ELUCIDATION OF THE MOLECULAR MECHANISMS OF *ORTHOSIPHON ARISTATUS* EXTRACT IN NON-ALCOHOLIC FATTY LIVER DISEASE

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by

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonate
ACAA2	Acetyl-coenzyme A acyltransferase 2
ACACA (ACC)	Acetyl-CoA carboxylase alpha
ACADL	Acyl- CoA dehydrogenase, long chain
ACADS	Acyl- CoA dehydrogenase
ACLY	ATP citrate lyase
ACOX1	Acyl- CoA oxidase 1, palmitoyl
ACTB	Actin, beta
ADIPOQ	Adiponectin, C1Q and collagen domain containing
AEU	Animal experimental unit
Akt1	Akt serine/threonine kinase 1
Akt2	Akt serine/threonine kinase 2
ALB	Albumin
ALT	Alanine transaminase
AMPK	Adenosine 5′monophosphate-activated protein kinase
ANOVA	Analysis of Variance
APOE	Apolipoprotein E
AST	Aspartate transaminase
ATCC	American-type culture collection
ATP	Adenosine 5'-triphosphate
B2M	Beta-2-microglobulin
BATMAN-TCM	Bioinformatics analysis tool for the molecular mechanism of traditional Chinese medicine
BAX	BCL2 associated X, apoptosis regulator
BCL2L1	BCL2-like 1
BCL2L11	BCL2 like 11
BDH1	3-hydroxybutyrate dehydrogenase, type 1
BSA	Bovine serum albumin
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CASP7	Caspase 7

CASP8	Caspase 8
CCL2	C-C motif chemokine ligand 2
CD36	CD36 molecule (thrombospondin receptor)
CD68	CD68 molecule
cDNA	Complementary DNA
CO_2	Carbon dioxide
COL1A1	Collagen, type I, alpha 1
COX1	Cytochrome c oxidase subunit I
COX2	Cytochrome c oxidase subunit II
COX3	Cytochrome c oxidase III
CPT1A	Carnitine palmitoyltransferase 1A (liver)
CPT2	Carnitine palmitoyltransferase 2
CRP	C-reactive protein
CYCS	Cytochrome c, somatic
DL	Drug-likeness score
DPP-4	Dipeptidyl peptidase-4
DPPH	1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-2,4,6-trinitrophenyl) hydrazyl
EDTA	Ethylenediaminetetraacetic acid
EOA	Ethanolic extract of *O. aristatus*
EFOA	Ethanolic fraction of the ethanolic extract of *O. aristatus*
ERN1	Endoplasmic reticulum to nucleus signalling 1
FA	Fatty acid
FABP4	Fatty acid binding protein 4
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FDR	False discovery rate
FGF19	Fibroblast growth factor 19
FRAP	Ferric ion reducing antioxidant power
G6PC	Glucose-6-phosphatase, catalytic subunit
GAE	Gallic acid equivalents
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLP-1	Glucagon-like peptide 1

GLUT4	Glucose transporter-4
GO	Gene ontology
GSK3A	Glycogen synthase kinase 3 alpha
GSK3B	Glycogen synthase kinase 3 beta
H&E	Hematoxylin and eosin
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HDL	High-density lipoprotein
HFD	High-fat diet
HGDC	Human genomic DNA contamination
HMGCL	3-hydroxymethyl-3-methylglutaryl-coa lyase
HMGCR	3-hydroxy-3-methylglutaryl-coa reductase
HMOX1	Heme oxygenase 1
HNF4A	Hepatocyte nuclear factor 4 alpha
HOMA-IR	Homeostasis model assessment for insulin resistance
HPLC	High-performance liquid chromatography
HPRT1	Hypoxanthine phosphoribosyl transferase 1
HSC	Hepatic stellate cells
IC_{50}	The half-maximal inhibitory concentration
IκBKβ	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL1β	Interleukin 1, beta
IL6	Interleukin 6
INSR	Insulin receptor
IPA	Ingenuity pathway analysis
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
Ki	Inhibition constant
LC/MS	Liquid chromatography-mass spectrometry
LD50	Median lethal dose
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MAPK10	Mitogen-activated protein kinase 10
MAPK8	Mitogen-activated protein kinase 8

MAPK9	Mitogen-activated protein kinase 9
MDA	Malondialdehyde
MetS	Metabolic syndrome
MLXIPL	MLX interacting protein-like
MMP9	Matrix metallopeptidase 9
MTOR	Mechanistic target of rapamycin
MTTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NAMPT	Nicotinamide phosphoribosyl transferase
ND	Normal diet
NFKB1	Nuclear factor kappa B subunit 1
NR1H3	Nuclear receptor subfamily 1 group H member 3
NR1H4	Nuclear receptor subfamily 1 group H member 4
OB	Bioavailability score
OMIM	Online Mendelian Inheritance in Man database
OXCT2	3-oxoacid CoA transferase 2
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PLIN2	Perilipin 2
PNPLA3	Patatin-like phospholipase domain containing 3
PPARA	Peroxisome proliferator-activated receptor alpha
PPARΓ	Peroxisome proliferator-activated receptor gamma
PPARΓC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PPC	Positive PCR control
PRKAA1	Protein kinase AMP-activated catalytic subunit alpha 1
PTPN1	Protein tyrosine phosphatase non-receptor type 1
QTOF	Quadrupole Time-of-Flight
QUICKI	Quantitative insulin sensitivity check index
RA	Rosmarinic acid
RELA	RELA proto-oncogene, NF-κB subunit
ROS	Reactive oxygen species

RPLP0	Ribosomal protein, large, P0
RTC	Reverse transcription control
RT-PCR	A real-time reverse transcription–polymerase chain reaction
RXRα	Retinoid X receptor alpha
SD	Standard deviation
SERPINE1	Serpin family E member 1
SIRT1	Sirtuin 1
SLC27A5	Solute carrier family 27 (fatty acid transporter), member 5
SREBF1	Sterol regulatory element binding transcription factor 1
TAG	Triglycerides
TC	Total cholesterol
TCMID	Traditional Chinese medicine integrated database
TLR4	Toll-like receptor 4
TLR9	Toll-like receptor 9
TMRM	Tetramethylrhodamine methyl ester
TNF	Tumour necrosis factor
XBP1	X-box binding protein 1
$\Delta\psi m$	Mitochondria membrane potential

LIST OF APPENDICES

ELUSIDASI MEKANISME MOLEKUL EKSTRAK *ORTHOSIPHON ARISTATUS* DALAM PENYAKIT HATI BERLEMAK BUKAN ALKOHOLIK

ABSTRAK

Penyakit hati berlemak bukan alkohol (NAFLD) adalah bentuk penyakit hati yang paling biasa, bermula dengan steatosis hepatic yang mudah dan berpotensi membawa kepada keradangan hati, fibrosis, dan sirosis. Walaupun banyak penyelidikan tentang NAFLD, tiada terapi standard yang diluluskan untuk itu setakat ini. Bahan semulajadi berpotensi berfungsi sebagai pilihan terapeutik alternatif atau sokongan. *Orthosiphon aristatus* (Blume) Miq, tanaman tradisional di Asia Tenggara, menjanjikan kerana potensinya untuk mengurangkan kegemukan dan keadaan hiperglikemia. Pelbagai komponen kimia dalam ekstrak etanol *O. aristatus* (EOA) telah dikenal pasti dalam kajian sebelumnya, menunjukkan sifat antioksidan yang kuat dan penggunaan etnofarmakologi yang luas. Kajian ini menilai kesan anti-NAFLD potensi EOA menggunakan pelbagai kaedah *in vitro, in vivo,* dan *in silico*. Ini termasuk memberi makan tikus C57BL/6 dengan diet tinggi lemak, menginduksi NAFLD dalam sferoid 3D dari sel HepG2 dan LX-2 menggunakan asid palmitik-oleik, dan menggunakan pelbagai alat bioinformatik, seperti dokking molekul, untuk mengenal pasti sifat farmakokinetik dan farmakodinamik senyawa bioaktif EOA. Penemuan kami mengenal pasti 20 sebatian bioaktif yang bersesuaian dengan 45 sasaran yang berkaitan dengan NAFLD. Tikus yang diberi makan dengan ekstrak etanol *O. aristatus* yang distandardkan (400 mg/kg) selama lapan minggu menunjukkan pengekangan perkembangan NAFLD. Penurunan signifikan dalam enzim hati seperti alanine

