean) NASH-HCC. **B-C.** Body weight curve and final body weight. **D.** Liver to body weight ratio. **E-F.** epididymal and inguinal white adipose tissue masses (eWAT and iWAT). **G-J.** Serum alanine aminotransferase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP) and total cholesterol (TCHOL) over time of CDAHFD-feeding. CDAHFD-feeding did not induce weight gain but induced hepatomegaly and liver damage. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. ** $P < 0.01$, **** $P < 0.0001$. n.s: non-significant.

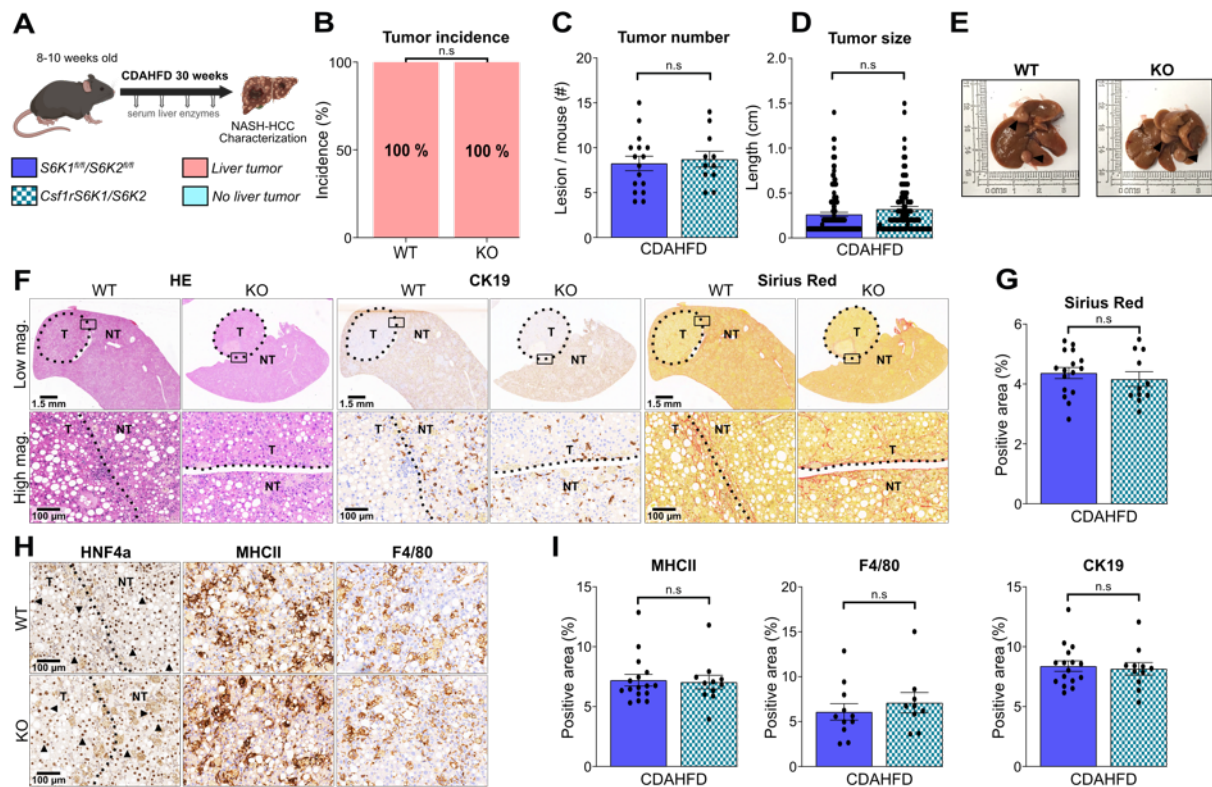


Figure 27. Myeloid S6K1 and S6K2 are dispensable for fibrotic, non-obesogenic NASH-HCC (2/2). **A.** Experimental scheme. 8-10 week old, CsflrS6K1/S6K2 and Cre-negative littermate mice received choline deficient L-amino acid defined high fat diet (CDAHFD) during 30 weeks to induce (lean) NASH-HCC. **B.** Liver tumor incidence; red-positive, blue-negative. Myeloid S6K1/S6K2 were dispensable for liver tumor incidence. **C & D.** Liver tumor number and size per mouse, respectively. **E.** Representative macroscopic liver pictures. Black arrow-tumor. **F-I.** Representative liver immunohistochemistry and hematoxylin-eosin (HE), collagen type IV (ColIV), liver fibrosis (Sirius Red), antigen presenting cells (MHCII), macrophages (F4/80), proliferating cells (Ki67) and quantifications. FOV: field of view. Scale bars 1 mm and 100 μ m as indicated. Data are expressed as mean $\pm$ SEM. Statistical significance was calculated using two-way analysis of variance with Tukey's multiple comparison test. Tumor incidence statistical significance was calculated using Chi-square test. n.s: non-significant.

9 Discussion

The epidemiological burden of obesity related diseases continues to grow in the world. For decades now, many people in affluent countries with considerable food security, have consumed excess energy beyond their daily requirements, that is, a positive energy balance. As a result, overweight and obesity-related diseases, have risen in prevalence. Thus, the metabolic syndrome, encompassing multiple metabolic diseases is now a main driver of disease in the world. Further, the so called –hepatic manifestations– of the metabolic syndrome, the non-alcoholic fatty liver disease (NAFLD) and its inflammatory evolution, non-alcoholic steatohepatitis (NASH), now affect around a quarter of the adult population in the world^{3,101}. Both NAFLD and NASH are important risk factors for the development of hepatocellular carcinoma (HCC), the main form of primary liver cancer which is the 6th most diagnosed cancer and the 3rd leading cause of cancer-related deaths in the world¹¹⁸.

There are presently no pharmaceutical compounds approved for treatment of NAFLD or NASH^{110,111}, leaving its treatment to be based in lifestyle interventions aimed at weight loss^{3,99}, so far the most effective regressor of NASH histological features. However, lifestyle interventions have limited long term effectivity as most patients regain any body weight initially lost. Thus, there is a pressing need to unravel the pathophysiological mechanisms driving the constellation of diseases under the umbrella of the metabolic syndrome, most of which stem from a chronic positive energy balance.

Nutrients are sensed by all cells through the mechanistic target of rapamycin (mTOR), a nutrient rheostat that initiates anabolic processes in response to a positive energy balance¹³⁸. Hepatocytes transiently deal with excess nutrients by storing carbohydrates as glycogen and lipids as triglycerides, however, upon chronic positive energy balance, excess lipids lead to hepatocyte damage, cell death and liver inflammation^{93,95,163}. In consequence, liver macrophages respond to hepatic damage by activating and recruiting several immune cells, driving inflammation. Moreover, macrophages play a key role in liver fibrosis as they secrete cytokines that activate hepatic stellate cells (HSC) to produce excess extracellular matrix that conforms liver fibrosis^{15,25}. Importantly, macrophages are exposed to the same excess nutrients that leads to hepatocyte damage; however, the role this might have in NAFLD/NASH is unknown.

mTOR inhibition has shown to recapitulate the beneficial effects of calorie restriction (i.e. negative energy balance)^{138,149,150}, however, systemic mTOR inhibition has several undesired side effects including liver damage, insulin resistance and immune-suppression^{148,173}. In line, one of the few clinical uses of systemic mTOR inhibition is that of an immunosuppressant where its use has been associated with reduced liver fibrosis^{152,153}; however, its negative effects prevent it from being a viable NASH therapy.

Consequently, targeting downstream effectors of mTOR and or limiting the inhibition to specific tissues or cell types is an ongoing effort to retain the beneficial effects of mTOR inhibition without undesirable effects^{154,155}. For instance, partial inhibition of mTOR in hepatocytes ameliorates NAFLD¹⁷⁴. However, confounding evidence comes from hepatocyte-specific upstream inhibition of mTORC1 (through deletion of *Raptor*) which accelerated liver damage and increased the incidence of HCC in mice¹⁵¹. Meanwhile, mTOR inhibition in non-parenchymal cells has been reported to be beneficial. In line, myeloid-specific mTORC1 inhibition (through deletion of *Raptor*) improved obesity-induced liver steatosis and inflammation in mice fed a high-fat diet (HFD)¹⁶⁴.

Targeting downstream effectors of mTOR, global deletion of the S6 kinase 1 (S6K1) has shown to recapitulate the beneficial effects of systemic mTOR inhibition, namely, increase lifespan, protect from diet-induced obesity, and improve glucose homeostasis^{156,159}, thereby avoiding the undesirable effects of upstream mTOR inhibition. In line, AAV-mediated knockdown of S6K1/S6K2 in hepatocytes recapitulated the beneficial effects of the S6K1 global knockouts by protecting mice from HFD-induced insulin resistance and liver steatosis¹⁶⁰. Nevertheless, the role of S6K1/S6K2 in macrophages in the liver, and whether they represent a therapeutic target is unknown.

