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Positive Allosteric Modulation of the $GABA_A$ Receptor exhibits Hepatoprotective Potential as a First-in-Class Treatment Approach for NAFLD

Dissertation

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Summary

Non-alcoholic fatty liver disease (NAFLD) has become one of the most common liver diseases worldwide, with a global prevalence of approximately 25% in the general population. NAFLD encompasses a broad continuum with different pathological entities and is recognised as the hepatic manifestation of the metabolic syndrome. The underlying pathogenesis is a multifactorial interplay of metabolic alterations, including lipid accumulation and insulin resistance as primary drivers. Our research on hepatic energy metabolism demonstrated that mitochondrial functionality can subsequently adapt to metabolic challenges by increased respiration up to an advanced stage of NAFLD, accompanied by oxidative and endoplasmic reticulum (ER) stress. In the following, molecular pathways of inflammation, apoptosis, and fibrogenesis are activated and promote pathogenic progression.

Considering the strong association between obesity and the pathogenesis of NAFLD, dietary interventions and weight loss play a key role in the prevention, especially in early childhood. Accordingly, the present work investigated the efficacy of a special dietary intervention, mimicking human milk, fed to mice in early life. We demonstrated that the so-called Concept diet with its large, phospholipid-coated lipid droplets improved hepatic mitochondrial respiration, oxidative capacity, and lipid metabolism acutely and under an obesogenic environment later in the life. Although early-life nutrition can have a long-lasting impact on adult metabolic health as a preventive attempt, dietary treatment approaches might not be sufficient for NAFLD patients in most cases.

Due to the complexity of NAFLD, a multitarget approach of combined pharmaceuticals may be required. However, not even one specific pharmacological therapy for NAFLD has been approved yet. Therefore, we additionally explored an innovative pharmacological strategy with the focus on the gamma-aminobutyric acid (GABA)ergic system as therapeutic target. The thioacrylamide molecule HK4 was designed to act as a positive allosteric modulator of the $GABA_A$ receptor, which was confirmed by patch clamping, Ca^{2+} influx measurements, and assays with non-competitive antagonist $GABA_A$ channel blockers. Our research highlighted that HK4 effectively prevented palmitic acid-induced ER stress, inflammation, DNA fragmentation, and cell death, particularly apoptosis. The beneficial effects of HK4 appear to be mediated by modifications of the transcription factors NF-κB and STAT3. Comprehensive 3'-mRNA sequencing analyses revealed a lipotoxicity-induced transcriptional dysregulation by palmitic acid and a restoration of the initial gene expression pattern by HK4. Several gene clusters, comprising oxidative phosphorylation, mitochondrial dysregulation, protein ubiquitination, apoptosis, and cell cycle regulation, were primarily affected by HK4 in a protective manner. Underlying mechanisms of the GABAergic signalling and key upstream regulators orchestrating metabolic stress responses could be placed in the overall context of the mode of action.

Taken together, our findings support a better understanding of the pathophysiological mechanisms during NAFLD progression and the benefit of pharmacological interference of the $GABA_A$ receptor. While dietary interventions and weight loss remain the cornerstone of NAFLD treatment, additional pharmacological approaches are urgently needed. We highlighted hepatoprotective effects of positive allosteric modulators of the $GABA_A$ receptor, implicating a great therapeutic potential as a first-in-class treatment concept for NAFLD.

Zusammenfassung

Die nichtalkoholische Fettlebererkrankung (NAFLE) zählt mit einer Prävalenz von 25 % zu einer der häufigsten Lebererkrankungen weltweit. Die NAFLE umfasst ein breites Kontinuum an pathologischen Entitäten und wird als hepatische Manifestation des metabolischen Syndroms betrachtet. Die zugrundeliegende Pathogenese ist ein multifaktorielles Zusammenspiel verschiedener Stoffwechselveränderungen mit der Lipidakkumulation und der Insulinresistenz als Haupttreiber. Unsere Forschung zum Energiestoffwechsel der Leber konnte zeigen, dass sich die mitochondriale Funktionalität bis zu einem fortgeschrittenen Stadium der NAFLE an die metabolischen Herausforderungen anpassen kann, einhergehend mit zellulärem Stress. In Folge werden Signalwege der Inflammation, Apoptose und Fibrogenese induziert, die das Fortschreiten der Erkrankung fördern.

Aufgrund des engen Zusammenhangs zwischen Adipositas und der Pathogenese von NAFLE spielen Ernährungsmaßnahmen, insbesondere im Kindesalter, eine Schlüsselrolle bei der Prävention. Daher wurde in der vorliegenden Arbeit die Wirkung einer speziellen Muttermilch-imitierenden Diät an jungen Mäusen untersucht. Wir konnten zeigen, dass die großen, phospholipidbeschichteten Lipidtröpfchen in der so genannten Concept-Diät die hepatische mitochondriale Atmung, die oxidative Kapazität und den Lipidstoffwechsel akut und unter adipogenen Einflüssen im späteren Leben verbessert. Obwohl die frühkindliche Ernährung einen langanhaltenden, präventiven Einfluss auf die metabolische Gesundheit im Erwachsenenalter haben kann, reichen diätetische Behandlungsansätze für NAFLE-Patienten meist nicht aus.

Auf Grund der Komplexität von NAFLE, ist möglicherweise ein vielfältiger Ansatz mit kombinierten Arzneimitteln erforderlich. Bisher wurde jedoch noch keine spezifische pharmakologische Therapie zugelassen. Daher haben wir uns zusätzlich auf eine innovative pharmakologische Strategie konzentriert, die das gamma-Aminobuttersäure (GABA)erge System adressiert. Das Thioacrylamid-Molekül HK4 wurde als positiver allosterischer Modulator des $GABA_A$-Rezeptors konzipiert, bestätigt durch Patch-Clamp und Ca^{2+} Einstrom-Messungen sowie Analysen mit nicht-kompetitiven antagonistischen $GABA_A$-Rezeptor-Blockern. Unsere Forschungsergebnisse zeigten, dass HK4 den durch Palmitinsäure-induzierten Stress des endoplasmatischen Retikulums, Inflammation, DNA-Fragmentierung und Zelltod, insbesondere Apoptose, effektiv verhinderte. Diese positiven Wirkungen werden potentiell durch Modifikationen der Transkriptionsfaktoren NF-κB und STAT3 vermittelt. Umfassende 3'-mRNA-Sequenzierungs-Analysen bestätigten Lipotoxizität-induzierte Dysregulationen der Transkription und die Wiederherstellung des ursprünglichen Genexpressionsmusters durch HK4. HK4 beeinflusste insbesondere Gencluster der oxidativen Phosphorylierung, mitochondrialen Dysregulation, Ubiquitinierung von Proteinen, Apoptose und Regulierung vom Zellzyklus. Die zugrundeliegenden Mechanismen der GABAergen Signaltransduktion und übergeordneten Regulatoren, die insbesondere metabolische Stressreaktionen steuern, konnten in den Kontext der Wirkungsweise eingeordnet werden.

Insgesamt tragen unsere Ergebnisse zu einem besseren Verständnis der pathophysiologischen Mechanismen bei der Progression der NAFLE und der pharmakologisch nützlichen Rolle der Modulation des $GABA_A$-Rezeptors bei. Während diätetische Maßnahmen und eine Gewichtsreduktion für die Behandlung von NAFLE grundlegend bleiben, sind zusätzliche pharmakologische Ansätze dringend erforderlich. Wir haben hepatoprotektive Effekte von positiven allosterischen Modulatoren des $GABA_A$-Rezeptors hervorgehoben, die ein großes therapeutisches Potenzial als first-in-class Behandlungskonzept für NAFLE darstellen.

List of Abbreviations

ALT	Alanine aminotransferase
ASK1	Apoptosis signal–regulating kinase 1
AST	Aspartate aminotransferase
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
BMI	Body mass index
CB	Cannabinoid receptor
CCL	C-C motif chemokine ligand
CCl_4	Carbon tetrachloride
CCR	C-C motif chemokine receptor
CHOP	CCAAT/enhancer-binding protein homologous protein
CK-18	Cytokeratin-18
CNS	Central nervous system
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DEG	Differentially expressed gene
DNL	*De novo* lipogenesis
ECM	Extracellular matrix
EMA	European Medicines Agency
ER	Endoplasmic reticulum
FA	Fatty acid
FDA	U.S. Food and Drug Administration
FXR	Farnesoid x receptor
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HFD	High-fat diet
HSC	Hepatic stellate cell
HSP	Heat shock protein
IL	Interleukin
IRE1α	Inositol-requiring enzyme 1 alpha
JNK	C-Jun N-terminal kinase
LDL	Low-density lipoprotein
MAFLD	Metabolic dysfunction-associated fatty liver disease

MPC	Mitochondrial pyruvate carrier
MUFA	Monounsaturated fatty acid
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAM	Negative allosteric modulator
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NEFA	Nonesterified fatty acid
NF-κB	Nuclear factor kappa B
OCA	Obeticholic acid
OXPHOS	Oxidative phosphorylation
PA	Palmitic acid
PAM	Positive allosteric modulator
PARP	Poly (adenosine diphosphate-ribose) polymerase
PDI	Protein disulfide isomerase
PERK	Eukaryotic translation initiation factor 2 alpha kinase 3
PI	Propidium iodide
PKCε	Protein kinase c epsilon
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SAF	Steatosis activity fibrosis
SCD1	Stearoyl-CoA-desaturase 1
SREBP-1c	Sterol regulatory element binding-protein-1c
STAT	Signal transducers and activators of transcription
STZ	Streptozotocin
SYVN1	Synoviolin 1
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TBPS	Tert-butylbicyclophosphorothionate
TCA	Tricarboxylic acid cycle
TGFβ	Transforming growth factor beta
THR	Thyroid hormone receptor
TNFα	Tumour necrosis factor alpha
TP53	Tumour suppressor 53
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling
UPR	Unfolded protein response
VLDL	Very-low-density lipoprotein
WSD	Western-style diet

List of Figures

Table of contents

1. Introduction

1.1. Definition of NAFLD

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease characterised by excessive fat deposition with the presence of steatosis in more than 5% of hepatocytes according to histological analysis [1]. For the diagnosis of NAFLD, an absence of secondary causes, such as drug-induced or hepatitis C virus-associated fatty liver or excessive alcohol consumption (≥30 g per day for men and ≥20 g for women), is required. The metabolic liver disease refers to a broad spectrum of liver damages, including steatosis, non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. While the non-alcoholic fatty liver (NAFL) is hallmarked by simple steatosis, NASH is characterised by additional hepatocyte injury, including inflammation and ballooning with or without fibrosis (Figure 1) [2].

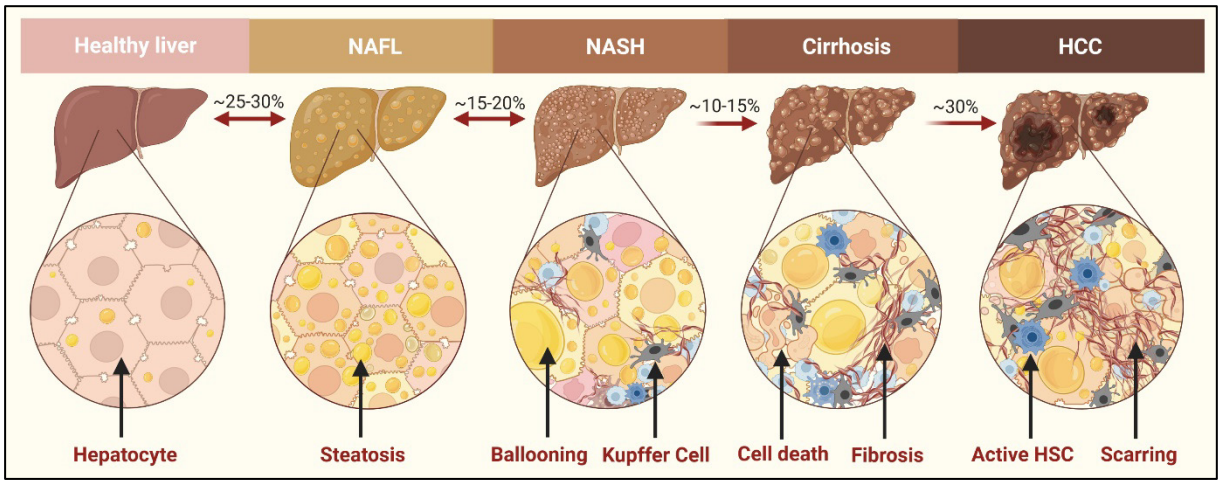

Fig. 1 | Disease spectrum of non-alcoholic fatty liver disease. The main feature of non-alcoholic fatty liver (NAFL) is hepatocellular steatosis. Non-alcoholic steatohepatitis (NASH) is distinguished from simple steatosis by additional hepatocellular ballooning and inflammation induced by cytokine- and chemokine-secreting Kupffer cells, in the presence or absence of fibrosis. Overproduction of fibrotic matrix proteins by hepatic stellate cells (HSCs) triggers the progress towards cirrhosis and hepatocellular carcinoma (HCC). Created by author with Biorender.com.

NAFLD is recognised as the hepatic manifestation of the metabolic syndrome, a systemic disorder of energy homeostasis often accompanied by visceral adiposity [3,4]. The presence of the metabolic syndrome carries an increased risk for cardiovascular diseases and type 2 diabetes mellitus (T2DM) [5]. Strong evidence verifies the association of NAFLD with metabolic risk factors or components of the metabolic syndrome, such as high waist circumference (≥94/≥80 cm for European men/women), high arterial pressure (≥130/85 mmHg or treated for hypertension), high fasting glucose levels (≥100 mg/dl or treated for T2DM), high serum triacylglycerol (TAG) levels (>150 mg/dl), or low levels of high-density lipoprotein (HDL) cholesterol (<40/50 mg/dl for men/women) [1]. To highlight the contribution of metabolic dysfunctions to NAFLD progression, the current nomenclature has been recently reassessed [6]. The concept of 'metabolic dysfunction-associated steatotic liver disease'

(MASLD) has been proposed as a new definition that has the advantage of being distinct from alcohol-related steatotic liver, the combined etiology, or cryptogenic steatotic liver disease. In addition to a better identification of patients, it avoids stigmatisation [6]. However, the impact of a MAFLD diagnosis (rather than NAFLD) on the progression of liver-related and extra-hepatic complications associated with NAFLD needs to be further analysed [7].

Although presence of the metabolic syndrome remains the strongest predictor, additional risk factors such as genetic predispositions and environmental factors are associated with the development and progression of NAFLD [8,9]. In fact, NAFLD has a heritable component estimated to be between 35% and 61%, as assessed in twin studies [10]. Especially single nucleotide polymorphisms of the patatin-like phospholipase domain-containing 3 or transmembrane 6 superfamily member 2 are linked with the severity of steatosis and fibrosis and favour susceptibility to liver damages [8,11]. Environmental factors associated with NAFLD include intrauterine nutrient supply, early infant feeding, dietary habitats, activity level, and socioeconomic and sociodemographic characteristics. Therefore, high consumption of cholesterol, fructose, and saturated fatty acids (FAs) instead of a diet rich in polyunsaturated fatty acids (PUFAs), fibres, and antioxidants promotes the development of NASH by modulating TAG metabolism, insulin sensitivity, and oxidative damage [12]. Unhealthy diet-related dysbiosis of the intestinal microbiota also represents a powerful trigger for the pathogenic progression of NAFLD [13,14]. Studies focusing on physical activity, have documented extremely low exercise levels and a sedentary lifestyle in patients with NAFLD compared with healthy individuals [15]. In addition, socioeconomic and sociodemographic factors also display an impact on the disease severity. However, acculturation, education, health-care use, and income may not directly correlate with NAFLD, as shown in a multicentre trial [16]. These data point to a region-specific interaction between modifiable and non-modifiable environmental factors and genetic predisposition in NAFLD development, while the exact contribution of each component remains unclear [15].

1.2. Epidemiology of NAFLD

The prevalence of NAFLD mirrors the global burden of the metabolic syndrome, while the number alarmingly continues to rise [15,17]. Currently, the prevalence of NAFLD affects around 25% of the population worldwide, although a recent meta-analysis even estimated a worldwide prevalence of 32.4% [18,19]. NAFLD is most common in the Middle East (31.79%) and South America (30.45%), followed by Asia (27.37%), North America (24.13%), and Europe (23.71%), while the lowest prevalence was reported in Africa (13.48%), mainly influenced by aforementioned environmental and genetic risk factors [20,21]. Regarding the different pathological entities in the NAFLD continuum, the risk of progressing from simple NAFL to more severe NASH fluctuates around 15-20% (Figure 1) [22]. The overall prevalence of NASH in the general population is currently estimated between 1.5% and 6.5% [20]. NASH has significant fibrogenic potential. Notably, 10-15% of NASH cases usually progress to cirrhosis. Conversely, a regression in 25% of NASH cases in general has been observed as a consequence of lifestyle-induced weight loss [21,23]. Around 30% of patients with NASH-related cirrhosis will develop HCC in their lifespan, predicting a 2% risk per year [24,25].