aminotransferase dan aspartate transaminase, serta tahap serum glukosa, insulin, kolesterol total, trigliserida, dan lipoprotein berdensiti rendah telah diperhatikan. Dari segi histologi, EOA mengurangkan penumpukan lemak di dalam hepatosit dan mengurangkan keparahan NAFLD. Notabene, tahap serum IL-6 dan TNFα juga menurun, menunjukkan aktiviti anti-radang. Fraksi polar EOA secara signifikan ($p<0.001$) mengurangkan penumpukan lemak intraselular dalam sferoid 3D selepas 24 jam rawatan, konsisten dengan hasil antioksidan yang diperhatikan secara in vitro. Kesan anti-NAFLD EOA dapat dikaitkan dengan tindakan gabungan beberapa sebatian, sasaran, dan komponen multiselular, seperti pengaturan metabolisme lipid dan laluan isyarat insulin. Oleh itu, penemuan kami sangat menyarankan untuk pengembangan lanjutan EOA sebagai agen pelindung semulajadi potensial terhadap NAFLD dan.hiperkolesterolemia.

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease, beginning with simple hepatic steatosis and potentially leading to hepatic inflammation, fibrosis, and cirrhosis. Despite extensive research on NAFLD, there is no approved standard therapy for it as of now. Natural agents could potentially serve as alternative or supportive therapeutic options. *Orthosiphon aristatus* (Blume) Miq, a traditional plant in Southeast Asia, holds promise due to its potential to mitigate obesity and hyperglycaemic conditions. A plethora of chemical constituents in the ethanolic extract of *O. aristatus* (EOA) have been identified in previous studies, underscoring its potent antioxidant properties and broad ethnopharmacological usage. This study evaluated the potential anti-NAFLD effects of the EOA using a variety of *in vitro*, *in vivo*, and *in silico* methods. This included feeding C57BL/6 mice a high-fat diet, inducing multilineage 3D spheroids of HepG2 and LX-2 cells using palmitic-oleic acid induced-NAFLD, and employing various bioinformatics tools, such as molecular docking, to identify the pharmacokinetic and pharmacodynamic properties of the bioactive compounds of the EOA. Our findings identified 20 bioactive compounds corresponding to 45 potential NAFLD-related targets. Mice fed with the standardized EOA (400 mg/kg) for eight weeks showed inhibited NAFLD progression. Significant reductions in liver enzymes such as alanine aminotransferase and aspartate transaminase, as well as serum levels of glucose, insulin, total cholesterol, triglycerides, and low-density lipoprotein were observed. Histologically, EOA reduced

fat accumulation within hepatocytes and alleviated NAFLD severity. Notably, serum levels of IL-6 and TNFα also decreased, suggesting anti-inflammatory activity. Polar fractions of EOA significantly (p<0.001) reduced intracellular fat accumulation in 3D spheroids after 24 hours of treatment, consistent with the antioxidant results observed in the *in vitro*. The anti-NAFLD effects of EOA could be attributed to the combined action of multiple compounds, targets, and multicellular components, such as regulation of lipid metabolism and insulin signalling pathway. Therefore, our findings strongly advocate for further development of EOA as a potential natural protective agent against NAFLD and hypercholesterolemia.

CHAPTER 1

INTRODUCTION

1.1 Background

Non-alcoholic fatty liver disease (NAFLD) is a major growing health problem affecting 25-30% of people worldwide (Younossi, 2019). NAFLD is characterized by the accumulation of excess liver fat (steatosis) that accounts for more than 5% of liver weight and can cause persistent liver enzyme abnormalities associated with hepatomegaly (Stefan *et al.*, 2019). In addition, NAFLD patients could progress to an advanced stage i.e., non-alcoholic steatohepatitis (NASH), which is characterized by the presence of inflammation with or without fibrosis. Eventually, NASH can progress to fibrosis, cirrhosis, and hepatocellular carcinoma (Vancells Lujan *et al.*, 2021).

Pathophysiologically, NAFLD is considered part of the Metabolic Syndrome (MetS) and is positively associated with insulin resistance (IR), obesity, hyperlipidaemia and hypertension (Younossi *et al.*, 2019). In addition, the development of NAFLD is attributed to many complex factors including impaired lipid homeostasis (lipogenesis and lipolysis), lipotoxicity, oxidative stress (imbalance between pro-oxidative and antioxidant chemicals leading to liver cell damage), increased production and release of pro-inflammatory cytokines, gut microbiota (gut bacteria), glucose and insulin intolerance, mitochondrial dysfunction and apoptosis (Alshawsh *et al.*, 2022; Torres *et al.*, 2012).

Criticism of the NAFLD condition has led to resources and research funding being diverted to support studies attempting to identify the high-risk NAFLD population. Further studies are focused on understanding the mechanism of progression and in turn identifying potential molecular targets for treatment. To date, there is no approved anti-NAFLD drug and treatment options are based on

prophylactic strategies involving diet and lifestyle changes and physical activity (Mascaró *et al.*, 2022). Several emerging potential therapies based on antioxidant effects have been studied and developed. Considering the multiple mechanisms underlying NAFLD, medicinal plants with their variety of bioactive composition could target multiple pathways of the disease progression (Xu *et al.*, 2020).

Orthosiphon aristatus (Blume) Miq. or Java tea (family Lamiaceae), known locally as Misai Kucing in Malaysia, has been used in folk remedies for years. It is mainly distributed in tropical Southeast Asian countries such as Malaysia, Indonesia and Thailand and has been introduced to western countries since the early 20th century (Chung *et al.*, 2020). Traditionally, the leaves of *O. aristatus* have been used as a tea in various regions of Southeast Asia and are believed to improve health and cure various types of diseases and ailments such as diabetes, kidney stones, atherosclerosis, cystitis, nephritis, hepatitis, syphilis, rheumatism, hypertension and gonorrhoea (Ashraf *et al.*, 2018). This herb has gained great interest nowadays due to its wide range of pharmacological effects such as antioxidant activity, antiangiogenic activity, and diuretic and hypoglycaemic effects (Chung *et al.*, 2020). A previous study reported the beneficial effects of *O. aristatus* in alleviating hyperglycaemia and improving lipid profiles in diabetic rats. Another study provided experimental evidence for the anti-obesity and anti-inflammatory effects of *O. aristatus* (Seyedan *et al.*, 2017).

