

Non-alcoholic fatty liver disease: Immunological mechanisms and current treatments

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Abstract

Non-alcoholic fatty liver disease (NAFLD) causes significant global disease burden and is a leading cause of mortality. NAFLD induces a myriad of aberrant changes in hepatocytes at both the cellular and molecular level. Although the disease spectrum of NAFLD is widely recognised, the precise triggers for disease progression are still to be fully elucidated. Furthermore, the propagation to cirrhosis is poorly understood. Whilst some progress in terms of treatment options have been explored, an incomplete understanding of the hepatic cellular and molecular alterations limits their clinical utility. We have therefore reviewed some of the key pathways responsible for the pathogenesis of NAFLD such as innate and adaptive immunity, lipotoxicity and fibrogenesis, and highlighted current trials and treatment options for NAFLD patients.

Key Words: Liver; Fat; Inflammation; Mitochondria; Immune system

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Core Tip: Non-alcoholic fatty liver disease (NAFLD) is a significant global disease burden and a leading cause of mortality causing aberrant changes in hepatocytes. Although the disease spectrum is widely recognised, precise triggers for disease progression remain poorly understood. Whilst some progress has evolved in terms of treatment, there are still no approved pharmacological therapies for NAFLD treatment due to the incomplete understanding of the hepatic cellular and molecular alterations in disease pathogenesis.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFL) (NAFLD) is the most common cause of liver disease globally which occurs due to an excessive accumulation of fat in the liver in the absence of secondary causes or other liver disease aetiologies[1]. NAFLD encompasses the spectrum of disease from simple NAFL, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and in many cases hepatocellular carcinoma (HCC)[1]. Due to increasing trends of sedentary lifestyles and dietary choices the prevalence of NAFLD continues to rise[1].

NAFLD has a significant global disease burden (882 million cases in 2017)[2], with cases likely to be higher due to poor screening of high risk asymptomatic populations. Furthermore, inaccurate disease progression predication markers and a lack of available licenced therapeutics hinders the treatment of NAFLD. Management of NAFLD is largely based on lifestyle modifications, but many newer therapies are being evaluated (discussed later). Further understanding into the mechanisms of disease progression, diagnostic biomarkers and therapeutic intervention should help mitigate burden of disease. Finally, liver disease combination therapies are becoming increasingly popular. This review will highlight some of the recent developments in NAFLD and aims to define the pathological features associated with disease progression including the immunological mechanisms as well as the current therapeutic interventions used for NAFLD.

Epidemiology and prevalence

Global reports relating to the epidemiology of NAFLD have estimated a global prevalence of NAFLD between 25% and 35% [3-5], with Europe as high as 30% [6], 35% in South American countries[6,7], and 35% in North America[6]. During the period of 1991 to 2019, trend analysis shown that increasing global prevalence increased yearly by 0.7%, rising from 21.9% in 1991 to 37.3% in 2019[5].

NAFLD has a global impact on health care systems due to its high rates of morbidity and mortality[8]. NASH is a particularly prevalent cause of chronic liver disease which in turn leads to cirrhosis and HCC, whereas NAFLD has been noted as the biggest cause of HCC in the United States, France, and the United Kingdom, with NAFLD-related HCC predicted to increase globally alongside the rise in obesity[3]. In the United States, by 2030, almost 49% of the total population is projected to be obese[3].

Globally, the phenotype of patients with NAFLD appears to be men of a mean age of 51.7 years old, with obesity and/or type 2 diabetes[6]. A linear increase prevalence of NAFLD, diabetes and metabolic disorders have also been documented[9,10], and especially occurs in those with central obesity, diabetes and metabolic syndrome[11,12]. Metabolic comorbidities include hypertension (37%) and metabolic syndrome (40%)[6]. NAFLD prevalence in type-2 diabetes mellitus (T2DM) is as high as 70% [13], and patients with T2DM also have a twofold increased risk of all-cause mortality [4,14]. Prevalence of NAFLD in patients with morbid obesity also rises to 90% [4]. Therefore, prevalence of NAFLD poses a significant global health burden requiring clinical attention.

Histopathology and disease spectrum

NAFLD is the most common cause of liver dysfunction with a high association for obesity and insulin resistance (IR). The spectrum of NAFLD can lead to progressive NASH, fibrosis, and lastly HCC and liver failure. NAFLD is a complex disease which results from environmental causes as well as polygenic background and risk factors (Figure 1). Although the molecular mechanisms underlying disease progression are complex, the histological spectrum of disease has been well described[15].

NAFLD is defined as a disease of the liver which is characterised by macrovesicular fat deposition and storage (> 5% of the hepatocytes) due to dysregulation in the mechanisms of fat synthesis and utilisation by the liver[7,16]. Steatosis is predominantly graded on a four-step scale, from 0 to 3. Grade 0 is defined as a normal liver containing fat in < 5% of hepatocytes[17,18]; grade 1 occurs when fat deposits occur in < 33% of hepatocytes, and grade 2 when fat occurs in 33%-66% [17,18]; grade 3 is the final stage in the spectrum of steatosis which occurs when > 66% hepatocytes contain fat[17,18]. The most important histological feature of NASH is hepatocyte ballooning and lobular inflammation as well as a steatotic liver. Hepatocyte ballooning is the second histological feature of NASH. Hepatocyte ballooning is defined by a clear, flocculent, not vacuolar cytoplasm with a ballooned shape. Inflammation occurs in a lobular pattern in NASH, containing Kupffer cells (KC), aggregates of neutrophils, and Mallory-Denk bodies[15]. NASH is defined as a chronic state of inflammation whereby hepatic stellate cells (HSCs) transform into myofibroblasts[19]. These transformed cells produce extracellular collagen matrix[19]. Normally fibrogenesis is a wound healing process. However, in NAFLD, sustained and progressive insults occurring over many years causes unregulated fibrogenesis[19]. Initially collagen deposits form in the perisinusoidal space. As collagen bundles form, architectural remodelling occurs which can lead to cirrhosis and HCC. Fibrosis stage 1 (F1) occurs when mild perisinusoidal/pericellular fibrosis is documented without septa[20]. Stage two occurs when fibrosis occurs in perisinusoidal/pericellular and portal/periportal regions with few septa (F2)[20]. Stage three (F3) is seen when numerous septa are documented, also known as bridging fibrosis[20]. Lastly, stage four is a cirrhotic liver[20]. An overview of NAFLD progression is shown in Figure 1.

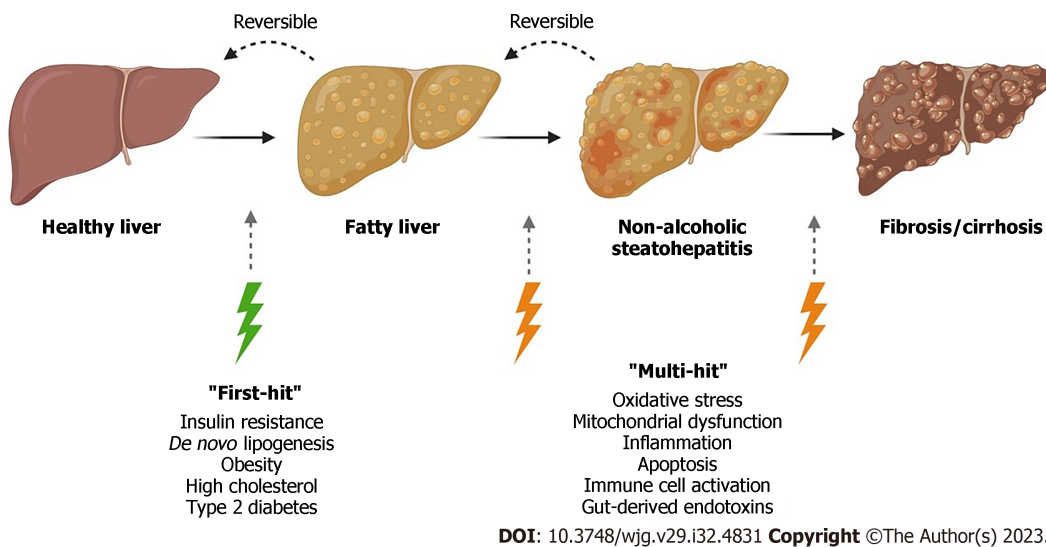


Figure 1 Overview of the current understanding of non-alcoholic fatty liver disease progression. Non-alcoholic fatty liver disease (NAFLD) encompasses a range of liver damage, from simple accumulation of fat in liver cells, named steatosis, to more severe forms of the diseases such as steatohepatitis, involving inflammation which can lead to fibrosis and cirrhosis. In the “multiple-hit” theory of progression, the first cause or “first-hit” in NAFLD is insulin resistance, obesity, type 2 diabetes, and metabolic syndrome. As the first hit occurs, free fatty acids are stored in the liver as triglycerides, resulting in simple steatosis. Disease progresses when multiple factors, or “multi-hits”, such as oxidative stress, inflammatory mediators, apoptosis, and mitochondrial dysfunction cause liver damage (created with BioRender.com).