As mentioned, individuals with NAFLD have a high frequency of various metabolic comorbidities. Among NAFLD patients, 51% are heavily obese, 23% suffer from T2DM, 69% have dyslipidaemia, 39% have arterial hypertension, and 43% are concerned with metabolic syndrome [20]. Observations from a different perspective predict that among individuals with T2DM, more than 55% show NAFLD-associated conditions [26,27]. Based on the global diabetes prevalence (10.5%) in 20-79-year-old people in 2021, around 5.8% (~300 million people) of the population worldwide suffer from both T2DM and NAFLD [27,28]. T2DM seems to accelerate the progression of NAFL to NASH with advanced fibrosis or even to cirrhosis [7]. In contrast, data on NAFLD in type 1 diabetes mellitus (T1DM) are far less comprehensive. A study by Targher et al. including 250 individuals with T1DM, showed a prevalence of NAFLD in 44%, assessed by ultrasound examination [29]. However, from 30% up to 50% of diagnosed NAFLD among individuals with T1DM have also been reported [30–32]. Accordingly, NAFLD occurs not only in overweight people but also in non-obese or lean individuals [33]. This newly identified growing subset of 'lean NAFLD' has been initially described in Asian populations [15]. Using region-specific body mass index (BMI) thresholds, the prevalence of lean NAFLD has been reported to be 7% in the United States, 12% in Greece, and 20% in India [34–36]. Compared to overweight or obese NAFLD patients, lean NAFLD patients are usually younger and have fewer obesity-related comorbidities, especially a lower prevalence of the metabolic syndrome (2–48% vs 22-64%) [15,37]. Independent of the BMI or the subset of NAFLD, the disease is increasingly diagnosed in children and adolescents, with a prevalence of 8% in the general population and 34% in clinics for paediatric obesity [38–40]. Due to the worldwide rise in the incidence of metabolic risk factors, the prevalence of NAFLD will continuously increase. From a future perspective of the disease, the number of NAFLD patients in the United States is projected to grow by around 20% to 100 million in 2030 [41]. The number of patients with NASH, in particular, is predicted to increase by more than 60% from 17 to 27 million in the next decade in the United States and thus becoming the leading cause of liver cirrhosis, transplantation for HCC, and end-stage liver disease. Even more dramatic is the predicted 168%-increase in decompensated cirrhosis onsets and the 137%-increase in HCC cases by 2030 [41]. Since NAFLD patients have an increased risk for liver-related death and cardiovascular complications compared to sex- and age-matched individuals without NAFLD, the mortality rate is rising in parallel [42].

NAFLD not only implies a tremendous personal clinical burden but also contributes to a significant economic burden [21]. In several European countries, a budget of up to 1163 € is estimated as the annual (direct) medical cost per patient with NAFLD, while in the United States, a yearly cost of $1613 per patient and thus $103 billion in total has been calculated [41,43]. Since the economic costs of NASH mainly result from progressed stages of the disease, earlier diagnosis and care of NASH patients could massively improve the wellbeing and reduce future healthcare costs [44]. Moreover, the actual prevalence of NAFLD and associated costs might be underestimated since it is not feasible to conduct liver biopsies in studies of the general population owing to cost, ethical, and practical considerations [44]. Therefore, appropriate diagnostics are essential.

1.3. Diagnosis of NAFLD

NAFLD remains clinically silent until the advanced stages. Thus, clinicians should have a high index of suspicion to diagnose the disease, especially for people at high risk [45]. In general, routine screening is not recommended by current guidelines [1,46]. A liver biopsy with subsequent histopathological evaluation is ranked as the gold standard with the benefit of evaluating precisely the amount and localisation of steatosis, inflammation, ballooning, or fibrosis [47,48]. Although there is variability in the histological evaluation among pathologists, a liver biopsy is the only procedure reliably differentiating NAFL from NASH [1,49]. The latter requires a joint presence of steatosis, ballooning, and lobular inflammation. However, the diagnosis is not dependent on a single histological feature but rather involves assessing multiple features in constellation [1,50]. The most common histological scoring systems for diagnosis are the NAFLD activity score (NAS) and the Steatosis Activity Fibrosis (SAF) score. The NAS, developed by the NASH Clinical Research Network, comprises histological features, which are evaluated semi-quantitatively like steatosis (0–3), lobular inflammation (0–3), hepatocellular ballooning (0–2), and separately fibrosis (0–4) or features, which are recorded as present-or-absent like Mallory's hyaline [50]. Compared to the NAS, the SAF score from the European Fatty Liver Inhibition of Progression Consortium includes fibrosis as part of the score and differentiates more accurate steatosis from necroinflammation, features with a distinct pathogenesis [51].

On the other hand, liver biopsies are invasive, might lead to severe complications, and cause high costs. Therefore, suitable mice models are of high interest for research purposes and non-invasive tests are being increasingly in focus to improve the diagnosis and prognosis across the NAFLD spectrum in humans. Non-invasive tests combine blood-based tests, methods assessing the physical property of the liver tissue, and imaging methods:

(I) Different blood tests can help to identify liver damage, although the classification is sometimes ambiguous. Therefore, it is not recommended to use a single biomarker for NAFLD identification [52]. A common approach to assess steatosis by circulating biomarkers is mainly based on the concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), apolipoprotein A1, bilirubin, cholesterol, haptoglobin, ferritin, γ-glutamyl transferase, and TAG in combination with other parameters such as BMI, waist circumference, fasting glucose, or serum insulin [52]. For the evaluation of apoptosis and inflammation in a progressed NAFLD stage with blood-based tests the concentration of C-X-C motif chemokine ligand 10, C-reactive protein, cytokeratin-18 (CK-18), interleukin (IL)-6, IL-1, and tumour necrosis factor alpha (TNFα) are mainly assessed. To distinguish between a fibrotic from a non-fibrotic liver, additional biomarkers such as hyaluronic acid, laminin, propeptides of collagen type II and III, metalloproteinases, and related inhibitors are intended to detect extracellular matrix (ECM) turnover or fibrogenic phenotypes [52,53].

(II) Magnetic resonance elastography and ultrasound-based methods, like transient elastography, allow an evaluation of the physical property of the level, such as stiffness and fibrosis, liver attenuation, or the viscosity degree of the tissue. Sonographic signs of NAFLD include bright hepatic echoes, increased hepatorenal echogenicity, and vascular blurring of the portal or hepatic veins.

(III) Imaging techniques can assess the liver anatomy and might help to identify signs of inflammation, swelling, or scarring. Among them, ultrasonography, computed tomography, magnetic resonance imaging, or spectroscopy methods are used to reliably diagnose steatosis or – to a certain degree – also fibrosis.
Other approaches focusing on genetic risk factors, omics- or gut microbiota-based markers need to be further developed to rise as promising methods for evaluating NAFLD [52].

1.4. Molecular Mechanisms of the NAFLD Pathophysiology

NAFLD, considered as the hepatic manifestation of the metabolic syndrome, is a complex multi-factorial disease. Its underlying pathogenesis is thought to be a multiple-hit process of lipid accumulation, insulin resistance, oxidative stress, and inflammation in combination with systemic and environmental triggers, genetic susceptibility, and microbiome-related factors. In brief, the primary drivers for the development of NAFL are based on the imbalance between food consumption and energy expenditure, leading to the expansion of depots in adipose tissue and ectopic lipid accumulation in the liver (steatosis) [54]. Excessive lipid availability leads to the formation of lipotoxic species and promotes insulin resistance, playing a pivotal role in the pathogenic switch of fatty liver [55,56]. To compensate for lipid oversupply, mitochondrial β-oxidation is upregulated in hepatocytes. However, loss of mitochondrial adaption results in reactive oxygen species (ROS) formation and progression to NASH [56,57]. Upcoming prolonged cellular stress is an initiator of inflammatory response and cell death [58]. Inflammatory signals from tissue-resident macrophages, the Kupffer cells, are implicated in the activation of hepatic stellate cells (HSCs) and the progression of fibrogenesis [54] (Figure 2).

1.4.1. Hepatic Lipid Accumulation

A healthy liver can adapt to the metabolic tasks of energy storage and supply. Therefore, the concentration of hepatocellular free FAs is balanced between delivery, lipid synthesis, storage in lipid droplets, oxidation, as well as export of TAGs as VLDLs [59,60]. However, this equilibrium is disrupted in people with fatty liver since the absolute amount of FA supply is strongly increased [9]. Donnelly et al. quantified sources of hepatic and plasma TAG supply in NAFLD and determined that 59% of the TAG amount arose from non-esterified fatty acids (NEFAs) originated from adipose tissue, 26% from *de novo* lipogenesis (DNL), and 15% directly from dietary intake [59].
Adipose tissue can store a limited amount of lipids. When the capacity of subcutaneous adipose tissue to expand through hyperplasia is exceeded, excess NEFAs are released into the bloodstream [61]. The insufficient storage ability of subcutaneous adipose tissue and subsequent lipid spillover cause lipid deposition in visceral adipose tissue and other lean tissues not designed for lipid accumulation [61]. Therefore, obesity-related adipose dysfunction and impaired insulin-mediated suppression of lipolysis result in the development of ectopic lipid deposition, specifically in the liver [62,63]. Thus, the major proportion of hepatocellular TAGs in NAFLD patients derives from circulating NEFAs of adipose tissue

through both passive diffusion and active transport. Not surprisingly, related transporters are reported to be upregulated in obesity [9,64].

The second major source of hepatocellular TAG supply during NAFLD is DNL, a process converting excess carbohydrates (such as glucose or fructose) into FAs before they are esterificated to TAG (Figure 2). Conversion of long-chain saturated FAs and the intermediate diacylglycerol (DAG) into TAG limits their lipotoxic property [9]. The enzymes responsible for DNL can be stimulated by glucose via carbohydrate response element-binding protein or by insulin via sterol regulatory element binding-protein-1c (SREBP-1c) [56,65]. Thus, liver-specific overexpression of SREBP-1c in mice results in a constant activity of DNL, associated with hepatic insulin resistance and adiposity [66]. In line, humans with NAFLD show a threefold higher DNL rate than healthy individuals [67]. A remarkable increase in sugar consumption has been recognised in obese individuals with NAFLD, as well as an evidence-based association of hepatic fat accumulation and development of NASH with the consumption of sugar-sweetened beverages [68,69].

The third source of hepatocellular TAGs in NAFLD derives from dietary FAs, which enter the liver through the uptake of intestinally derived chylomicron remnants (Figure 2) or through spillover into the plasma NEFA pool [59]. In contrast to the FA supply by chylomicron remnants, VLDLs can export lipids from the liver to peripheral tissues. However, this process is impaired in individuals with NASH [56,70].

Although lipid accumulation is described as a hallmark of NAFLD, it is considered an adaptive response. However, when the metabolic plasticity of the hepatocyte is exhausted, meaning that the liver's capacity to handle FAs and other energy substrates is overwhelmed, it leads to an accumulation of toxic lipid species [71–73]. Among them, saturated FAs, DAG, ceramides, lysophosphatidylcholine species, and cholesterol are known to provoke lipotoxicity or insulin resistance in hepatocytes [74].

1.4.2. Hepatic Insulin Resistance

Insulin regulates substantial hepatic glucose and lipid metabolism via direct and indirect mechanisms [63]. Activation of hepatic insulin receptors through portal insulin activates DNL and glycogen synthesis and downregulates gluconeogenic enzymes. At the same time, peripheral insulin promotes glucose uptake in skeletal muscle and inhibits lipolysis in adipose tissue [63]. Thus, disruption of insulin signalling exhibits severe alterations of glucose and lipid homeostasis, suggesting that hepatic insulin resistance is a central event in the development of NAFLD and associated systemic pathological metabolic conditions [63,75]. Moreover, as one of the strongest risk factors, T2DM seems to accelerate the progression of NAFL to NASH [7]. Thus, mouse models of obesity, T2DM and NASH all showed increased whole body insulin resistance. However, differences in hepatic insulin expression were recognised among the groups. Only in NASH, hepatic insulin resistance has been confirmed on molecular level by decreased phosphorylation of hepatic Akt^{T308} and Akt^{S473} [76].

Considering the tight association between ectopic lipid deposition and hepatic insulin resistance, the lipid-mediated crosstalk between adipose tissue and the liver profoundly affects insulin sensitivity [77]. Accumulation of the lipotoxic mediator DAG is hypothesised

as the root cause of hepatic insulin resistance, although multiple models for the development of hepatic insulin resistance in NAFLD have been proposed [78,79]. DAG prompts impairment of the hepatic insulin signalling cascade by activating protein kinase C epsilon (PKCε) (Figure 2) [80,81]. Translocation of activated PKCε to the cell membrane reduces phosphorylation of insulin receptor substrate 2 and impairs Akt2 signalling. The main consequences are reduced hepatic glycogen synthesis and insufficient suppression of hepatic endogenous glucose production due to gluconeogenic protein expression [81]. In parallel, peripheral skeletal muscle insulin resistance due to intramyocellular ectopic lipid deposition reduces muscle glycogen synthesis and redirects carbohydrates to the liver [63].

Interestingly, selective hepatic insulin resistance in mouse models of T2DM results in a more severe metabolic defect than total insulin resistance demonstrated in liver-specific insulin receptor knockout mice. In a selective hepatic insulin resistance state, insulin fails to suppress gluconeogenesis but continues to activate lipogenesis in the liver. Thus, it exhibits a combination of hyperinsulinemia, hyperglycaemia, and hypertriglyceridemia in the liver, enhancing the pathogenic switch from fatty liver more rapidly than total hepatic insulin resistance [82,83]. However, *in vivo* studies provide evidence that hepatic lipogenesis is increased mainly by excessive lipid supply from peripheral tissue and that its conversion to TAG occurs in an insulin-independent manner rather than by hepatic insulin directly [84,85].

Evidence underlines the strong association between intracellular fat accumulation, hepatic insulin resistance, and the development of NAFLD. However, insulin sensitivity is not per se negatively influenced by a high amount of fat but rather depends on the type of FAs taken up with the diet and circulating in the plasma. The most frequent dietary and plasma FAs are palmitic acid (PA) (C16:0, a saturated FA) and oleic acid (C18:1, a monounsaturated fatty acid [MUFAs]). They represent approximately 28% and 31% of total plasma NEFAs, respectively. Interestingly, in contrast to PA, oleic acid induces beneficial effects on insulin sensitivity [86,87].

1.4.3. Mitochondrial Dysfunction in the Liver

Hepatic energy metabolism is orchestrated in the mitochondria by substrate conversion via β-oxidation, tricarboxylic acid cycle (TCA), and adenosine triphosphate (ATP) synthesis through oxidative phosphorylation (OXPHOS). In addition, mitochondria are the primary source of ROS formation, which is tightly regulated by antioxidant defence mechanisms under healthy conditions [88,89]. Mitochondria can respond quickly to metabolic alterations and maintain the balance of intrahepatic lipid metabolism to a certain extent [90]. In fact, upregulation of mitochondrial respiration as a compensatory (protective) mechanism has been reported in obese insulin-resistant humans with or without NAFL compared to healthy individuals [57,91]. In line, mouse models of obesity and NASH favours an enhanced hepatic mitochondrial function, as reflected by elevated β-oxidation. Whereas mouse models of diabetes adapt to metabolic challenges by both β-oxidation and TCA flux in our study [76]. The type of lipids not only has an impact on insulin sensitivity but also plays a role in mitochondrial functionality, as shown in a mouse model with early-life feeding of large, phospholipid-coated lipid droplets [14].

However, prolonged augmented respiration might be bioenergetically inefficient. This hypothesis is supported by signs of mitochondrial uncoupling and leaking respiration, accumulated oxidative stress, and decreased OXPHOS complex expression, challenging the hepatocellular antioxidant defence mechanism [57]. As a result, a vicious circle during NASH progression arises, consisting of increased lipid flux into the mitochondria, disrupted fat homeostasis, accelerated insulin resistance, mitochondrial damage, and high production of ROS (Figure 2) [92,93]. Koliaki and Roden illustrated several changes in the hepatic energy metabolism in individuals with NAFL and NASH regarding their oxidative phosphorylation capacity, β-oxidation, respiration rates and electron transport chain complex activities: While the maximal respiration rate of isolated mitochondria was up to five-fold higher in obese humans with or without NAFL compared to lean individuals, people with NASH featured up to 40% lower maximal respiration, associated with greater hepatic insulin resistance, mitochondrial uncoupling, leaking activity, and oxidative stress [57,89]. However, contrasting findings have been shown in a mouse model for lean NASH in early course with increased β-oxidation-derived mitochondrial respiration from high-resolution respirometry [76]. Furthermore, reduced activity of respiratory chain complexes besides elevated TNFα levels has been described as a sign of systemic inflammation in humans with NASH [94]. Reduced stability of oxidative phosphorylation subunits has also been demonstrated in a diet-induced mouse model of NAFLD [95]. Of note, elevated ROS production was associated with increased antioxidant capacity only in NAFL since mechanisms to cope with excess ROS generation may be insufficient in NASH [57,64]. Accordingly, during the progression from NAFL to NASH, the so-called 'hepatic mitochondrial flexibility' is subsequently lost.

1.4.4. Endoplasmic Reticulum Stress in the Liver

Hyperlipidaemia and mitochondrial dysfunction-associated ROS production can induce endoplasmic reticulum (ER) stress and provoke the accumulation of unfolded or misfolded proteins in the ER lumen of hepatocytes [96]. The subsequent activated unfolded protein response (UPR) aims to restore ER homeostasis by activating different signalling pathways. As an adaptive mechanism to cope with ER stress, the UPR mediates the deceleration of protein synthesis and degradation of misfolded proteins and switches on the expression of inflammation-related genes and chaperones [96,97]. Besides the protein homeostasis regulation, ER stress can also disrupt hepatic lipid metabolism by upregulating SREBP-1c-mediated lipogenesis, reducing VLDL assembly and promoting insulin resistance in the liver and adipose tissue through the folding of ER membrane-localised intermediators [96].

Monitoring UPR in eukaryotic cells reveals an initiation by three primary transmembrane sensors, namely inositol-requiring enzyme 1 alpha (IRE1α), eukaryotic translation initiation factor 2 alpha kinase 3 (PERK), and activating transcription factor 6 (ATF6), intersecting with a variety of stress signalling and inflammatory systems [97]. All three arms of the UPR are distinctly altered in mouse models of NASH and related comorbidities and associated with hepatic mitochondrial respiration [76]. Regulation of the different sensors might be mediated by dedicated protein disulfide isomerase (PDI) members, catalysing oxidative protein folding beside their role as chaperones (Figure 2) [98,99]. Thus, PDI is a promising biomarker for

dysregulated liver metabolism, underlined by a strong protein downregulation in hepatocytes during acute lipotoxicity-induced injury and a switch towards a prominent upregulation in HCC liver tissue of individuals with a more severe stage of NAFLD [100,101].

In response to mild ER stress, the UPR allows cells to survive by regulating protein homeostasis and avoiding the luminal accumulation of misfolded proteins via ubiquitin proteasome-mediated protein degradation. Proteasomal ubiquitination plays a pivotal role in the progression of NAFLD. However, the proteasome activity is remarkably reduced in both dietary and genetic mouse models of obesity, which contributes to ER stress response and the development of hepatic insulin resistance [102]. An impaired proteasomal degradation is also remarked in the differential expression of E3 ubiquitin-protein ligase synoviolin 1 (*SYVN1*) in the presence of lipotoxic stimuli [103]. The upstream regulator SYVN1 can ameliorate hepatic steatosis, enhance insulin sensitivity in db/db mice, influence cell cycle regulation, DNA repair, and apoptosis, and regulate the ubiquitination process through tumour suppressor 53 (TP53) [103–105].