1.2 Problem statement

1.2.1 Study rationale

Despite the high prevalence of NAFLD, the underlying pathophysiology progression is not fully illustrated due to the complexity of confounding factors, and there are no approved drugs to treat NAFLD or NASH (Eslam *et al.*, 2020). On the other hand, traditional medicine is widely used to prevent and manage NAFLD/NASH, as they are readily available in the community, and having high variety of compound composition that gives them the ability to target several disorders. This study spotted light on *O. aristatus* against NAFLD, as it is widely distributed throughout the tropical regions, especially in Southeast Asia. Besides wide ethnopharmacological use, for example, anti-diabetics, kidney protective, anti-oedema, anti-inflammatory, anti-oxidants, and anti-hypertensive. Furthermore, *O. aristatus* has potent antioxidant activity that is driven by the different variety of phytoconstituents, including flavonoids, diterpenes, triterpenes, saponins, and caffeic acids derivatives, which make potent antioxidants (Ashraf *et al.*, 2018; Bakar *et al.*, 2018). Moreover, a recent systematic review has shown that investigations into the antidiabetic effects and mechanisms of *O. aristatus* have mainly focused on the effects of extracts, particularly 50% ethanol extract and aqueous extract (Wang *et al.*, 2022). Also, previous research found that the ethanolic (100%) extract used by maceration is high in phenols and flavonoids (rosmarinic acid; RA) with high antioxidant activity (Saidan *et al.*, 2015). Therefore, illustrating the effects of *O. aristatus* ethanol extract as an anti-NAFLD *in vivo*, *in vitro* and *in silico* might effectively contribute to the management of NAFLD by recognising new active compounds and novel targets for potential drug development. The flowchart of this study is available in **Figure 1.1.**

1.3 Hypothesis

1. The ethanolic extract of *O. aristatus* will show anti-NAFLD effects among *in vitro*, *in vivo*, and *in silico* approaches.
2. The anti-NAFLD effects of the ethanolic extract of *O. aristatus* are due to its potent antioxidant activity.
3. The anti-NAFLD effects of the ethanolic extract of *O. aristatus* may be mediated by the flavonoid rosmarinic acid.
4. The anti-NAFLD effects of the ethanolic extract of *O. aristatus* are mediated by multiple mechanisms, including anti-inflammatory, and anti-oxidative.

1.4 Objectives

1.4.1 General objective

This study's main objective is to investigate the inhibitory effect of *O. aristatus* extract against the progress of NAFLD, and elucidation of its mechanisms of action.

1.4.2 Specific objectives

1. To evaluate the anti-NAFLD effect of *O. aristatus* using mice fed a high-fat diet (*in vivo*).
2. To determine the most effective fraction using palmitic-oleic acid-induced NAFLD in HepG2 Cells (*in vitro*).
3. To investigate the molecular mechanisms of action of the most effective fraction of *O. aristatus* using palmitic-oleic acid-induced NAFLD in a human multilineage 3D spheroid (*in vitro*).
4. To explore the molecular mechanisms of action of *O. aristatus* bioactive compounds using bioinformatics tools (*in silico*).

1.5 Study scope

There is increase interest in NAFLD and its more advanced stage, NASH, because of its growing impact on world health. To date, there is no proved drug to treat NAFLD. In view of this situation, the present study used several approaches of the pharmaceutical sciences, including pharmacology, drug discovery, and bioinformatics, to shedding light on the deleterious effects of one of the Malaysian traditional medicinal herbs on the treatment of NAFLD.

This study employed various models, such as mono and multilineage 3D co-culture of liver cells, to investigate the study objectives. In addition, a C57BL/6 mice model was used, which were fed a high-fat diet for sixteen weeks, and different agents of interest were administered during the last eight weeks. To further explore the mechanism of action, bioinformatics tools such as molecular docking, target prediction, and network pharmacology were also utilized.

This novel study demonstrates the utility of using *O. aristatus* in the treatment of NAFLD (particularly in the early silent stages) and suggested using it with conventional therapy to reduce the side effects of the chemical drugs or increase their effect. Furthermore, opens the way to further isolate and design a novel active anti-NAFLD drug candidate based on the *O. aristatus* bioactive compositions.

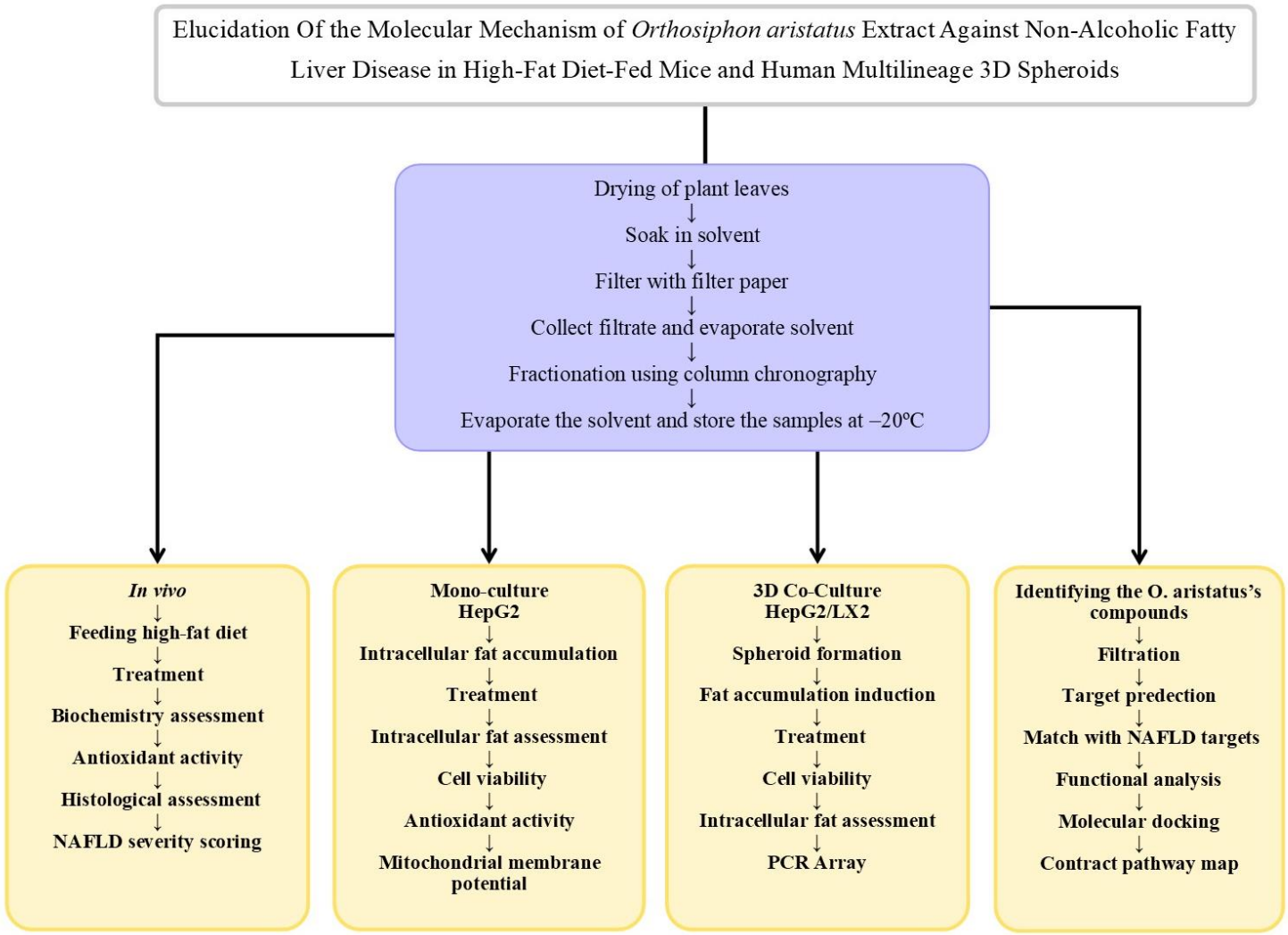

Figure 1.1. The study flow chart diagram

CHAPTER 2

LITERATURE REVIEW

2.1 Non-alcoholic fatty liver disease

Due to its increasing impact on global health, there has been a surge of interest in NAFLD and its advanced stage i.e., NASH. According to the 2019 Global Burden of Disease results, the prevalence of NAFLD was 16.61% (95% CI: 14.91-18.53) worldwide and 23.84% (95% CI: 14.91-18.53) in Malaysia (Seattle, 2020). Additionally, a cross-sectional study conducted in 2018 found that the prevalence of NAFLD among urban Malaysian adults was 37.4%. The prevalence of NAFLD was higher in males (48.3%) than females (27.3%) and in Indians (61.1%) and Malays (51.1%) than in Chinese (34.2%) (Khammas *et al.*, 2019). In addition, NAFLD prevalence varies widely across Asian countries, such as China (12.5–38%), Japan (23–26%), Korea (27%), Taiwan (12–51%), Hongkong (28%), and India (9–32%). This variation could be due to the wide geographical distribution of Asia, regional differences in economic development, diet and lifestyle between different countries and also within countries, and possible genetic influences (Mitra *et al.*, 2020).