Therefore, I studied the causal effects of myeloid S6K1/S6K2 in NASH, liver fibrosis and NASH-HCC using chronic and acute models of liver insults. To do so, I generated two mouse lines with macrophage-specific deletion of S6K1 and S6K2. Due to known limitations of macrophage-specific genetic manipulations stemming from the diverse macrophage populations and their ability to replenish distal macrophage pools, I utilized the *Csf1r*-Cre recombinase to target all myeloid cells early in their differentiation. This Cre has been reported to target liver resident Kupffer cells as well as monocyte-derived macrophages. I additionally utilized *Lysm*-Cre recombinase to target mature macrophages, that is liver Kupffer cells. Importantly, I validated the deletion of S6 kinases at baseline (i.e. young healthy mice) both, in mouse macrophages (F4/80⁺) in the liver and in bone marrow derived macrophages (BMDMs) from both mouse lines.

9.1 Human and mouse NAFLD/NASH myeloid cells have upregulated mTOR-S6K signaling

The search for NASH therapeutics has questioned whether targeted mTOR inhibition could represent a therapeutic target in NASH. However, as explained above, there is confounding evidence of the utility of this approach in hepatocytes. In contrast, preclinical macrophage targeted mTOR inhibition seemed to yield more consistent results in NAFLD/NASH. Thus, to further understand whether such approach could be exploited, in the present work, I could demonstrate that the mTOR-S6K axis is upregulated in macrophages in both human and mice NASH by immunofluorescence-based screening of liver samples. Further, by investigating the status of myeloid mTOR-S6K in human liver samples with diverse NAFLD-Activity Scores (NAS), I found a positive correlation between disease severity and the proportion of macrophages with increased mTOR signaling. Moreover, I could also show that mTOR-S6K is upregulated in polarized macrophages, and this was particularly stronger in classical pro-inflammatory macrophages.

Investigating whether mTOR-S6K signaling was necessary for macrophage polarization, I could not observe a causal link between S6K1/S6K2 signaling and macrophage polarization *ex vivo*, given that BMDMs lacking S6K1/S6K2 did not show altered mRNA levels of pro-inflammatory and anti-inflammatory genes compared with control BMDMs upon stimulation with LPS or IL-4. In contrast, Byles *et al* showed that activation of mTOR (through *Tsc1* deletion) in BMDMs potentiated LPS-induced upregulation of classical 'M1' pro-inflammatory cytokines and chemokines while dampening the expression of classical 'M2' anti-inflammatory target genes¹⁶¹.

In contrast, in their hands, mTORC1 inhibition by rapamycin treatment exacerbated IL-4-induced upregulation of M2 target genes and was associated with decreased phosphorylation of S6K1; thus, they could not confirm the causality of mTORC1 inhibition on macrophage polarization they reported¹⁶¹. Notably, rapamycin treatment would be closest to my model with loss of S6 kinases (mTOR inhibition), albeit macrophage specific rather than systemic like rapamycin. Nevertheless, my data demonstrate that the effects of rapamycin are independent of S6K1/S6K2 since BMDMs derived from either *Csf1r*S6K1/S6K2 or *LysM*S6K1/S6K2 mice did not show any alterations in IL4-induced expression of M2 target genes. However, there might be mechanistic differences upstream of S6K1/S6K2 that further differentiate rapamycin treatment from my model. Moreover, chronic rapamycin treatment has been shown to inhibit mTORC2, which is not directly related to S6 kinases, thus, perhaps the observations of Byles *et al* with regards to potentiated anti-inflammatory gene expression could be controlled by mTORC2 inhibition.

9.2 Myeloid S6K1/S6K2 are dispensable for diet-induced obesity and NASH

Systemic mTOR inhibition, as well as global deletion of the mTOR effector –S6K1– protect from diet induced-obesity^{156,159}. The latter further improves glucose homeostasis and insulin sensitivity in mice¹⁵⁹. Thus, I aimed to understand whether modulation of mTOR-S6K signaling in macrophages might recapitulate some aspects of the global deletion of S6K1 or systemic mTOR inhibition with rapamycin. Namely, I investigated whether myeloid S6K1/S6K2 play a role in diet-induced obesity and NASH development.

In disagreement with previous reports of myeloid-specific mTORC1 inhibition (Lysm-Cre x Raptor^{fl/fl})¹⁶⁴, S6K1/S6K2 deletion in macrophages did not affect WD-induced weight gain, body composition nor glucose homeostasis. Moreover, I did not observe myeloid-specific S6K1/S6K2 deletion to affect insulin sensitivity. However, elsewhere, myeloid-specific mTORC1 inhibition displayed improved insulin sensitivity when challenged by an ITT¹⁶⁴. Nevertheless, a major difference in the two studies is that in the present work I assessed these parameters in the context of a chronic model of WD-induced NASH that closely recapitulates human NASH histopathology in C57BL6J-background mice⁹, while Jiang *et al* studied the effects of myeloid-specific mTORC1 inhibition under short-term (10-12 weeks) feeding of a high-fat diet (HFD) which does not induce NASH, but rather NAFLD¹⁶⁴. Moreover, their inhibition of mTORC1 is through deletion of *Raptor*, therefore upstream mTOR inhibition (similar to rapamycin), meaning that there might be confounding effectors in the signaling cascade due to the site of inhibition. Nevertheless, the same could be argued of my model with mTOR-effector inhibition through the deletion of the S6 kinases. In any case, the different diet model and lack of inflammatory milieu might represent the biggest limitation for direct comparison of both results. However, if both datasets are considered, then the improved insulin sensitivity reported upon inhibition of mTORC1 by Jiang *et al*, must be orchestrated independently from the S6 kinases and or is limited to the context of NAFLD rather than NASH.

Furthermore, my experimental data revealed that the lack of S6K1/S6K2 in monocytes/macrophages did not result in modulation of any of the histological characteristics of NASH, that is, liver steatosis, inflammation, and hepatocyte ballooning. In contrast, myeloid-specific deletion of mTORC1 (Lysm-Cre x Raptor^{fl/fl}) in the context of NAFLD (through HFD-feeding) attenuated steatosis and inflammation¹⁶⁴, while exacerbating steatosis, inflammation and markers of liver damage in NASH (WD-feeding)¹⁶⁵. This, together with my data, further suggests that myeloid mTOR-signaling may have divergent roles at the different stages of NAFLD/NASH and that these effects are independent of myeloid S6K1/S6K2, likely orchestrated upstream from the S6 kinases. Put in the context of whether macrophage S6 kinases represent a therapeutic target for NASH, although the available evidence is

heterogeneous, given the small effects observed for and against their inhibition, macrophage S6 kinases might not be a viable therapeutic target for NASH and associated metabolic disruptions.

9.3 Loss of myeloid S6K1/S6K2 dampened liver fibrosis in two independent models

Macrophages play a key role in the activation of hepatic stellate cells (HSC) to ultimately produce liver fibrosis¹⁵. In my research for the present thesis, the most important consequence of the loss of myeloid S6K1/S6K2 was observed in liver fibrosis, both in the context of chronic WD-feeding for 30 weeks (NASH), and independently in a toxin (CCl₄)-based model of acute liver fibrosis.

In the chronic diet experiment, I could observe lower fibrosis in Csf1rS6K1/S6K2 or LysMS6K1/S6K2 mice fed a WD for 30 weeks, which recapitulates a state of NASH. However, we did not observe this phenomenon following 12 months of WD or 30 weeks of CDAHFD feeding at a time point where tumors are also evident, suggesting that myeloid S6K1/S6K2 may play a more prominent role in earlier phases of liver fibrosis. Thus, it is likely that dominant pathways override the effects of myeloid S6K1/S6K2 at later stages of NASH-HCC.

I additionally assessed whether myeloid S6K1/S6K2 play a role in a toxin (CCl₄)-model of liver fibrosis. In this model, the deletion of S6K1/S6K2 in myeloid cells resulted in a significant reduction in liver fibrosis, which was accompanied by lower expression of matrix metalloproteinases but without regulation of fibrogenic genes. Moreover, I observed a reduction in fibrosis (Sirius red) concomitant with increased HSC activation (α SMA) both in NASH and in CCl₄, suggesting that loss of myeloid S6K1/S6K2 rather delays fibrogenesis since activation of hepatic stellate cells precedes collagen deposition in multiple contexts including liver transplants, viral hepatitis and CCl₄-induced liver fibrosis¹⁷⁵⁻¹⁷⁸.

To date, none of the studies conducted on myeloid-specific mTORC1 deletion studied the effects on liver fibrosis. This has been seemingly out of the scope of said publications and has traditionally been relegated in the context of long-term models. In part perhaps due to their elevated costs. Nevertheless, it was demonstrated that mTOR inhibitors sirolimus and everolimus were potent antifibrotic agents in an experimental model of liver fibrosis (bile duct ligation) in rats, thus showing that targeting the mTOR-S6K pathway may hold antifibrotic value¹⁷⁹. Further long-term studies on the effects of mTOR inhibition should be assayed to confirm or deny their usability in the treatment of liver fibrosis. However, their systemic nature as presently used will inevitably require either tissue specific and or downstream targeting efforts to circumvent the undesirable effects associated to these rapalogs.