In response to chronic ER stress, the UPR is limited to orchestrating the recovery of homeostasis [106]. Like mitochondrial flexibility, the adaptive UPR is overwhelmed by a certain extent of prolonged ER stress in the progression of NASH [96]. ATP deficiency due to an impaired mitochondrial respiration rate contributes to the inhibition of energy-dependent ubiquitin-proteasome degradation, accumulating misfolded proteins and exacerbating ER stress [95]. Failure to preserve ER function contributes to ROS overproduction, inflammation, and apoptosis (Figure 2) [97,107].

1.4.5. Liver Inflammation

Inflammation is an adaptive response triggered by infections or tissue injury, which results in the secretion of inflammatory mediators essential for tissue repair and cell defence mechanisms [108]. In the absence of pathogens, a so-called sterile inflammation defines inflammatory responses triggered by a variety of endogenous molecules. Those triggers originate from extrahepatic tissues, such as the gut or adipose tissue, or directly from intrahepatic tissue [109]. Increased visceral adipose tissue secretes chemokines, cytokines, and a disturbed profile of pro-inflammatory adipokines (low adiponectin, high leptin and TNFα levels) to induce liver inflammation and hepatic insulin resistance [109]. Intrahepatic triggers arise from lipotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress, innate immune responses, and cell death pathways [109]. During NAFLD progression, a variety of damage-associated molecular patterns (DAMPs), including ATP, free RNA and DNA, heat shock protein (HSP), hepatokines, and extracellular vesicles, among others, are released by damaged hepatocytes [110]. Appropriate wound healing fails under persistent inflammatory activity, linking metabolic stress and subsequent hepatocyte death with stimulation of fibrogenesis [74,111]. Therefore, it is not surprising that inflammation contributes to the development of NASH [109].

In response to chronic ER stress, the aforementioned transmembrane sensors can activate c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NF-κB) signalling, which control the transcriptional activation of pro-inflammatory and pro-apoptotic pathways (Figure 2) [96].

Studies have implicated that JNK activation mediates steatohepatitis, apoptosis, as well as insulin resistance in dietary and genetic mouse models of obesity through phosphorylation of insulin receptor substrate 1 [112,113]. Furthermore, genetic evidence demonstrates a causal link between increased JNK activity and T2DM in humans [112,114]. Thus, not only hepatic lipid accumulation but also inflammatory events are commonly associated with hepatic insulin resistance [56]. Interestingly, JNK activation can, in turn, affect nuclear factor kappa B (NF-κB) signalling and vice versa [115]. Since the NF-κB-JNK crosstalk is crucial for transmitting pro-inflammatory signalling, a vicious cycle of liver injury, cellular stress, and inflammation exists between JNK-dependent hepatocyte death and NF-κB-induced inflammation [96,115]. Persistent hepatocyte-specific phosphorylation or activation of the transcription factor NF-κB has been shown in HepG2 cells upon lipotoxic stimulus as well as in mouse models of NAFLD and humans with NASH [101,116,117]. Accordingly, unresolved ER stress in PA-treated Huh7 cells triggers apoptosis by affecting the pro-apoptotic transcription factor CCAAT/enhancer-binding protein homologous protein (CHOP), which upregulates pro-apoptotic Bax or Bad and potentially suppresses anti-apoptotic B-cell lymphoma-2 (Bcl-2) expression (Figure 2) [118].

In response to DAMP-mediated hepatocyte injury, activated Kupffer cells release chemokines and cytokines, including IL-1β, IL-6, C-C motif chemokine ligand (CCL) 2, and TNFα via Toll-like receptor response [109]. In turn, this release leads to the recruitment of other inflammatory immune cells. The expression of cytokines and their receptor subunits are elevated in humans suffering from NASH with severe fibrosis and in hepatocytes with PA-induced lipotoxicity [103,119,120].

1.4.6. Hepatocellular Apoptosis

Hepatocellular death is one of the key triggers of NAFLD progression, characterised by subsequent development of inflammation, fibrosis, cirrhosis, and HCC [107]. Increased levels of ALT and AST are useful indicators of hepatocyte cell death, suggesting monitoring patients with liver damage [107]. Different modes of cell death (apoptosis, autophagy, necroptosis, necrosis, and pyroptosis) are activated during NAFLD progression and might interact or have redundant roles in triggering specific cell death responses through distinct mechanisms [121]. Apoptosis is the primary driver of hepatocyte cell death in NASH [122,123]. This highly organised process is characterised by cellular rounding and shrinking, phosphatidylserine exposure on the cellular surface, a dense cytoplasm, nuclear condensation and fragmentation, and formation of cytoplasmic blebs and apoptotic bodies [121,124,125]. During NAFLD, hepatocytes can undergo apoptotic cell death via an extrinsic or intrinsic pathway, whereas molecules of one pathway might influence the other [126,127]. Lipid overload, DNA damage, ER stress, or hypoxia can trigger the intrinsic apoptotic pathway. In detail, underlying JNK activation engages apoptosis by phosphorylation of the anti-apoptotic protein Bcl-2 or by activation of pro-apoptotic Bax, which causes mitochondrial outer membrane permeability as a point of no return (Figure 2) [107,128]. Membrane perturbation leads to the release of cytochrome c, followed by activation of initiator caspase 9 and executioner caspase 3/7. Similar to the intrinsic apoptotic pathway, the TNF- and Fas-receptor-mediated extrinsic signalling

pathway is also increased in the liver of individuals with NASH [72]. Downstream of the receptor activation, the initiator caspase 8 is activated in an autocatalytic way. Finally, caspase 3/7 is activated, which is considered an irreversible step towards apoptotic cell death [124]. Notably, the intrinsic and extrinsic signalling pathways converge on the same target, initiated by the cleavage of executioner caspase 3/7 (Figure 2) [124].

Apoptosis can be confirmed in PA-treated hepatocytes with the terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) method, a common procedure to identify DNA strand breaks *in situ* [101,129]. Poly-adenosine diphosphate-ribose polymerase (PARP)-1 also plays a central role in damaged DNA by catalysing the polymerisation of adenosine diphosphate-ribose units to label single-strand breaks (Figure 2). Both active and cleaved PARP-1 can arise as a marker for apoptosis [130]. In line, PA-treated hepatocytes as well as livers from individuals with NASH display activated PARP-1 levels [101,131]. Furthermore, liver cells under lipotoxic-induced injury or in NAFLD patients also display impaired expression of signal transducers and activators of transcription (STAT) 5, which regulates cell cycle, proliferation and survival, among others [103,132].

Evidence suggests that in progressed NASH stage, there is a shift from apoptotic cell death towards necroptotic signalling, an intermediate form between apoptosis and necrosis. In clinical studies, necroptosis is underlined by characteristic upregulation of receptor-interacting serine/threonine kinase 3 expression in the livers of individuals with NASH [124,133]. Morphologically, necroptosis is characterised by a translucent cytoplasm, irregular chromatin condensation into small patches, organelle swelling, increased cell volume, and plasma membrane disruption [134]. In contrast, necrosis is a more rupturing and uncontrolled autolytic loss of cellular integrity, distinguishable by cell swelling, disruption of cell membranes and cytoplasmic changes [135].

1.4.7. Hepatic Fibrosis

Liver fibrosis is characterised by the excessive accumulation of ECM proteins as a result of hepatic wound healing in response to repeated liver injury [136]. The intimate crosstalk between injured hepatocytes, chemokine- and cytokine-releasing Kupffer cells, and HSCs triggers matrix protein deposition [137]. As a central event of fibrogenesis, quiescent HSCs transdifferentiate into proliferative, fibrogenic myofibroblasts in response to intra- and extracellular signals [138]. Extracellular stimuli comprise signals of injured hepatocytes and Kupffer cells, secreting a variety of the aforementioned DAMPs, DNA fragments, and ROS, as well as IL-1β, IL-6, CCL2, and TNFα (Figure 2) [74,139].

Once myofibroblasts are transformed, HSCs are the main producer of ECM proteins, as discovered in a genetic model with liver injury [140]. The ECM includes mainly collagens (type I, II, IV), fibronectin, elastin, laminin, hyaluronan, and proteoglycans and is accumulated around six times more in NASH than under normal conditions [136]. In line, liver alpha smooth muscle actin protein content and total liver hydroxyproline, as well as liver collagen content from sirius red staining, and liver fibrosis score from histopathological analysis were significantly higher in NASH mouse model, as assessed in our study [76]. ECM continuously accumulates due to impaired degradation and inhibited ECM-removing matrix

metalloproteinases [141]. As a consequence of ECM overproduction, fibrous scars are formed that distort the liver's structure and cause hepatocellular dysfunction, liver failure, increased resistance to blood flow, and elevated pressure in the portal vein [136].

Fibrosis markers and treatment approaches are of high research interest since the liver fibrosis stage predicts the long-term prognosis of NASH, as examined in a 33-year follow-up study [42]. Thus, mortality essentially dependents on the fibrosis stage at the initial diagnosis and increases exponentially [42,142]. Interestingly, the depletion of Kupffer cells has been shown to ameliorate inflammation and HSC activation in a fibrosis mouse model [143]. Additionally, neutralisation of transforming growth factor beta (TGFβ), a potent NASH-specific fibrogenic trigger, might also ameliorate pre-existing hepatic fibrosis in rats [144].

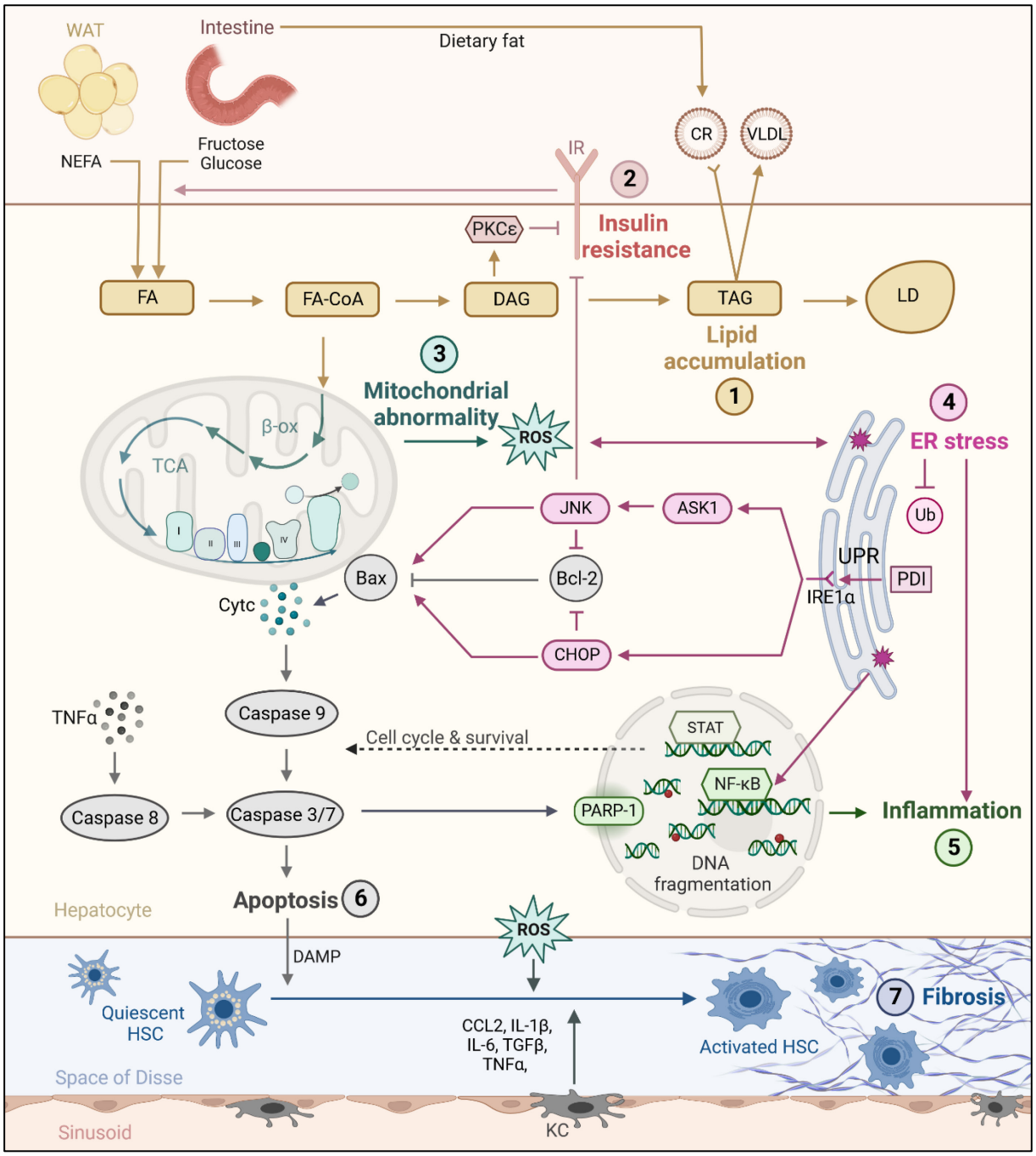

Fig. 2 | Molecular pathogenic mechanisms during NAFLD progression. (1) Central to the pathogenesis is the vast availability of fatty acids (FAs) from non-esterified fatty acids (NEFAs) of white adipose tissue (WAT) and from *de novo* lipogenesis of dietary glucose or fructose, which is driven by

sterol regulatory element binding-protein-1c (SREBP-1c). Dietary fat can also enrich the hepatic triacylglycerol (TAG) pool through the uptake of an intestinal derived chylomicron remnant (CR). If the very-low-density lipoprotein (VLDL) export is limited, TAG accumulates in lipid droplets (LDs). (2) Conversion of FAs to TAG via lipotoxic mediator diacylglycerol (DAG) might substantiate insulin resistance by promoting translocation of protein kinase C epsilon (PKCε) and phosphorylation of the insulin receptor (IR) tyrosine kinase. (3) Elevated lipid availability induces transient mitochondrial adaption with elevated β-oxidation (β-ox), tricarboxylic acid cycle (TCA), and adenosine triphosphate synthesis. Prolonged adaption to the FA overload initiates mitochondrial dysfunction and excessive reactive oxygen species (ROS) production when hepatic mitochondrial flexibility is exhausted. (4) Overwhelming endoplasmic reticulum (ER) stress impairs proteasomal ubiquitination (Ub), promoting the accumulation of misfolded proteins in the ER lumen and disruption of the unfolded protein response (UPR). Failure to preserve ER function contributes to insulin resistance via c-Jun N-terminal kinase (JNK), inflammation, and apoptosis via CCAAT/enhancer-binding protein homologous protein (CHOP). (5) Subsequently, nuclear factor kappa B (NF-κB) is activated and transcribes pro-inflammatory genes, while family members of signal transducers and activators of transcription (STAT) might regulate the cell cycle, proliferation, and survival. (6) Bax-induced cytochrome c (Cytc) release triggers an intrinsic apoptosis cascade, while external stimuli from tumour necrosis factor alpha (TNFα) also enhance caspase 3/7 activation. Upon caspase cleavage, activated poly-adenosine diphosphate-ribose polymerase-1 (PARP-1) arises as a marker for DNA fragmentation. (7) As a response to hepatocyte injury, a damage-associated molecular pattern (DAMP) is released into the extracellular space. Subsequently, activated Kupffer cells (KCs) synthesize inflammatory cytokines and chemokines. In turn, activated hepatic stellate cells (HSCs) promote fibrogenesis. ASK1: Apoptosis signal–regulating kinase 1; Bcl-2: B-cell lymphoma-2; CCL2: C-C motif chemokine ligand 2; CoA: Coenzyme A; IL: Interleukin; IRE1α: Inositol-requiring enzyme 1 alpha; PDI: Protein disulfide isomerase; TGFβ: Transforming growth factor-β. Created by author with Biorender.com.

1.5. Treatment of NAFLD

Although several drugs have been evaluated in clinical trials, no specific pharmacological therapy has been approved for the treatment of NAFLD. The absence of accurate and inexpensive imaging tools, the lack of adequate non-invasive biomarkers and an incomplete understanding of the multi-systemic disease contribute to the limited efficacy of current clinical management, which primarily combats the consequences of metabolic risk factors [145]. Besides ongoing drug development, weight loss and lifestyle changes remain the cornerstone of NASH treatment since genetic predispositions and several environmental factors are not modifiable yet [46].

1.5.1. Dietary Intervention

Regarding the close association between NAFLD and obesity, lifestyle intervention-mediated weight loss is key for preventing and treating NAFLD [1,46]. Lifestyle modifications based on decreased energy intake and/or increased physical activity improve biochemical and histological hepatic parameters in obese individuals with NASH [146]. Evidence from a histologically controlled trial with 293 individuals with NASH suggests that modest weight loss (>7%) is needed to achieve resolution of biopsy-proven NASH, while significant weight

loss (≥10%) is required to trigger fibrosis regression or beneficial effects in the presence of unfavourable risk factors [23]. Greater weight loss might improve histologic parameters even more [23,146]. Surprisingly, a lifestyle intervention-induced weight loss of 3–10% in individuals with lean NAFLD also leads to the resolution of hepatic steatosis [147].

Metabolic functions can be affected not just by the amount of dietary lipids but also by the type of lipids [14]. Thus, saturated FA-enriched diets may promote inflammation, insulin resistance, and cardiovascular risk. At the same time, a higher intake of unsaturated FAs in the form of MUFAs or especially PUFAs has been reported to exert beneficial metabolic effects [148–150]. Furthermore, certain dietary interventions like the Mediterranean diet might reduce the degree of steatosis and show beneficial effects in NAFLD even without body weight reduction [150].