Progression of the fibrosis stage to a cirrhotic scarred liver can vary between individuals, with cirrhosis occurring up to 15 to 20 years after initial diagnosis[19,21]. The median survival rate for patients with compensated cirrhosis is approximately 9 to 12 years, however, patients with decompensated cirrhosis have a significantly lower median survival rate of approximately 2 years[22]. Patients in the compensated stage are frequently asymptomatic, and often remain undiagnosed[22]. Therefore, early detection of cirrhotic patients who are still in the compensated stage is crucial, as early diagnosis could prevent or slow down disease progression. The onset of decompensated cirrhosis occurs when symptoms such as ascites, hepatic encephalopathy, and/or gastroesophageal variceal haemorrhage are found[22]. Histologically, cirrhosis is diagnosed when a disconnection of the hepatocytes from the central vein occurs as well as capillarisation of the liver sinusoids[19]. These modifications that occur to the liver structure and integrity lead to elevated intravascular resistance within the portal system and decreased hepatic perfusion, ultimately causing a loss of liver function[19]. Hence, timely diagnosis and management of cirrhosis is required to improve patient outcomes.

IR in NAFLD

Many studies have shown that metabolic dysfunction and IR play a significant role in the development of NAFLD. Various animal and clinical studies suggest that chronic low grade inflammation may play a role in the development of IR and NAFLD as well as extrahepatic complications such as cardiovascular diseases, T2DM, and renal dysfunction[23]. IR is a complex state by which the skeletal muscle, liver, and adipose tissue become less sensitive to insulin and its metabolic effects[24]. IR is related to obesity, hypertension, hyperglycaemia and metabolic syndrome. During a carbohydrate-rich diet, excess glucose is converted into fatty acids *via* lipogenesis, using acetyl-CoA which is generated from glycolysis-driven pyruvate[25]. These fatty acids are then incorporated into very low-density lipoproteins (VLDL) for transport to white adipose tissue for storage[25]. Accumulation of lipids in adipocytes trigger downstream signalling of pathways including c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB), leading to production of proinflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-6[26,27]. JNK regulates the production of proinflammatory cytokines, karyomitosis, and cellular apoptosis, and therefore, contributes to inflammation and IR. Research has proposed activation of JNK also accelerates lipid accumulation, thus, exacerbating liver injury. JNK-1 deficiency in adipose tissue shows protection from development of hepatic steatosis and promotes glucose intolerance, insulin clearance, IR, and hepatic steatosis[24]. Kluwe *et al*[28] also showed that in a mouse model, inhibition of JNK lead to modulation of fibrosis in hepatocytes. In a high fat diet (HFD) model of NAFLD, IR, liver injury and increased autophagy were documented[29]. However, JNK inhibition decreased autophagy and IR[29]. Therefore, JNK signalling plays a significant role in NAFLD progression.

The hormone adiponectin is secreted by adipocytes and is associated with improved insulin sensitivity. Adiponectin-mediated signalling is also able to stimulate fatty acid β-oxidation, glucose utilisation and uptake, as well as suppression of fatty acid synthesis[30]. Adiponectin can also inhibit the glycerol 3 phosphate (G3P) pathway; however, research has shown that during NAFLD serum levels of adiponectin are reduced[30]. Another metabolite of the G3P pathway is fatty acyl-CoA which is involved in mitochondrial β-oxidation. In patients with NAFLD, an increase in mitochondrial β-oxidation can lead to a state of oxidative stress due to increased substrate delivery to the mitochondrial electron transport chain (ETC), increasing reactive oxygen species (ROS) and damaging mitochondrial DNA[30,31]. Although β-oxidation is upregulated, ATP levels are decreased in NAFLD due to reduced activity of ETC complexes I and IV[32], and have been