9.4 Infiltrating monocytes are a limitation of macrophage studies

Upon chronic challenge, resident Kupffer cells are replenished by infiltrating monocytes during the course of NASH progression, and this constitutes a limitation for macrophage targeted genetic modifications^{28,134}. Therefore, I investigated the efficiency of Cre recombinase-mediated deletion of S6K1/S6K2 by the Lysm-Cre and Csf1r-Cre promoters in two time and disease stages. Importantly, Csf1r is expressed from the early stages of monocyte differentiation while Lysm is expressed predominantly in mature macrophages¹⁷¹. Using multiplex immunofluorescence, I could confirm that Csf1r promoter induced an efficient and continued deletion of S6K1/S6K2 in both resident Kupffer cells as well as in monocytes throughout the course of NASH while this was impaired in LysmS6K1/S6K2 mice. This suggests that for long-term chronic studies involving liver injury, Csf1r-Cre mediated deletion is the superior option unless one wants to study specifically the role of liver resident Kupffer cells in mild-acute rather than chronic models.

9.5 Myeloid S6K1/S6K2 are dispensable for liver carcinogenesis

Liver fibrosis is an important risk factor for the development of HCC^{3,99,117,127}, and tumor-associated macrophages have been shown to play an important role in tumor development¹⁷². In my research, deletion of myeloid S6K1/S6K2 did not modulate the liver inflammatory milieu but did lead to diminished liver fibrosis, both, acutely in a toxin (CCl₄) model of liver injury and in a chronic (WD-feeding) model of NASH. Therefore, I investigated whether myeloid S6K1/S6K2 mediated modulation of liver fibrosis might a role in NASH-HCC development using two distinct models of NASH-HCC in obese and lean mice.

However, although both models induced profound liver damage and tumorigenesis in accordance with the literature, macrophage-specific deletion of S6K1/S6K2 did not impact tumor incidence, tumor number or size in either obese (WD-feeding) or lean-fibrotic (CDAHFD-feeding) mice. Moreover, in both chronic settings, liver fibrosis was no longer modulated by the loss of myeloid S6K1/S6K2. Notably, my findings on unchanged tumorigenesis between KO and control mice are partially in conflict with reports of prolonged mTORC1 inhibition (both systemic and hepatocyte specific) inducing enhanced liver tumorigenesis by Umemura *et al*¹⁵¹. Nevertheless, apart from the cell-type studied, a further detriment for the comparison between our studies is that Umemura and co. employed a mixed model of NASH-HCC where high-fat diet-feeding is combined with a potent liver carcinogen early in life (Diethylnitrosamine), which leads to relatively early tumorigenesis after 6 to 7 months of HFD-feeding, at the expense of the spontaneity in tumorigenesis impaired by chronic models.

10 Concluding remarks

Overall, my data on the role of myeloid mTOR-S6K signaling through the mTOR-effectors – S6K1/S6K2– in liver metabolic pathologies has two concluding remarks.

First, that myeloid S6K1/S6K2 are dispensable for obesity and its metabolic effects, namely, glucose and insulin sensitivity. In the same context, the macrophage S6 kinases are also dispensable for the development of NASH and NASH-HCC, including their histological characteristics like steatosis, inflammation and hepatocyte damage.

Second, in line with reports of systemic mTOR inhibition moderating liver fibrosis, loss of myeloid S6K1/S6K2 seem to play a role in modulating liver fibrosis in the context of NASH and CCl₄-liver injury. This seems to be regulated through a mechanism involving extracellular tissue remodeling but requires further investigation to be confirmed. Moreover, the effects of myeloid S6K1/S6K2 appear to be limited to early stages of liver fibrosis (independent of NASH), as two long-term chronic models of liver injury did not recapitulate the fibrosis protection observed before.

Taken together, targeted inhibition of myeloid S6K1 and S6K2 might hold potential therapeutic target in early NAFLD-NASH stages, but this requires further study and human data to be validated. This is of utmost importance as several factors are presently driving the burden of liver fibrosis and its consequences in our society.

11 References

- 1 Tiniakos, D. G., Kandilis, A. & Geller, S. A. Tityus: a forgotten myth of liver regeneration. *J Hepatol* **53**, 357-361 (2010). <https://doi.org/10.1016/j.jhep.2010.02.032>
- 2 Michalopoulos, G. K. & Bhushan, B. Liver regeneration: biological and pathological mechanisms and implications. *Nat Rev Gastroenterol Hepatol* **18**, 40-55 (2021). <https://doi.org/10.1038/s41575-020-0342-4>
- 3 Rinella, M. E. *et al.* AASLD Practice Guidance on the clinical assessment and management of nonalcoholic fatty liver disease. *Hepatology* **77**, 1797-1835 (2023). <https://doi.org/10.1097/HEP.0000000000000323>
- 4 Eslam, M. *et al.* A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *J Hepatol* **73**, 202-209 (2020). <https://doi.org/10.1016/j.jhep.2020.03.039>
- 5 Yang, L. L. in *Anesthesia for Hepatobiliary Surgery and Transplantation* (eds Zoka Milan & Chula Goonasekera) 15-40 (Springer International Publishing, 2021).
- 6 Rogers, A. B. & Dintzis, R. Z. in *Comparative Anatomy and Histology* (eds Piper M. Treuting & Suzanne M. Dintzis) 193-201 (Academic Press, 2012).
- 7 Betts, J. G. *et al.* in *Anatomy and Physiology 2e* (OpenStax, 2022. Available at: <https://openstax.org/books/anatomy-and-physiology-2e/pages/23-6-accessory-organs-in-digestion-the-liver-pancreas-and-gallbladder?query=liver&target=%7B%22index%22%3A0%2C%22type%22%3A%22search%22%7D#fs-id2279526> (Accessed 20.09.2023)).
- 8 Wang, Z. *et al.* Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure. *Am J Clin Nutr* **92**, 1369-1377 (2010). <https://doi.org/10.3945/ajcn.2010.29885>
- 9 Gallage, S. *et al.* A researcher's guide to preclinical mouse NASH models. *Nat Metab* **4**, 1632-1649 (2022). <https://doi.org/10.1038/s42255-022-00700-y>
- 10 Nevzorova, Y. A., Boyer-Diaz, Z., Cubero, F. J. & Gracia-Sancho, J. Animal models for liver disease - A practical approach for translational research. *J Hepatol* **73**, 423-440 (2020). <https://doi.org/10.1016/j.jhep.2020.04.011>
- 11 Brown, Z. J., Heinrich, B. & Greten, T. F. Mouse models of hepatocellular carcinoma: an overview and highlights for immunotherapy research. *Nat Rev Gastroenterol Hepatol* **15**, 536-554 (2018). <https://doi.org/10.1038/s41575-018-0033-6>
- 12 Schulze, R. J., Schott, M. B., Casey, C. A., Tuma, P. L. & McNiven, M. A. The cell biology of the hepatocyte: A membrane trafficking machine. *J Cell Biol* **218**, 2096-2112 (2019). <https://doi.org/10.1083/jcb.201903090>
- 13 Haubrich, W. S. Disse of the space of Disse. *Gastroenterology* **127**, 1684 (2004). <https://doi.org/10.1053/j.gastro.2004.10.021>
- 14 Fanti, M., Gramignoli, R., Serra, M., Cadoni, E., Strom, S. C. & Marongiu, F. Differentiation of amniotic epithelial cells into various liver cell types and potential therapeutic applications. *Placenta* **59**, 139-145 (2017). <https://doi.org/10.1016/j.placenta.2017.03.020>
- 15 Vonderlin, J., Chavakis, T., Sieweke, M. & Tacke, F. The Multifaceted Roles of Macrophages in NAFLD Pathogenesis. *Cell Mol Gastroenterol Hepatol* **15**, 1311-1324 (2023). <https://doi.org/10.1016/j.jcmgh.2023.03.002>
- 16 Kotsiliti, E. *et al.* Intestinal B cells license metabolic T-cell activation in NASH microbiota/antigen-independently and contribute to fibrosis by IgA-FcR signalling. *J Hepatol* **79**, 296-313 (2023). <https://doi.org/10.1016/j.jhep.2023.04.037>
- 17 Crispe, I. N. The liver as a lymphoid organ. *Annu Rev Immunol* **27**, 147-163 (2009). <https://doi.org/10.1146/annurev.immunol.021908.132629>
- 18 Bonnardel, J. *et al.* Stellate Cells, Hepatocytes, and Endothelial Cells Imprint the Kupffer Cell Identity on Monocytes Colonizing the Liver Macrophage Niche. *Immunity* **51**, 638-654 e639 (2019). <https://doi.org/10.1016/j.immuni.2019.08.017>