In addition to these direct dietary effects, early postnatal feeding in mice considerably affects adult body composition and metabolic health across their lifespan [151]. A high saturated FA intake during pregnancy contributes to insulin resistance, impaired hepatic mitochondrial metabolism, upregulated lipogenesis, oxidative stress, and inflammatory pathways in adult offspring, especially when newborns have been exposed to a HFD postnatally [150,152,153]. On the other side, a healthy diet in postnatal life can even attenuate the metabolic impact of an adverse maternal diet in mouse offspring. In particular, a PUFA-rich diet in postnatal life protects against Western-style diet (WSD)-induced adiposity complications in rodents by reducing the number of hypertrophic adipocytes, reducing fat accumulation, and causing a healthier plasma lipid profile and plasma glucose homeostasis [151]. Thus, patterns of dietary lipids during pre- and postnatal life can program offspring metabolic health. In turn, susceptibility to NAFLD in childhood and accelerating progression to NASH is affected [154,155]. Oosting et al. highlighted the contribution of the physical properties of dietary lipids to this nutritional programming [156]. Thus, a so-called Concept diet has been newly designed with large, phospholipid-coated lipid droplets, mimicking human milk lipid composition [157]. Similar to other animal feeds and infant milk formula, the Concept diet contains several types of FAs, carbohydrates like sugars or polysaccharides, cholesterol, and proteins mixed with fibres, vitamins, and minerals [14]. Several studies reported a beneficial effect of early life feeding with the Concept diet and its large lipid droplet coated with milk fat globule membrane fragments on metabolic deterioration due to improved regulation of mitochondrial oxidative capacity and function in murine adipose and liver tissue in later life [158,159].

1.5.2. Pharmacological Treatment

Since NAFLD is a complex multi-factorial disease with individual variability, a multitarget approach of combined pharmaceuticals may be required [17]. Additionally, precision medicine based on the exact pathophysiological classification in the NAFLD continuum might maximise the therapeutic benefits and minimise adverse effects [160]. Accordingly, current research approaches focus on various compound classes with different modes of action (Figure 3) to reduce steatosis, insulin resistance, inflammation, and cell death in parallel to NASH-related progression, secondary risk factors, and overall mortality [161].

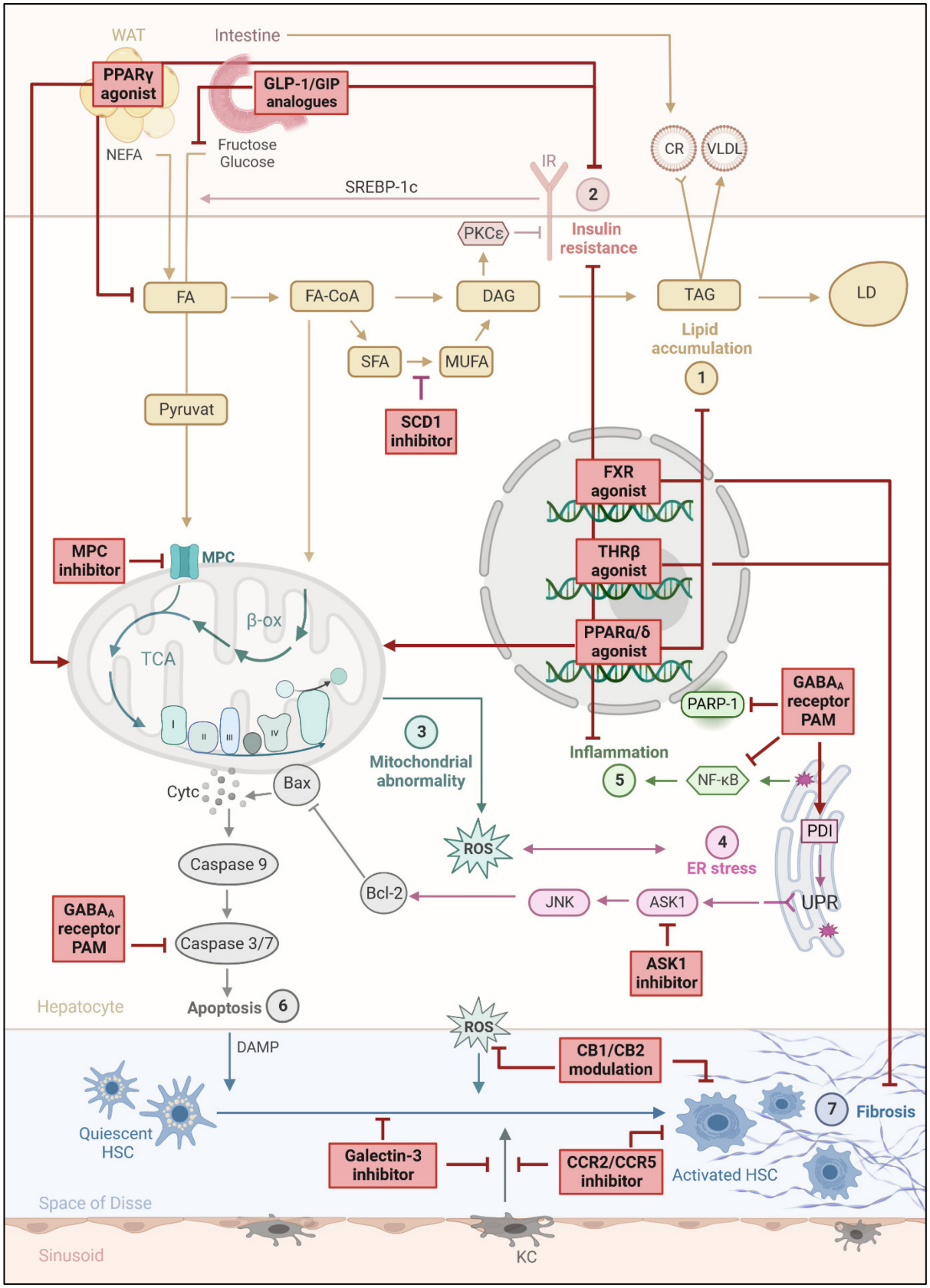

Fig. 3 | Potential pharmacological targets to treat non-alcoholic fatty liver disease. Several pharmacological agents, including farnesoid x receptor (FXR) agonist, stearoyl-Coenzyme A-desaturase 1 (SCD1)-inhibitor, peroxisome proliferator-activated receptor (PPAR) γ or α/δ agonist, and thyroid hormone receptor beta (THRβ) affect lipid metabolism and fatty acid (FA) β-oxidation (β-ox) to augment accumulation of FAs. Furthermore, the mitochondrial pyruvate carrier (MPC) inhibitor, designed as a second-generation insulin sensitiser, reduces pyruvate flow into the tricarboxylic acid

(TCA) cycle and, thus, might alter the ability of hepatic mitochondria to fuel *de novo* lipogenesis. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) receptor agonists enhance insulin sensitivity and reduce appetite, thereby supporting body weight loss and avoiding hepatic fat accumulation. Apoptosis signal–regulating kinase 1 (ASK1) inhibitor might prevent further progression of apoptosis, inflammation, and fibrosis signalling. Using an antagonist against the galectin-3 protein found in immune cells is predicted to inhibit fibrogenesis. A dual antagonist of C-C motif chemokine receptor 2 and 5 (CCR2/CCR5) is expected to reduce the recruitment and infiltration of pro-inflammatory immune cells and, on top, inhibit the proliferation of collagen-producing hepatic stellate cells (HSCs). Cannabinoid receptor (CB) 1 antagonists and CB2 agonists are able to attenuate hepatic oxidative stress parameters and fibrogenesis. An innovative positive allosteric modulator (PAM) of the gamma-aminobutyric acid $(GABA)_A$ receptor reduces apoptosis by preventing inflammation, DNA damage, and endoplasmic reticulum (ER) stress. To do so, $GABA_A$ receptor PAM targets protein disulfide isomerase (PDI), poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1), nuclear factor kappa B (NF-κB), and caspase 3/7. Bcl-2: B-cell lymphoma-2; CoA: Coenzyme A; CR: Chylomicron remnant; Cytc: Cytochrome c; DAG: Diacylglycerol; DAMP: damage-associated molecular pattern; IR: insulin receptor tyrosine kinase; JNK: C-Jun N-terminal kinase KC: Kupffer cell; LD: Lipid droplet; MUFA: Monounsaturated fatty acid; NEFA: Non-esterified fatty acid; PKCε: Protein kinase C epsilon; ROS: Reactive oxygen species; SFA: saturated fatty acid; SREBP-1c: Sterol regulatory element binding-protein 1c; TAG: Triacylglycerol; UPR: Unfolded protein response; VLDL: Very-low-density lipoprotein; WAT: White adipose tissue. Created by author with Biorender.com.

However, no drug has yet been approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA) for the specific treatment of NASH [162]. The FDA mandates the attainment of a single NASH endpoint (either improvement of at least one stage in fibrosis without worsening of NASH or improvement in NASH resolution without worsening of fibrosis), while the guidelines from the EMA necessitate efficacy in both these endpoints [163,164]. This could potentially postpone approval in the European market.
In the absence of an approved therapy for NAFLD, restricted use of the anti-diabetic drugs pioglitazone and vitamin E has been endorsed by the European and American Association for the Study of Liver Diseases for the treatment of biopsy-proven NASH in selected individuals [1,46]. Both pharmaceuticals show beneficial effects on hepatic steatosis, lobular inflammation, and ballooning score without resolution of fibrosis [165]. Furthermore, the peroxisome proliferator-activated receptor (PPAR)γ agonist pioglitazone improves insulin resistance mainly by stimulating adipocyte differentiation, increasing adiponectin secretion, and decreasing adipocyte FA release (Figure 3) [56,166]. However, adverse effects such as osteoporosis, bladder cancer, and heart failure, aside from increased body weight, might be associated with the intake of pioglitazone. At the same time, the antioxidant vitamin E might cause an increased risk of mortality, haemorrhagic stroke, and prostate cancer [167,168]. Therefore, off-label use should be limited, especially in NASH individuals without diabetes. Thus, research for new therapeutic options should be encouraged. Remarkably, some drug categories comprise medications that have diverse mechanisms of action, despite having a common therapeutic goal, while certain drugs of different categories show overlapping properties.

1.5.2.1. Pharmacological Therapies targeting Lipid Metabolism

Accumulated cytotoxic lipid species due to impaired lipid metabolism and excessive lipid availability are critical drivers of insulin resistance, inflammation, and subsequent fibrosis progression. Lipid-lowering approaches show an additional beneficial effect in reducing cardiovascular events. Farnesoid x receptor (FXR) agonists, stearoyl-CoA-desaturase 1 (SCD1)-inhibitors, thyroid hormone receptor (THR) β agonists, and PPARα/γ/δ agonists are currently under evaluation as they affect lipid metabolism (Figure 3).

FXR agonists

FXR-mediated gene expression is involved in lipid and glucose homeostasis, insulin signalling, bile acid production, and immune responses [169]. FXR activation mostly ameliorates hyperlipidaemia by lowering triglyceride content in the liver and reducing hepatic expression of transcription factor SREBP-1c as well as bile acid production (Figure 3) [170]. Thus, FXR signalling modulation beneficially reduces obesity and associated complications in mouse models [169]. The front-runner obeticholic acid (OCA) from Intercept Pharmaceuticals is a semi-synthetic bile acid with agonistic action at the nuclear FXR receptor and has already been approved for the treatment of primary biliary cholangitis. OCA improved insulin sensitivity, reduced inflammation associated with sustained improvement of non-invasive biomarkers (e.g. ALT, AST, γ-glutamyl transferase, Fibrosis-4 Score), and remarkably exerted anti-fibrotic effects in phase II- and phase III-studies including individuals with NAFLD [171,172]. However, OCA still failed to significantly improve characteristic features of NASH in the liver [173]. FDA's Gastrointestinal Drugs Advisory Committee recently voted against the accelerated approval of OCA for treatment of NASH with stage 2 or 3 fibrosis due to concerns about the drug's safety. In addition, some daunting side effects, such as elevated low-density lipoprotein (LDL) and decreased HDL cholesterol levels, as well as higher rates of pruritus, appear to be associated with the use of OCA [172].

SCD1 inhibitors

SCD1 is the key enzyme for the biosynthesis of MUFAs from saturated FAs, triggering lipid storage (Figure 3). Therefore its downregulation is associated with reduced hepatic lipogenesis, enhanced insulin sensitivity, and lipid oxidation [174]. Thus, liver-specific SCD1 knockout mice were protected from high carbohydrate diet-induced hepatic lipid accumulation [175]. Administration of the SCD1 inhibitor aramchol from Galmed Pharmaceuticals reduced liver fat content associated with metabolic improvements in individuals with NAFLD after three months [176]. The protective effect could have been verified in a phase IIb-trial, where aramchol promoted NASH resolution and also fibrosis stage reduction in a dose-response manner [177]. A phase III-study is currently running to evaluate the efficacy and safety of aramchol in individuals with NASH.

THRβ agonists

The transcription factor THRβ is highly expressed in hepatocytes and mainly regulates glucose and lipid metabolism. In detail, the activation of THRβ results in decreased cholesterol and

TAG levels, improvement of insulin sensitivity, promotion of liver regeneration, and reduction of apoptosis *in vivo* [178]. Resmetirom (MGL-3196) from Madrigal Pharmaceuticals reduced lipid content along with NASH resolution in patients with biopsy-proven NASH in a phase II-trial [178]. A phase III-study was examining the effect of resmetirom in individuals with NASH and fibrosis, showing an achievement in both primary endpoints (NASH resolution and fibrosis). FDA has accepted for review the New Drug Application for resmetirom for the treatment of adult patients with NASH with liver fibrosis. So far, resmetirom is the most promising drug fulfilling regulations also of the European market. Although the liver-specific THRβ isoform has the benefit of minimising THRα-associated side effects in the heart or bone, other adverse effects like diarrhoea and nausea have been determined in clinical studies [179].

PPARα/γ/δ agonists

PPARα signalling action results in increased β-oxidation, thus it reduces TAG levels in the liver, increases HDL levels, and ameliorates hepatic lipotoxicity, while PPARγ is mainly expressed in adipocytes, where it regulates their differentiation and lipid metabolism [180]. Furthermore, PPARγ improves insulin sensitivity due to increased adiponectin expression [181]. PPARδ activation specifically exerts anti-inflammatory effects and addresses β-oxidation (Figure 3). Elafibranor (GFT-505) is a dual PPARα/δ agonist of GENFIT, pivotal in lipid metabolism and innate immunity. In several phase IIa/IIb-studies, elafibranor improved metabolic features of NASH, including plasma lipids and glucose homeostasis, peripheral and hepatic insulin resistance, and reduced liver inflammation [182–184]. However, elafibranor recently failed to fulfil the predefined primary endpoints of NASH resolution without worsening fibrosis in a phase III-trial. Lanifibranor is a next-generation pan-PPAR agonist modulating key metabolic, inflammatory, and fibrogenic pathways in the pathogenesis of NASH with higher efficacy than single or dual PPAR agonists [185]. Currently, a phase III-study is being conducted to evaluate the effect of lanifibranor in NASH patients with liver fibrosis stage 2 or 3.

1.5.2.2. Pharmacological Therapies targeting Insulin Resistance

Insulin resistance is one of the main drivers of NAFLD and plays a pivotal role in the pathogenic switch from fatty liver. NAFLD exacerbates hepatic and peripheral insulin resistance, followed by the release of pro-inflammatory cytokines, promoting the development of T2DM [56]. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) receptor agonists, as well as second-generation insulin sensitisers are key therapies currently under examination.

GLP-1 and GIP analogues

The incretins GLP-1 and GIP are generated through the proteolytic processing of a glucagon precursor and released by the gastrointestinal tract mainly in response to glucose [186]. Incretins or GLP-1/GIP analogues primarily stimulate glucose-dependent insulin secretion besides enhancing insulin sensitivity, inhibiting hepatic glucose production, and lowering liver transaminases [187]. GLP-1 additionally slows gastric emptying and reduces appetite,

supporting body weight loss and avoiding hepatic fat accumulation (Figure 3) [186]. It has also been demonstrated that GLP-1 suppresses hepatic lipogenesis through cyclic adenosine monophosphate-activated protein kinase action and exerts anti-inflammatory effects in a normoglycemic rat model [188]. However, GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 in only a few minutes. This is why Novo Nordisk designed the incretin-mimetic liraglutide with a longer half-life. Liraglutide is an approved pharmacological treatment for T2DM, which showed additional improvement in NASH resolution and body weight in a phase II-study with NASH patients [187]. Unlike the required daily injection of liraglutide, the GLP-1 agonist semaglutide can be administered subcutaneously once a week. Notably, treatment with semaglutide resulted in a higher percentage of individuals with NASH resolution than the placebo treatment in a phase II-trial, although it missed fibrosis improvement [189]. Besides those monotherapies, a dual activation of GLP-1 and GIP can be achieved with tirzepatide from Eli Lilly. Tirazepatide improved NASH-related biomarkers and adiponectin levels in humans with T2DM and achieved a substantial and sustained reduction in body weight after long-term application in obese humans in phase II-studies [190]. However, these results await validation in larger phase III-trials.

MPC inhibitors

Compared to other glitazones, the second-generation insulin sensitiser MSDC-0602K from Cirius Therapeutics showed minimised side effects. MSDC-0602K primarily targets the mitochondrial pyruvate carrier (MPC) (Figure 3), besides a low affinity for PPARγ. The MPC is a prime element in controlling mitochondrial respiration in the liver. While the MPC is highly expressed in diet-induced obesity mouse models, its elimination is protective against HFD-induced liver damage [191,192]. Furthermore, inhibiting MPC reduces stellate cell activation, lipogenesis, and gluconeogenesis [193]. MSDC-0602K substantially attenuated fibrosis in mice fed a diet high in fructose, trans-fatty acids, and cholesterol [194]. Although histological evaluation did not show a reduction in the NAS, MSDC-0602K improved glucose metabolism and liver enzymes in a phase IIb-trial in individuals with NASH and fibrosis [195].

1.5.2.3. Pharmacological Therapies targeting Inflammation

Activated inflammatory cells and circulating cytokines contribute to hepatocyte injury and subsequently, to pathophysiological complications. Therefore, inflammation is considered a substantial feature in evaluating NAFLD. Anti-inflammatory approaches targeting the innate immune system via C-C motif chemokine receptor 2 and 5 (CCR2/CCR5) antagonists have been examined in clinical trials.