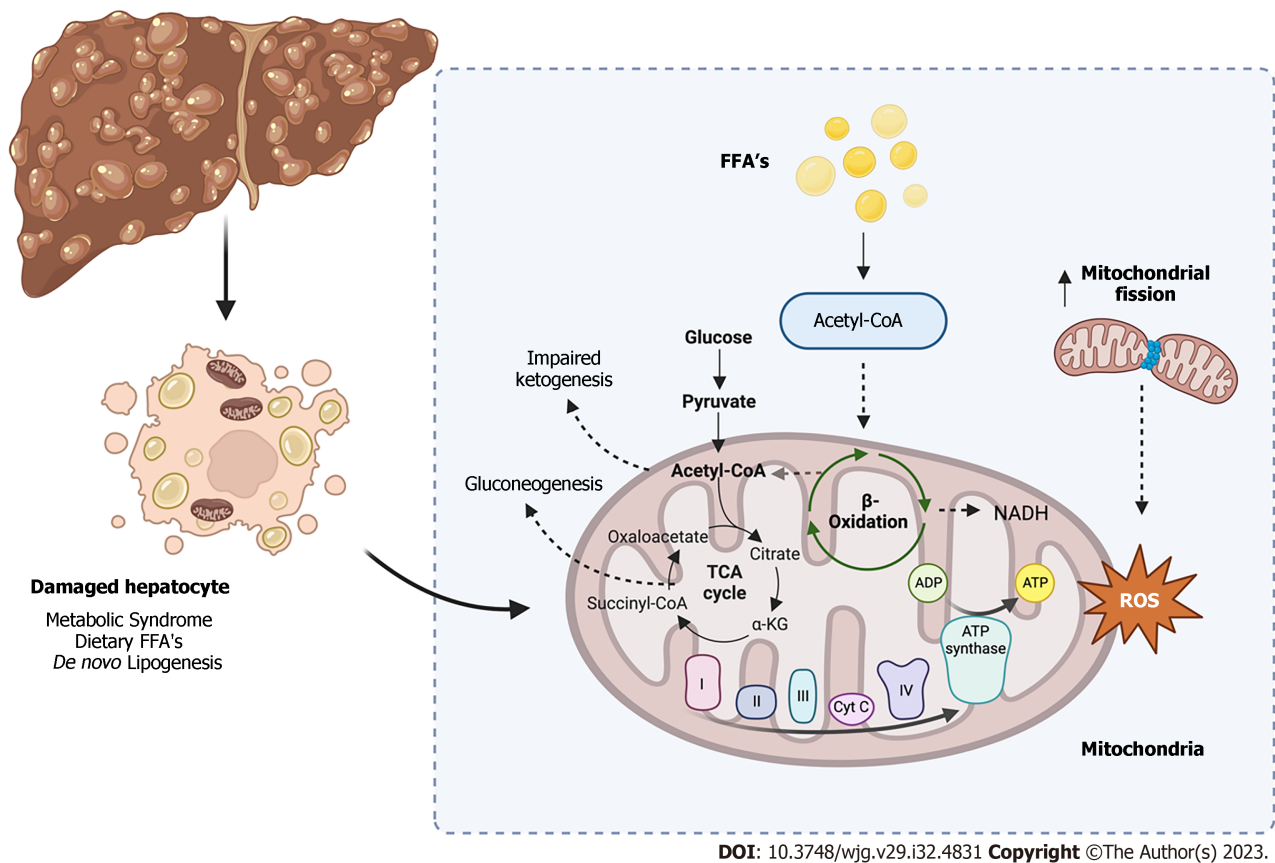


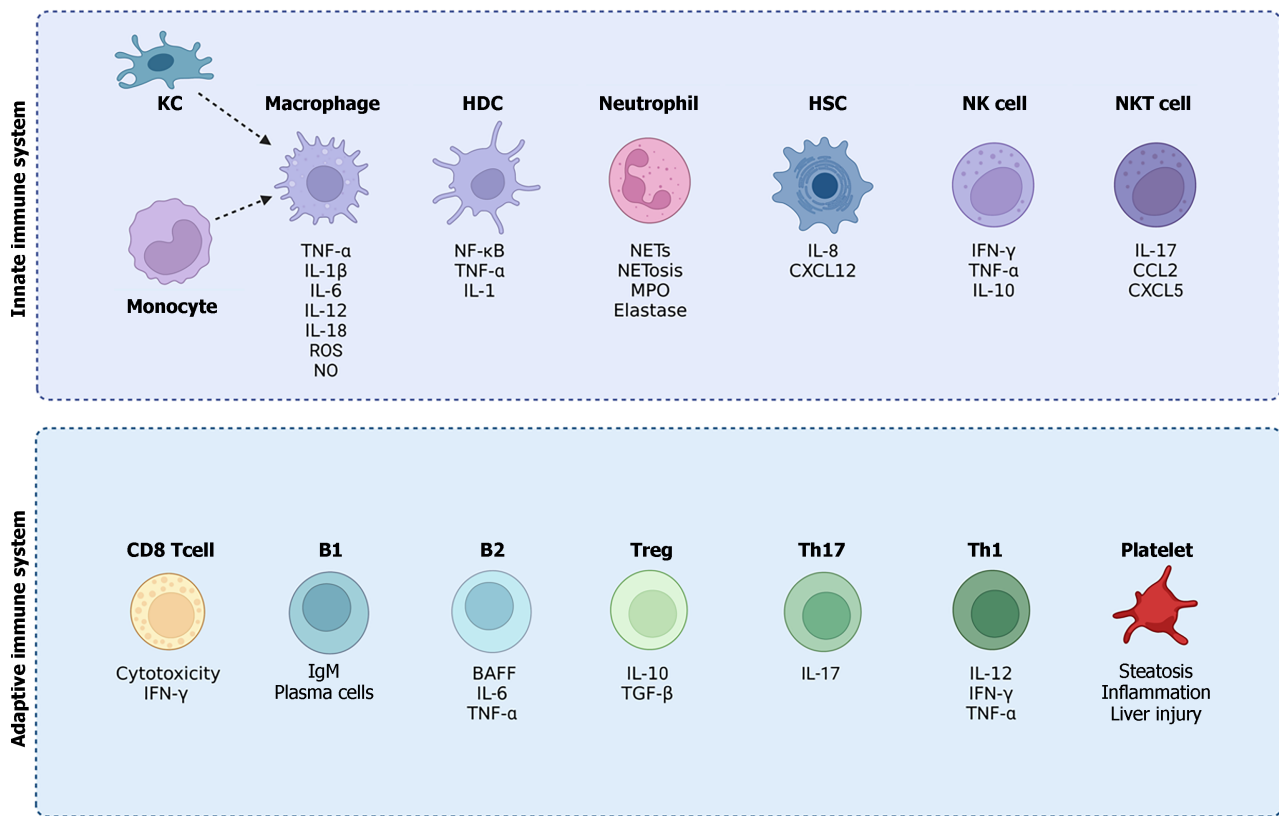
Figure 2 Overview of mitochondrial dynamics in non-alcoholic fatty liver disease. The accumulation of triglycerides and free fatty acids (FFAs) in the liver are converted to fatty acyl-CoA, which is then transported to the mitochondria for β -oxidation, generating acetyl-CoA. However, increased accumulation of FFA can cause insufficient hepatic β -oxidation, triggering inflammation and oxidative stress. Acetyl-CoA enters the mitochondrial tricarboxylic acid cycle allowing continuation of gluconeogenesis. The by-product nicotinamide adenine dinucleotide, produced through β -oxidation are transported to the electron transport chain (ETC) for oxidative phosphorylation. Disruption and dysfunction of the ETC can cause electron leakage and hepatocyte damage leading to reactive oxygen species production and oxidative stress. Accumulation of FFAs can cause mitochondrial damage, resulting in increased mitochondrial fission and degradation *via* mitophagy processes (created with BioRender.com). ROS: Reactive oxygen species; NADH: Nicotinamide adenine dinucleotide; TCA: Tricarboxylic acid; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate.

correlated to levels of disease progression. Therefore, modifications to mitochondrial function can exacerbate disease progression in NAFLD. Hyperinsulinemia alongside increased levels of ROS also leads to an imbalance between mitochondrial fission and fusion proteins such as dynamin-related protein 1 (Drp1) and mitofusin-2 proteins (Mfn2)[33]. It is possible that an excessive mitochondrial fission in the liver plays an important role in NAFLD progression. Increases in the Drp1-to-Mfn2 ratio causes enhanced mitochondrial fission which in turn causes reduced endoplasmic reticulum association, decreases oxidative phosphorylation capacity, and elevated mitochondrial ROS production[33], which can exacerbate disease state. Therefore, it is plausible that a reduction in mitochondrial oxidative capacity as well as the impairment of metabolic fuels provides a plausible relationship between mitochondrial dysfunction, lipotoxicity and IR. Therefore, metabolic health and mitochondrial function are fundamental to progression of NAFLD (Figure 2).

The inflammasome pathway and macrophages are known to play a significant role in the development of IR. Production of IL-1 β and IL-18 can be regulated by the inflammasome pathway, which contains complexes including nucleotide-binding oligomerization domain (NOD)-, leucine-rich repeats-and pyrin domain-containing protein 3 (NLRP3) and activation of caspase-1[34]. In NAFLD, inflammasome formation can be activated upon a variety of stress stimuli such as damage-associated molecular patterns, pathogen-associated molecular patterns, for example, lipopolysaccharide (LPS) from the gut-liver axis, mitochondrial ROS, endoplasmic reticulum stress, and ROS[34-36]. Activation of the NLRP3 inflammasome has shown to contribute to disease progression from steatosis to NASH[34,37]. mRNA levels of NLRP3 inflammasome components such as NLRP3, caspase-1, IL-1 β , and IL-18 were also found to be significantly upregulated in NAFLD patients[37-39]. Therefore, targeted modulation of the inflammasome will lead to improved insulin signalling and mitochondrial function.

Immunological Mechanisms

In chronic liver disease, the activation of the immune response can both restore tissue function as well as cause tissue injury. Therefore, an amplified immune response may lead to organ dysfunction. The following section describes the involvement of various immune cell types in the pathogenesis of NAFLD (Figure 3).



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Figure 3 Overview of innate and adaptive immune cells involved in non-alcoholic fatty liver disease (NAFLD). Activation of immune cells can promote inflammation and liver injury in NAFLD. The innate immune system cells include Kupffer cells, monocytes, and macrophages as well as hepatic dendritic cells, neutrophils and natural killer cells. Natural killer T cells provide a bridge between both the innate and adaptive immune system. The adaptive immune system includes T cells such as CD8 T cells and CD4 T cells (Th1, Th17 and regulatory T cells). The adaptive immune system also comprises of B1 and B2 cells as well as platelets. Although the contribution of immune cells to the development of NAFLD has been explored, the crosstalk between the distinctive immune cell subsets in the pathogenesis of NAFLD requires further investigation (created with BioRender.com). KC: Kupffer cells; TNF- α : Tumour necrosis factor-alpha; IFN- γ : Interferon- γ ; IL: Interleukin; ROS: Reactive oxygen species; NO: Nitric oxide; NETs: Neutrophil extracellular traps; NF- κ B: Nuclear factor-kappa B; HDC: Hepatic dendritic cells; MPO: Myeloperoxidase; HSC: Hepatic stellate cells; CXCL: C-X-C motif chemokine ligand; BAFF: B cell activating factor; TGF- β : Transforming growth factor- β ; NK: Natural killer; NKT: Natural killer T cells; Treg: Regulatory T cells.

Innate immune system in NAFLD: During NAFLD, the innate immune system is involved in the activation of resident KCs, with recruitment of innate immune cells such as neutrophils, monocytes, natural killer (NK) cells and natural killer T (NKT) cells[40], and inflammation is further exacerbated *via* the production of cytokines, (e.g. TNF- α , IL-1 β , IL-6, IL-12, IL-18), chemokines, nitric oxide (NO) and ROS[40-42].

Macrophages: The main role of macrophages in the liver is predominantly for immunoregulatory and detoxifying functions, however, alterations in macrophage phenotypes and dynamics have been reported to be involved in the pathogenesis of NAFLD[43,44]. Liver resident KCs are a large population of macrophages as well as other subsets of macrophages such as monocyte-derived macrophages and liver capsular macrophages[45]. M1 macrophages have a pro-inflammatory phenotype and are induced by mediators such as LPS and interferon- γ (IFN- γ) and their activation causes secretion of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α [46,47]. On the other hand, M2 macrophages have an anti-inflammatory phenotype and are induced by Th2 cytokines such as IL-4 and IL-13, causing secretion of anti-inflammatory factors such as IL-10 and transforming growth factor- β (TGF- β)[46,47]. M2-type macrophages have also been reported to induce M1-type KCs apoptosis decreasing disease progression[48]. Therefore, the balance between M1 and M2 macrophages is important for homeostasis in the liver.