- 19 Theurl, I. *et al.* On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat Med* **22**, 945-951 (2016). <https://doi.org/10.1038/nm.4146>
- 20 Hua, Z. *et al.* Contamination of Aflatoxins Induces Severe Hepatotoxicity Through Multiple Mechanisms. *Front Pharmacol* **11**, 605823 (2020). <https://doi.org/10.3389/fphar.2020.605823>
- 21 Wang, L. Q. *et al.* Perfluoroalkyl substance pollutants activate the innate immune system through the AIM2 inflammasome. *Nat Commun* **12**, 2915 (2021). <https://doi.org/10.1038/s41467-021-23201-0>
- 22 Costello, E. *et al.* Exposure to per- and Polyfluoroalkyl Substances and Markers of Liver Injury: A Systematic Review and Meta-Analysis. *Environ Health Perspect* **130**, 46001 (2022). <https://doi.org/10.1289/EHP10092>
- 23 Miura, A., Hosono, T. & Seki, T. Macrophage potentiates the recovery of liver zonation and metabolic function after acute liver injury. *Sci Rep* **11**, 9730 (2021). <https://doi.org/10.1038/s41598-021-88989-9>
- 24 Scott, C. L. *et al.* Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun* **7**, 10321 (2016). <https://doi.org/10.1038/ncomms10321>
- 25 Tacke, F., Puengel, T., Loomba, R. & Friedman, S. L. An integrated view of anti-inflammatory and antifibrotic targets for the treatment of NASH. *J Hepatol* **79**, 552-566 (2023). <https://doi.org/10.1016/j.jhep.2023.03.038>
- 26 Winau, F. *et al.* Ito cells are liver-resident antigen-presenting cells for activating T cell responses. *Immunity* **26**, 117-129 (2007). <https://doi.org/10.1016/j.immuni.2006.11.011>
- 27 Ficht, X. & Iannacone, M. Immune surveillance of the liver by T cells. *Sci Immunol* **5** (2020). <https://doi.org/10.1126/sciimmunol.aba2351>
- 28 Malehmir, M. *et al.* Platelet GPIIb α is a mediator and potential interventional target for NASH and subsequent liver cancer. *Nat Med* **25**, 641-655 (2019). <https://doi.org/10.1038/s41591-019-0379-5>
- 29 Heymann, F. & Tacke, F. Immunology in the liver--from homeostasis to disease. *Nat Rev Gastroenterol Hepatol* **13**, 88-110 (2016). <https://doi.org/10.1038/nrgastro.2015.200>
- 30 Li, X., Ramadori, P., Pfister, D., Seehawer, M., Zender, L. & Heikenwalder, M. The immunological and metabolic landscape in primary and metastatic liver cancer. *Nat Rev Cancer* **21**, 541-557 (2021). <https://doi.org/10.1038/s41568-021-00383-9>
- 31 Pfister, D. *et al.* NASH limits anti-tumour surveillance in immunotherapy-treated HCC. *Nature* **592**, 450-456 (2021). <https://doi.org/10.1038/s41586-021-03362-0>
- 32 Wolf, M. J. *et al.* Metabolic activation of intrahepatic CD8 $^{+}$ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. *Cancer Cell* **26**, 549-564 (2014). <https://doi.org/10.1016/j.ccell.2014.09.003>
- 33 Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. *World Health Organ Tech Rep Ser* **854**, 1-452 (1995).
- 34 Keys, A., Fidanza, F., Karvonen, M. J., Kimura, N. & Taylor, H. L. Indices of relative weight and obesity. *J Chronic Dis* **25**, 329-343 (1972). [https://doi.org/10.1016/0021-9681\(72\)90027-6](https://doi.org/10.1016/0021-9681(72)90027-6)
- 35 WHO. *Malnutrition in women*, <<https://www.who.int/data/nutrition/nlis/info/malnutrition-in-women>> (Accessed 28.09.2023)> (
- 36 CDC. *Defining Adult Overweight & Obesity*, <<https://www.cdc.gov/obesity/basics/adult-defining.html>> (Accessed 28.09.2023)> (
- 37 Caleyachetty, R. *et al.* Ethnicity-specific BMI cutoffs for obesity based on type 2 diabetes risk in England: a population-based cohort study. *Lancet Diabetes Endocrinol* **9**, 419-426 (2021). [https://doi.org/10.1016/S2213-8587\(21\)00088-7](https://doi.org/10.1016/S2213-8587(21)00088-7)
- 38 Humphreys, S. The unethical use of BMI in contemporary general practice. *Br J Gen Pract* **60**, 696-697 (2010). <https://doi.org/10.3399/bjgp10X515548>

39 CDC. *Body Mass Index: Considerations for Practicioners*,
 <<https://www.cdc.gov/obesity/downloads/bmiforpractitioners.pdf> (Accessed 29.09.2023)> (

40 WHO. *Obesity and overweight*, <<https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight> (Accessed 28.09.2023)> (2021).

41 Global_Obesity_Federation. *World Obesity Atlas 2023*,
 <<https://data.worldobesity.org/publications/?cat=19> (Accessed 29.09.2023)> (2023).

42 Our_World_in_Data. *What share of adults are overweight?*,
 <<https://ourworldindata.org/obesity> (Accessed 01.10.2023)> (2016).

43 Hou, J., He, C., He, W., Yang, M., Luo, X. & Li, C. Obesity and Bone Health: A Complex Link. *Front Cell Dev Biol* **8**, 600181 (2020). <https://doi.org/10.3389/fcell.2020.600181>

44 Csige, I. *et al.* The Impact of Obesity on the Cardiovascular System. *J Diabetes Res* **2018**, 3407306 (2018). <https://doi.org/10.1155/2018/3407306>

45 McGill, H. C., Jr. *et al.* Obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation* **105**, 2712-2718 (2002).
<https://doi.org/10.1161/01.cir.0000018121.67607.ce>

46 Reaven, G. M. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* **37**, 1595-1607 (1988). <https://doi.org/10.2337/diab.37.12.1595>

47 Kahn, S. E., Hull, R. L. & Utzschneider, K. M. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444**, 840-846 (2006). <https://doi.org/10.1038/nature05482>

48 Alcala, M. *et al.* Increased inflammation, oxidative stress and mitochondrial respiration in brown adipose tissue from obese mice. *Sci Rep* **7**, 16082 (2017).
<https://doi.org/10.1038/s41598-017-16463-6>

49 Pi-Sunyer, X. The medical risks of obesity. *Postgrad Med* **121**, 21-33 (2009).
<https://doi.org/10.3810/pgm.2009.11.2074>

50 Bhaskaran, K., Douglas, I., Forbes, H., dos-Santos-Silva, I., Leon, D. A. & Smeeth, L. Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5.24 million UK adults. *Lancet* **384**, 755-765 (2014). [https://doi.org/10.1016/S0140-6736\(14\)60892-8](https://doi.org/10.1016/S0140-6736(14)60892-8)

51 Yumuk, V. *et al.* European Guidelines for Obesity Management in Adults. *Obes Facts* **8**, 402-424 (2015). <https://doi.org/10.1159/000442721>

52 Thow, A. M., Rippin, H. L., Mulcahy, G., Duffey, K. & Wickramasinghe, K. Sugar-sweetened beverage taxes in Europe: learning for the future. *Eur J Public Health* **32**, 273-280 (2022).
<https://doi.org/10.1093/eurpub/ckab211>

53 Silver, L. D. *et al.* Changes in prices, sales, consumer spending, and beverage consumption one year after a tax on sugar-sweetened beverages in Berkeley, California, US: A before-and-after study. *PLoS Med* **14**, e1002283 (2017). <https://doi.org/10.1371/journal.pmed.1002283>

54 WHO. *WHO acceleration plan to stop obesity*,
 <<https://www.who.int/publications/i/item/9789240075634> (Accessed 01.10.2023)> (2022).