CCR2/CCR5 antagonists

The receptor CCR2 is located on macrophages, while CCR5 has been found on hepatic stellate cells of mouse models and in human NASH patients [196]. Upon CCR2 receptor activation (mainly through monocyte chemoattractant protein 1), activated Kupffer cells secrete pro-inflammatory cytokines and chemokines, attracting other inflammatory cells like monocytes. CCR5 receptor activation triggers hepatic stellate cells in producing fibrotic matrix. Thus, dual

inhibition of CCR2 and CCR5 is expected to reduce the recruitment, migration, and infiltration of pro-inflammatory immune cells and, on top, inhibit the proliferation of collagen-producing hepatic stellate cells (Figure 3) [196]. In a clinical IIb-trial with humans suffering from NASH, the CCR2/CCR5 antagonist cenicriviroc from Tobira Therapeutics reduced short-term fibrosis progression. However, after long-term exposure of two years, the mean fibrosis resolution was no longer significant among the treatment groups [74]. In line, a phase III-study with cenicrivirox was terminated early due to a lack of efficacy in improving fibrosis based on the results of the interim analysis.

1.5.2.4. Pharmacological Therapies targeting Apoptosis

Apoptosis is one key trigger of NAFLD progression since releasing DAMPs and apoptotic bodies stimulates fibrosis progression and worsens NASH prognosis. Inhibition of apoptosis showed promising effects by reducing hepatocyte cellular damage and ALT, AST, and CK-18 fragment levels [197]. Therefore, pharmaceuticals addressing apoptosis are increasingly being developed, although long-term treatment should be considered with caution. In fact, the application of caspase inhibitors (like GS-9450) or the apoptosis signal–regulating kinase 1 (ASK1) inhibitor selonsertib in clinical trials has been terminated due to drug-induced liver toxicity concerns or insufficient improvement.

ASK1 inhibitors

ASK1 is activated as a consequence of various stress signals such as ROS, TNFα, and pathogens from oxidative and ER stress. ASK1 belongs to the mitogen-activated protein kinase 3 family, triggering phosphorylation and activation of p38 and JNK. Subsequently, stress response pathways are stimulated that worsen hepatic inflammation, apoptosis, and fibrosis [198]. The selective ASK1 inhibition with the drug selonsertib from Gilead ameliorated metabolic parameters associated with NASH, as well as liver steatosis, inflammation, and fibrosis in a murine NASH model [199]. However, in a clinical trial, selonsertib only improved fibrosis in some individuals, which led to early termination of the phase III-study that included individuals with bridging (F3) fibrosis [200].

1.5.2.5. Pharmacological Therapies targeting Fibrosis

Fibrosis links liver injury with cirrhosis and predicts the long-term prognosis of NASH. Thus, it is the primary cause of liver-related death in patients with NAFLD [42]. Since advanced liver fibrosis is reversible in patients, researchers have been encouraged to develop anti-fibrotic drugs. However, targeting the TGFβ signalling pathway with broad biological functions induces non-desirable side effects that override therapeutic benefits [201]. Modulating the cannabinoid receptor (CB) 1 and 2 also represented successful strategies, although immense side effects led to withdrawal. So far, a galectin-3 antagonist is tested in phase IIb/III-trials.

CB1 antagonists and CB2 agonists

Further research might consider dual targeting of CB1 and CB2, which are expressed on the cellular surface of HSCs and hepatocytes from patients with NAFLD [202,203]. As described

in our previous review, pharmacological blockade of the CB1 receptors attenuates hepatic oxidative stress parameters and metabolic alterations, substantiating the therapeutic potential of CB1 receptor antagonists to prevent NAFLD pathogenesis [202]. Oppositely, CB2 knockout mice showed pronounced liver fibrogenesis when repeatedly exposed to carbon tetrachloride (CCl_4), suggesting a protective effect of CB2 agonistic modulation [204]. However, rimonabant and other CB1 inhibitors have driven depression and suicidal ideation when used for NAFLD-associated metabolic complications, although they have initially proven safe in several clinical trials. Consequently, this led to abrupt withdrawal. In a renaissance of the CBs, adapted CB1 receptor antagonists and CB2 receptor agonists without blood-brain barrier penetration might appear as therapeutic targets for NAFLD and metabolic-associated complications.

Galectin-3 antagonists

Galectin-3 is a β-galactoside-binding lectin that regulates cell proliferation and differentiation, angiogenesis, adhesion, and apoptosis [138]. Increased levels of galectin-3 enhance toxin-induced liver fibrosis in mice and is associated with NASH [205]. In contrast, galectin-3-deficient mice showed attenuated CCl_4-induced myofibroblast activation and matrix production compared to wild-type animals [138]. It has been proposed that TGFβ-stimulated HSC activation and procollagen deposition requires galectin-3, although its precise role is unknown [206]. Belapectin is a carbohydrate that inhibits the galectin-3 protein found in immune cells, thus inhibiting fibrogenesis (Figure 3) [161]. Galectin-3 inhibition by belapectin from Galectin Therapeutics ameliorated fibrosis and cirrhosis *in vivo* [207]. In a phase IIb-study, a one-year application of belapectin infusion showed no significant difference concerning primary endpoints of hepatic venous pressure gradient and fibrosis. However, a subgroup analysis of patients without oesophageal varices at inclusion yielded a significant reduction under the higher body weight-adjusted dosage versus placebo [205]. Thus, more research is needed to fully understand the effectiveness of galectin inhibitors in NAFLD treatment. In this context, a phase IIb/III-study has been enrolled to evaluate the efficacy and safety of belapectin in individuals with NASH cirrhosis.

1.5.2.6. Innovative Pharmacological Approach targeting $GABA_A$ Receptor

The gamma-aminobutyric acid (GABA) receptor and its inhibitory neurotransmitter ligand GABA are widely distributed in the central nervous system (CNS). In the last decades, the GABAergic system of peripheral tissues such as the pancreas or the liver also obtained increasing attention [208,209]. Minuk et al. demonstrated that the liver likely plays an important role in regulating its GABA concentration since the GABA uptake by hepatocytes is saturable and time-dependent, while an uptake by Kupffer cells or endothelial cells is denied [210]. Overall, a substantial GABA concentration of 250 nmol/g of liver tissue has been estimated in humans [211]. Remarkably, the glutamic acid decarboxylase (GAD), responsible for GABA synthesis, is not continuously active inside hepatocytes. Instead, GAD is rather upregulated during hepatic regeneration, development, or functional processing [211]. Thus, the GABA release concentration in lean mice is about 100 μmol/mg, while this concentration is nearly doubled (180 μmol/mg) in obese mice [212].

A protective effect of GABA in the liver has been broadly demonstrated in various studies with ethanol-induced hepatocyte cell death, D-galactosamine-, or TNFα-induced liver injury *in vitro* and *in vivo* [213–216]. GABA also protects against fluoride-induced alterations in liver function, potentially attributed to its antioxidative property [217]. Furthermore, the administration of GABA-coupled nanoparticles in partially hepatectomised rats improves hepatic regeneration and proliferation, while increased GABAergic activity in malignant hepatocytes causes cell growth inhibition [218,219]. In addition, GABA attenuates liver insulin resistance in T2DM mice and their offspring by regulating hepatic insulin signalling and gluconeogenesis pathways [220]. Furthermore, GABA improves insulin resistance in streptozotocin (STZ)-induced diabetic rats on HFD, probably through modulation of glucose transporter 4 and glucagon receptor gene expression [221]. In contrast, it has also been reported that GABA synthesis, catalysed by GABA transaminase, might result in hyperinsulinemia, insulin resistance, and hyperphagia in the liver of obese mice when acting on the parasympathetic nervous system [222]. In general, a growing body of evidence suggests a link between GABA and its protective effects in metabolic disease, most likely through the control of cell-cycle activation, proliferation, differentiation, and reduced chromosomal abnormalities.

GABA receptors comprise the ionotropic $GABA_A$ or $GABA_C$ and the metabotropic $GABA_B$ receptor [208]. Our research focuses on the $GABA_A$ receptor, a pentameric Cl^- channel with a diverse subunit composition ($\alpha_{[1-6]}$, $\beta_{[1-3]}$, $\gamma_{[1-3]}$, δ, ε, θ, and π), and its signalling [208]. Besides the binding sites for positive allosteric modulators (PAMs) (so-called benzodiazepine site) and for agonists/antagonists (orthosteric GABA site) in the extracellular domain, there is a third major binding site of the $GABA_A$ receptor in the transmembrane domain for negative allosteric modulators (NAMs) (picrotoxin site) [223]. Thus, $GABA_A$ receptor activation can be substantially regulated in the presence of PAMs by enhancing the affinity and/or the efficacy of $GABA_A$ receptor agonists. To avoid CNS side effects but take advantage of $GABA_A$ receptor-mediated protective effects, novel PAMs from thioacrylamide structures have been designed with extreme low blood–brain barrier penetration [224]. The protective effects of these thioacrylamide molecules have already been validated in β-cells [225,226]. Upon binding and activation of the $GABA_A$-receptor in pancreatic β-cells, insulin secretion is stimulated through depolarisation by Cl^- efflux and subsequent Ca^{2+}-dependent signalling [227]. Notably, GABAergic signalling plays a potential role in β-cell survival and α- to β-cell transdifferentiation, suggesting a promising strategy for the treatment of diabetes [226,228].

Although convincing data also suggest favourable cytoprotective effects of GABA signalling in the liver, its effect there has not yet been studied. Opposite to β-cells, $GABA_A$ receptor activation in hepatocytes most likely results in Cl^- influx and thereby hyperpolarises the cell membranes, although depolarisation has been suggested in dysfunctional liver metabolism [212,229–231]. Thus, it is of high research interest for us to analyse downstream signalling mechanisms and resulting hepatic effects of a thioacrylamide molecule and $GABA_A$ receptor PAM, the so-called HK4, on liver injury.

Taken together, NAFLD comprises complex pathophysiological mechanisms closely related to metabolic features of T2DM and obesity. Understanding the molecular alterations in the

disease progression using suitable animal models or well characterised patients is essential to identify predictive biomarkers and therapeutic targets. Since no specific pharmacological therapy has been approved to treat NAFLD, weight reduction and dietary intervention, especially in early life, are still key in preventing NAFLD. However, targeting the GABAergic system with positive allosteric modulation by HK4 may provide a promising therapeutic opportunity for NAFLD.

1.6. Aims of Thesis

NAFLD is recognised as the hepatic manifestation of the metabolic syndrome, causing a global public health burden. The severe and complex liver disease encompasses a broad continuum with different pathological entities, tightly associated with obesity and T2DM. Dysregulated lipid homeostasis and accumulated lipotoxic species, as well as insulin resistance are the main drivers triggering the progression from simple steatosis (NAFL) to the severe entity steatohepatitis (NASH) and ultimately HCC. Underlying pathophysiological mechanisms further affect mitochondrial dysfunction, inflammation, ER stress, and hepatocellular cell death.

- The first study of the thesis provides extensive knowledge on the development of the hepatometabolic features in NAFLD to understand disease progression, applied in the introduction. Conflicting results have been reported specifically for hepatic energy metabolism in humans with NAFLD and related comorbidities over time. Thus, as a first aim we investigated hepatic mitochondrial respiration by high-resolution respirometry among others in murine models of obesity, diabetes, or NASH mice during the early course of hepatometabolic diseases. By this, we also examined mechanisms linking hepatic mitochondrial adaption to metabolic alterations, comprising changes in lipid metabolism, insulin sensitivity, oxidative and ER stress, inflammation, and fibrosis besides disrupted mitochondrial biogenesis. Comprehensive research on the different features of hepatic energy metabolism enables us to consider specific treatment options for different phases of metabolic liver disease.

Considering the lack of approved pharmacological interventions and the tight association between obesity and NAFLD pathogenesis, diet composition and weight loss play a key role in treating NAFLD. Saturated FA-enriched diets and lipotoxic species promote inflammation, insulin resistance, and cardiovascular risk, while a healthy PUFA-rich diet in postnatal life can protect against obesity-related complications. Especially early-life feeding may have a long-lasting impact on metabolic health.

- Therefore, our second aim was to monitor the programming of metabolic health with a special early-life diet intervention to prevent later life WSD-induced hepatic lipid and energy dysregulation, an important step in the development of NAFLD. We hypothesised that postnatal exposure to the Concept diet mimicking human milk lipids would improve hepatic mitochondrial function and lipid metabolism, thereby protecting the mice from WSD-induced metabolic deterioration later in life. To resolve the underlying question, we focused on the effect on insulin resistance, TCA cycle, β-oxidation-related hepatic oxidative capacity using high-resolution respirometry, mitochondrial dynamics, lipotoxic mediators (DAG) in subcellular compartments, and systemic oxidative stress.

NAFLD is gaining considerable interest due to tremendous rising predicted numbers in the next decades and the lack of pharmacological interventions. Accordingly, a variety of compound classes with different modes of action are in research focus with the aim to improve lipotoxicity-induced insulin resistance, hepatocellular damage, and cell death, as well as associated risk factors and overall mortality. However, no specific pharmacological therapy has been approved yet for the treatment of NAFLD.

- For that reason, the third aim was to determine whether novel PAMs of the $GABA_A$ receptor are protective pharmacological tools against lipotoxic-induced injury. First, we concentrated on providing the specificity of HK4 as an allosteric modulator of the $GABA_A$ receptor. Then, we investigated the hepatocellular metabolic effects of HK4 *in vitro* and *in vivo*, specifically focusing on apoptosis, inflammation, and ER stress interactions to evaluate HK4s' potency as a first-in-class drug.

The beneficial effects of HK4 on ER stress, inflammation, DNA damage, and apoptotic cell death appeared to be mediated by changes in gene expression through the downregulation of NF-κB phosphorylation and STAT3 activation. Although the protective effects caused by HK4 were well examined, the underlying detailed molecular mechanisms remained puzzling, especially how the diverse pathways interacted.

- Therefore, the fourth aim of this thesis was to explore the underlying mechanisms of the protective effects of HK4 on PA-induced lipotoxicity in hepatocytes at the transcriptional level. This was conducted by comprehensive 3'-mRNA sequencing analyses of injured hepatocytes treated with HK4. With *in silico* analyses, we performed a functional characterisation of lipotoxicity-induced gene regulation and expanded nodal points of mitochondrial respiration, cell cycle, apoptosis, and ubiquitination. Furthermore, we investigated potential interactions within these networks and identified upstream regulators of the differentially expressed genes. Finally, we translated these novel targets in the context of NAFLD to other *in vitro* and *in vivo* settings by evaluating detected biomarkers.

Study 1

Alterations of hepatic energy metabolism in murine models of obesity, diabetes and fatty liver diseases

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eBioMedicine, 2023

Study 2

Dietary lipid droplet structure in postnatal life improves hepatic energy and lipid metabolism in a mouse model for postnatal programming

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Pharmacological research, 2022

Study 3

Positive allosteric γ-aminobutyric acid type A receptor modulation prevents lipotoxicity-induced injury in hepatocytes *in vitro*

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Diabetes, obesity & metabolism, 2022

Study 4

Positive allosteric $GABA_A$ receptor modulation counteracts lipotoxicity-induced gene expression changes in hepatocytes *in vitro*

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6. Discussion

6.1. Hepatometabolic Features of NAFLD Pathophysiology

NAFLD is tightly and mutually associated with obesity and T2DM, therefore metabolic features might overlap, complicate diagnosis, and affect its disease progression [232]. In fact, the impact of the reassessed MAFLD concept , highlighting the contribution of those metabolic dysfunctions to the progression of NAFLD, should be given more consideration [7]. Most conflicting results have been reported for hepatic mitochondrial functionality in humans with obesity, T2DM or NAFLD [89,93,233]. According to distinct methods, hepatic oxidative capacity was heavily increased in obese people independent of NAFLD severity, but unchanged in T2DM and even decreased in NASH when compared to lean humans [57,234]. Likewise, mitochondrial oxidation was found to be doubled or unchanged in NAFLD, while the specific diagnosis of NASH or higher fibrosis scores correlated with reduced mitochondrial respiration [235–237]. In line, obese people with NASH showed impaired activity of mitochondrial complexes, rate-limiting enzymes of fatty acid oxidation and mitochondrial structural abnormalities [91,94,238]. In summary, most studies suggest that mitochondrial plasticity enables the metabolism to adapt to obesity- and diabetes-related metabolic challenges until a progressed stage of NAFLD [57,93].