During NAFLD, macrophages can recognise and respond to stimuli through pattern recognition receptors such as membrane-bound toll-like receptors (TLRs), such as TLR4 and TLR9, and cytoplasmic NOD-like receptors [43,45,49]. LPS translocated from the gut can activate TLR4 and TLR9, and this recognition of stimuli can then result in inflammation *via* secretion of proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-12, and IL-18, as well as molecules such as ROS and NO. Activation of TLR9 can induce the release of IL-1 β from KCs, which occurs during processes such as lipid accumulation, fibrinogenesis and cell death[50]. In NAFLD patients, TLR4 expression is often correlated with hepatic inflammation and fibrosis[51]. There are also many advantages to the activation of macrophages due to their immunosuppressive effects and secretion of anti-inflammatory cytokines, however, they also have pro-fibrinogenic effects. Activation of M2-type macrophages can release TGF- β 1 and IL-13, leading to a fibrotic response of liver remodelling and tissue repair[52].

During IR, circulating free fatty acids (FFA) can also directly activate KCs causing activation of the stress-response kinases (JNK1 and JNK2) producing pro-inflammatory cytokines and extracellular vesicles (EVs), stimulating macrophage activation. EVs have been studied in their contribution to pathobiology of NAFLD, and have been found to mediate inflammation[53,54]. Hepatocytes can release EVs containing cargoes which activate signalling pathways[53,54]. Some EVs can carry mitochondrial DNA, which in mice and humans have been shown to activate macrophages *via* TLR9 [53,54]. Activation of TLR9 then causes inflammation through resultant downstream activation of NF- κ B-dependent pro-inflammatory cytokines in macrophages[53,54]. Macrophage activation and inflammation may also occur *via* EV formation in response to lipid-associated toxicity[55]. Therefore, both harmful and beneficial macrophage phenotypes can co-exist during NAFLD, and the balance between these phenotypes must be considered for therapeutic strategies. Both cellular and molecular macrophage targets for therapy may provide a new perspective as well as the adoptive transfer therapies.

Neutrophils: Neutrophil infiltration during NASH has been documented in both patients and in mouse models. In the early stages of NAFLD, recruitment of neutrophils occurs *via* chemokines such as C-X-C motif chemokine ligand (CXCL) 1, IL-8, and CXCL2[56]. Research has shown that various neutrophil specific components are released in NAFLD. Neutrophil elastase is a major inflammatory protease which can be released by neutrophils. In a mouse model of NAFLD, elastase suppression has been shown to improve disease severity[57]. As well as elastase, neutrophil proteinase-3 has also been reported as elevated in NASH and both the levels of proteinase-3 and elastase were correlated with liver fibrosis [58]. Elastase has also been shown to play a role in metabolic dysfunction and IR, and therefore, targeting or improving IR and metabolic disease may improve inflammation in NAFLD. Myeloperoxidase (MPO), a neutrophil derived enzyme has been shown to become increased in NASH. It is thought that both neutrophils and MPO may cause activation of HSCs inducing liver fibrosis and inflammation in NASH[59,60]. Plasma levels of MPO have been found to be correlated with fibrosis in NAFLD patients[61]. More research is required to determine how neutrophils contribute to disease pathogenesis in NAFLD.

NKT cells: NKT cells provide a bridge between the innate and adaptive immune system and express both NK cell and T cell markers[40]. NKT cells become activated upon antigen presentation by CD1d which can be expressed by a variety of cells such as hepatocytes, macrophages, dendritic cells, and B cells[40]. Pro-inflammatory cytokines such as IL-4 and IFN- γ are secreted after NKT activation causing further tissue injury. Although there is conflicting evidence, during steatosis levels of NKT cells become reduced, whereas in NASH and fibrosis they are increased[40]. This has been shown by *in vivo* studies where NKT cells were elevated in the blood and liver of patients with moderate-to-severe steatosis[62]. During early-stage NAFLD such as fatty liver, the phenotype of NKT cells demonstrate a pro-inflammatory Th1 cytokines profile such as IFN- γ , whereas, in advanced end stage disease, they have a profibrotic role[40].

NK cells: NK cells have a cytotoxic role and can attack cells *via* perforin-mediated pathways or cell-cell interactions, for example Fas. NK cells can also act as regulatory cells by releasing various cytokines and chemokines, such as IFN- γ , TNF- α , and IL-10 as well as growth factors[40]. NK cell-associated cytotoxic ligands, such as TNF-related apoptosis-inducing ligand (TRAIL), NK group 2 member D, and major histocompatibility (MHC) class I chain-related protein A and B mRNAs, have been reported to be elevated in obese NASH patients[63]. NK cells therefore may possibly promote inflammation and hepatocyte apoptosis *via* TRAIL secretion[63], leading to progression of fibrosis.

Dendritic cells: Hepatic dendritic cells (HDCs), which highly express MHC-class II molecules and CD45 are important immune cells in the liver which have migratory capabilities as well as production of cytokines such as TNF- α , IFN- γ , IL-2, IL-4, and IL-6[64]. Three distinct subsets of HDCs have been described in experimental models of HDCs: Lymphoid, myeloid and plasmacytoid[64]. They play a crucial role in the progression of metabolic steatohepatitis bridging lipid metabolism and inflammation. HDCs can shift from a tolerant state to an active state, triggering an inflammatory process [65]. In an immune tolerant state, immature HDCs secrete IL-10, and TGF- β , as well as limit T cell expansion[64]. In a tolerant state they suppress inflammasome activation, maintaining homeostasis in the liver. This regulatory role of HDC in NASH can therefore restrict inflammation as well as clear apoptotic cells and necrotic debris[65]. Additionally, they are involved in lipid storage within the liver and are important for antigen presentation and induction of inflammatory pathways[64]. Recent studies suggest that HDCs have antifibrogenic effects by activating metalloproteinases, such as matrix metalloproteinase 9 which has been found to be involved in regression of fibrosis as well as remodelling of the extracellular matrix.

On the other hand, in an active state HDCs cause inflammation and liver damage as well as contribute to fibrosis. In an active or inflammatory state, mature recruit macrophages to the liver and can activate the NF- κ B pathway as well as produce pro-inflammatory cytokines such as TNF- α and IL-1, contributing to the inflammatory microenvironment[64]. In humans, a subset of HDCs (CD11C + cDC2) have been shown to play an important role in development of fibrosis and have been positively correlated with metabolic steatohepatitis[64]. HDC's in a mouse model of metabolic associated fatty liver disease (MAFLD) have shown that in a matured form they produce more inflammatory cytokines and may be responsible for proinflammatory responses[65]. HDC may therefore play dual roles in both the suppression and progression of disease state.

Adaptive immune system in NAFLD: The adaptive immune system is defined by antigenic specificity and immunological memory and includes predominantly B and T lymphocytes. Experimental research has suggested that sustained immune responses activated by oxidative stress-related antigens can affect the pathogenesis of disease *via* activation of CD4+ T-cells, which, in turn, stimulate macrophage M1 responses and liver CD8+ T- and NKT cell recruitment[66]. Experimental *in vitro* data as well as *in vivo* data supports adaptive immunity in NAFLD disease progression.

T cells: Conventional T cells have been well studied in their involvement in the pathogenesis of NAFLD. The main CD4+ T-cell are divided into Th1, Th2, and Th17 populations characterised by production of specific cytokines. Recruitment of CD4+ T-cell in the liver have been reported to be increased in individuals diagnosed with NASH[67-71],

as well as in animal models fed a high calorie diet[72]. Upon presentation to inflammatory stimuli, CD4+ T cells can differentiate into Th17 cells[73], a subset of pro-inflammatory T helper cells defined by their ability to produce IL-17. In animal models of NAFLD, in the liver it has been documented that the Th-17 phenotype was favoured, promoting inflammation[74]. Human studies in obese and overweight patients have also shown an increase in the Th17 population[74]. The IL-17 family of cytokines have been implicated in the progression of fatty liver disease through interference of the insulin signalling pathway[75]. In mouse models, deficiency of IL-17A, IL-17F or IL-17A receptor (IL-17RA) results in increased steatosis but reduced steatohepatitis[76,77]. In HFD mouse model, neutralisation or IL-17RA deficiency as well as treatment with anti-IL-17mAb therapy has shown to protect mice from diet-induced liver injury *via* improvement of lipid accumulation, suppressing KCs activation, decreased pro-inflammatory cytokines levels and inhibition downstream of NF- κ B signalling[40,78]. IL-17 has also been shown to activate the signal transducer and activator of transcription 3 pathway in HSCs causing progression of fibrosis in the liver[79]. These studies indicate the involvement of IL-17 in the pathogenesis of NAFLD.