The majority of patients with fatty livers have a low liver-related death rate, while approximately 20% of NAFLD cases have NASH that may progress to cirrhosis (20%-45%), a well-recognized hepatocellular carcinoma (HCC) risk factor (Dam-Larsen, 2004). In a large United States healthcare database study between 2002 and 2008, NAFLD was the most common underlying HCC risk factor (59%), followed by diabetes (36%) and hepatitis C infection (22%) (Sanyal *et al.*, 2010). In a large longitudinal study (173643 patients with diabetes and 650620 patients without) with a follow-up of 10-15 years, NAFLD incidence was higher among patients with diabetes (incidence rate 18.13 *vs* 9.55 per 10000 person-years, respectively, $P < 0.0001$) (El-

serag *et al.*, 2004). NAFLD is present in up to 90% of all obese persons and up to 70% of type 2 diabetes mellitus (T2DM) patients (El-Serag, 2011).

The economic healthcare burden of NAFLD/NASH is large. The annual direct medical costs associated with NAFLD in 2016 were estimated to be $103 billion in the USA and €35 billion in Europe (Hara *et al.*, 2020). It was expected by 2030 NAFLD burden could rise to $1.005 trillion (Puri and Fuchs, 2019). In a health economics analysis using claims data in 2017, the annual per-person cost of a new NAFLD diagnosis was $7,804 and the $3,789 annual per-person cost for long-term management (Allen *et al.*, 2018). Total costs were highest in patients aged between 45 and 65 years (Hara *et al.*, 2020). This increasing disease prevalence will impose an increasing economic burden and will be accompanied by an increasing number of patients with cirrhosis and end-stage liver disease requiring liver transplantation and an alarming increase in hepatocellular carcinoma (Younossi *et al.*, 2016).

2.1.1 Progression of NAFLD

NAFLD is a disease that has very variable rates of progression and clinical manifestations between individuals. NAFLD is an umbrella term that encompasses a continuum of liver diseases that vary in severity of the injury, beginning with the early stage of NAFLD, which is simply hepatic steatosis (fatty liver), followed by NASH, a more serious process involving inflammation and hepatocyte damage (steatohepatitis); typically, NASH is accompanied by pericellular fibrosis, which can progress to cirrhosis (Siddiqui *et al.*, 2018). Despite this, hepatic steatosis does not usually cause harm but can lead to severe liver damage, including cirrhosis, if it worsens. High levels of fat in the liver are also linked to an increased risk of serious health problems, such as diabetes, high blood pressure and kidney disease (Alshawsh et al., 2022; Lindenmeyer and McCullough, 2018). Hepatic steatosis, or NASH, can be strongly suspected in a person

based on imaging and clinical features (such as the presence of metabolic comorbidities and abnormal laboratory tests), but most patients with NASH are asymptomatic for decades with no clinically relevant findings, therefore histologic evaluation is required to diagnose NASH (Siddiqui *et al.*, 2018). The importance of managing NAFLD stems from the various contributing factors, including the environment, microbiome, metabolism, comorbidities, and genetic risk factors (Singh *et al.*, 2015).

2.1.2 Risk factors of NAFLD

The presence of MetS in a person is the most important risk factor for NAFLD/NASH. MetS are defined differently, but typically include increased waist circumference (i.e., obesity), hyperglycaemia, dyslipidaemia, and high blood pressure (Huang, 2009). The association between NAFLD and features of MetS may be bidirectional, particularly in diabetes and hypertension, meaning that MetS not only increases NAFLD risk but also several features and comorbidities of MetS may be exacerbated by NAFLD. Thus, effective treatment of NAFLD/NASH could have the added benefit of improving MetS properties. MetS also contributes significantly to the development of cardiovascular disease and all-cause mortality in NAFLD patients (Käräjämäki *et al.*, 2017).

Among the features of MetS, diabetes mellitus has the clearest biological link to the progression of NAFLD, and up to 75% of people with T2DM have NAFLD. In individuals with diabetes and NAFLD, the prevalence of NASH and advanced fibrosis is also increased compared to non-diabetic individuals with NAFLD (Bazick *et al.*, 2015). IR has been recognized for many years as an integral part of NAFLD pathogenesis and worsens as the disease progresses (**Figure 2.1**) (Godoy-Matos *et al.*, 2020). IR is characterised by reduced glucose disposal in non-hepatic tissues, including adipose tissue and muscle (Bugianesi *et al.*, 2005). In adipose tissue, IR results in the

inappropriate release of fatty acids through dysregulated lipolysis that further contributes to impaired insulin signalling throughout the body (Loomba *et al.*, 2012). Studies have shown metabolic cross-talk between adipose tissue and the liver. Adiponectin, IL-6 and other peptides released from adipose tissue have protective and pro-inflammatory effects on the liver (Sabio *et al.*, 2008). Another link between liver and adipose tissue defects may be the enzyme dipeptidyl peptidase 4, which can promote IR (Ghorpade *et al.*, 2018). In addition, NAFLD has been associated with changes in arterial stiffness, myocardial remodelling, renal disease and heart failure (Valbusa *et al.*, 2017). There is also evidence that the renin-angiotensin-aldosterone system's antagonism, a pathway that contributes to high blood pressure, may also improve NAFLD/NASH and liver fibrosis (Pelusi *et al.*, 2016).

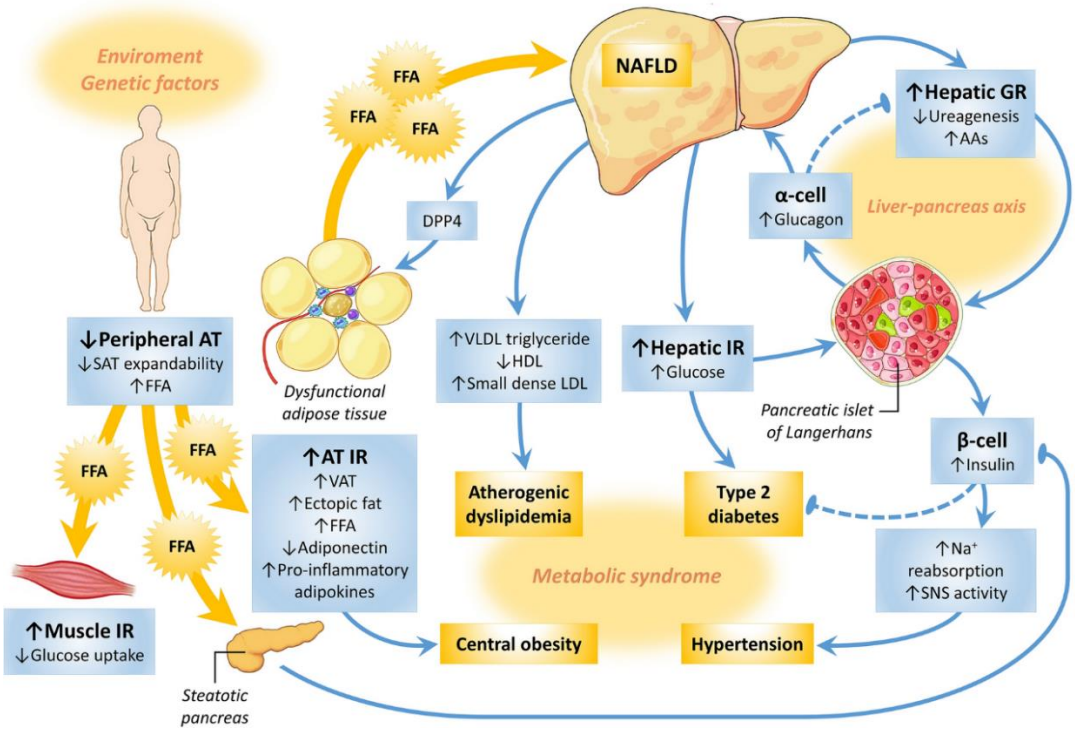

Figure 2.1. The figure shows the pathophysiology of non-alcoholic fatty liver disease (NAFLD) as a continuum from obesity to metabolic syndrome and diabetes. Source: (Godoy-Matos *et al.*, 2020): The source is distributed under the Creative Commons CC BY license, which allows the unrestricted use, distribution, and reproduction of the original work.