We tested different mice receiving either STZ or placebo and then HFD or regular chow diet for several weeks, yielding control, diabetes [STZ and regular chow diet], obesity [placebo and HFD] and diabetes-related NASH [STZ and HFD] models [76]. Our research study confirms that hepatic mitochondrial oxidation is upregulated during the development of diet-induced obesity and diabetes with or without NASH in the face of comparable whole-body insulin resistance. Additionally, our study provides comprehensive information on the different features of hepatic energy metabolism amongst the closely related metabolic disorders. The mechanisms linking hepatic mitochondrial adaptation to metabolic alterations comprise changes in (I) lipid metabolism, (II) insulin sensitivity, (III) mitochondrial mass or biogenesis, (IV) oxidative stress, (V) ER stress, (VI) inflammation, and (VII) fibrosis in mouse models of obesity, T2DM and NAFLD [76] and are briefly highlighted in the following: (I) Lipid metabolism (lipid uptake, lipolysis, lipid synthesis, DNL, lipid efflux) is distinctively affected in each mouse model [76]. As a shared commonality, mitochondrial acetyl-CoA carboxylase 2 expression is reduced in all groups and coupled with an increase of the β-oxidation-rate limiting enzyme carnitine palmitoyltransferase 1 A, underlining the association with β-oxidation-linked respiration. Acetyl-CoA carboxylase 2 regulates malonyl-CoA, which in turn inhibits carnitine palmitoyltransferase 1 A [239]. Increased lipolysis in liver and white adipose tissue was observed specifically in the T2DM group and correlated with hepatic TCA-derived respiration [76]. (II) All models have been linked to whole-body insulin resistance, as discussed in previous studies, while its manifestation in the liver was observed in our study to occur only gradually [76,240,241]. However, insulin resistance at the level of whole-body (including adipose tissue and skeletal muscle) may have a greater impact on hepatic energy metabolism regarding increased lipid flux to the liver [76,242]. Although the causal

relationship between mitochondrial function and insulin resistance is still controversially discussed, hypoinsulinemia in the T2DM and NASH group likely predicts the elevated hepatic respiratory function, as assessed in severe hyperglycemic and hypoinsulinemic mice [243]. In contrast, exposure to insulin implied a reduction in mitochondrial biogenesis-related genes, mitochondrial DNA, and ATP production [244]. (III) Mitochondria are dynamic organelles, which might additionally adapt by fission, fusion, posttranslational modifications or structural organisations besides a constant mass, as described in our study [76,245,246]. (IV) During progressed NAFLD stages elevated oxidative metabolism correlates with greater ROS production and impaired antioxidative defense mechanisms [57,247,248]. The efficient hepatic mitochondrial coupling and antioxidant defense mechanisms might explain the absence of hepatic oxidative stress, while systemic oxidative stress was increased in our T2DM and NASH models [76]. Moreover, OXPHOS gene expression positively correlated with expression of genes involved in ROS generation and scavenging in obese human with T2DM [249]. This study indicates that mainly obesogenic metabolism induces hepatic oxidative stress, partly attributable to ROS generation from OXPHOS pathway [249]. (V) Given the close association between the ER and the mitochondria and the observed correlation between markers of UPR and mitochondrial respiration, it is plausible to speculate that interorganelle cross-talk may play a role in the progression of NAFLD [76,250]. Our study confirms a BiP translocation into mitochondria in an ER-stress cell environment, like in the T2DM and NASH mice model [76,251]. In support, various metabolic diseases exhibit ER stress and selectively activate specific arms of UPR [76,96]. While ATF6 is preferably activated by membrane protein expression, IRE1α and PERK signalling pathways exhibit a more rapid response to the release of Ca^{2+} from the ER [252,253]. (VI) Elevated inflammation has been linked to maximal coupled β-oxidation- and TCA-derived respiration, assessed with higher TNFα expressions in all groups [76]. Based on other studies, TNFα exhibits a direct inhibitory effect on mitochondrial respiration [254,255]. (VII) There was a positive correlation observed between fibrogenesis and TCA-linked respiration. Hepatic wound healing in response to repeated liver injury results in excessive accumulation of ECM proteins as a central event of fibrogenesis [256]. Thus, alpha smooth muscle actin, a marker of fibrosis, is highly upregulated in our NASH group. HSCs exhibit aerobic glycolysis and elevated levels of glycolytic enzymes [257]. This metabolic profile has the potential to generate TCA substrates that can support mitochondrial respiration in the neighbouring hepatocytes under NAFLD-related conditions [76].

Hepatic mitochondrial plasticity might resemble an important feature of human NASH. In addition, our diabetes-related NASH model followed a characteristic disease pattern, starting with early steatosis and impaired hepatic insulin resistance, advancing to the development of hepatocellular nodules [76,258]. Therefore it is not surprising that this group showed the highest NAS, characterised by increased liver steatosis, hepatocellular ballooning, inflammatory cytokines, and liver fibrosis marker [76]. In contrast, the only low increasing body weight or the low fat mass compared to obese mice is a strong limitation of the model. Rather, it closely resembles the phenotype of lean NAFLD or the novel clustered severe insulin-resistant diabetes endotype, which features higher risk for NAFLD and its progression [259]. In addition, the metabolic situation of insulin deficiency coupled with suppression of

adipose tissue lipolysis and hepatic insulin resistance in our NASH mouse model might also remind of the severe insulin-deficient diabetes endotype [76,259]. Currently available animal models of NAFLD or, in particular, NASH, show inherent specific advantage but also disadvantage. Therefore, continuous improvement of better simulations and models are necessary to enable a complete understanding of human NASH pathogenesis, allowing development of crucial therapeutics [260].

6.2. Dietary Treatment Approach with the Concept Diet

The global prevalence of NAFLD has dramatically increased in parallel with the epidemic of obesity, especially among children [39,261]. A rapidly growing prevalence of pediatric NAFLD has been observed, along with a higher potential for progression to advanced stages, since overweight children are more likely to develop NAFLD in adulthood than their normal-weight peers [39,262]. Thus, the early-life environment can already program metabolic health in a negative manner, for example, with foetal over- or undernutrition or in a positive manner, for example, with breastfeeding [261,263,264]. Notably, breastfed children are less likely to be overweight and less prone to develop diabetes later in life [265,266]. Furthermore, breastfeeding in general is fundamental to the growth, development and health of children, while the antibodies in the human milk help to protect the infant against many common childhood illnesses. Thus, breastfeeding is strongly recommended by WHO for all children except for a few medical conditions.

The Concept diet has been inspired by human milk to mimic its supramolecular lipid droplet structure [157]. Human milk typically contains larger lipid droplets than infant milk formula and consists of a TAG-rich core surrounded by a trilayer of phospholipids, which integrated cholesterol, glycoproteins, enzymes, and other membrane proteins [267]. Accordingly, the novel Concept diet was composed of large lipid droplets with an aimed volume weighted mode diameter of 3-5 μm and coated by milk fat globule membrane fragments [157]. In contrast, the size of the lipid droplets from the isocaloric control diet only reached 0.47 μm on average [14]. While several studies already reported a beneficial effect of the Concept diet on metabolic health, we highlighted the role of the Concept diet specifically on mitochondrial function and lipid metabolism directly after its exposure as well as later in life [14,156,158,268].

Protective effects of the Concept diet on mitochondrial function

We examined the direct effects of the Concept diet on mitochondrial function and its long-term protective impact. Concept feeding directly enhanced mitochondrial fusion and maximum hepatic TCA-cycle-linked mitochondrial oxidative capacity, while it further improved maximum uncoupled β-oxidation-linked respiration later in WSD-fed adult mice [14]. These results indicate that the Concept diet can influence the programming of hepatic mitochondrial function in a way that maximum oxidative capacity is increased. Since ROS production has been implicated in NAFLD progression, we would have expected a rise in ROS levels in WSD-fed mice, expressed as H_2O_2 emission. However, based on this result, we suggest an improved antioxidant activity combined with higher oxidative capacity in the presence of the Concept diet in early life as a protective mechanism [14]. The Concept diet also

favoured mitochondrial dynamics and biogenesis, assessed by more mitochondrial fusion proteins. This hints at an improvement in mitochondrial function and supports our theory of an increased oxidative phosphorylation capacity in these mice [76,269]. Accordingly, plasma lipid peroxides increased only during WSD challenge of control mice but not in mice fed the Concept diet in early life. We speculate that a preferred β-oxidation or a decreased VLDL secretion in the latter can alter lipid metabolism and prevent from harmful lipid peroxides.
Adaption of mitochondrial oxidative capacity to an increased lipid availability during WSD challenge points towards "mitochondrial flexibility" in obese mice in the early stage of NAFLD. This finding is similar in humans suffering from obesity [57]. Thus, feeding of the Concept diet featured higher hepatic mitochondrial function from various substrates, adjusting to short-term as well as long-term changes in substrate availability. This effect seemed independent of mitochondrial biogenesis, including mitochondrial content or mass [14]. Despite beneficial effects on mitochondrial function, we can suggest a protective effect of early life exposure to the Concept diet on WSD-induced impairment of hepatic lipid metabolism, which is in line with previous evidence [156,268].

Protective effects of the Concept diet on lipid metabolism

After short-term exposure in early life, mice fed the Concept diet showed increased lean body mass and, after a WSD challenge, reduction in fat mass in later adulthood, suggesting a protective effect of the Concept diet on lipid metabolism [14]. We hypothesise that the lipid structure of this diet may positively influence lipolysis in the intestine, postprandial absorption, and availability of FAs in the body with longer-term consequences on hepatic bioenergetics [14]. However, the exact mechanisms are still under investigation.
Regarding the lipid profile, it is striking that Concept feeding prevented a WSD-induced increase in membrane and cytosolic DAG levels [14]. Since membrane DAG prompts impairment of the hepatic insulin signalling cascade by activating PKCε, Concept feeding seemed to protect from insulin resistance with a redistribution of the lipid profile in the liver tissue [85]. We hypothesize that the Concept diet protects from metabolic abnormalities, like WSD-induced lipotoxicity, by triggering the sequestration of DAG into neutral lipid storage sites (lipid droplets) as a compensatory mechanism [14]. Alternatively, the Concept diet might exert effects on DAG expression by affecting epigenetic modulation of genes involved in lipid metabolism and mitochondrial function, as shown in the human liver after bariatric surgery [270]. Despite the beneficial effect on insulin signalling, lower DAG levels also protected from restricted mitochondrial respiration [14]. This association was strengthened by a dose-dependent decrease of state 3 mitochondrial respiration in the presence of DAG [271].
Higher levels of certain ceramide species (C16, C18, C22, and C24) have been linked to fatty liver disease and decreased mitochondrial function in response to diets with a fat content of 45% to 60% [272]. The increased ceramide levels in the liver tissue of mice fed with Concept diet in early life were only seen in the membrane fractions and therefore being most likely originated directly from the ceramides of the milk fat globule membrane fragments used to coat the lipid droplets in the Concept diet. Despite the elevated ceramide levels, no differences in key metabolic outcomes have been observed. Therefore, we conclude that elevated

ceramides in combination with the Concept diet are not linked to any metabolic impairments also regarding long-term consequences [14].
The Concept diet did not significantly affect NEFA levels and fasting glycemia, and the effects of the WSD on insulin sensitivity is only partly prevented, this might be due to the short exposure time to WSD and absent dysregulations. Extending the WSD challenge enables the prediction of a more distinct phenotype. Thus, dietary intervention with the Concept diet in mice challenging subsequently with WSD for 12 weeks has been shown to favour decreased fasting plasma leptin, glucose, and lipid levels [156]. In line, previous studies in mice with postnatal feeding of the Concept diet additionally reported reduced fat accumulation and adipocyte hypertrophy, and improved metabolic profile in adulthood with WSD challenge [156,268].

Nonetheless, the exact mechanisms underlying the Concept diet-mediated regulation of mitochondrial respiration, oxidative stress response, and lipid metabolism are still unknown. Thus, it is inconclusive whether the size of dietary lipid droplets, the phospholipid coating of these droplets, or their combined effects are responsible for the impact of the Concept diet on mitochondrial oxidative activity and lipid metabolism. However, a previous study has demonstrated that neither an increase in droplet size alone nor phospholipid coating of small lipid droplets had any positive impact on the programming of adult body composition. Combining both was necessary to prevent fat mass accumulation in response to WSD challenge in adulthood [273]. It is worth to investigate if the beneficial effects of the Concept diet might further be improved by introducing a trilayer coating, similar to the surrounding of the lipid core in human milk.
In conclusion, the early life feeding of the Concept diet might present a promising approach to improve hepatic oxidative capacity, mitochondrial fusion, and lipid profiles in early and late life. Thus, it plays a role in programming adult metabolic health to be resilient in an obesogenic environment and might be considered as an approach to prevent the development of NAFLD. The potential protective effects of the Concept diet could be attributed to its lipid structure and physical characteristics resembling those of human milk. To further understand the underlying mechanisms, future studies should investigate the interaction between the lipid composition, physical structure, and other molecular processes involved in the progression of NAFLD.
The study with the postnatal Concept feeding shows that prevention is a promising strategy, although a substantial treatment concept is crucial when children and adults have been diagnosed for NAFLD. For the moment, weight loss and lifestyle changes remain the cornerstone of NASH treatment, those are, however, not very successful in long-term application since personal efforts and beliefs highly influence them. Thus, treatment beyond lifestyle changes and weight loss is necessary to reduce the significant disease burden currently observed [274]. Particularly since well-retained subsequent changes in histological outcomes are necessary additionally to induced improvements in NASH-related features [23]. Therefore, innovative pharmacological approaches are needed in addition to lifestyle interventions.

6.3. Pharmacological Treatment Approach with $GABA_A$ Receptor PAM

The discovery of the $GABA_A$ receptor in hepatocytes has led to the suggestion that the GABAergic system might also play an important role in the liver. Although the precise role of the $GABA_A$ receptor and downstream signalling machinery in the liver remains to be determined, evidence underlines its protective effect in lipotoxicity-induced hepatocellular injury [211]. Allosteric modulation of the $GABA_A$ receptor confirmed those beneficial effects against liver toxicity in several *in vitro* and *in vivo* models [101,225,226]. Therefore, targeting the GABAergic system with positive allosteric modulators may provide a promising therapeutic opportunity for NAFLD. The following chapter describes in detail the allosteric modulator function of the thioacrylamide HK4 on the $GABA_A$ receptor, the resulting hepatoprotective effects in lipotoxicity-induced hepatocellular injury, and the potential underlying mechanisms.

6.3.1. Positive Allosteric Modulation of the $GABA_A$ Receptor

Positive allosteric modulation of the $GABA_A$ receptors has been extensively used since the early 20th century as intravenous applied anaesthetics, which dampen neuronal activity in the brain [275]. Nowadays benzodiazepines are mostly used for anticonvulsant properties in epilepsy, spasticity, or anxiety, panic disorders, and insomnia [276].

To avoid those central nervous system side effects mediated by $GABA_A$ receptors, novel thioacrylamide molecules have been designed to both positively modulate $GABA_A$ receptor signalling and show low blood-brain barrier penetration [224]. A blood-brain barrier penetration has been excluded with liquid chromatography and mass spectrometry analysis of cerebrospinal fluid and brain tissues from rats treated with HK4 and its derivative HK3 (data not published). We proved an allosteric modulation activity of HK4 by performing patch-clamp experiments with HEK-293 cells expressing different subunit compositions. HK4 elicited currents in the presence of GABA through $GABA_A$ receptors containing $\alpha_{1\text{-}3}$, β_3, and γ_2 subunits. Thus, there is no doubt that HK4 is able to activate the majority of $GABA_A$ receptors in an allosteric manner since most $GABA_A$ receptors comprise two α subunits, two β subunits, and one γ (or δ) subunit [208]. Similar to our results, a dose-dependent potentiation of the PAM diazepam in the presence of GABA in HEK-293 cells stably transfected with the $\alpha_1\beta_3\gamma_2$ $GABA_A$ channel has been described [277]. This underlines the suitability of patch-clamp experiments with transfected HEK-293 cells for PAM activity measurements on $GABA_A$ receptors.

To exclude the possibility that HK4 only activates stably transfected $GABA_A$ receptors, we additionally measured HK4 signalling among native $GABA_A$ receptors. Regarding different cell models, INS-1E β-cells are suitable to measure GABA signalling by tracking Ca^{2+} influx with the fluorescent ratiometric dye Fura-2. Unlike neurons or pancreatic α-cells, GABAergic activity in β-cells causes membrane depolarisation through Cl^- efflux [227]. According to substantial evidence, the voltage-dependent Ca^{2+} channel subsequently opens and activates phosphoinositide 3-kinase/Akt-mediated cell growth and survival signalling pathways [227]. We were able to confirm a depolarising effect in INS-1E β-cells when GABA or HK4 were injected [101]. These results may indicate that GABAergic signalling through agonistic action

or additional allosteric modulation might help to preserve β-cell mass and prevent diabetes [227]. The specific allosteric action of HK4 has been demonstrated in the INS-1E β-cell model in the presence of the $GABA_A$ receptor antagonist bicuculline and the non-competitive $GABA_A$ channel blocker tert-butylbicyclophosphorothionate (TBPS). While bicuculline did not abolish HK4-induced depolarisation in β-cells, the high-affinity ligand TBPS for negative allosteric modulation was able to block HK4-mediated Ca^{2+} influx [101]. Regarding the complete channel inhibition by TBPS, we can speculate that signalling from NAMs might have a more dominant impact on GABA channel activity than from PAMs [278]. There are equivocal findings on whether agonistic binding is required for proper NAM or PAM signalling activity; however, the presence of GABA was confirmed in the supernatant of our INS-1E and HepG2 cell models [101]. Moreover, the presence of both GAD and $GABA_A$ receptor subunits in rat hepatocytes suggests that a functional auto- or paracrine GABAergic signalling exists in the liver [215]. Further, we demonstrated that the anti-apoptotic effect of HK4 in HepG2 cells has been extinguished in the presence of the NAM picrotoxin or the complete channel blocker TBPS, suggesting a $GABA_A$ receptor-mediated action of HK4.

Whether $GABA_A$ receptor activation in hepatocytes causes depolarisation (Cl^- efflux) or hyperpolarisation (Cl^- influx) is still a topic of ongoing debate [211,212]. However, slightly more evidence reports a hyperpolarisation-based GABA signalling theory in hepatocytes [215,216,231,279]. It has also been suggested that $GABA_A$ receptors might coordinate hepatocyte depolarisation and hyperpolarisation dependent on metabolic disorders, such as steatosis or insulin resistance [212]. In this context, it has been hypothesised that lipid accumulation in hepatocytes leads to increased TCA cycle substrates, whereas α-ketoglutarate can be converted by GABA transaminases to GABA, thus regulating membrane potential [212]. Regarding our Ca^{2+} influx experiments in hepatocytes, neither hyperpolarisation nor depolarisation upon HK4 binding could have been verified. We can only deny a significant voltage-dependent Ca^{2+} influx upon HK4 administration in hepatocytes. It might also be feasible that $GABA_A$ receptor responses switch from a hyperpolarising to depolarising direction following repeated activation, which has been assessed in neurons [280,281].

Several studies have substantiated the effect of the $GABA_A$ receptor PAMs in neurons, especially of benzodiazepine and barbiturate [275]. Those PAMs are known for their anaesthetic use, since neurons are less excitable when hyperpolarised by Cl^- influx. There is evidence that benzodiazepine facilitates the $GABA_A$ receptor-mediated potential in an allosteric manner by increasing the frequency of Cl^- channel opening, whereas the PAM barbiturate increases the duration of Cl^- channel opening in the presence of an additional agonist [223,282]. Regarding our results, it remains unclear if our PAM HK4 addresses frequency or duration of the $GABA_A$ receptor opening.

Nevertheless, we were able to validate the expression of pharmacologically relevant $GABA_A$ receptor subunits, such as α1, α3, α5, β2/β3, and γ2 in HepG2 cells. Recently we also discovered the presence of several subunits in the HSC line LX-2, implicating a dual targeting potential (data not published).

6.3.2. Hepatoprotective Effects of the $GABA_A$ Receptor PAM

Our research demonstrated that the newly designed thioacrylamide molecules address highly innovative physiological pathways to protect hepatocytes by reducing ER stress, inflammation, DNA fragmentation, and overall cell death or, more specifically, apoptosis. We focused mainly on hepatoprotective functions, since 70% of cell mass in the liver consists of hepatocytes [283]. However, recent findings suggest also a regression of hepatic fibrosis in activated HSC *in vitro* as well as in CCl_4-intoxicated mice by a thioacrylamide derivative.