55 Grunvald, E. *et al.* AGA Clinical Practice Guideline on Pharmacological Interventions for Adults With Obesity. *Gastroenterology* **163**, 1198-1225 (2022).
<https://doi.org/10.1053/j.gastro.2022.08.045>

56 Apovian, C. M. *et al.* Pharmacological management of obesity: an endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* **100**, 342-362 (2015).
<https://doi.org/10.1210/jc.2014-3415>

57 Wilding, J. P. H. *et al.* Once-Weekly Semaglutide in Adults with Overweight or Obesity. *N Engl J Med* **384**, 989-1002 (2021). <https://doi.org/10.1056/NEJMoa2032183>

58 Loomba, R. *et al.* Semaglutide 2.4 mg once weekly in patients with non-alcoholic steatohepatitis-related cirrhosis: a randomised, placebo-controlled phase 2 trial. *Lancet Gastroenterol Hepatol* **8**, 511-522 (2023). [https://doi.org/10.1016/S2468-1253\(23\)00068-7](https://doi.org/10.1016/S2468-1253(23)00068-7)

59 Neovius, M. *et al.* Health care use during 20 years following bariatric surgery. *JAMA* **308**, 1132-1141 (2012). <https://doi.org/10.1001/2012.jama.11792>

- 60 Sjostrom, L. *et al.* Effects of bariatric surgery on mortality in Swedish obese subjects. *N Engl J Med* **357**, 741-752 (2007). <https://doi.org/10.1056/NEJMoa066254>
- 61 Karlsen, T. I. *et al.* Health related quality of life after gastric bypass or intensive lifestyle intervention: a controlled clinical study. *Health Qual Life Outcomes* **11**, 17 (2013). <https://doi.org/10.1186/1477-7525-11-17>
- 62 Burza, M. A. *et al.* Long-term effect of bariatric surgery on liver enzymes in the Swedish Obese Subjects (SOS) study. *PLoS One* **8**, e60495 (2013). <https://doi.org/10.1371/journal.pone.0060495>
- 63 Chang, S. H., Stoll, C. R., Song, J., Varela, J. E., Eagon, C. J. & Colditz, G. A. The effectiveness and risks of bariatric surgery: an updated systematic review and meta-analysis, 2003-2012. *JAMA Surg* **149**, 275-287 (2014). <https://doi.org/10.1001/jamasurg.2013.3654>
- 64 Speakman, J. R. Thrifty genes for obesity, an attractive but flawed idea, and an alternative perspective: the 'drifty gene' hypothesis. *Int J Obes (Lond)* **32**, 1611-1617 (2008). <https://doi.org/10.1038/ijo.2008.161>
- 65 Neel, J. V. Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? *Am J Hum Genet* **14**, 353-362 (1962).
- 66 Campos, A., Port, J. D. & Acosta, A. Integrative Hedonic and Homeostatic Food Intake Regulation by the Central Nervous System: Insights from Neuroimaging. *Brain Sci* **12** (2022). <https://doi.org/10.3390/brainsci12040431>
- 67 Pontzer, H. Constrained Total Energy Expenditure and the Evolutionary Biology of Energy Balance. *Exerc Sport Sci Rev* **43**, 110-116 (2015). <https://doi.org/10.1249/JES.0000000000000048>
- 68 O'Neal, T. J., Friend, D. M., Guo, J., Hall, K. D. & Kravitz, A. V. Increases in Physical Activity Result in Diminishing Increments in Daily Energy Expenditure in Mice. *Curr Biol* **27**, 423-430 (2017). <https://doi.org/10.1016/j.cub.2016.12.009>
- 69 Pontzer, H. Energy Constraint as a Novel Mechanism Linking Exercise and Health. *Physiology (Bethesda)* **33**, 384-393 (2018). <https://doi.org/10.1152/physiol.00027.2018>
- 70 NIH. *Metabolic syndrome*, <<https://www.nhlbi.nih.gov/health/metabolic-syndrome> (Accessed 01.10.2023)> (2022).
- 71 Dobrowolski, P. *et al.* Metabolic syndrome - a new definition and management guidelines: A joint position paper by the Polish Society of Hypertension, Polish Society for the Treatment of Obesity, Polish Lipid Association, Polish Association for Study of Liver, Polish Society of Family Medicine, Polish Society of Lifestyle Medicine, Division of Prevention and Epidemiology Polish Cardiac Society, "Club 30" Polish Cardiac Society, and Division of Metabolic and Bariatric Surgery Society of Polish Surgeons. *Arch Med Sci* **18**, 1133-1156 (2022). <https://doi.org/10.5114/aoms/152921>
- 72 Grundy, S. M. *et al.* Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* **112**, 2735-2752 (2005). <https://doi.org/10.1161/CIRCULATIONAHA.105.169404>
- 73 Alberti, K. G., Zimmet, P. & Shaw, J. Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med* **23**, 469-480 (2006). <https://doi.org/10.1111/j.1464-5491.2006.01858.x>
- 74 Saklayen, M. G. The Global Epidemic of the Metabolic Syndrome. *Curr Hypertens Rep* **20**, 12 (2018). <https://doi.org/10.1007/s11906-018-0812-z>
- 75 American_Heart_Association. *What is Metabolic Syndrome?*, <<https://www.heart.org/-/media/files/health-topics/answers-by-heart/what-is-metabolic-syndrome.pdf> (Accessed 01.10.2023)> (
- 76 Mayo_Clinic. *Metabolic Syndrome*, <<https://www.mayoclinic.org/diseases-conditions/metabolic-syndrome/symptoms-causes/syc-20351916> (Accessed 01.10.2023)> (
- 77 Stephens, C. R. *et al.* The Impact of Education and Age on Metabolic Disorders. *Front Public Health* **8**, 180 (2020). <https://doi.org/10.3389/fpubh.2020.00180>

- 78 Wright, J. T., Jr. *et al.* Clinical outcomes by race in hypertensive patients with and without the metabolic syndrome: Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). *Arch Intern Med* **168**, 207-217 (2008). <https://doi.org/10.1001/archinternmed.2007.66>
- 79 Fappa, E., Yannakoulia, M., Pitsavos, C., Skoumas, I., Valourdou, S. & Stefanadis, C. Lifestyle intervention in the management of metabolic syndrome: could we improve adherence issues? *Nutrition* **24**, 286-291 (2008). <https://doi.org/10.1016/j.nut.2007.11.008>
- 80 Del Corral, P., Bryan, D. R., Garvey, W. T., Gower, B. A. & Hunter, G. R. Dietary adherence during weight loss predicts weight regain. *Obesity (Silver Spring)* **19**, 1177-1181 (2011). <https://doi.org/10.1038/oby.2010.298>
- 81 Dansinger, M. L., Gleason, J. A., Griffith, J. L., Selker, H. P. & Schaefer, E. J. Comparison of the Atkins, Ornish, Weight Watchers, and Zone diets for weight loss and heart disease risk reduction: a randomized trial. *JAMA* **293**, 43-53 (2005). <https://doi.org/10.1001/jama.293.1.43>
- 82 Svetkey, L. P. *et al.* Comparison of strategies for sustaining weight loss: the weight loss maintenance randomized controlled trial. *JAMA* **299**, 1139-1148 (2008). <https://doi.org/10.1001/jama.299.10.1139>
- 83 Wing, R. R. & Hill, J. O. Successful weight loss maintenance. *Annu Rev Nutr* **21**, 323-341 (2001). <https://doi.org/10.1146/annurev.nutr.21.1.323>
- 84 Wing, R. R. & Phelan, S. Long-term weight loss maintenance. *Am J Clin Nutr* **82**, 222S-225S (2005). <https://doi.org/10.1093/ajcn/82.1.222S>
- 85 Hill, J. O. & Commerford, R. Physical activity, fat balance, and energy balance. *Int J Sport Nutr* **6**, 80-92 (1996). <https://doi.org/10.1123/ijsn.6.2.80>
- 86 Hall, K. D. *et al.* Quantification of the effect of energy imbalance on bodyweight. *Lancet* **378**, 826-837 (2011). [https://doi.org/10.1016/S0140-6736\(11\)60812-X](https://doi.org/10.1016/S0140-6736(11)60812-X)
- 87 Roach, P. J., Depaoli-Roach, A. A., Hurley, T. D. & Tagliabracci, V. S. Glycogen and its metabolism: some new developments and old themes. *Biochem J* **441**, 763-787 (2012). <https://doi.org/10.1042/BJ20111416>
- 88 Acheson, K. J., Schutz, Y., Bessard, T., Anantharaman, K., Flatt, J. P. & Jequier, E. Glycogen storage capacity and de novo lipogenesis during massive carbohydrate overfeeding in man. *Am J Clin Nutr* **48**, 240-247 (1988). <https://doi.org/10.1093/ajcn/48.2.240>
- 89 Murray, R. K., Bender, D. A., Botham, K. M., Kennelly, P. J., Rodwell, V. W. & Weil, P. A. in *Harper's Illustrated Biochemistry* Ch. 16, 131-142 (McGraw Hill, 2009).
- 90 Murray, R. K., Bender, D. A., Botham, K. M., Kennelly, P. J., Rodwell, V. W. & Weil, P. A. in *Harper's Illustrated Biochemistry* Ch. 25, 212-223 (2009).
- 91 Nguyen, P. *et al.* Liver lipid metabolism. *J Anim Physiol Anim Nutr (Berl)* **92**, 272-283 (2008). <https://doi.org/10.1111/j.1439-0396.2007.00752.x>
- 92 Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D. & Parks, E. J. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* **115**, 1343-1351 (2005). <https://doi.org/10.1172/JCI23621>
- 93 Feldstein, A. E. *et al.* Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway. *Hepatology* **40**, 185-194 (2004). <https://doi.org/10.1002/hep.20283>
- 94 Roskams, T. *et al.* Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol* **163**, 1301-1311 (2003). [https://doi.org/10.1016/S0002-9440\(10\)63489-X](https://doi.org/10.1016/S0002-9440(10)63489-X)
- 95 Gastaldelli, A. & Cusi, K. From NASH to diabetes and from diabetes to NASH: Mechanisms and treatment options. *JHEP Rep* **1**, 312-328 (2019). <https://doi.org/10.1016/j.jhepr.2019.07.002>
- 96 Korenblat, K. M., Fabbrini, E., Mohammed, B. S. & Klein, S. Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. *Gastroenterology* **134**, 1369-1375 (2008). <https://doi.org/10.1053/j.gastro.2008.01.075>