Genetically, NAFLD is found to be associated with many genetic variants (Zhao *et al.*, 2020), for example, single-nucleotide polymorphism in the PNPLA3 gene, which regulates lipolysis of hepatocyte lipid droplets (Bruschi *et al.*, 2017). The risk-associated PNPLA3-I148M variant is resistant to normal proteasomal degradation and accumulates on lipid droplets, interfering with lipolysis (BasuRay *et al.*, 2017). Interestingly, NASH risk in patients with this variant is only maximized when it coexists with obesity, demonstrating the additive effects of genetic and environmental factors in this disease (Stender *et al.*, 2017).

Alteration of gut microbial diversity (i.e., dysbiosis) in response to various environmental cues (choline deficiency, obesity etc.) is observed in NAFLD. In such a condition, growth and type of microbial strains vary and it is observed as a dynamic process in patients with confounding variables which affect the disease progression). Furthermore, this group of patients with concurrent MetS showed similar diversity abundance. Hence, gut microbiota composition and species richness in addition to the classical prognosis factors may play a significant role in the stage detection and severity of NAFLD (Ni *et al.*, 2022). Human studies document a gut microbiome among patients with NASH that is less complex than that of healthy subjects (Schnabl and Brenner, 2014) and indicate that weight loss also alters the microbiome (Liu *et al.*, 2017). The bacterial proteins were reported to function as ligands for G protein-coupled receptors (Cohen *et al.*, 2017), modulate the gut-liver axis through intestinal farnesoid X receptor signalling and release fibroblast growth factor 19 (FGF19), which regulates bile acid synthesis and also lipid and glucose metabolism (Schubert *et al.*, 2017). Dysbiosis could also lead to increased intestinal permeability, which can amplify many of these gut-derived effects (Ferolla *et al.*, 2014).

2.1.3 Pathogenesis of NAFLD

The "two-hit theory" is a conceptual framework used to explain the development and progression of non-alcoholic fatty liver disease (NAFLD). NAFLD is a condition characterized by the accumulation of excess fat in the liver in individuals who do not consume significant amounts of alcohol. The two-hit theory proposes that the development of NAFLD involves two distinct mechanisms or "hits" that contribute to liver damage (Peng *et al.*, 2020). First hit: Steatosis (Fat Accumulation) The first hit refers to the initial accumulation of fat in the liver, known as steatosis. It occurs when there is an excessive uptake and synthesis of fatty acids in the liver or a reduced ability to break down and export them. This can be caused by factors such as obesity, insulin resistance, high levels of free fatty acids in the blood, or dietary factors. The accumulation of fat in the liver can lead to hepatocyte injury and inflammation, although it may not necessarily progress to advanced liver disease. In some individuals, the liver can tolerate the excess fat without significant consequences. However, in others, the presence of additional factors can trigger the progression to more severe stages of NAFLD. Second hit: Inflammation and Progression The second hit refers to additional factors that promote inflammation and contribute to the progression of NAFLD. These factors can include oxidative stress, mitochondrial dysfunction, gut-derived endotoxins, and release of pro-inflammatory cytokines. These events can cause hepatocyte injury, leading to inflammation and the development of non-alcoholic steatohepatitis (NASH). NASH is a more advanced form of NAFLD characterized by liver inflammation, hepatocyte injury, and fibrosis (scarring). Fibrosis can progress to cirrhosis, a severe condition in which the liver tissue becomes extensively scarred, impairing its function. The second hit can also increase the risk of liver cell death (apoptosis) and promote the development of liver cancer (hepatocellular carcinoma) in some cases (Buzzetti *et al.*,

2016). Many molecular pathways contribute to the development of NAFLD (**Figure 2.2**), and it is not even certain that hepatic steatosis always precedes NASH. In addition, the pathogenic drivers are unlikely to be the same in all patients. Therefore, both the mechanisms leading to disease and their clinical manifestations are very heterogeneous (Alonso *et al.*, 2017).

In defining the pathogenetic drivers of hepatic steatosis and NASH, a useful conceptual framework is that the liver's capacity to handle the primary metabolic energy substrates, carbohydrates, and fatty acids, is overwhelmed, leading to the accumulation of toxic lipid species **(Figure 2.2)** (Rios *et al.*, 2021). By overloading fatty acids with high intake or by compromising their metabolism, they can serve as substrates for the formation of lipotoxic species that induce hepatocellular damage (**Figure 2.2**) (Reccia *et al.*, 2017), which is characterised by endoplasmic reticulum stress, a dysfunctional unfolded protein response, inflammasome activation, activation of apoptotic pathways, inflammation, and an enhanced wound response (Friedman *et al.*, 2018; Mota *et al.*, 2016). It was suggested that NAFLD progression is likely to depend on the complex interplay between environmental factors and genetic predisposition, via multiple mechanisms that involve liver crosstalk with other organs and tissues, especially the gut and adipose tissue (Corte *et al.*, 2014). Furthermore, the accumulation of extracellular matrix in the liver leads to progressive fibrosis, cirrhosis, hypertension and liver failure (Lade *et al.*, 2014). Fibrogenesis is driven by signalling from stressed or injured hepatocytes and activated Kupffer cells (KC; hepatic macrophages), resulting in the activation of resident hepatic stellate cells into myofibroblasts to produce matrix proteins faster than they are degraded (George *et al.*, 2003).