Regulatory T cells (Tregs), a subset of cells which promote immune tolerance and facilitate tissue repair and express the transcription factor forkhead box P3. Although there is limited data available, the number of hepatic Tregs have been documented to be decreased in animal models of NAFLD[80]. In humans, the levels of Tregs in the liver and the circulation of patients with NAFLD is also reported to be decreased[80]. Also, in a NASH mouse model, induced by a HFD and endotoxin challenge, a decrease in liver Tregs was documented, however, transfer of Tregs into mice showed reduced liver injury and inflammation, through decreased expression of TNF- α [81].

B cells: Until recently, the role of B cells in the pathogenesis of NAFLD was less understood. B cells are highly specific antibody producing cells of the adaptive immune system. B cells contribute to approximately 6% of intrahepatic cells[40, 82]. B cells produce the B cell activating factor (BAFF), a cytokine controlling the process of B cell survival and maturation [83,84]. Liver biopsies from patients with NASH have been shown to contain both B and T cells in the inflammatory infiltrates [73,85,86].

In mouse models, B cells have been shown to become activated in NASH, concomitantly with the onset of steatohepatitis and thus, maturing to plasma blasts and plasma cells[73,86]. Another study has also shown that BAFF signalling increased IR in an NAFLD model as well as promoting fatty liver[87]. Whereas, in mice models of NASH, B2 cell responses upregulate BAFF[73]. Levels of BAFF in patients with NASH appear to occur at a higher level than with patients with fatty liver, therefore it has been proposed BAFF levels correlate with severity of steatohepatitis fibrosis[88]. BAFF receptor-deficient mice showed an improvement in HFD-induced obesity and IR, as well as a reduction in the number of B cells and a decrease in serum immunoglobulin G level[40]. The involvement of B cells in the progression of liver disease can also be due to production of pro-inflammatory mediators and antigen-presentation[89,90]. In patients with NASH, MHC class II molecules become upregulated causing inflammatory infiltration and recruitment of CD4+ and CD8+ T cells to the liver[73], whereby Th1 CD4 + T cell activation and IFN- γ production occurs[86]. Therefore, this research suggests B cells and BAFF play an important role in NAFLD pathogenesis and can contribute to pathogenesis of NAFLD *via* autoimmune hepatitis and liver fibrogenesis [87,90,91].

Serum levels of IgA has been found to be associated with patients with NASH and levels of IgA can predict advanced liver disease progression[92]. Patients with HCC have also been documented to have higher serum IgA[93-95]. In both mouse models and human studies, liver resident IgA accumulation in hepatic cells has been associated with chronic inflammation and fibrosis[93]. Regression of HCC has also been documented when IgA is inhibited, causing reactivation of CD8+ T cell function[93,94].

B cell deficiency also has been shown to improve development of NASH. The mechanisms and signalling of B cell activation in NASH is *via* myeloid differentiation early response protein 88[40,96]. Studies have shown that elimination of myeloid differentiation early response protein 88 on B cells reduced hepatic T cell-mediated inflammation and fibrosis, but had no effects on steatosis[40,96]. Therefore, B cells show involvement in the pathogenesis of NAFLD, and manipulation on B cells may provide a therapeutic opportunity for NAFLD treatment. An overview of innate and adaptive immune cells involved in NAFLD is shown in [Figure 3](#).

Diagnostic procedures

Most commonly a combination of clinical history, laboratory findings, biopsies and radiological testing are used in the diagnosis of NAFLD. The clinical diagnosis of NAFLD is usually considered when aminotransferases become elevated or imaging on the liver detects high hepatic fat[97]. Both the American and European guidelines agree that suspicion of fibrosis warrants a confirmatory liver biopsy[98].

Current biomarkers: Liver function tests, particularly alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, can indicate liver damage in NAFLD/NASH[12,99]. These levels may be elevated by two to four times the normal limit. Mild elevations in aminotransferase levels may be present in lower stages of the disease, but routine tests may appear normal. Alkaline phosphatase and gamma-glutamyl transferase (GGT) levels may be up to three times the upper limit even in the absence of advanced disease[100]. A serum GGT cut-off value of 96.5 can predict advanced fibrosis with a sensitivity of 83% and specificity of 69%[101].

Several different biomarker panels have been described in their use to assess liver fat. These include the liver fat score (LFS) (sensitivity 86%, specificity 71%), hepatic steatosis index (sensitivity 92.5%, specificity 92.4%), fatty liver index (FLI) (sensitivity 87%, specificity 86%) and the steatotest (sensitivity 95.5%, positive predictive value 97.0%)[102]. LFS is calculated using a patients serum AST/ALT ratio, fasting serum AST level, fasting serum insulin level, any presence of metabolic syndrome and diagnosed diabetes mellitus. Studies have shown that a score greater than -0.640 can predict NAFLD with a sensitivity of 86% and specificity of 71%. The FLI uses waist circumference, body mass index (BMI), triglyceride, and GGT and this was initially developed to detect fatty liver in western countries.

Screening tools: The fibrosis-4 scoring system uses a patient's age, platelet count, AST, and ALT to determine a liver fibrosis score and can help to predict advanced fibrosis. A score < 1.45 defines a negative predictive value or low probability of advanced liver fibrosis[103]. Scores of A score > 3.25 indicated a higher likelihood and has a positive predictive value of 65%, and specificity of 97% at predicting advanced fibrosis[103]. Recently, a new FAST™ screening tool has been used which comprises a combination of FibroScan® parameters such as liver stiffness measurement and controlled attenuation parameter as well as AST. This screening tool has been shown to have diagnostic accuracy when predicting those at risk of NASH. The FAST™ screening tool may provide a better non-invasive algorithm for diagnosis of NASH. FAST™ performed better than other non-invasive algorithms for the diagnosis of at-risk NASH[104,105]. Results have shown that the FAST score had the highest area under curve for the most high-risk NASH criteria as well as liver stiffness showing a consistently acceptable performance in predicting NASH[106].

Emerging biomarkers: Various non-invasive biomarkers for NAFLD have been developed and validated over the last 20 years, however, there is a need to develop and validate new biomarkers for NAFLD which encompass IR, inflammation and fibrogenesis[107-109].

Cell Death: As well as current markers such as cytokeratin 18, necroptosis is a form of cell death which is characterised by changes such as organelle swelling, plasma membrane damage, and release of cellular contents[110]. Proteins such as receptor-interacting protein kinase (RIPK) family members such as RIPK1 and RIPK3, and mixed lineage kinase domain-like (MLKL) are involved in the process[111]. During NAFLD, TLR4 ligands such as LPS cause activation of the TLR4 receptor causing activation of proteins such as RIPK3 and MLKL leading to necroptosis[112]. In patients with MAFLD, necroptosis components such as RIPK3 have been shown to be upregulated, therefore, it may be important to consider markers of necroptosis in NAFLD.

MicroRNAs (miRNAs): miRNAs are non-coding RNAs which play a role in gene expression regulation[113] and have been implicated as potential biomarkers in NAFLD. Research has shown that miRNAs such as miR-21, miR-34a, miR-122, and miR-451 have been found to be upregulated in NAFLD patients[114-117]. Various studies have also shown that profiles of miRNAs in NAFLD patients can differentiate between disease stage. In NAFLD and NASH, miR-122, miR-192, miR-19a, miR-19b, miR-125b, and miR-375 have been found to be increased greater than 2-fold, whereas continuous upregulated expression of miR-122, miR-192, and miR-375 was found in NASH patients[118,119]. Furthermore, higher expression of miR-122 and miR-21 have been reported in people with high fasting blood glucose, obesity and fatty liver infiltrations[119,120]. Therefore, these findings suggest that miRNAs may be promising biomarkers for NAFLD and metabolic disease, in particular miR-122, miR-192, and miR-34a, which have been shown to be correlated with severity of NAFLD.

EVs: Growing evidence suggests that EVs play a significant role pathological disease including those associated with obesity and metabolic syndrome. Obesity, IR, T2DM and NAFLD have all been linked to changes in the abundance and phenotype of circulating EVs[121] and therefore circulating EVs and their composition may be a candidate biomarker for NAFLD. EVs contain substances such as genetic material including miRNAs[122]. It has been documented that in humans, the number of AD-EVs is correlated with IR in overweight people[123], as well as visceral AD-EV number correlated with liver injury measured by ALT and AST and metabolic syndrome[124]. Circulating EVs have also been shown to become elevated during the progression of NASH and have reported to be correlated with histological findings [125]. Research has also shown that EVs from a NAFLD model in mice contained miR-122 and miR-192[125]. In cirrhotic patients, plasma hepatocyte-EVs were found to contain elevated levels of CK-18[126]. EVs from visceral adipose tissue can exacerbate disease in NAFLD by causing further inflammation, fibrosis and IR. Both pro- and anti-fibrotic EVs have been documented in the liver and therefore it is plausible to investigate the differences in the pro- and anti-fibrotic phenotypes to track disease progression and likelihood of fibrosis development. Future research should consider the use of EVs in monitoring metabolic dysfunction and IR in NAFLD, with focus on EV phenotypes, cargo and cell specific markers.