- 97 Ozcan, U. *et al.* Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **306**, 457-461 (2004). <https://doi.org/10.1126/science.1103160>
- 98 Zhang, X. Q., Xu, C. F., Yu, C. H., Chen, W. X. & Li, Y. M. Role of endoplasmic reticulum stress in the pathogenesis of nonalcoholic fatty liver disease. *World J Gastroenterol* **20**, 1768-1776 (2014). <https://doi.org/10.3748/wjg.v20.i7.1768>
- 99 European Association for the Study of the, L., European Association for the Study of, D. & European Association for the Study of, O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* **64**, 1388-1402 (2016). <https://doi.org/10.1016/j.jhep.2015.11.004>
- 100 Kleiner, D. E. *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41**, 1313-1321 (2005). <https://doi.org/10.1002/hep.20701>
- 101 Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L. & Wymer, M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64**, 73-84 (2016). <https://doi.org/10.1002/hep.28431>
- 102 Estes, C., Razavi, H., Loomba, R., Younossi, Z. & Sanyal, A. J. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. *Hepatology* **67**, 123-133 (2018). <https://doi.org/10.1002/hep.29466>
- 103 Ando, Y. & Jou, J. H. Nonalcoholic Fatty Liver Disease and Recent Guideline Updates. *Clin Liver Dis (Hoboken)* **17**, 23-28 (2021). <https://doi.org/10.1002/cld.1045>
- 104 Chalasani, N. *et al.* The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* **67**, 328-357 (2018). <https://doi.org/10.1002/hep.29367>
- 105 Younossi, Z. M. *et al.* The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *J Hepatol* **71**, 793-801 (2019). <https://doi.org/10.1016/j.jhep.2019.06.021>
- 106 Quek, J. *et al.* Global prevalence of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in the overweight and obese population: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* **8**, 20-30 (2023). [https://doi.org/10.1016/S2468-1253\(22\)00317-X](https://doi.org/10.1016/S2468-1253(22)00317-X)
- 107 Harrison, S. A. *et al.* Prospective evaluation of the prevalence of non-alcoholic fatty liver disease and steatohepatitis in a large middle-aged US cohort. *J Hepatol* **75**, 284-291 (2021). <https://doi.org/10.1016/j.jhep.2021.02.034>
- 108 Younossi, Z. M. *et al.* Nonalcoholic Steatohepatitis Is the Most Rapidly Increasing Indication for Liver Transplantation in the United States. *Clin Gastroenterol Hepatol* **19**, 580-589 e585 (2021). <https://doi.org/10.1016/j.cgh.2020.05.064>
- 109 Sterling, R. K. *et al.* Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* **43**, 1317-1325 (2006). <https://doi.org/10.1002/hep.21178>
- 110 Medicines Output European Public Assessment Reports, <[https://www.ema.europa.eu/en/medicines/download-medicine-data#european-public-assessment-reports-\(epar\)-section](https://www.ema.europa.eu/en/medicines/download-medicine-data#european-public-assessment-reports-(epar)-section)> (2022).
- 111 Final Guidance Document, <<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/technical-specifications-submitting-clinical-trial-data-sets-treatment-noncirrhotic-nonalcoholic>> (2022).
- 112 Karim, G. & Bansal, M. B. Resmetirom: An Orally Administered, Smallmolecule, Liver-directed, beta-selective THR Agonist for the Treatment of Non-alcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis. *touchREV Endocrinol* **19**, 60-70 (2023). <https://doi.org/10.17925/EE.2023.19.1.60>
- 113 Madrigal_Pharmaceuticals. Positive Topline Phase 3 MAESTRO-NAFLD-1 Data Demonstrate Resmetirom was Safe, Well-Tolerated and Provided Statistically Significant Improvements in Key Measures of Liver and Cardiovascular Health, <<https://ir.madrigalpharma.com/news->

- [releases/news-release-details/positive-topline-phase-3-maestro-nafld-1-data-demonstrate](#) (Accessed 01.10.2023)> (2023).
- 114 Madrigal_Pharmaceuticals. *Madrigal Receives Breakthrough Therapy Designation from FDA for Resmetirom and Completes Enrollment of the Phase 3 MAESTRO-NASH Biopsy Trial*, <<https://ir.madrigalpharma.com/news-releases/news-release-details/madrigal-receives-breakthrough-therapy-designation-fda> (Accessed 01.10.2023)> (2023).
- 115 Ratzliff, V. & Charlton, M. Rational combination therapy for NASH: Insights from clinical trials and error. *J Hepatol* **78**, 1073-1079 (2023). <https://doi.org:10.1016/j.jhep.2022.12.025>
- 116 Puengel, T. et al. Combined Therapy with a CCR2/CCR5 Antagonist and FGF21 Analogue Synergizes in Ameliorating Steatohepatitis and Fibrosis. *Int J Mol Sci* **23** (2022). <https://doi.org:10.3390/ijms23126696>
- 117 Singal, A. G. et al. AASLD Practice Guidance on prevention, diagnosis, and treatment of hepatocellular carcinoma. *Hepatology* (2023). <https://doi.org:10.1097/HEP.0000000000000466>
- 118 Sung, H. et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **71**, 209-249 (2021). <https://doi.org:10.3322/caac.21660>
- 119 International Consensus Group for Hepatocellular NeoplasiaThe International Consensus Group for Hepatocellular, N. Pathologic diagnosis of early hepatocellular carcinoma: a report of the international consensus group for hepatocellular neoplasia. *Hepatology* **49**, 658-664 (2009). <https://doi.org:10.1002/hep.22709>
- 120 Llovet, J. M. et al. Hepatocellular carcinoma. *Nat Rev Dis Primers* **7**, 6 (2021). <https://doi.org:10.1038/s41572-020-00240-3>
- 121 Gallage, S. et al. The therapeutic landscape of hepatocellular carcinoma. *Med (N Y)* **2**, 505-552 (2021). <https://doi.org:10.1016/j.medj.2021.03.002>
- 122 Anstee, Q. M., Reeves, H. L., Kotsiliti, E., Govaere, O. & Heikenwalder, M. From NASH to HCC: current concepts and future challenges. *Nat Rev Gastroenterol Hepatol* **16**, 411-428 (2019). <https://doi.org:10.1038/s41575-019-0145-7>
- 123 Bhurwal, A. et al. Inverse Association of Coffee with Liver Cancer Development: An Updated Systematic Review and Meta-analysis. *J Gastrointest Liver Dis* **29**, 421-428 (2020). <https://doi.org:10.15403/jgld-805>
- 124 Simon, T. G., Duberg, A. S., Aleman, S., Chung, R. T., Chan, A. T. & Ludvigsson, J. F. Association of Aspirin with Hepatocellular Carcinoma and Liver-Related Mortality. *N Engl J Med* **382**, 1018-1028 (2020). <https://doi.org:10.1056/NEJMoa1912035>
- 125 Islam, M. M., Poly, T. N., Walther, B. A., Yang, H. C. & Jack Li, Y. C. Statin Use and the Risk of Hepatocellular Carcinoma: A Meta-Analysis of Observational Studies. *Cancers (Basel)* **12** (2020). <https://doi.org:10.3390/cancers12030671>
- 126 Plaz Torres, M. C., Jaffe, A., Perry, R., Marabotto, E., Strazzabosco, M. & Giannini, E. G. Diabetes medications and risk of HCC. *Hepatology* **76**, 1880-1897 (2022). <https://doi.org:10.1002/hep.32439>
- 127 European Association for the Study of the Liver. Electronic address, e. e. e. & European Association for the Study of the, L. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* **69**, 182-236 (2018). <https://doi.org:10.1016/j.jhep.2018.03.019>
- 128 Cancer_Research_UK. *Survival for primary liver cancer*, <<https://www.cancerresearchuk.org/about-cancer/liver-cancer/survival> (Accessed 01.10.2023)> (
- 129 Finn, R. S. et al. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med* **382**, 1894-1905 (2020). <https://doi.org:10.1056/NEJMoa1915745>
- 130 FDA. *FDA approves atezolizumab plus bevacizumab for unresectable hepatocellular carcinoma*, <<https://www.fda.gov/drugs/resources-information-approved-drugs/fda->