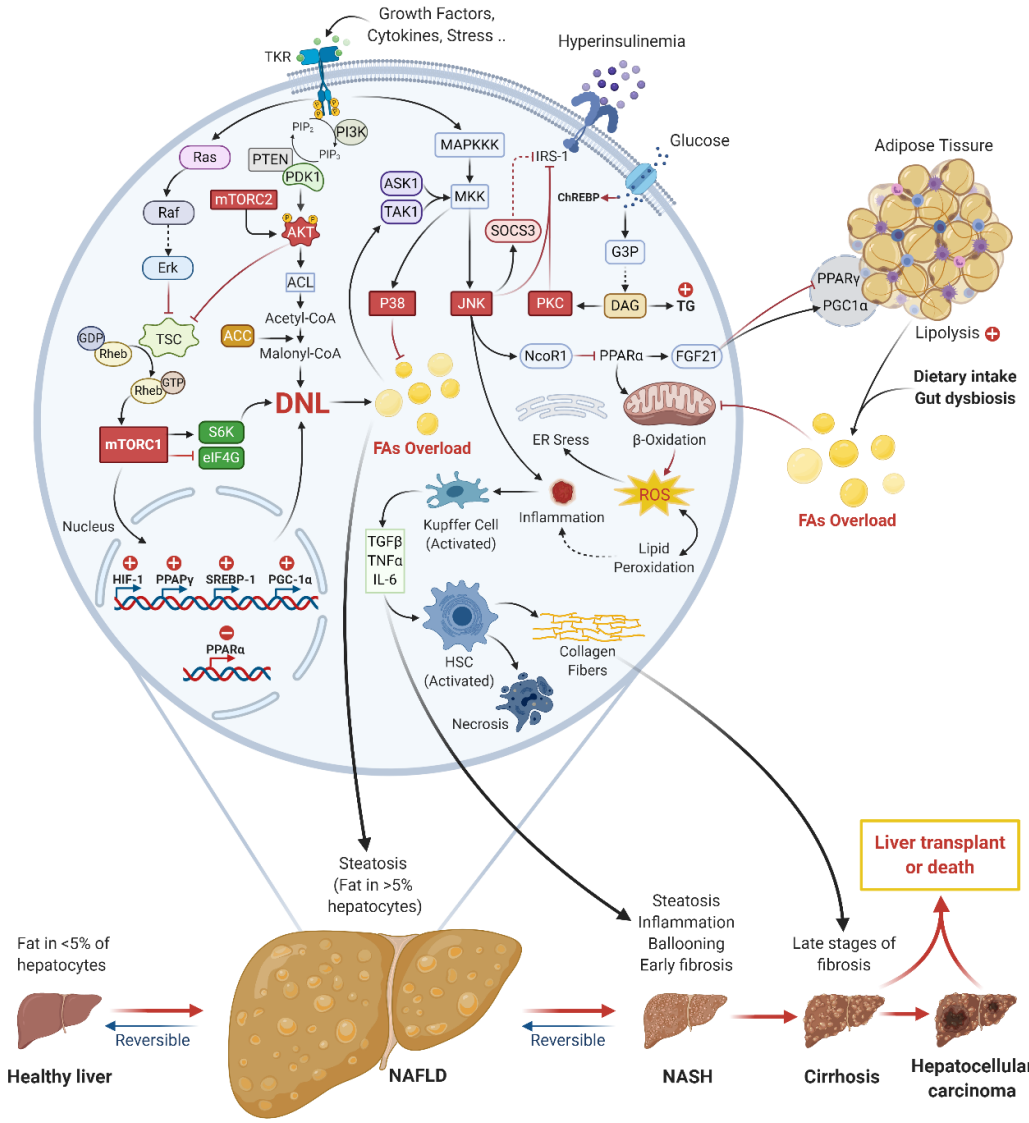

Figure 2.2. The cellular drivers of hepatic steatosis and NASH. Extracellular stimuli (e.g., FFAs overload, stress, and cytokines) can trigger many activities of the intracellular Kinases. JNK1/2 activation contributes to the development of hepatic steatosis. Within the hepatocyte, JNK1/2 is activated by MKK4/7 in response to cellular stress or FFAs overload. Also, JNK signalling inhibits IRS-1 causing IR. JNK1/2 up-regulates NcoR1 which will disrupt PPARα, a regulator of FGF21 expression, which activates adipocyte PGC1α and inhibits PPARγ. Also, increase the production of DAG, which inhibits IRS1 by activating PKC. DNL is increased by Akt, which is directly activated by mTORC2 and inhibited by PKC in response to increased free fatty acids. Also, activated Akt will activate mTORC1 by affecting the tuberous sclerosis complex (TSC). The activated mTORC1 enhances the expression of many lipid-related genes (e.g., SREBP-1, PGC-1α), besides activates of S6K, thus enhancing hepatic fat accumulation. Source: (Alshehade *et al.*, 2022)

2.1.4 The currently available treatments of NAFLD

Lifestyle modifications and treatment of underlying metabolic conditions should be performed in all NAFLD patients, while pharmacological treatment is mainly given to patients with biopsy-proven NASH and fibrosis (Jeznach-Steinhagen *et al.*, 2019). The Mediterranean diet is recommended, along with reducing calorie intake by 500-1000 kcal, to reduce hepatic steatosis and IR (Fraser *et al.*, 2008). Weight loss, induced by diet and physical activity, is also crucial for improving liver histology and even resolving NASH or fibrosis (Promrat *et al.*, 2010). Moderate physical activity for 150-200 min/week is recommended, even in patients with advanced chronic liver disease. Weight loss of at least 3-5% of body weight is recommended, and weight loss of 10% is associated with a greater decrease in portal pressure in compensated cirrhosis with portal hypertension (Hickman *et al.*, 2004).

No drug treatment has been approved by the Food and Drug Administration for NAFLD, but a few drugs are being studied with promising results. Vitamin E and the PPARγ ligand Pioglitazone are recommended for selected patients by the European- and American Association for the Study of the Liver. Vitamin E has anti-oxidative effects and has shown promising results in trials. It was shown that 800 IU once daily of Vitamin E resulted in a significantly higher rate of NASH improvement in non-diabetic patients. Possible side effects of Vitamin E include an increased bleeding risk, prostate cancer, heart failure, and haemorrhagic stroke (Paternostro and Trauner, 2022). Pioglitazone has shown improvement in liver histology, and 47% showed a resolution of definite NASH. Pioglitazone treatment has positive side effects such as improvement in insulin sensitivity and diabetic control but can cause negative side effects such as weight gain, fluid retention, and bone loss (AJ Sanyal et al., 2010).

Furthermore, several drugs have shown promising results in treating NAFLD in clinical trials, including GLP-1 agonists, SGLT2 inhibitors, and FXR ligands. GLP-1 agonists such as semaglutide and liraglutide have been shown to increase NASH resolution in patients with biopsy-confirmed NASH and reduce fibrosis (Rong *et al.*, 2023). However, their use is not recommended outside their labelled indications of treating diabetes mellitus and obesity. DPP-IV inhibitors have shown disappointing results and are not recommended for NAFLD patients. SGLT2 inhibitors have consistently shown improvement in imaging-based biomarkers and reduction of liver transaminases (Mirarchi *et al.*, 2022). FXR ligands such as obeticholic acid have shown improved liver histology and fibrosis in NAFLD patients but have an unfavourable effect on the lipid profile (Radun and Trauner, 2021). Aldafermin, an FGF19 mimetic, showed improved hepatic fat content but no improvement in fibrosis or NASH resolution after 6 months; long-term study results are awaited (Harrison, Neff, et al., 2021). FGF-21 mimetic Pegbelfermin reduced hepatic fat and liver transaminases, and improved lipid profiles, while Efruxifermin demonstrated promising results in a Phase IIa study, leading to further trials (Harrison, Ruane, et al., 2021). PPAR agonists have had mixed results, with Seladelpar improving liver enzymes but not hepatic fat, Elafibranor failing to meet its primary endpoint in a Phase III trial, Saroglitazar improving ALT and hepatic fat but not NAS, and Lanifibranor showing promise in a Phase 2b trial (Harrison SA, Ratziu V, 2020). Unfortunately, anti-inflammatory and anti-fibrotic therapies like Selonsertib, Cenicriviroc, and Simtuzumab have not demonstrated significant benefits in their respective studies (Loomba *et al.*, 2021). For advanced chronic liver disease, management focuses on liver-related complications and preventing hepatic decompensation (Paternostro and Trauner, 2022).

2.1.5 Pre-clinical experimental models of NAFLD

Pre-clinical animal models are essential to identify novel drug targets. Many studies using a variety of different NAFLD animal models have been published in recent years. All these models have their specific advantages and disadvantages. In terms of pathophysiology, it should be noted that the relatively high diversity between individual models can make it difficult to translate the results to human diseases. Therefore, it is a challenge to identify and develop clinically relevant and reliable NAFLD models that allow the generation of valid, reproducible and translational results (Jahn et al., 2019; Oligschlaeger and Shiri-Sverdlov, 2020).

2.1.5(a) *In vivo* models

The most commonly used animal models for studying NAFLD are summarized in **Table 2.1**, which also gives an overview of induction methods and experimental outcomes (Jahn *et al.*, 2019).

