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# Non-alcoholic fatty liver disease: the interplay between metabolism, microbes and immunity

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**Non-alcoholic fatty liver disease (NAFLD) has emerged pandemically across the globe and particularly affects patients with obesity and type 2 diabetes. NAFLD is a complex systemic disease that is characterised by hepatic lipid accumulation, lipotoxicity, insulin resistance, gut dysbiosis and inflammation. In this review, we discuss how metabolic dysregulation, the gut microbiome, innate and adaptive immunity and their interplay contribute to NAFLD pathology. Lipotoxicity has been shown to instigate liver injury, inflammation and insulin resistance. Synchronous metabolic dysfunction, obesity and related nutritional perturbation may alter the gut microbiome, in turn fuelling hepatic and systemic inflammation by direct activation of innate and adaptive immune responses. We review evidence suggesting that, collectively, these unresolved exogenous and endogenous cues drive liver injury, culminating in liver fibrosis and advanced sequelae of this disorder such as liver cirrhosis and hepatocellular carcinoma. Understanding NAFLD as a complex interplay between metabolism, gut microbiota and the immune response will challenge the clinical perception of NAFLD and open new therapeutic avenues.**

NAFLD affects up to 25% of the world's population, representing the most prevalent liver disease[1]. This disease presents with varying phenotypic aspects ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, liver cirrhosis and hepatocellular carcinoma. In the majority of patients with NAFLD, liver histology is characterized by simple steatosis, whereas up to 30% of patients exhibit inflammation and/or fibrosis[1]. Patients with NAFLD and simple steatosis but especially with NASH or liver fibrosis develop long-term complications that result in increased mortality[2], mostly from cardiovascular diseases and malignancies[3]. Medical therapy remains an unmet need for NAFLD[4,5], which is partly reflected by the strong need for liver transplantation for this disorder in the US and Europe[6].

Whereas obesity and related disorders are by far the most common conditions associated with NAFLD, this disease can also be observed in lean individuals[7]. Hepatic steatosis occurs after exposure to various hepatotoxins (for example, drugs such as amiodarone, corticosteroids, tamoxifen and many others), consequent to infection or in genetic liver disorders, indicating that hepatic steatosis reflects a response toward hepatic stressors. Another stressor that was recognized early is type 2 diabetes (T2DM), a major risk factor for NAFLD[8]. As obesity and metabolic dysfunction remain key clinical features of NAFLD, a new name has recently been proposed: metabolic-associated fatty liver disease[9,10]. A consensus on an appropriate definition of metabolic-associated fatty liver disease is challenging[11], because the pathophysiology is complex and involves heterogeneous exogenous cues, including nutritional factors and lifestyle, and endogenous cues, such as lipogenesis, lipotoxicity, insulin resistance, cell death and an altered gut microbiome[12]. Collectively, these cues converge on induction of systemic chronic organ inflammation, which particularly fuels many features of NAFLD[13]. Furthermore, various genetic variants influence the risk for developing NAFLD as, for example, evidenced by an association of a single-nucleotide polymorphism (rs738409, I148M) in *PNPLA3* (encoding the lipid droplet protein patatin-like phospholipase domain-containing protein 3) with severity of NAFLD[14].

Current knowledge indicates that pathophysiology of this disease is focussed mainly on metabolic dysfunction and lipotoxicity. In this article, we will discuss three (in our opinion) cornerstones of the pathophysiology of NAFLD: metabolic dysfunction, altered gut microbiome and dysregulated innate and adaptive immunity. We propose a concept for how metabolic perturbation, dysbiosis and liver-damaging immunity establish a self-amplifying vicious circle in NAFLD and how this crosstalk fosters evolution toward NASH and complicated NAFLD.

## Metabolic dysfunction in NAFLD

Metabolic dysfunction such as hepatic steatosis is considered an important early step in the pathogenesis of NAFLD. Accumulation of liver fat, which is most commonly observed in cases of obesity or T2DM, not only constitutes a first hit in this disease, but toxicity exerted by certain lipids might also drive important further steps of this disease such as inflammation, liver injury and insulin resistance.