- [approves-atezolizumab-plus-bevacizumab-unresectable-hepatocellular-carcinoma](#) (Accessed 01.10.2023)> (2020).
- 131 Kazankov, K. *et al.* The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Nat Rev Gastroenterol Hepatol* **16**, 145-159 (2019). <https://doi.org/10.1038/s41575-018-0082-x>
 - 132 Tacke, F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol* **66**, 1300-1312 (2017). <https://doi.org/10.1016/j.jhep.2017.02.026>
 - 133 Miura, K., Yang, L., van Rooijen, N., Ohnishi, H. & Seki, E. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. *Am J Physiol Gastrointest Liver Physiol* **302**, G1310-1321 (2012). <https://doi.org/10.1152/ajpgi.00365.2011>
 - 134 Tran, S. *et al.* Impaired Kupffer Cell Self-Renewal Alters the Liver Response to Lipid Overload during Non-alcoholic Steatohepatitis. *Immunity* **53**, 627-640 e625 (2020). <https://doi.org/10.1016/j.immuni.2020.06.003>
 - 135 Guilliams, M. *et al.* Spatial proteogenomics reveals distinct and evolutionarily conserved hepatic macrophage niches. *Cell* **185**, 379-396 e338 (2022). <https://doi.org/10.1016/j.cell.2021.12.018>
 - 136 Krenkel, O. *et al.* Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. *Hepatology* **67**, 1270-1283 (2018). <https://doi.org/10.1002/hep.29544>
 - 137 Guilliams, M., Mildner, A. & Yona, S. Developmental and Functional Heterogeneity of Monocytes. *Immunity* **49**, 595-613 (2018). <https://doi.org/10.1016/j.immuni.2018.10.005>
 - 138 Saxton, R. A. & Sabatini, D. M. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* **168**, 960-976 (2017). <https://doi.org/10.1016/j.cell.2017.02.004>
 - 139 Kim, J., Kundu, M., Viollet, B. & Guan, K. L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* **13**, 132-141 (2011). <https://doi.org/10.1038/ncb2152>
 - 140 Lee-Fruman, K. K., Kuo, C. J., Lippincott, J., Terada, N. & Blenis, J. Characterization of S6K2, a novel kinase homologous to S6K1. *Oncogene* **18**, 5108-5114 (1999). <https://doi.org/10.1038/sj.onc.1202894>
 - 141 Fenton, T. R. & Gout, I. T. Functions and regulation of the 70kDa ribosomal S6 kinases. *Int J Biochem Cell Biol* **43**, 47-59 (2011). <https://doi.org/10.1016/j.biocel.2010.09.018>
 - 142 Pardo, O. E. & Seckl, M. J. S6K2: The Neglected S6 Kinase Family Member. *Front Oncol* **3**, 191 (2013). <https://doi.org/10.3389/fonc.2013.00191>
 - 143 Pende, M. *et al.* S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* **24**, 3112-3124 (2004). <https://doi.org/10.1128/MCB.24.8.3112-3124.2004>
 - 144 Yang, L. *et al.* The mTORC1 effectors S6K1 and 4E-BP play different roles in CNS axon regeneration. *Nat Commun* **5**, 5416 (2014). <https://doi.org/10.1038/ncomms6416>
 - 145 Brown, E. J. *et al.* A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **369**, 756-758 (1994). <https://doi.org/10.1038/369756a0>
 - 146 Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S. H. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78**, 35-43 (1994). [https://doi.org/10.1016/0092-8674\(94\)90570-3](https://doi.org/10.1016/0092-8674(94)90570-3)
 - 147 Sabers, C. J. *et al.* Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J Biol Chem* **270**, 815-822 (1995). <https://doi.org/10.1074/jbc.270.2.815>
 - 148 Unnikrishnan, A., Kurup, K., Salmon, A. B. & Richardson, A. Is Rapamycin a Dietary Restriction Mimetic? *J Gerontol A Biol Sci Med Sci* **75**, 4-13 (2020). <https://doi.org/10.1093/gerona/glz060>
 - 149 Miller, R. A. *et al.* Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. *Aging Cell* **13**, 468-477 (2014). <https://doi.org/10.1111/accel.12194>

- 150 Wilkinson, J. E. *et al.* Rapamycin slows aging in mice. *Aging Cell* **11**, 675-682 (2012).
<https://doi.org/10.1111/j.1474-9726.2012.00832.x>
- 151 Umemura, A. *et al.* Liver damage, inflammation, and enhanced tumorigenesis after persistent mTORC1 inhibition. *Cell Metab* **20**, 133-144 (2014).
<https://doi.org/10.1016/j.cmet.2014.05.001>
- 152 Klintmalm, G. B. & Nashan, B. The Role of mTOR Inhibitors in Liver Transplantation: Reviewing the Evidence. *J Transplant* **2014**, 845438 (2014). <https://doi.org/10.1155/2014/845438>
- 153 Fernandez-Yunquera, A. *et al.* Everolimus immunosuppression reduces the serum expression of fibrosis markers in liver transplant recipients. *World J Transplant* **4**, 133-140 (2014).
<https://doi.org/10.5500/wjt.v4.i2.133>
- 154 Wassarman, D. R., Bankapalli, K., Pallanck, L. J. & Shokat, K. M. Tissue-restricted inhibition of mTOR using chemical genetics. *Proc Natl Acad Sci U S A* **119**, e2204083119 (2022).
<https://doi.org/10.1073/pnas.2204083119>
- 155 Ehinger, Y., Zhang, Z., Phamluong, K., Soneja, D., Shokat, K. M. & Ron, D. Brain-specific inhibition of mTORC1 eliminates side effects resulting from mTORC1 blockade in the periphery and reduces alcohol intake in mice. *Nat Commun* **12**, 4407 (2021).
<https://doi.org/10.1038/s41467-021-24567-x>
- 156 Selman, C. *et al.* Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* **326**, 140-144 (2009). <https://doi.org/10.1126/science.1177221>
- 157 Smith, M. A. *et al.* Ribosomal S6K1 in POMC and AgRP Neurons Regulates Glucose Homeostasis but Not Feeding Behavior in Mice. *Cell Rep* **11**, 335-343 (2015).
<https://doi.org/10.1016/j.celrep.2015.03.029>
- 158 Irvine, E. E. *et al.* Genetic deletion of S6k1 does not rescue the phenotypic deficits observed in the R6/2 mouse model of Huntington's disease. *Sci Rep* **9**, 16133 (2019).
<https://doi.org/10.1038/s41598-019-52391-3>
- 159 Um, S. H. *et al.* Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431**, 200-205 (2004).
<https://doi.org/10.1038/nature02866>
- 160 Bae, E. J. *et al.* Liver-specific p70 S6 kinase depletion protects against hepatic steatosis and systemic insulin resistance. *J Biol Chem* **287**, 18769-18780 (2012).
<https://doi.org/10.1074/jbc.M112.365544>
- 161 Byles, V. *et al.* The TSC-mTOR pathway regulates macrophage polarization. *Nat Commun* **4**, 2834 (2013). <https://doi.org/10.1038/ncomms3834>
- 162 Weichhart, T., Hengstschlager, M. & Linke, M. Regulation of innate immune cell function by mTOR. *Nat Rev Immunol* **15**, 599-614 (2015). <https://doi.org/10.1038/nri3901>
- 163 Liu, K. *et al.* Lipotoxicity-induced STING1 activation stimulates MTORC1 and restricts hepatic lipophagy. *Autophagy* **18**, 860-876 (2022). <https://doi.org/10.1080/15548627.2021.1961072>
- 164 Jiang, H., Westerterp, M., Wang, C., Zhu, Y. & Ai, D. Macrophage mTORC1 disruption reduces inflammation and insulin resistance in obese mice. *Diabetologia* **57**, 2393-2404 (2014).
<https://doi.org/10.1007/s00125-014-3350-5>
- 165 Liu, W. *et al.* Macrophage Raptor Deficiency-Induced Lysosome Dysfunction Exacerbates Nonalcoholic Steatohepatitis. *Cell Mol Gastroenterol Hepatol* **7**, 211-231 (2019).
<https://doi.org/10.1016/j.jcmgh.2018.09.011>
- 166 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012). <https://doi.org/10.1038/nmeth.2019>
- 167 Bankhead, P. *et al.* QuPath: Open source software for digital pathology image analysis. *Sci Rep* **7**, 16878 (2017). <https://doi.org/10.1038/s41598-017-17204-5>
- 168 Itoh, M. *et al.* Hepatic crown-like structure: a unique histological feature in non-alcoholic steatohepatitis in mice and humans. *PLoS One* **8**, e82163 (2013).
<https://doi.org/10.1371/journal.pone.0082163>