Table 2.1. The common animal models of NAFLD

Type of Mouse Model	Findings	References
Dietary Models		
High-fat diet (HFD)	The C57BL6/J mice were administered with an induction method consisting of HFD containing 60% fat, which led to the induction of obesity, insulin resistance, and hyperlipidemia after 10-12 weeks. Long-term exposure of 36 weeks showed either no or minimal signs of inflammation and fibrosis, while chronic feeding of 80 weeks resulted in hepatic steatosis, cell injury, portal and lobular inflammation, and fibrosis. However, it is important to note that the effects on liver pathology were highly variable and difficult to reproduce, and a long feeding period was necessary to achieve the desired outcomes.	(Chen *et al.*, 2019; Velázquez *et al.*, 2019)
Atherogenic diet	An induction method consisting of a diet containing 1.25% cholesterol and 0.5% cholate was found to increase plasma and liver lipid levels. Over a period of 6-24 weeks, the mice showed the induction of NASH with hepatocellular ballooning in a time-dependent manner. This method is mainly used as an animal model of atherosclerosis and does not result in weight gain or insulin resistance.	(Oligschlaeger and Shiri-Sverdlov, 2020)
High-fat atherogenic diet	An induction method involving a high-fat diet containing 1.25% cholesterol and 0.5% cholate was found to exacerbate NASH features, including hepatic insulin resistance, oxidative stress, and activation of hepatic stellate cells.	(Montandon *et al.*, 2019)
High-fat/high-cholesterol diet (HFC)	The induction method involved a HFC diet containing 21% milk butter and 0.2% cholesterol. In C57BL/6 mice fed with a short-term HFC diet, only steatosis was observed. However, male hyperlipidemic mice showed severe hepatic inflammation but no steatosis after seven days. In C57BL/6 mice fed with an HFC diet for seven months, the development of obesity, hepatomegaly, hepatic steatosis, and varying degrees of steatohepatitis was observed. It is important to note that this model lacks the human phenotype of obesity.	(Wouters *et al.*, 2010)
High-fat/high-cholesterol/ high-fructose diet (AMLN)	An induction method involving a diet containing 40% high-fat and 22% fructose, supplemented with approximately 18% trans-fat and 2% cholesterol, resulted in marked steatosis, moderate lobular inflammation, and hepatocellular ballooning in C57BL/6 and *ob/ob* mice after 26-30 weeks.	(Kristiansen *et al.*, 2016)

Gubra amylin diet (GAN)	An induction method involving a high-fat diet (40 kcal-%, with 0% trans-fat and 46% saturated fatty acids by weight), fructose (22%), sucrose (10%), and cholesterol (2%) was found to result in more pronounced weight gain and a highly similar phenotype of biopsy-confirmed fibrotic NASH in C57BL/6 and *ob/ob* mice after 8-16 weeks.	(Boland *et al.*, 2019)
High-fat/high-fructose/high-cholesterol	An induction method composed of 41% fat, 30% fructose, and 2% cholesterol was found to induce NASH in various models. However, the effects on liver pathology were highly variable.	(Abe *et al.*, 2019)
Soybean-oil-based Western-type diet	An induction method involving a western-type diet containing 25g/100g n-6-PUFA-rich soybean oil with or without 0.75% cholesterol was found to induce hepatic steatosis, inflammation, and fibrosis, weight gain, insulin resistance, hepatic lipid peroxidation, and oxidative stress in C57BL/6 mice after long-term exposure of 20 weeks.	(Henkel *et al.*, 2017)
High-caloric cholesterol-free HFD	An induction method consisting of lard (21g/100g)/soy-bean oil (3g/100g)/5% fructose in drinking water was found to induce only mild steatosis with no signs of hepatic inflammation and fibrosis.	(Henkel *et al.*, 2017)
Choline-deficient diet	C57BL/6 mice were fed with a high-fat diet (45% of calories) for 8 weeks, followed by choline-deficient (or choline-supplemented) diets during the final 4 weeks. This method was found to result in amplified liver fat accumulation while improving glucose tolerance.	(Raubenheimer *et al.*, 2006)
Methionine/choline-deficient diet (MCD)	An induction method involving a diet lacking methionine and choline but containing high sucrose (40%) and moderate fat (10%) was found to induce severe steatohepatitis with elevated serum AST and ALT levels after 2 weeks. After 10 weeks, additional Kupffer cell infiltration and irreversible fibrosis were observed. However, no signs of insulin resistance were noted within 1.5-4 weeks. It is important to note that this method lacks the human metabolic profile of systemic insulin resistance and may result in substantial weight loss.	(Montandon *et al.*, 2019)
Choline-deficient L-amino acid-defined diet (CDAA)	An induction method involving a choline-deficient L-amino acid-defined diet containing carbohydrates (68.5%), proteins (17.4%), and fats (14%) was found to induce fatty liver followed by mild features of NASH in C57BL/6J mice within a few weeks. After more than 20 weeks, mild-to-moderate fibrosis and insulin resistance were observed. However, this method does not recapitulate the metabolic features of human NAFLD.	(Van Herck *et al.*, 2017)

Choline-deficient L-amino acid-defined diet on a high-fat diet (CDAHFD)	An induction method consisting of a choline-deficient, L-amino acid-defined, high-fat diet containing 60 kcal% fat and 0.1% methionine by weight was found to result in excessive liver fat accumulation, increased circulating liver enzymes, and progressive hepatic fibrosis.	(Matsumoto *et al.*, 2013)
High-fat/high-fructose diet (ALIOS)	An induction method involving a high-fat diet with fructose-containing drinking water and additional administration of a low weekly dose of intraperitoneal carbon tetrachloride (CCl_4) was found to result in substantial steatosis with necro-inflammatory changes and increased ALT levels after 16 weeks. No difference in steatosis degree or ALT levels was observed when compared to the group without additional fructose. This method was able to induce progressive stages of human-like fatty liver disease.	(Tsuchida *et al.*, 2018)
Diet-induced animal model of non-alcoholic fatty liver disease (DIAMOND)	An induction method involving a high-fat/carbohydrate diet (Western diet) with 42% kcal from fat, containing cholesterol (0.1%), with a high fructose/glucose solution (23.1 g/L d-fructose +18.9 g/L d-glucose) was found to result in sustained obesity, liver injury, dyslipidemia, and insulin resistance for up to 52 weeks after 16 weeks of exposure.	(Santhekadur *et al.*, 2018)
High-fat diet + glucose/ fructose-enriched drinking water	An obesogenic diet containing 35.5% w/w crude fat (58 kJ%), 22.8 MJ/kg = 5.45 kcal/g, and fructose (55% w/v) and glucose (45% w/v) enriched drinking water was found to induce pro-inflammatory/fibrogenic states in C57BL/6 mice. However, voluntary wheel running was found to prevent these effects induced by a HFD.	(Gehrke *et al.*, 2019)
Genetic Models		
Leptin deficiency (*ob/ob*)	Leptin-deficient (*ob/ob*) mice are predisposed to developing NASH and fibrosis, but not when maintained on a regular chow diet. When treated with a high-fat/high-fructose/high-cholesterol diet, these mice lack the ability to spontaneously develop hepatic inflammation. However, after 12-26 weeks, increased adiposity, total cholesterol, and elevated plasma liver enzymes were observed upon a diet high in trans-fat (40%), fructose (22%), and cholesterol (2%). Treatment with a high-fat/high-fructose/high-cholesterol diet resulted in the development of metabolic, histologic, and transcriptomic features similar to human NASH.	(Abe *et al.*, 2019)