Protective effects of HK4 on ER stress

We investigated the impact of PA and HK4 treatment on signalling pathways that arise from ER stress, interconnected by dynamic regulation [284]. PDI members acquire an important regulatory role during ER stress, as they mediate responses of the three major transmembrane sensors of UPR, catalyse oxidative protein folding, and act as chaperones [98,99,285]. We identified an impaired PDI protein expression in the presence of PA and a counter-regulation by HK4. However, there is a scope for interpretation since the critical role of PDI in both opposing pro-survival or cell death-triggering pathways in the context of ER stress and apoptosis remains controversial [286]. The function of PDI depends on its subcellular localisation, the degree of cellular damage, or the redox environment [98,287]. On the one hand, inhibition of PDI is a promising strategy to treat cancerous tissue due to a specific increased vulnerability of cancer cells, which have a higher rate of protein synthesis than normal cells [288]. On the other hand, activation of PDI might be a promising treatment strategy for neurodegenerative diseases, which are characterised by abnormal post-translational modification of PDI, leading to a compromised chaperone activity [98,289]. Nevertheless, impaired PDI expression is a helpful tool for evaluating dysregulated liver metabolism. Remarkably, a strong protein downregulation of PDI is observed in hepatocytes with acute lipotoxicity-induced injury and a switch towards a prominent upregulation in HCC liver tissue of individuals with severe stages of NAFLD [100,101]. Regarding our results, the PA-induced low expression of PDI might explain the lacking repair mechanisms within the UPR, resulting in high levels of ER stress. Our findings are supported by a clinical study of Lee et al., describing lower levels of PDI and increased expression of ER stress-associated transcription factors in liver tissues of patients with NAFLD [290].

PDI does not just regulate ER stress but might also play a role in inflammatory responses and apoptosis [291,292]. Higuchi et al. demonstrated a regulatory effect of PDI on NF-κB-dependent gene expression in a macrophage cell line [291]. We can confirm an inverse relation between PDI and NF-κB in our study. In addition, Data from Na et al. indicated that overexpression of cytosolic PDI can counter-regulate stimulated cell death, although PDI is a substrate for caspase 3/7 and will be cleaved sooner or later [292]. We verified an association of impaired PDI protein expression and caspase 3/7 activity in the presence of PA and an attenuation by HK4. Therefore, we hypothesise that PA-induced activation of caspase 3/7 cleaves PDI, resulting in functional loss and ER stress-mediated inflammation. Furthermore, we assume that the anti-apoptotic activity of HK4 is able to prevent PDI cleavage, resulting in anti-inflammatory and pro-survival activity [98,101]. In this context, low levels of PDI,

accumulated ER stress, inflammation, and risk for apoptosis pointed toward a vicious circle during NAFLD progression, which can be interrupted by HK4.

In line with our results, Gu et al. reported that saturated FAs exert high lipotoxic potential and trigger ER stress in HepG2 cells in contrast to unsaturated FAs [293]. Remarkably, PA-induced ER stress and apoptosis resolved in upregulated gene and protein expression of PDI downstream targets IRE1α and CHOP [293]. In parallel, gene expression of the transmembrane sensor *IRE1A* and the pro-apoptotic transcription factor *CHOP* are strongly increased in our sequencing analysis when treated with PA and nearly completely prevented when preincubated with HK4. In contrast to our observation, CHOP induction coincided with anti-apoptotic Bcl-2 expression in NASH tissues [290]. However, other anti-apoptotic markers clearly promoted a dysregulation of apoptotic signalling in the presence of CHOP, suggesting a complex connection between ER stress and apoptotic processes in NASH tissues [290]. Data from a recently published study indicated that CHOP expression interacts with the $GABA_B$ receptor by blocking the assembly of receptor subunits in the ER and obstructing the supply of new receptors to the cell surface [294]. To the best of our knowledge, no association of CHOP and $GABA_A$ receptor has been studied. However, evidence allows for the hypothesis that HK4 might counteract the CHOP-mediated downregulation of $GABA_A$ receptors by enhancing the intensity of $GABA_A$ receptor signalling, a property of allosteric modulation.

Impaired ER stress and apoptosis signalling during hepatocellular injury have been associated with loss of proteasome function, suggesting that proteasomal ubiquitination is a predominant hallmark in the progression of NAFLD [295]. Consequently, inactivated components of the ubiquitin-proteasome pathway trigger ER stress and apoptotic cell death in the liver of individuals with NAFLD [296]. Given the fact that decreased mitochondrial respiration and ATP deficiency contribute to the inhibition of the ubiquitin-proteasome pathway, it is not surprising that proteasome- and ubiquitination-related genes are differentially expressed in our dataset after PA exposure or HK4 preincubation [95,101]. Among them, the HK4-induced upregulation of *HSPA1B*, an HSP family member-coding gene, has been proposed to trigger an anti-inflammatory response in Kupffer cells during metabolic abnormalities [297,298]. Only limited research reveals the possible regulatory effects of GABA on HSP expression. It has been described that exogenous GABA administration upregulates HSP gene expression, whereas application of an inhibitor or GABA biosynthesis diminishes HSP gene expression under stress conditions in plants [299]. Controversial, oxidative stress-mediated inactivation of the GABAergic system in *Caenorhabditis elegans* is suggested to enhance the activity of the heat shock stress response [300]. These findings suggest that the expression of HSP may not serve as a reliable biomarker for assessing GABAergic dysfunction. Further research is needed for a better understanding of the role of HSP and its interaction with the GABA system in the pathogenic progress of NAFLD.

In summary, our discovery that HK4 could prevent PA-induced dysregulation of the ER chaperone PDI or ER stress-related gene expression might be perceived as a promising therapeutic approach for NAFLD. However, further research on GABA-related regulation of ER stress is necessary to bridge the gap towards the clinical application of HK4.

Protective effects of HK4 on inflammation

Another key finding of a present study is that HK4 prevented PA-induced inflammation. The transcription factor NF-κB was monitored in our hepatocellular injury model as a master regulator of inflammation and cell death [115]. Substantial evidence also suggests that NF-κB is an important biomarker for metabolic disease since downstream signalling exhibits hepatic insulin resistance [56]. Under oxidative stress conditions, NF-κB-regulated pathways are also critically involved in the initiation of hepatocyte apoptosis [115]. Genetic studies have revealed a complex network regulated by NF-κB, thus promising anti-inflammatory approaches for the intervention in chronic liver disease might consider targeting NF-κB subunits directly, upstream activators, NF-κB-activating kinases, as well as pathways modulated or genes regulated by NF-κB [115]. There is evidence that the counteraction of TP53 with NF-κB might even improve pathophysiological conditions associated with NAFLD [301,302]. We discovered an increased ratio of the phosphorylated (active) NF-κB subunit p65 compared to its unphosphorylated state in the presence of PA, while HK4 was able to prevent it. In line with the protein expression of phosphorylated NF-κB subunit p65, the gene expression of NF-κB subunit p100 was also upregulated when exposed to PA and effectively prevented when preincubated with HK4. Thus, the present studies figured out that PA induces hepatocellular inflammation by promoting phosphorylation of NF-κB, while preincubation with HK4 enhances the cell death-preventive function of NF-κB and protects from pro-inflammatory gene expression.

Identical to our findings, an upregulation of NF-κB has been reported in HepG2 cells after exposure to PA but also in the liver of several obese mice models and patients with NASH [116,117,303,304]. Several studies have substantiated the hypothesis of a preventive effect of the GABAergic system on NF-κB signalling [305,306]. For example, GABA supplementation decreased NF-κB signalling in pigs through action on ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors, thus alleviating liver inflammation [306]. Additionally, GABA administration also reduced mRNA expression levels of NF-κB and improved cell viability in lactating ruminants [305]. Consistent with the anti-inflammatory effect of HK4, the $GABA_A$ receptor PAM alprazolam augmented the ability of GABA to inhibit inflammatory T-cell responses *in vitro* [225]. It has recently been highlighted that GABA administration also inhibits IL-1β production in macrophages and alleviates inflammatory responses [307]. Our sequencing analysis strengthens the anti-inflammatory role of GABAergic signalling since HK4 prevented aberrant gene expression levels of the IL-related receptors *IL17RC* and *IL1R2* [103].

Even though an inverse correlation between GABAergic activity and NF-κB levels has been observed, it should be noted that NF-κB serves a dual function, ensuring both pro-inflammatory and anti-apoptotic responses [115]. As a double-edged sword in the context of liver inflammation, a slight upregulation of NF-κB protects hepatocytes from cell death, while constant activation promotes secretion of inflammatory mediators [115]. Although acute inflammatory responses might be beneficial in eradicating pathogens or regeneration after massive liver injury, a chronic response leads to a permanent activation of HSCs and associated wound-healing processes. Subsequently, HSCs promote the secretion of other

inflammatory mediators and worsen the fibrosis stage, which can straightforwardly result in cirrhosis and HCC [115,306]. Therefore, maintaining a delicate balance is crucial since an impaired activation of the NF-κB pathway may lead to increased inflammation or insufficient protection from cell death. Our data clearly point towards a pro-inflammatory function of NF-κB when assessed to a lipotoxic stimulus, while HK4 might trigger a cell death-preventive function of NF-κB in hepatocytes. The anti-inflammatory activity of HK4 attracts much attention for drug development for NAFLD.

Protective effects of HK4 on DNA damage

Additionally, we investigated the role of HK4 on DNA fragmentation during lipotoxic injury. PARP-1 is a notable feature of DNA damage since it is implicated in several DNA repair pathways and labels damaged single-strand breaks [130]. In line, PA-treated hepatocytes and livers from mice on HFD and individuals with NASH display activated PARP-1 levels [131,308]. Along with an increasing apoptosis rate due to overwhelming cellular stress and DNA damage, PARP-1 is cleaved by caspase 3/7 (Figure 2), resulting in activity loss [309]. Therefore, increasing levels of active PARP-1 may represent an effort to counterbalance accumulating DNA damage, while cleaved PARP-1 levels might indicate persistent cytotoxic effects paired with apoptosis. Thus, proteolytic cleavage of PARP-1 has been used for several decades as a marker of chemotherapy-induced apoptosis [310]. Upon PA treatment, upregulated expression levels of cleaved PARP-1 have been observed in HepG2 cells [101,308]. Preincubation of HK4 led to a significantly reduced expression of PARP-1, suggesting lower levels of DNA damage together with a decreased rate of apoptosis in the presence of HK4. A study by Hata et al. has demonstrated similar findings, implying that GABA signalling reduced DNA damage from ongoing oxidative stress, assessed with a reduced H2AX expression [213]. GABA administration generally represents a promising tool for decreasing DNA damage through the regulation of ROS [212].

As a second approach, we assessed DNA strand breaks *in situ* with the TUNEL method. In the presence of HK4, only a few hepatocytes were TUNEL-positively stained, suggesting a strong preventive effect of HK4 on PA-induced DNA fragmentation and a reliable readout by PARP-1 expression and TUNEL staining [101]. Furthermore, our sequencing analysis revealed that several upstream regulators of PA and HK4-modified differentially expressed genes (DEGs) are involved in DNA repair and orchestrate the cellular response to DNA damage. Taken together, our findings suggest that the thioacrylamide molecule HK4 has great potential to prevent DNA damage occurring in NAFLD.

Protective effects of HK4 on apoptosis

HK4 protects against lipotoxicity in HepG2 cells and human primary hepatocytes by reducing the activity of caspase 3/7 and the expression of cleaved caspase 7 in response to PA. Substantial evidence has pinpointed caspase 3/7 as mediators of apoptotic signals during liver injury. According to Feldstein et al., active caspase 3/7-mediated apoptosis is increased in NASH and is strongly associated with NAFLD progression [123]. These findings are consistent with a prior study demonstrating that GABA prevented hepatic necrosis and apoptosis by decreasing caspase 3 activity and increasing the expression of anti-apoptotic Bcl-2 in an acute

liver injury model [213]. In contrast, Lee at el. observed no significant differences in cleaved caspase 3 levels in normal and NASH tissues, although cytochrome c, Bcl-2, and Bim protein expression was aberrant [290].

Apoptosis-related protein and gene expression also showed partly contrasting results in our studies. 3'mRNA sequencing analysis revealed that gene expression of *CASP3* and *CASP7* was downregulated after 7h of exposure to PA, while protein expression was upregulated after 24h. At first glance, PA-induced downregulation of *CASP9* gene expression might also be contradictory. However, Kakisaka et al. reported that ballooned hepatocytes in NASH might display diminished expression of caspase 9 [311]. Thus, the link between transcriptional responses and protein abundance of apoptosis-related components considering time shift differences remains complex and unclear. Additionally, the concordance of gene expression and the related protein expression is still a matter of debate since post-transcriptional and translational regulation have an underestimated impact on determining protein concentrations [312].

Expression of other genes and proteins was consistent in our dataset and crucial for protecting cells from lipotoxicity [103]. Among them, the pro-apoptotic gene *BAX* was downregulated, and the anti-apoptotic gene *BCL2L11* was upregulated in the presence of PA and HK4. These findings are in parallel with results from Ji et al., indicating that liver steatosis influences the expression of those pro- and anti-apoptotic proteins [313,314].

In addition, ALT and AST levels have also been used as an indicator of hepatocyte cell death since they are produced in hepatocytes and released into the bloodstream when liver cells are damaged [107]. HK4 demonstrated a significant hepatoprotective activity in an acute CCl_4-induced injury mice model, accompanied by improvement in ALT and AST levels and hepatocyte necrosis scores (data not published). Thus, the hepatoprotective results could have been successfully translated to an *in vivo* setting.

Given that apoptosis is a fundamental driver of NAFLD progression, anti-apoptotic therapies are suggested as promising pharmacological approaches. In line, an inhibitor of caspase 1, 8, and 9 reduced ALT and CK-18 levels in a phase II-study including patients with NASH [197]. Therefore, cell death inhibition might be a rational approach in the acute application; however, concerns about the cell-specificity and safety of long-term application persist [9]. Apoptosis of hepatocytes, for example, can contribute to inflammation and fibrogenesis, while apoptosis of HSCs might help to resolve liver fibrosis and should not be inhibited [109,315]. Regarding our data, we cannot exclude side effects or safety concerns in human NASH; however, long-term application of HK4 has been tested in mice in a 90-day repeated-dose toxicity study. No HK4-related lesions, associated adversity or toxicity were identified at concentrations up to 10 mg/kg (data not published).

Distinct forms of programmed cell death pathways may be triggered during NAFLD progression, depending on the underlying disease entity [124]. Thus, evidence suggests a shift from apoptotic cell death towards necroptotic or necrotic signalling in progressed NASH stage [124,133]. Accordingly, not only apoptotic but also necroptotic hepatocytes have been verified in our lipotoxicity cell model over time [101]. It has been demonstrated that both late apoptotic (secondary necrotic) and necroptotic cells show a positive staining with the intercalation agent

propidium iodide (PI). However, staining morphology and intensity of the PI staining are distinguishable between the two distinct cell death pathways [316]. Currently, the different cell death forms underlying specific liver disease stages are extensively examined for the purpose of developing new biomarkers and specific therapeutic strategies [124]. The results above demonstrate an important protective role of HK4 in lipotoxicity-induced cell death, including apoptosis and necroptosis, suggesting a great therapeutic potential of the $GABA_A$ receptor PAMs for NAFLD.

6.3.3. Mechanistic Action of the $GABA_A$ Receptor PAM

As emphasised before, it is still an open debate whether GABAergic signalling leads to depolarisation or hyperpolarisation [211,212]. We might favour hyperpolarisation since slightly more evidence exists for this [215,216,231,279]. Previously, it has been demonstrated that membrane hyperpolarisation beneficially mediates the protection of ER stress upon acute hepatocyte injury [216]. Under the assumption of a voltage-mediated membrane hyperpolarisation by the ionotropic $GABA_A$ receptor, we can postulate two mechanistic actions focusing on either a direct ionotropic regulating effect on proteins or an indirect gene-regulating effect.

Overall, HK4-induced hyperpolarisation may prevent intracellular Ca^{2+} accumulation since Ca^{2+} channels are typically activated in response to depolarising action potential [317]. Depolarising signals normally trigger both Ca^{2+} influx from extracellular space and Ca^{2+} release from the ER lumen. Ca^{2+} signalling is a crucial and complex process that simultaneously discriminates among many functions. It is well exemplified that Ca^{2+} modulates hepatic bile secretion, cell proliferation, metabolism, and apoptosis amongst others [318]. Thus, Ca^{2+} signalling-associated defects, especially Ca^{2+} enrichment, have been implicated in the progression of NAFLD [318]. In detail, lipotoxicity can disrupt ER function by altering ER structure and Ca^{2+} homeostasis, affecting chaperone functionality [319,320]. Sustained ER injury and UPR activation can no longer ensure proper protein-folding but rather induce apoptosis through the mitochondrial cytochrome c-related cell death pathway [317]. Notably, disturbed Ca^{2+} levels also affect the expression of pro-apoptotic transcription factor CHOP and Bcl-2 family members, which is linked to FA-induced hepatocellular death and progression of NAFLD [314,317].

However, assuming $GABA_A$ receptor activation induces hyperpolarisation, HK4 might counteract intracellular Ca^{2+} accumulation and prevent lipotoxicity-induced disrupted Ca^{2+} signalling. In turn, HK4 might increase the expression and activity of ER chaperones, enhancing proper protein folding and degradation of misfolded proteins as an adaptive mechanism to combat ER stress and relaunch Ca^{2+} homeostasis. We provide clear evidence of a protective effect of HK4, specifically on PA-induced ER stress and intrinsic apoptosis pathway, regarding PDI and caspase 3 and 7 protein expression. Thus, maintaining regulated Ca^{2+} signalling diminishes apoptosis and cellular damage through GABAergic signalling. Furthermore, there is evidence that GABA signalling attenuates the pro-apoptotic IRE1α-ASK1-JNK pathway in the liver of ethanol-stimulated mice and subsequently upregulates components of the GABAergic system as a protective feedback response [216].

Taken together, hepatic injury may alter Ca^{2+} homeostasis in the liver, while GABAergic signalling can resolve Ca^{2+}-related dysregulation by enhancing proper protein folding of chaperones and even boosting its autocrine activation.