## Obesity and T2DM: key underlying disorders in NAFLD

It is now well established that NAFLD is accompanied by metabolic dysfunction, obesity and obesity-related disorders such as T2DM in >90% of patients[15]. The global obesity pandemic, which has occurred over the past 3 decades, paved the way for this dramatic increase in NAFLD[4]. The obesity pandemic has been parallelled by overconsumption of (hidden) calorie-enriched food, such as sweetened beverages and fructose-enriched corn syrup, oversized meals and a sedentary lifestyle, which also represent risk factors for T2DM. This may explain a strong association between NAFLD and T2DM (that is, >70% of patients with T2DM have NAFLD and >20% of patients with NAFLD have or will develop T2DM)[16,17]. Therefore, many overlapping pathophysiological aspects exist between NAFLD and T2DM. NAFLD occasionally also develops in lean individuals, which likely indicates an independent pathophysiology (or cause). However, similar long-term consequences apply to these patients,

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as recently demonstrated by patients with 'lean NASH' with increased risk for hepatic or extrahepatic disease and metabolic comorbidities[7], stressing the link to metabolic dysfunction.

## Hepatic lipid and sugar metabolism in NAFLD

Hepatic steatosis is a complex process with several contributors. Whereas an increased influx of lipids or free fatty acids (FFA) into the liver[18] and increased de novo lipogenesis (DNL), typically observed in association with hepatic insulin resistance[19], play a key role, the decrease in fatty acid oxidation and lipid export from the liver (and other mechanisms) might also contribute[20,21]. The global increase in NAFLD is parallelled by overconsumption of calories derived from fat and sugar[22]. Dietary FFA and glycerol participate in hepatic triglyceride synthesis via hepatocellular long-chain fatty acids bound to coenzyme A (CoA) (they form fatty acyl-CoA). Visceral adipose tissue (VAT) also contributes to hepatic steatosis. Increased delivery of FFA from VAT to the liver promotes hepatic steatosis, hepatic insulin resistance and dyslipidaemia. The extent to which VAT contributes to NAFLD remains unclear, although there is a significant association between VAT volume and hepatic steatosis[23]. Importantly, aside from VAT, subcutaneous adipose tissue (SAT) also reflects a major source of FFA flooding the liver in obesity[24]. More than 25% of FFA coming to the liver are derived from SAT, and this percentage is increased in the case of adipose insulin resistance.

Fructose overconsumption plays a major role in the aetiology of NAFLD. Dietary sugars and especially excessive fructose consumption induce lipogenesis, and fructose is largely metabolized into triglycerides via DNL, thereby also reflecting a major energy source[25]. It has been demonstrated in mice that microbiota-derived Toll-like receptor (TLR) agonists, such as endotoxin, drive NAFLD without influencing fructose-1-phosphate and cytosolic acetyl-CoA[26]. Fructose consumption in this study induced DNL, NASH and hepatocellular carcinoma, parallelled by intestinal barrier dysfunction and epithelial endoplasmic reticulum (ER) stress. Mechanistically, fructose induced endotoxaemia and activated MyD88-mediated inflammatory processes in liver myeloid cells, thereby triggering tumour necrosis factor (TNF) synthesis. TNF furthermore promoted fructose-driven lipogenesis, demonstrating how an inflammatory signal affects lipogenesis[26].

## Lipotoxicity and liver injury

**Free cholesterol and sphingolipids.** It has long been assumed that lipids are hepatotoxic and propagate inflammatory responses. Whereas triglycerides were previously considered major culprits in lipotoxicity, recent evidence indicates rather that cholesterol and sphingolipids are equally involved in lipid-induced hepatic inflammation. Hepatic free cholesterol (FC) acts in a lipotoxic fashion, thereby potentially contributing to disease progression[27]. Levels of liver cholesterol and the transcriptional regulators YAP–TAZ are substantially increased in NAFLD livers both in mice and humans during disease progression from simple steatosis toward inflammation and fibrosis[28,29]. The importance of this pathway even in liver fibrosis has been substantiated in further studies by targeting acid ceramidase, which inhibits YAP–TAZ[30]. The interaction between FC and TAZ is an excellent example of how FC might act in a lipotoxic manner and cause 'sterile inflammation' without involving microbial cues. An earlier study by Fernandez-Checa and colleagues highlighted a key role for FC in NASH by showing that FC but not FFA or triglycerides sensitizes mice to TNF- and Fas-induced steatohepatitis[31].