- 169 Ioannou, G. N., Haigh, W. G., Thorning, D. & Savard, C. Hepatic cholesterol crystals and crown-like structures distinguish NASH from simple steatosis. *J Lipid Res* **54**, 1326-1334 (2013). <https://doi.org:10.1194/jlr.M034876>
- 170 Vuppalanchi, R., Nouredin, M., Alkhouri, N. & Sanyal, A. J. Therapeutic pipeline in nonalcoholic steatohepatitis. *Nat Rev Gastro Hepat* **18**, 373-392 (2021). <https://doi.org:10.1038/s41575-020-00408-y>
- 171 Shi, J., Hua, L., Harmer, D., Li, P. & Ren, G. Cre Driver Mice Targeting Macrophages. *Methods Mol Biol* **1784**, 263-275 (2018). https://doi.org:10.1007/978-1-4939-7837-3_24
- 172 Zheng, H. *et al.* Targeting tumor-associated macrophages in hepatocellular carcinoma: biology, strategy, and immunotherapy. *Cell Death Discov* **9**, 65 (2023). <https://doi.org:10.1038/s41420-023-01356-7>
- 173 Lamming, D. W. *et al.* Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* **335**, 1638-1643 (2012). <https://doi.org:10.1126/science.1215135>
- 174 Gosis, B. S. *et al.* Inhibition of nonalcoholic fatty liver disease in mice by selective inhibition of mTORC1. *Science* **376**, eabf8271 (2022). <https://doi.org:10.1126/science.abf8271>
- 175 Venturi, C. *et al.* Relevance of activated hepatic stellate cells in predicting the development of pediatric liver allograft fibrosis. *Liver Transpl* **22**, 822-829 (2016). <https://doi.org:10.1002/lt.24412>
- 176 Varma, S. *et al.* The histological quantification of alpha-smooth muscle actin predicts future graft fibrosis in pediatric liver transplant recipients. *Pediatr Transplant* **21** (2017). <https://doi.org:10.1111/petr.12834>
- 177 Akpolat, N., Yahsi, S., Godekmerdan, A., Yalniz, M. & Demirbag, K. The value of alpha-SMA in the evaluation of hepatic fibrosis severity in hepatitis B infection and cirrhosis development: a histopathological and immunohistochemical study. *Histopathology* **47**, 276-280 (2005). <https://doi.org:10.1111/j.1365-2559.2005.02226.x>
- 178 Komatsu, Y., Waku, T., Iwasaki, N., Ono, W., Yamaguchi, C. & Yanagisawa, J. Global analysis of DNA methylation in early-stage liver fibrosis. *BMC Med Genomics* **5**, 5 (2012). <https://doi.org:10.1186/1755-8794-5-5>
- 179 Patsenker, E. *et al.* Potent antifibrotic activity of mTOR inhibitors sirolimus and everolimus but not of cyclosporine A and tacrolimus in experimental liver fibrosis. *J Hepatol* **55**, 388-398 (2011). <https://doi.org:10.1016/j.jhep.2010.10.044>

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Next is of course my daily supervisor, and mentor (in many topics, not just science), Dr. Suchira Gallage, the time has come! I can't believe the time went by (I'm lying of course I can believe it, we all felt it go by like water, sometimes boiling water, but we like to pretend time doesn't move (cannot put emojis here, but please use your imagination and sprinkle some along the text). –Anyway, back to the many thanks section–. I thought of making a list of things to thank you for but it would be too long so I'll keep it short. First and foremost, thank you for your scientific teachings, I truly have absorbed a lot from you! Special remarks to the dramatic teachings of course, now I preach the gospel of getting plasmid maps and knowing your tools, the gospel of "n", that indicates that the n must be big as fortune allows. The gospel of come let's do something quickly (gesturing hand for urgency) and it taking 6 hours longer than anticipated because the gospel of coffee and a side challenge got on the way. The gospel of let's correct this manuscript/grant/report/letter/etc. but let us discuss investments first. We went through so much together in the last years, both personally and professionally that it's hard to recount the many things I have learned and gotten from you. Nevertheless, thank you for it all! thank you truly!

Of course, to the 1, 2, 3 team; for this exercise let's pretend I'm 3, but we know clear as day that I'm sometimes 2. Occasionally. Sometimes 3. Let us go one by one porfa. So, the author wishes to thank Dr. Mirian FV. for her assistance and critical feedback over the last 4.5 years of PhD. Many thanks!. You thought that was it! ay hija pues que te puedo decir, muchas gracias por todo hija, sin ti este evento no habría terminado bien sinceramente. Probablemente habría terminado antes too be fair, pero it is what it is. Acentos? Nobody knows them. Many thanks for all your motivation hija, we somehow took each other, unaware of life as we were, and pushed and supported each other along the way. I think we have both learned so much from each other, the good, the bad, the criminal, el chicken teriyaki, etc. But most of all, the laughs. You put it more eloquently in your many thanks section, but I wish

everyone has a Mirian in their life because it is, 100% good for health -restrictions apply if she moves to the 4th floor again, porfa. Thank you for making sure to give racatanga cuando comia como el perro, por controlar los detallitos de la atencion al detalle y todo el trabajo de consultoria de los ultimos años. Muchas vivencias hija, a tu edad se entiende, pero a la mía? Not really. Hicimos de todo, cosas personales, cosas profesionales (colores, inyecciones, racatangas, feedback, quien te crees, salidas, yellow bins, comidas, asuntos legales e internacionales, memes, etc., todo eso, lloveit). Tenemos que continuar over here and over there. Gracias por que te importaban las cosas como la inscripcion y la hoja azul. Y por darme el racatanga necesario para que organizara esos asuntos. Siempre que se requiera te consigo el wiswi y le ponemos azucar al gusto. Hija muy bueno el tiempo contigo, lo demas fatal pero las risas a tope nunca faltaron en esa sala de estar. Hija porfavor toma agua como lo indica el creador. Eat antes de hacer ejercicio, comida solida, las comidas de neonato no cuentan porfa. Gracias!

Svenja S. hija! ay, shall we continue in Spanish C12? I know you can but I actually cannot. Svenja Svenja Svenja, many thanks to you too for the last years, with bonus points for the last few months, first when we became orphans in the living room, and then with your continuous remote burden support. Let it be known that many expats come to Germany and find it hard to make German friends (allegedly because they already have their groups since school, but I suspect it has something to do with the DB haha), you took us both la otra hija and I and warmly welcomed us in your life. Now you cannot get rid of us (Y bin and all that). Maybe you didn't have a choice pero it is what it is and we win some, we lose some. Naja let us get serious. Many thanks for your patience, your non-racatanga approaches that balance the 1, 2, 3 team, of course special thanks for the "hi – ya" comments, I live for those, I can in fact feel p53 being upregulated each time. Bonus points for helping keeping me social and well behaved, the uuuuy moments when we spilled the beans, for personally financing my tea addiction last winter because you were the only responsible adult in the row bringing water and tea -tumtum- (Mirian will likely say, lack of foundation/hearsay here haha), thank you for giving me the push I needed in the last months and for reminding me how to do basic things of life without having to google them, (post stamps have no business being so complicated). And lastly, many thanks for being there to deliver and execute the burden of the documents, you know the ones, I owe you a tesla for that, but let us negotiate it. Lastly, many thanks for being a good friend! The PhD times would have been worse without you! I'm happy we 3 found each other there. Please book your loss of p53 sessions over here ASAP and kindly bring number 1. Always 2 or 3, occasionally 1, sometimes 2.

Tania AR., tanto que invertimos hija, pero tambien a ti muchas gracias por todo tu apoyo y feedback los ultimos años. Todos los momentos de decompression que son siempre tan necesarios. Los momentos de musica, de pushear, de figurar la truth y de risas tambien. Muchas gracias hija! Hija ya se acabó! Lo que sigue y adelante. Dr. Enrico F., Enriquño, many thanks to you too for all the moments of fun and decompression with the good, the bad and the ugly variety of coffee we enjoyed and suffered along the years. Thank you as well for your feedback and fun moments in the living room. Let us keep in touch, I'll organize a decent coffee. Whether I will find it, nobody knows. Mr. Ing. Vikas, Vikas, Vikas, many thanks to you too for your kind feedback, organizing the laughs and encouraging us to relax. Dr. Jakob J., JJ, many thanks to you too for putting up with us in the lab!, Many thanks for being our role model of commitment and hard work even when it was all S. We shall fake it till we make it. Tabea B., Mrs. B&B, many thanks to you for all your protocol optimizations and carefully considered questions that had me reconsidering all my SOPs. Your strong commitment came through even after I witnessed you being let go so many times. Many thanks for all the good times and the laughs! Phil H., not safe at all, we still do not know what your status is to be fair. Please give us an update. In any case, many thanks to you too for all the laughs, the kind feedback on advanced computer analysis. Dr. Tobias R., Many thanks to you too for your kind feedback during and after the lab reports! A true scientist if I ever saw one. Dr. Xin L. Chaofan F. and Peng S., you guys are also my reference of hard work! Many thanks to you too for all the scientific input and all the laughs. I keep educating people about fuxie everywhere I go. Charles K. many thanks to you too for being a role model of professionalism above all. Many thanks for all the laughs as well with bonus points at Tania's birthday with the color lights. Aikaterini K. many thanks for all the laughs too and for kindly assisting me with transport whenever possible. It made staying in the lab too late or on the weekend a bit easier to be fair. Please go out and check the lights; thank you! Dr. Pierluigi R. many thanks for all your scientific input, you were always making the right questions at the right moment, it keeps one motivated.

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Many thanks of course to my family, to the ones on the planet and the ones no longer on it. For believing me when I said this would be for the best. For supporting me loudly and in silence. For being there. For all.

Thanks to myself as well, for enduring the good and the bad. For keeping open to the good and learning how to arrest the bad. For keeping cool when tested. For keeping up. For evolving. For it all.

Sincerely,

Jose Efren Barragan Avila.