Therapeutic interventions

Despite NAFLD being an extremely prevalent liver disease, no specific pharmacological interventions are currently Food and Drug Administration approved for treatment. Some therapeutic agents used as anti-diabetics, antilipidemic and natural bile treatments have previously been evaluated in their ability to treat liver disease, although they have limitations. Predominantly, lifestyle interventions including diet and exercise are most commonly used to treat NAFLD. The current therapeutic interventions used for NAFLD are outlined below including future potential agents such as sirtuins, antioxidants and vitamins.

Lifestyle modifications: A substantial amount of research indicated that changes to lifestyle are a primary approach for the treatment of NAFLD[127]. Diet changes and weight loss can reverse liver disease. Weight loss in the range of 5%-10% can provide beneficial effects to NAFLD patients *via* a reduction in NAFLD activity score (NAS)[128,129]. A weight reduction greater than 10% has also been shown to reduce the severity of fibrosis as well as resolution of NASH[12,129, 130].

European Association for the Study of the Liver (EASL), National Institute for Health and Care Excellence and American Association for the Study of Liver Diseases guidelines provide recommendations in terms of diet and physical activity for the management of NAFLD. These bodies recommend caloric restriction with a calorie deficit of 500-1000 kcal a day, as well as limiting the consumption of alcohol, fats and coffee. EASL also favours the Mediterranean diet, with studies indicating a reduction in liver fat in NAFLD patients[131]. Lifestyle modifications in terms of physical exercise can also positively effect liver fat content. Both resistance/weight training, high-intensity interval training and aerobic exercise have all equally been shown to reduce liver fat content; however, in men with NAFLD, high-intensity interval

training was the most effective in restoring hepatic fat, reducing hepatic stiffness and improving Kupffer cell function; key features of NASH[132].

Evidence has suggested that the ketogenic diet (KD) is an effective treatment for NAFLD. Ketogenesis is a metabolic process resulting in the production of ketone bodies, namely acetoacetate, beta-hydroxybutyrate (BHB), and acetone, which act as alternative energy sources[33,133]. The KD has been shown to change hepatic mitochondrial fluxes and redox state as well as significantly reducing liver fat content and hepatic IR[134]. These changes were found to be accompanied by an increase in the net hydrolysis of liver triglycerides, a decrease in endogenous glucose production, and lower serum insulin levels[134]. BHB has also been shown to interact with inflammasomes[135,136] and neutralise ROS [137], leading to a reduction in inflammatory cytokines and oxidative damage *via* its antioxidant capacity. These findings suggest a KD can contribute to the reversal of NAFLD through improvement of IR and cellular redox function.

Bariatric surgery: Bariatric surgery been shown to be an effective treatment for NAFLD improving overall liver health *via* facilitating weight loss, improving insulin sensitivity, and subsiding inflammation[138,139]. Roux-en-Y gastric bypass (RYGB) and laparoscopic sleeve gastrectomy (LSG) are the most prevalent bariatric procedures, which lead to significant weight loss and metabolic health improvements[140].

Several research studies have investigated the impact of bariatric surgery in NAFLD and have shown positive results. Studies have indicated that bariatric surgery resulted in significant improvements in liver enzymes and histology, with a decrease in liver fat and fibrosis. Results from two meta-analyses have shown that treatment of NAFLD using bariatric surgery resulted in a biopsy-confirmed resolution of steatosis (56%-66%), inflammation (45%-50%), ballooning degeneration (49%-76%), and fibrosis (25%-40%), as well as showing a decrease in NAS scoring[140-142]. Bariatric surgery has therefore proven to be effective in ameliorating NAFLD, however, it is important to clarify which type of surgery is most effective. A study by Baldwin *et al*[143] compared RYGB and LSG against its effectiveness at improving AST and ALT concentration, NAS and NAFLD fibrosis score. Overall, both procedures reduce AST and ALT levels, however, LSG showed slightly more favourable results[139]. Another study has shown NAS scoring reduced significantly in patients who underwent both surgery types 12-mo after the surgery[139]. RYGB patients had a more significantly decreased steatosis and superior improvement in plasma lipid profile[144]. Furthermore, bariatric surgery has been demonstrated to lower the risk of liver-related complications and death in individuals with NAFLD. Bariatric surgery can therefore be considered as a promising treatment option for those with NAFLD who are overweight or obese.

Insulin sensitising agents: IR is believed to play an essential role in the development and progression of NAFLD. In recent years, various insulin sensitizers such as biguanides, thiazolidinediones (TZDs), glucagon-like peptide-1 receptor agonists (GLP-1), and dipeptidyl peptidase 4 inhibitors have been investigated as potential therapeutic targets for NAFLD[145]. However, there are safety concerns associated with long-term use of these targets[145].

Biguanides: As the development of NAFLD is closely associated with IR, diabetes, hyperglycaemia and hyperlipidaemia, antidiabetic drugs are often utilised in the treatment of NAFLD[145]. Metformin is considered the first-line treatment for T2DM due to its ability to improve insulin sensitivity and promote weight loss without causing hypoglycaemia[145,146]. Although its mechanisms of action are not fully understood, it works by lowering hepatic glucose production[145]. Previous open-label studies have suggested that metformin may have a positive impact on hepatic steatosis and necroinflammation, although excessive weight loss may have confounded these results[30]. However, some studies have shown that metformin does not significantly improve the histological response in NAFLD [30,147], but improves liver function and BMI[148]. In a mouse model, metformin treatment showed improvements to the gut-liver axis *via* attenuation of the loss of tight junction proteins in the small intestine as well as reducing the increase of endotoxin levels in the portal circulation[149].

GLP-1: GLP-1 receptor agonists can alter IR by promoting weight loss *via* suppressing appetite and delaying gastric emptying[150]. In NAFLD patients, research has shown GLP-1 receptor agonists improve hepatic and adipose tissue IR, suppress de novo lipogenesis and oxidative stress as well as increased clearance of VLDL[30,150]. GLP-1 may also modulate the immune system *via* the reprogramming of macrophages to the M2 phenotype. In the exenatide study of cardiovascular event lowering trial, GLP-1 receptor agonists reduced cardiovascular risk[151] and visceral fat, improved glucose tolerance, body fat percentage and resting energy rate (NCT01144338). The trial semaglutide unabated sustainability in treatment of type 2 diabetes (NCT02054897) are a series of phase III clinical trials which suggest treatment with semaglutide has a higher effectiveness than other GLP-1 therapeutics in the reduction of HbA1c in patients with T2DM. In a phase 2 trial with patients diagnosed with NASH receiving semaglutide treatment of 0.1 mg (80 patients), 0.2 mg (78 patients), or 0.4 mg (82 patients), patients resulted in a significantly higher NASH resolution when compared to the placebo (NCT02970942)[152]. Although NASH resolution was significantly improved, no significant difference was shown in the fibrosis stage[152].

Fibroblast growth factor (FGF) based therapeutics: FGF analogs have been proposed to target steps of disease pathogenesis. FGF-21 treatments are currently in clinical development for the treatment of NASH[153,154] and data suggests that FGF21 is anti-fibrotic and has the potential to improve the metabolic syndrome and is effective in treating NASH. The BALANCED trial (NCT03976401) evaluated the effects of efruxifermin, a long-acting Fc-FGF21 fusion protein [155]. In this study, 80 patients were given either placebo ($n = 21$) or efruxifermin 28 mg ($n = 19$), efruxifermin 50 mg ($n = 20$) or efruxifermin 70 mg ($n = 20$) *via* weekly subcutaneous injection for 16 wk[155]. Treatment with efruxifermin was found to significantly reduced hepatic fat fraction (HFF) in patients with F1-F3 stage NASH[155]. FGF based compounds which are currently in phase II are efruxifermin (FGF-21), pegbelfirmin (FGF-21), aldafermin (FGF-19), pegozafermin (FGF-21) and BFK8588A (FGF-21), which have been shown to achieve a reduction in ALT levels[109].

Thyroid hormone receptor- β (THR β) 1 agonists: Currently, several different THR β specific agonists have been shown to produce positive therapeutic effects in both animal models and clinical trials for treatment of NAFLD. Treatment with

TG68, a novel THR β agonist, has positive effects on resolution of NAFLD *via* reduction in liver weight, hepatic steatosis, serum transaminases, and circulating triglycerides in a NAFLD model[156]. Resmetirom (MGL-3196) has also been found to significantly reduce hepatic lipid content and improve liver enzyme levels and plasma lipid levels in NASH patients [157], however, glucose or insulin levels remained unchanged. Another THR β agonist, VK2809 has been shown to reduce hepatic steatosis in a mouse model and in a phase II clinical trial reduced liver lipid content[158,159].