Leptin resistance (*db/db*)	*db/db* mice, which are deficient in the leptin receptor with dramatic elevations in circulating leptin concentrations, lack the ability to spontaneously develop hepatic inflammation and thus need to be combined with a nutritional model for NASH. Dietary intervention with a MCD diet for four weeks resulted in marked hepatic inflammation and fibrosis in these mice. However, this method does not reflect the natural etiology of NAFLD, and a second hit is required to induce NASH.	(Kennedy *et al.*, 2010)
MS-NASH (FATZO/Pco)	A fructose-supplemented diet administered for 20 weeks was found to result in hepatic steatosis, lobular inflammation, ballooning, and fibrosis in mice that spontaneously developed obesity.	(Sun *et al.*, 2019)
Apolipoprotein E2 knock-in (APOE2)	After replacing murine ApoE with the human APOE2 gene, mice were fed a HFC diet for 12 weeks, resulting in steatosis in conjunction with early but not sustained hepatic inflammation.	(Bieghs *et al.*, 2012)
ApoE deficiency (*ApoE*$^{-/-}$)	Complete deficiency in the murine ApoE gene resulted in abnormal glucose tolerance, hepatomegaly, weight gain, and the full spectrum of NASH after seven weeks of the Western diet, while lacking humanized lipoprotein profiles.	(Schierwagen *et al.*, 2015)
Low-density lipoprotein receptor deficiency (*Ldlr*$^{-/-}$)	Complete deficiency of the murine Ldl receptor, an important gene regulating the transport of non-modified lipids into macrophages, resulted in a resemblance to lifestyle-induced early-onset hepatic inflammation after three to twelve weeks of the HFC diet. The mice exhibited high and low levels of circulating low-density lipoprotein (LDL) and high-density lipoprotein (HDL), respectively, which closely mimicked the human lipoprotein profile, and the development of mild fibrosis.	(Bieghs *et al.*, 2012)
Microsomal prostaglandin E synthase 1 (mPGES1) deficiency	Mice with a global deletion of mPGES-133 were backcrossed on C57BL/6J and were found to exhibit a TNFα-dependent inflammatory response in murine liver, as well as an increased severity of diet-induced murine NASH.	(Henkel *et al.*, 2018)
Patatin-like phospholipase domain-containing 3 (PNPLA-3) knock-in	Mice carrying the I148M mutation in the Pnpla3 gene were fed a high-sucrose or HFD for four weeks, resulting in the accumulation of PNPLA3 on lipid droplets and the development of hepatic steatosis.	(Smagris *et al.*, 2015)

Transmembrane 6 superfamily member 2 knockdown (mTm6s2-shRNA8)	Short hairpin RNA knockdown of Tm6sf2 in the liver of C57BL/6J mice using adeno-associated virus resulted in increased hepatic fat content and decreased very low-density lipoprotein (VLDL) secretion.	(Kozlitina *et al.*, 2014)
Gankyrin liver-specific knockout (GLKO)	LoxP-Gank mice were backcrossed with Cre-Alb mice resulting in GLKO mice. Gankyrin was found to generally drive liver proliferation. After 6-7 months of HFD, a higher degree of hepatic steatosis was observed in GLKO mice compared to wild-type mice; however, GLKO mice were found to be protected against fibrosis development.	(Cast *et al.*, 2019)
Truncated mutation in Alström (Alms1) gene (*foz/foz*)	A mutation in the Alström gene ALMS1 caused an 11-base pair truncation, but the exact role of Alms1 was unknown. After 6 months of HFD, mice with the mutation developed MetS features such as obesity, hyperglycaemia/lipidaemia, and insulin resistance. These mice also spontaneously developed steatosis, hepatic inflammation, and fibrosis.	(Jiang *et al.*, 2019)
Fatty liver Shionogi	Severe liver steatosis, inflammation, advanced fibrosis, and spontaneous HCC were observed after backcrossing mice with *ob/ob* mice. The mice exhibited spontaneous development of hepatic inflammation with a rather mild degree of fibrosis, which was characterized by uncontrollable heterogeneity in disease onset.	(He *et al.*, 2015)
Hepatocyte-specific phosphatase and tensin homolog deficiency ($Pten^{-/-}$)	Steatosis, inflammation, and fibrosis in the liver were observed after 40 weeks of age in mice with PTEN deficiency specifically in the liver.	(Takakura *et al.*, 2018)
Augmenter of liver regeneration knock-out ($Alr^{-/-}$)	Steatohepatitis with hepatocellular necrosis, ductular proliferation, and fibrosis were induced 4-8 weeks after birth through liver-specific deletion of augmenter of liver regeneration.	(Van Herck *et al.*, 2017)
Melanocortin 4 receptor knockout ($Mc4r^{-/-}$)	A targeted disruption of the melanocortin 4 receptor was introduced in mice, which is a seven-transmembrane G protein-coupled receptor expressed in the hypothalamic nuclei. The mice developed simple steatosis, and upon feeding a HFD, human-like NASH developed with obesity, insulin resistance, and dyslipidaemia. After 20 weeks of HFD, the mice showed signs of moderate fibrosis, functionally mimicking the human NASH disease state.	(Yamada *et al.*, 2019)

Chemically-induced Models		
Carbon tetrachloride (CCL_4)	Following biweekly injections of CCl_4, Balb/C mice demonstrated increased circulating liver enzymes and dose-dependent progression of liver fibrosis after 6 weeks.	(Domitrović *et al.*, 2009)
Thioacetamide	Following the combination of a western-type diet and three times/week intraperitoneal injection of thioacetamide (75 mg/kg), C57BL/6 mice developed hepatic inflammation, severe diffuse fibrosis, and collagen deposition after 8 weeks.	(Santhekadur *et al.*, 2018)
Streptozotocin + high-fat diet (STAM)	After being injected with 200 μg streptozotocin at 2 days after birth and fed ad libitum with a high-fat diet at 4 weeks of age, mice developed hepatic inflammation, hepatocellular ballooning, progressive fibrosis, and HCC between 6-20 weeks of age. However, the mice also exhibited reduced body weight and insulin levels compared to those that were fed an HFD.	(Fujii *et al.*, 2013)

2.1.5(b) *In vitro* models

The biological models used for the *in vitro* study of NAFLD range from monolayer cell cultures to more complex 3D cultures (Müller and Sturla, 2019). The purpose is to recapitulate the biology of NAFLD and identify the specific pathways involved in its pathogenesis and to find useful therapeutic targets for drug development.

In addition to hepatocytes, various non-parenchymal cells such as KC, liver endothelial cells or hepatic stellate cells (HSC) can also influence liver biology (Müller and Sturla, 2019). A close look into the previous studies shows that researchers predominantly favoured 2D monocultures (59.4%) over more complex models (2D co-cultures (14%), spheroids (9.7%), organoids (7.3%), liver-on-a-chip (7.8%), collagen gel sandwiches (1.2%), and micropatterned cultures (0.6%). That could be resulting in the relatively high cost and need for experience with the special culture technic of the complex *in vitro* models. In addition, monocultures have been used widely in conjunction with additional *in vivo* experiments, or as a benchmark for more complex *in vitro* models (Ramos *et al.*, 2022). However, *in vitro* monoculture models of NAFLD do not accurately recapitulate the pathological mechanisms of liver disease and take advantage of the complex models, an increasing trend to adopt 3D *in vitro* models, particularly in on-chip cultures. This indicates that 3D culture systems are becoming increasingly relevant in this area (Pelechá *et al.*, 2022; Ramos *et al.*, 2022).

2.1.1(b)(i) Monoculture models

Depending on their origin, cell lines are either tumour-derived (commonly known as immortalized), primary cell lines, or pluripotent stem cells. The closest *in vitro* model to the human liver is primary human hepatocytes, as they show specific hepatic functions, such as metabolic detoxification of foreign compounds, glycogen synthesis and storage, lipid metabolism, urea and albumin production, and the