Besides this direct effect on ER stress- and apoptosis-related proteins via Ca^{2+} signalling, GABAergic activity might also comprise an indirect transcriptional effect on gene expression. We have previously demonstrated that the protective effects of HK4 come along with reduced protein expression of the transcription factors NF-κB and STAT3 [101]. Those two transcription factors regulate a large number of downstream genes related to cell proliferation, survival, stress responses, and inflammation to maintain homeostasis [321]. Interestingly, the omnipresent transcription factors NF-κB and STAT3 are mutually engaged in both positive and negative crosstalks [321]. While NF-κB expression has been linked to NAFLD and GABA signalling, it is important to note that high levels of STAT3 activated pro-apoptotic mechanisms by binding to the caspase 3 promoter [322]. Moreover, pre-emptive treatment with GABA coordinated anti-apoptotic effects in mice with severe acute liver damage through the STAT3 pathway, although interaction inversely correlated to our previously reported data [213]. We demonstrated that HK4 prevents PA-induced upregulation of STAT3 protein and *STAT5A* gene expression. STAT3 and STAT5A show similarities in controlling cell survival, differentiation, proliferation, and metabolism in response to extracellular stimuli [323]. Additionally, STAT5 is involved in regulating hepatic fat metabolism, while STAT3 has inflammatory properties and is involved in the stimulation of HSCs for collagen type I expression [132,324]. Thus, inactivation of the transcription factor STAT3 can potentially hinder the progression of hepatic fibrosis *in vivo*, suggesting great therapeutic potential [303]. Regarding the huge transcriptional impact of NF-κB and STAT3, it is obvious that the preventive effect of HK4 could also be mediated through a counteraction of the gene expression pattern induced by lipotoxic stimuli. This (second) hypothesis is underlined by a comprehensive transcriptomic analysis, which detected substantial modifications in gene expression. Those DEGs can be clustered into pathways pointing towards oxidative phosphorylation, mitochondrial dysregulation, protein ubiquitination, apoptosis, cell cycle control and regulation, which are all targeted in NAFLD. Several studies resembling pathophysiological processes in NAFLD have reported that these pathways regulate an adaptive response in hepatocytes [325–327]. The assigned expression of *CHOP*, *IRE1α*, *BAX*, *BCL2L11*, and *HSPA1B* especially underlines the protective effect of HK4 on ER stress, protein ubiquitination, and apoptosis at the transcriptional level. Furthermore, causal network analysis highlighted master regulators of PA and HK4-modified genes, providing insights into targeted downstream genes and underlying biological mechanisms. Among them, the E3 ubiquitin-protein ligase SYVN1 is involved in the ubiquitin-dependent degradation of misfolded proteins. SYVN1 negatively regulates the transcription factor and upstream regulator TP53, ensuring transcription of cell cycle regulation, senescence, DNA repair, and apoptosis [328]. In line, the other most significant upstream regulators, KDM5B, DDX5, and CAB39L, orchestrate the degradation of ER stress-induced misfolded proteins and DNA repair [101]. Remarkably, $GABA_A$ receptor-associated proteins (GABARAP, GABARAPL1, GABARAPL2) have also been proposed as master regulators with a trend towards an

activating action when being preincubated with HK4. GABARAPL1 is involved in $GABA_A$ receptor binding and might inhibit unwanted cell growth in HCC cell lines [329]. Thus, an upregulation can be interpreted as a positive feedback loop of the cell to enhance protective $GABA_A$ receptor signalling.

We can summarise from our sequencing analysis that preincubation of HK4 prevents PA-induced dysregulation and a NAFLD-related hepatocellular phenotype by restoring the initial gene expression pattern of untreated hepatocytes [101]. However, HK4 itself might not act as a transcription factor that enters the cell and targets gene expression. Instead, the $GABA_A$ receptor is located on the cell surface, and its signalling might lead to the activation of a transcription factor in the downstream cascades. Studies propose that the second messenger Ca^{2+} can induce differential gene expression by regulating transcription factors, thus combining both hypotheses [318,330]. Notably, inhibition of calcineurin leads to a decreased NF-κB action in hepatocytes, suggesting an interaction with Ca^{2+}-calmodulin signalling [331]. This hypothesis has been validated in Jurkat cells, where Ca^{2+} and calcineurin stimulate NF-κB and its DNA binding affinity [332,333]. Studies also confirm an association of Ca^{2+} with STAT3 signalling since Ca^{2+} signals elicited by IL-6 are necessary for persistent STAT3 activation [334]. It has been reported that a Ca^{2+} influx can activate Src family kinases in neurons, which are required for the Janus kinase-dependent phosphorylation of STAT3 [335]. Inhibition of Ca^{2+} flux or calmodulin-dependent kinase resulted in a lack of STAT phosphorylation and STAT-dependent gene activation [336]. Our data suggest that hyperpolarisation prevents disrupted Ca^{2+} homeostasis and, therefore, reduces expression of the transcription factors NF-κB and STAT3, leading to a differential gene expression pattern.

In conclusion, Ca^{2+}-related dysregulation might affect proper protein folding from chaperons and impair gene expression patterns through enhanced expression of the transcription factors NF-κB and STAT3. Thus, relaunching of disturbed Ca^{2+} homeostasis through hyperpolarisation is most likely the starting point of $GABA_A$ receptor downstream signalling. A direct protein-regulating and an indirect gene-regulating effect of the ionotropic $GABA_A$ receptor probably happens simultaneously. At the same time, their relative contribution may depend on the context of the signalling pathway.

6.4. Combined Therapeutic Approaches as a Perspective

Given the complexity of NAFLD pathophysiology with individually associated comorbidities, it is not surprising that the current treatment options show limited efficacy. Even if drug development for NAFLD is challenging, there is a clear consensus in the field regarding trial design, endpoints, compatibility, and targeting approaches. Considering individual pathogenesis and tailoring personalised treatment is crucial to increase response rates since different entities require distinct therapeutics. In fact, associated comorbidities, like obesity, dyslipidaemia, T2DM, and cardiovascular-related complications, as well as other factors, like intestinal microbiota diversity, endocrine disorders, sex, reproductive status, and physical activity contribute to NAFLD heterogeneity and should be characterised for optimal classification of patient subgroups [337]. Thus, there is an urgent need for more sensitive and specific, independently validated, and qualified non-invasive biomarkers with diagnostic but

also prognostic features, as well as therapeutics for NAFLD [54]. Innovative biomarkers are crucial to substitute liver biopsies, the most reliable diagnostic tool at the moment. Our comprehensive sequencing analysis revealed that plenty of gene expressions correlate with aberrant pathways triggering NAFLD and might ensure accurate identification in the future [338,339]. Machine learning tools utilising artificial intelligence will be increasingly capable of analysing huge amounts of data (genetics and environmental factors) and have the potential to identify accurate prognostication, select appropriate therapy, and predict treatment response [17].

Combined therapies, which address pleiotropic mechanisms with various metabolic risk factors in parallel, are more likely to improve NASH and associated comorbidities [340]. It is anticipated that a combination therapy approach focusing on precision medicine will be utilised in the future to manage progressive stages of NAFLD, similar to the treatment concepts for T2DM and hypertension [340]. Therefore, combination therapies are already under examination in clinical trials, even before the first monotherapy for NAFLD has been registered. The pathways that control NASH resolution combined with those targeting fibrosis offer promising opportunities for intervention. However, targeting the upstream drivers become even more important for the prediction of long term impact of downstream targets, according to several liver specialists [173]. Even if drugs showed insufficient effects or failed due to side effects, they might still be considered for coupling approaches, provided their application is optimised through microencapsulation. From a future perspective, development of innovative nanocarriers for targeted delivery might significantly improve efficiency and minimise side effects [341].

Obesity is one of the main risk factors for NAFLD. Hence, the prevention of steatosis highly influences disease progression. Thus, FXR agonists are promising candidates for combination therapies in NASH because of their strong ability to target steatosis but also insulin resistance, inflammation, or to a certain degree fibrosis [342]. The FXR agonist tropifexor has already been evaluated in combination with the CCR2/CCR5 antagonist cencriviroc in a phase II-study, while the FXR agonist cilofexor has been combined with the ASK1 inhibitor selonsertib or the acetyl-coenzyme A-carboxylase inhibitor firsocostat in a phase II-study. Notably, those studies combine a drug acting more upstream (lipid accumulation, insulin resistance) with a drug that targets downstream pathways (apoptosis, fibrosis) in the pathogenic progression of NAFLD, implicating a favourable dual targeting potential [340]. Although these therapeutic combinations did not pass primary endpoints, it has been demonstrated that long-term application of cilofexor and firsocostat improved NASH resolution, including ballooning, inflammation, and steatosis, and may evoke an anti-fibrotic effect [343]. Considering that cilofexor and firsocostat led to elevated serum triglyceride levels in some patients, the study protocol had been adjusted to include the triglyceride-lowering fenofibrate. Fenofibrate was able to mitigate hypertriglyceridemia associated with cilofexor and firsocostat application in patients with NASH [344]. FXR activation might also negatively influence plasma lipoprotein concentrations. Therefore, the front-runner OCA has been tested in combination with statins as a counterregulatory approach [345]. It has been shown that the OCA-dependent rise in LDL concentration can be ameliorated by atorvastatin, offering a potentially better treatment option

for NASH than OCA alone [345]. Several studies are ongoing to investigate additional combinations of medication classes, such as FXR agonists, PPARα/γ agonists, CCR2/CCR5 antagonists, bile acid metabolism-related substances, or an inhibitor of the leukotriene synthesis [346]. Even more promising might be the combination of the top targets, a THRβ with an FXR agonist, which is currently tested in a phase II-study with designed compounds from Terns Pharmaceuticals.

Unlike most steatosis-targeting drugs, HK4 acts more downstream in NAFLD pathogenesis. HK4 can be in focus as an anti-apoptotic drug that prevents ER stress and shows anti-inflammatory features in hepatocytes. Remarkably, recent data also displayed strong anti-fibrotic properties of a derivative of HK4 in a human hepatic stellate cell line and in a CCl_4-induced injury mice model (data not published). Therefore, thioacrylamides already exhibit dual functional activity in downstream pathways. Still, they are exceptional candidates for combination therapies with drugs targeting lipid accumulation or insulin resistance. Thus, coupling HK4 with an FXR, THRβ, or PPAR-pan agonist might even improve its pharmacological property. Given that HK4 targets extracellular $GABA_A$ receptor on the hepatocellular surface and FXR agonists address the intracellular nuclear receptor, separate administration of the drugs should be aspired. A linkage of those drugs might generate a loss of efficiency. Linking HK4 with a drug targeting steatosis or insulin resistance might only be favourable with extracellular receptor agonists. Thus, coupling with the antagonists of the CB1 receptor might result in an additional reduction of lipid accumulation, inflammation, and oxidative stress. Since studies suggest that endocannabinoids may promote liver fibrosis through the CB1 receptor, an appropriate CB1 receptor antagonist might even boost the potential anti-fibrotic effect of the $GABA_A$-receptor PAM [347]. The GLP-1 receptor might not be expressed on the surface of hepatocytes, contrary to what was initially assumed, so it is not aspired to couple HK4 with semaglutide. Nevertheless, the horizon for HK4 application might be expandable since HK4 may have the ability to target also other organs and cell types that are affected by inflammation, ER stress, apoptosis or fibrosis. Chronic kidney disease, for example, is closely related to NAFLD and shows similar cellular features [348]. In addition, expression of $GABA_A$ receptors has been identified in the kidney of multiple species, especially in the renal proximal tubules in the cortex [209,349]. This opens up new therapeutic applications for $GABA_A$ receptor PAMs.

6.5. Conclusion

NAFLD has become the most common chronic liver disease worldwide and represents a substantial clinical and economic burden [18]. During the past century, dramatic lifestyle modifications contributed to the growing prevalence of NAFLD in parallel to the rising epidemic of obesity and T2DM [28]. Thus, NAFLD is strongly associated with visceral adiposity and insulin resistance, considering NAFLD as the hepatic manifestation of the metabolic syndrome [3]. Due to the strong association, metabolic features of obesity, T2DM and NAFLD might overlap, complicating diagnosis and mutually influencing disease progression. The progressions are determined by dynamic and complex interactions of metabolic alterations, including lipid accumulation as a main driver in parallel to insulin

resistance and mitochondrial impairment. Since conflicting results have been reported for hepatic mitochondrial functionality, we investigate hepatic energy metabolism in appropriate mouse models. We were able to show that hepatic mitochondrial oxidation is upregulated during the early development of diet-induced obesity and diabetes with or without NASH. This mitochondrial adaption, also known as mitochondrial plasticity, allows the body to meet the bioenergetic needs in the liver. The adaption is operative not only in altered tissue-specific insulin sensitivity and lipid metabolism, but also in situations of systemic oxidative stress, hepatic ER stress, inflammation, apoptosis, and fibrogenesis. Understanding those underlying molecular mechanism of NAFLD progression is crucial to identify predictive biomarkers and therapeutic targets to achieve a personalised treatment approach, as the diagnosis of the specific NAFLD entity still ultimately depends on histopathologic evaluation.

Considering the tight association between obesity and NAFLD progression, dietary intervention and weight reduction are key for the prevention and treatment of NAFLD [23,54]. Therefore, the starting point of the present work evaluated the potency of a particular dietary intervention, mimicking human milk, in mice fed in early life in an attempt to reduce later life risk for adiposity, T2D and potentially also NAFLD. We examined for the first time that the so-called Concept diet, composed of large lipid droplets coated by milk fat globule membrane fragments, improved hepatic mitochondrial respiration, oxidative capacity, mitochondrial fusion, and lipid metabolism and might have prevented WSD-induced insulin resistance and other metabolic deterioration. These effects were observed immediately after exposure to the Concept diet and, interestingly, were maintained even under an obesogenic environment later in the life in our mouse model for nutritional programming. Although the exact mechanisms underlying Concept diet-mediated regulation of mitochondrial function and lipid metabolism remain elusive, we would recommend an early life-feeding intervention with the Concept diet to combat hepatic metabolic disorders, when breast-feeding is not ensured. In addition to previous evidence, we can conclude that early-life nutrition may have a long-lasting impact on adult metabolic health and should be considered a fundamental approach to prevent NAFLD. Protective effects of the Concept diet may be partly explained by its physical properties and lipid profile, which are similar to human breast milk lipids. Future studies should examine the interplay between lipid composition and physical structure, also regarding other downstream molecular pathogenic processes during NAFLD progression. Even though early-life feeding can show potential preventive properties, a personalised treatment concept is crucial once children and adults have been diagnosed for NAFLD. Lifestyle changes focusing on weight loss remain the cornerstone of NASH treatment. However, they have a low success rate in long-term application since personal efforts and beliefs highly influence them. Therefore, pharmacological therapies are needed in addition to lifestyle interventions to combat the clinical and economic burden of NAFLD. So far, no specific pharmacological therapy for NAFLD has been approved, but numerous compounds are currently being tested in clinical trials. Some of these trials show promising resolution of steatosis, inflammation, cell death, or fibrosis. Given the complexity of NAFLD with individual associated comorbidities, a multitarget approach with combined pharmaceuticals may be required [162].

Because of the growing global burden of NAFLD and the scarcity of licensed therapies, we focused on an innovative pharmacological strategy to treat or prevent NALFD with the aim of addressing several metabolic facets. Since growing evidence suggests a link between the GABAergic system and its protective effect in metabolic disease, novel thioacrylamide molecules have been designed to both positively modulate $GABA_A$ receptor signalling and show low blood-brain barrier penetration. Selective allosteric modulator activity of HK4 has been approved by patch clamping, calcium influx measurements, and assays with non-competitive antagonist $GABA_A$ channel blockers. Our research further demonstrated that HK4 beneficially addresses several pathophysiological pathways to protect hepatocytes by reducing ER stress, inflammation, DNA fragmentation, and cell death, specifically apoptosis. Remarkably, a derivative of HK4 even displayed anti-fibrotic properties, implicating a favourable dual targeting potential. Thus, $GABA_A$ receptor PAMs might not only prevent NASH resolution but also liver fibrosis to combat the metabolic burden of NAFLD. Taken together, positive allosteric modulation of the $GABA_A$ receptor has great therapeutic potential as a first-in-class treatment concept for NAFLD.

To bridge the gap towards clinical application of $GABA_A$ receptor PAMs, the underlying molecular mechanism by HK4 in lipotoxicity-induced hepatocellular injury has been further explored. Unfortunately, it remains elusive if GABAergic signalling and transcription factor activation are initiated by depolarisation or hyperpolarisation of the GABA Cl^- channel, as emphasised in the previous sections. However, the beneficial effects of HK4 appear to be mediated by changes in gene expression through modifications of the transcription factors NF-κB and STAT3. However, comprehensive 3'-mRNA sequencing analysis revealed substantial dysregulations at the transcriptional level in response to the lipotoxic stimulus PA and restoration of the initial gene expression pattern when preincubated with HK4. In summary, these differentially expressed genes can be clustered into pathways that are specific pathophysiological targets in NAFLD. TP53, KDM5B, DDX5, CAB39L, and SYVN1 were identified as key upstream regulators of PA and HK4-modified genes, orchestrating the metabolic and oxidative stress responses, including modulation of DNA repair and degradation of misfolded proteins. We can conclude that mitochondrial respiration, protein ubiquitination, apoptosis, and cell cycle regulation are major targets of HK4 to minimise hepatocyte lipotoxic injury through modification of gene expression. Targeting transcription factors responsible for DNA repair and ER stress might be a strategy to prevent lipotoxicity, a process that holds great therapeutic potential of HK4 for treating NAFLD.

Taken together, our findings may provide a better understanding of pathophysiological mechanisms during NAFLD progression. However, continuing research in this field will be crucial to identify new targets and biomarkers for the management of NAFLD. While dietary interventions and weight loss remain the cornerstone of NAFLD treatment, pharmacological approaches are urgently needed. A better understanding of the underlying mechanisms will support a clinical translation and, thus, an option for novel pharmaceutical combinations for a personalised treatment concept of this endemic disease. Our research paves the road for positive allosteric modulators of the $GABA_A$ receptor as a first-in-class treatment concept due to strong hepatoprotective effects.

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