Currently there are many ongoing clinical trials studying the effects of THR β agonists in NAFLD. The VOYAGE study is assessing VK2809 to determine its efficacy in the resolution of biopsy proven NASH (NCT04173065). The DUET study is also assessing the effects of orally administered TERN-501 and TERN-101 in presumed NASH (NCT05415722), whilst the LIFT study is assessing TERN-101 alone in NASH patients (NCT04328077)[160]. In a NASH mouse model, TERN-501 reduced steatosis as well as reducing serum total cholesterol, triglyceride and ALT levels[161].

Peroxisome proliferator-activated receptor- γ (PPAR- γ) agonists: PPAR- γ is a ligand-activated nuclear receptor and its activation causes insulin sensitization and enhances glucose metabolism. TZDs are a group of insulin sensitizers used to treat T2DM by action on PPAR- γ . PPAR agonists have been shown to modulate the innate immune response. PPAR- δ can promote anti-inflammatory polarization of macrophages and modulate their activation[162], whilst PPAR- γ ligands can inhibit the activation of macrophages and cytokine production (TNF- α , IL-6, and IL-1 β), thus, reducing inflammation [163]. Activated PPARs can also regulate immune cells (macrophages, DCs, T cells, and B cells) as well as decrease inflammatory cytokine production[163].

Pioglitazone, a TZD, has been used for treatment of NASH with its effects improving steatohepatitis, ballooning degeneration and lobular inflammation[164]. Pioglitazone (45 mg/d) for 6 mo has been shown in patients with prediabetes or T2DM to improve the fibrosis stage (NCT00227110)[165]. Elafibranor is a dual PPAR- α/δ agonist. PPAR- δ functions to regulate peroxisomal β -oxidation of FFA as well as improve insulin sensitivity, lipid and glucose homeostasis[30]. Several clinical trials have assessed the effects of elafibranor in improving histology in NASH patients such as RESOLVE-IT (NCT02704403), and the GOLDEN trial[166]. In both trials elafibranor showed no effect.

Sodium glucose cotransporter 2 (SGLT2) Inhibitors: Another class of drugs lower serum glucose levels *via* inhibition of the SGLT2 and promote weight loss. Studies have shown that SGLT2 inhibitors reduce ALT levels correlating with changes in bodyweight and glycaemic control[30], although further studies are required to identify whether SGLT2 inhibitors can prevent progression of NAFLD/NASH.

Current clinical trials are underway to assess the efficacy and effectiveness of SGLT2 inhibitors. A randomised clinical trial aims to compare the effect of the pioglitazone and empagliflozin combination on liver fat mass (NCT04976283). Another trial is investigating dapagliflozin in NASH (NCT05254626).

Farnesoid X receptor (FXR) agonists: The FXR is a nuclear receptor which is activated by bile acids. FXR inhibits the expression sterol regulatory element binding protein-1 and carbohydrate-responsive element-binding protein. FXR also enhances the clearance of high-density lipoprotein and VLDL in the liver and well as promoting hepatic regeneration. However, little is documented regarding the immune modulation in this class of drugs.

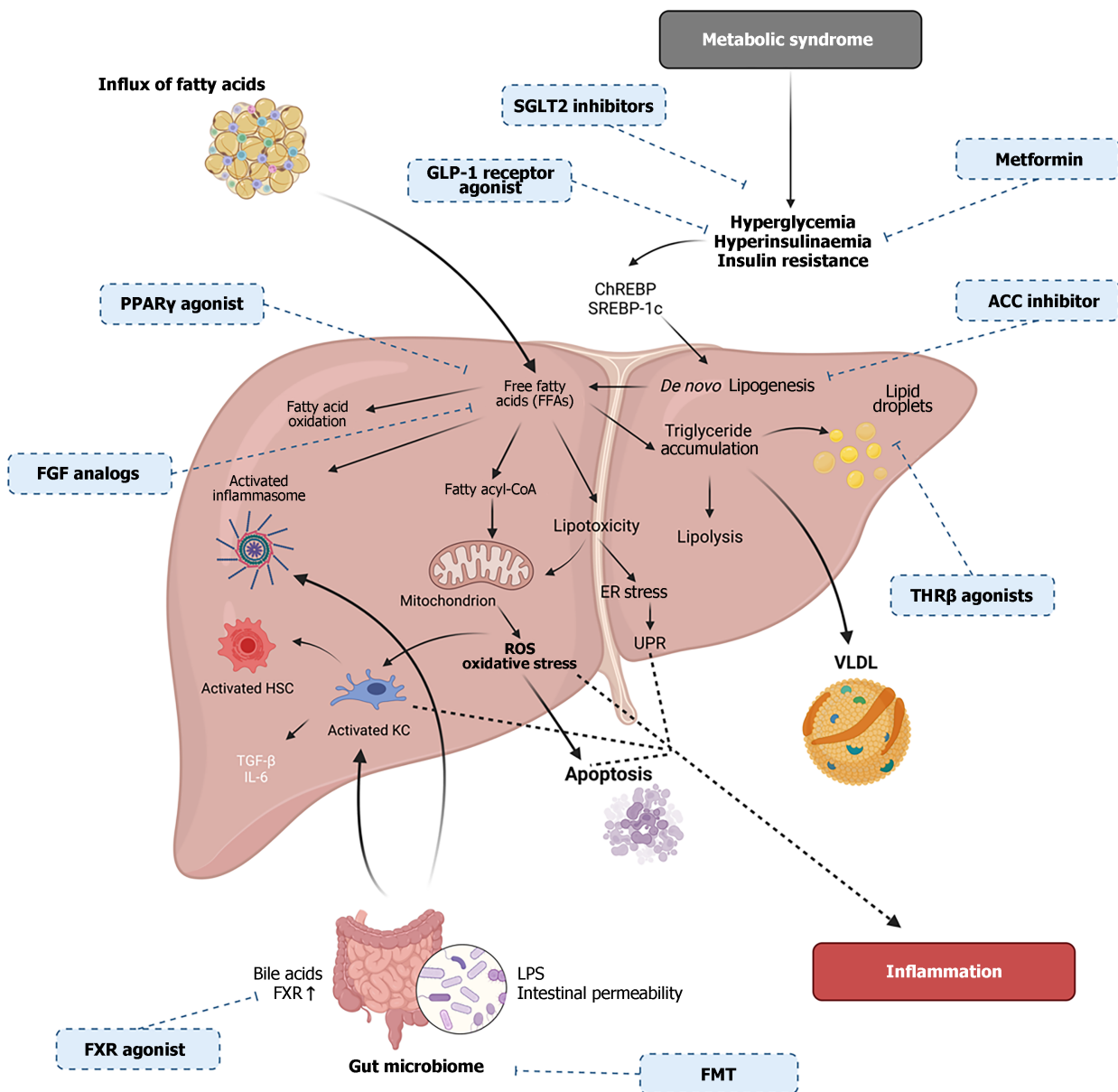
Obeticholic acid (OCA) is a synthetic bile acid which acts as a FXR agonist. The use of OCA for NASH is still under investigation, due to reported side effects. The FLINT trial (NCT01265498) was a multicentre, randomized, double-blind, placebo-controlled phase IIb study assessing the effects of 25 mg of OCA for 72 wk. Treatment with OCA was linked with a significant improvement fibrosis stage in the treated group (35% *vs.* 19%; $P = 0.004$), although there was no difference in rates of NASH resolution. A phase III trial (REGENERATE) is currently active to further assess the treatment of OCA in patients with NASH (NCT02548351).

Other FXR agonists, such as tropifexor, have been studied in the treatment of NASH in the FLIGHT-FXR study (NCT02855164). This 48-wk study using tropifexor found sustained decreases in ALT and liver fat content (measured by HFF using magnetic resonance imaging-estimated proton density fat fraction) during the therapy duration[167]. A Phase IIa (LIFT trial) studying TERN-101, a FXR agonist, showed that in a 12-wk controlled trial, significant improvements in cT1, a marker of fibro-inflammation was observed[160].

Other treatment approaches: Modulation of the gut microbiome: Gut dysbiosis is a common feature of NAFLD[168]. Whilst pre- and pro- biotics are being evaluated, faecal microbiota transplant (FMT) may provide an alternative approach. HFD-fed mice which received FMT from healthy donors showed a significant reduction in intracellular hepatic lipid and proinflammatory cytokines concentration (IFN- γ and IL-17)[169]. Small intestinal microbiota transplants from healthy lean individuals to obese individuals have reported improvements in insulin sensitivity in those patients with metabolic syndrome[170]. A phase I pilot study is currently underway to study FMT in patients with NASH (NCT02469272). FMT as a treatment for NASH patients seems to be both a safe and efficient treatment, although, more high quality studies, trials and follow-ups are required to verify its therapeutic potential[168].

Modulation of the immune system: Methods for modulating the immune system as potential therapies for NAFLD are currently under investigation. Various pleiotropic effects of platelets have recently been discovered in liver homeostasis and disease as platelets are also involved in inflammatory regulation. Anti-platelet therapy (APT) has been shown to reduce NASH pathogenesis in rats[171]. Evidence has shown that APT may have a protective effect in patients with NAFLD[172]. In the liver, platelet and neutrophils can interact leading to neutrophil extracellular trap formation[173]. APT has been shown to reduce both NASH and HCC development[174]. It has been observed that APT reduced platelet accumulation in hepatocytes as well as reducing immune cell interaction, which led to decreases in cytokine and chemokine release, thus attenuating macrovesicular steatosis and liver damage[174].

It is well documented that NASH can progress to HCC. Immunotherapy, including programmed cell death 1 (PD-1) treatment, has been approved for the treatment of HCC[175]. PD-1 can interfere with the immune response and contributes to the growth and expansion of cancer. It has been documented that in NASH there is a progressive accumulation of exhausted, unconventionally activated CD8+ PD1 + T cells[175] and data has shown that in HCC, immune



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Figure 4 Overview of the metabolic and molecular mechanisms occurring in non-alcoholic fatty liver disease pathogenesis with therapeutic targets. Non-alcoholic fatty liver disease (NAFLD) is a multifactorial disease, including metabolic syndrome, insulin resistance (IR) and gut microbiome changes. IR and metabolic syndrome cause increased free fatty acids (FFAs) which lead to endoplasmic reticulum stress, reactive oxygen species accumulation, oxidative stress, apoptosis, immune cell activation and inflammasome activation. Changes in the gut barrier can cause changes to bile acids and intestinal permeability causing lipopolysaccharides translocation from the gut causing immune activation, thus, inducing inflammation. FFA's can also activate transcription factors such as sterol regulatory binding protein-1c and carbohydrate response element binding protein causing the storage of FFAs as triglycerides in lipid droplets. Accumulated triglycerides can be exported as very low-density lipoprotein or oxidized by mitochondrial β -oxidation. Pharmacologic treatments for NAFLD are identified with corresponding drug mechanism of action (created with BioRender.com). ROS: Reactive oxygen species; LPS: Lipopolysaccharide; UPR: Unfolded protein response; FMT: Faecal microbiota transplant; VLDL: Very low-density lipoproteins; PPAR- γ : Peroxisome proliferator-activated receptor- γ ; KC: Kupffer cells; HSC: Hepatic stellate cells; FXR: Farnesoid X Receptor; SGLT2: Sodium-glucose cotransporter 2; SREBP-1c: Sterol regulatory binding protein-1c; ChREBP: Carbohydrate response element binding protein.

surveillance was impaired[175]. Several pharmaceutical agents have been developed to target PD-1 receptors. Pembrolizumab has shown significant enhancement in overall survival and progression-free survival although statistical significance was not met[176]. However, it is also thought that NASH-derived HCC may be less responsive to immune modulated therapy due to NASH-related aberrant T cell activation, causing damage to tissues[175,177,178]. A summary of current clinical trials is shown in Table 1; and an overview of the metabolic and molecular mechanisms occurring in NAFLD pathogenesis with therapeutic targets in Figure 4.

Table 1 Current active and recruiting clinical trials assessing therapeutics for non-alcoholic fatty liver disease

Drug name	Condition	Target	Clinical trial Number	Phase	Status	Primary endpoint
GH509	NASH NAFLD		NCT05784779	Phase Ib/II	Recruiting	Change in liver fat content assessed by MRI-PDFF
LUM-201	NASH NAFLD	Lipid accumulation	NCT05364684	Phase II	Recruiting	Change in intrahepatic lipid content measured by proton magnetic resonance spectroscopy
Choline	NAFLD	Choline deficiency	NCT05200156	N/A	Recruiting	Change in Thiobarbituric acid reactive substances serum level
Dasatinib and quercetin	Fibrosis	Senescence	NCT05506488	Phase I	Recruiting	Improvement of fibrosis NAFLD score based on histology after 21 wk
GSK4532990	NAFLD fibrosis	17 β -HSD	NCT05583344	Phase II	Recruiting	Improvement of fibrosis measured by clinical research network scoring
Lisinopril	NASH HCC	ACE inhibitor	NCT04550481	Phase II	Recruiting	Changes in fibrosis marker PRO-C3
Rencofilstat		Cyclophilin inhibitor	NCT05402371	Phase II	Recruiting	Improvement in fibrosis score CRN or NASH resolution
Lactobacillus reuteri GMNL-263 and GMNL-89 and lactobacillus rhamnosus GMNL-74	NAFLD	Gut microbiome	NCT05402449	N/A	Recruiting	Changes in serum ALT levels
Lubiprostone	NAFLD	Type 2 chloride channel activator	NCT05768334	Phase III	Recruiting	Changes in liver fat by measured by MRI-PDFF
Bacillus coagulans TC1711	NAFLD	Gut microbiome	NCT05635474	N/A	Recruiting	Changes measured by fibroscan
TVB-2640	NAFLD	FASN inhibitor	NCT04906421	Phase III	Active, not recruiting	Improvement in NAS and CRN scoring
ASC41		THR β agonist	NCT05462353	Phase II	Recruiting	Improvement in NAS score
Ketohexokinase inhibition	NAFLD		NCT05463575	Phase II	Recruiting	Insulin-mediated suppression of endogenous glucose production
PF-06865571/ PF-05221304	NAFLD NASH with fibrosis	DGAT2 inhibitor/ ACC inhibitor	NCT04321031	Phase II	Active, not recruiting	Resolution of NASH
ZED1227		TG2 inhibitor	NCT05305599	Phase II	Recruiting	Relative change of serum PRO-C3 levels
MK-3655	NASH	FGF21 agonist	NCT04583423	Phase II	Active, not recruiting	Resolution of NASH
MXP22 (probiotic and antioxidant capsule)	NAFLD	Gut microbiome	NCT05808049	N/A	Recruiting	Changes in steatosis measure by fibroscan
TERN 501/TERN-101	NASH	THR β agonist/ FXR agonist	NCT05415722	Phase II	Active, not recruiting	Relative change in liver fat content (MRI-PDFF)
MET642	NASH	FXR agonist	NCT04773964	Phase II	Active, not recruiting	Safety study to measure adverse events
VK2809	NASH	THR β agonist	NCT04173065	Phase II	Recruiting	Relative change in liver fat content (MRI-PDFF)

MRI-PDFF: Magnetic resonance imaging-estimated proton density fat fraction; NASH: Non-alcoholic steatohepatitis; FXR: Farnesoid X receptor; NAFLD: Non-alcoholic fatty liver disease; THR β : Thyroid hormone receptor- β ; NAS: Non-alcoholic fatty liver disease activity score; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; FASN: Fatty acid synthase; HSD: High-salt diet; CRN: Clinical research network; N/A: Not applicable.

CONCLUSION

NAFLD is a prevalent and progressive disease that can lead to liver damage and is strongly associated with obesity, IR,

and metabolic syndrome. Current treatments for NAFLD include lifestyle modifications such as diet and exercise, surgery, as well as medications to sensitise insulin. However, there is a need for effective and safe interventions that can directly target the underlying mechanisms of NASH/NAFLD in relation to IR, the gut microbiome and immunological mechanisms.

There is a growing body of evidence that NAFLD and metabolic syndrome are closely linked, and it is crucial for future research to prospectively evaluate interventions and therapeutics which both target improvement to liver outcomes as well as comorbidities associated with NAFLD (including cardiovascular diseases, T2DM, renal dysfunction). It is also essential to develop more refined and early risk stratification tools and biomarkers to identify individuals at the highest risk for NAFLD, considering that this condition affects a substantial portion of the world. Understanding the implications of metabolic signatures, chronic insulin signalling, mitochondrial dysfunction and cellular redox, is important for accurate prognosis and potential therapeutics.

Significant progress in the field has been made using bioinformatics to integrate intra- and extra-hepatic signals including gut and adipose interactions as well as patient information regarding lifestyle, nutrition, and comorbidities. The interplay between this multitude of factors provides promise for advancing therapeutics targeting immune regulation and mitochondrial function during progression of NAFLD. Advancements to the field should consider multidisciplinary approaches for the prevention, diagnosis, treatment, and care of patients with NAFLD.

FOOTNOTES

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