Aside from FC, sphingomyelin (SM), produced by sphingomyelin synthases (SMS), also provides another candidate for lipotoxicity. A high-fat and high-cholesterol diet increased SM and diacylglycerol concentrations in mouse livers, parallelled by increased expression of *sphingomyelin synthase 1* (*Sms1*), and this was also observed in patients with NASH[32]. FC upregulates *Sms1* in hepatocytes, and *Sms1* knockdown in these animals prevented NASH[32]. This study illustrates well how certain lipids may activate inflammatory pathways and suggests that SMS1 is a potential target for future therapies. Furthermore, ceramides are a central product in sphingolipid metabolism; intestinal ceramide production promotes hepatic steatosis[33]; and ceramide serum levels correlate with the presence of NASH[34]. Hepatic sphingolipid and ceramide concentrations also correlate with insulin resistance in human NASH[35,36], which may be related to a sensitizing effect of ceramide on insulin sensitivity[37], and, consistently, targeted ceramide degradation in a transgenic mouse model improved both hepatic steatosis and insulin sensitivity[38]. In conclusion, both preclinical and human studies support an important role for sphingolipids and ceramide in NAFLD (Fig. 1).

**FFA and the inflammasome.** Although most lipids accumulate in the steatotic liver as inert triglycerides, certain lipids, such as saturated fatty acids, diacylglycerols, ceramide, FC or SM, exert lipotoxicity. The nucleotide oligomerization domain-containing protein (NOD)-, LRR- and pyrin domain-containing protein (NLRP)3 inflammasome links lipid sensing with induction of inflammation, and this pathway was convincingly demonstrated to play a role in obesity and related disorders. The NLRP3 inflammasome is a multiprotein complex that mediates processing of caspase-1 and finally results in the release of mature interleukin (IL)-1β. Cytoplasmic receptors of the NOD-like receptor (NLR) family interact with the adaptor protein ASC, which recruits the precursor form of caspase-1. The NLRP3 inflammasome was shown to sense diverse stimuli ranging from microbial to nonmicrobial damage-associated molecular patterns (DAMPs) including adenosine triphosphate (ATP), uric acid, necrotic cells and various lipids[39]. For example, Wen and colleagues demonstrated that various FFA and especially palmitate (which shows elevated plasma concentrations after consuming a high-fat diet) activate NLRP3 in hematopoietic cells[40]. Activation of the NLRP3 inflammasome also links obesity with insulin resistance as demonstrated in *Nlrp3*$^{-/-}$ mice[41]. High-fat diet-induced insulin resistance was mitigated in these mice and parallelled by decreased levels of IL-1β, IL-18 and interferon (IFN)-γ expression in adipose tissue and the liver[41].

When at sufficiently high concentrations, saturated fatty acids may crystallize in macrophages or cause $K^+$ efflux, both of which may trigger NLRP3 activation[42,43]. While certain FFA activate NLRP3, ω-3 polyunsaturated fatty acids (and derivatives) or mono-unsaturated fatty acids conversely attenuate macrophage activation and might thereby limit inflammatory responses[44,45]. Oral administration of sulforaphane, a specific NLRP3 inhibitor, improves hepatic steatosis after a high-fat diet, and this effect was accompanied by inhibition of saturated fatty acid-induced activation of the NLRP3 inflammasome[46]. Finally, other inflammasome components, such as NLRP6 or NLR family CARD domain-containing protein 4 (NLRC4), have also been found to play a role in murine models of NAFLD[47]. In summary, the concept of lipotoxicity is now well established in NAFLD (Fig. 1). Nonetheless, 70–80% of patients with NAFLD never develop inflammation in a steatotic liver, indicating that host genetics and possibly specific dietary lipids exert protective or anti-inflammatory functions.

## ER stress

Lipotoxic lipids cause not only inflammasome activation and oxidative stress but also perturb ER functions such as lipid homoeostasis[48,49]. Perturbation of ER functions, collectively referred to as ER stress, orchestrates key cellular signalling events in obesity, T2DM[50] and liver diseases[51] with an increasingly recognized role in human NAFLD[52]. Distinct inflammatory signals are derived from the ER, involving activation of transcription factors nuclear

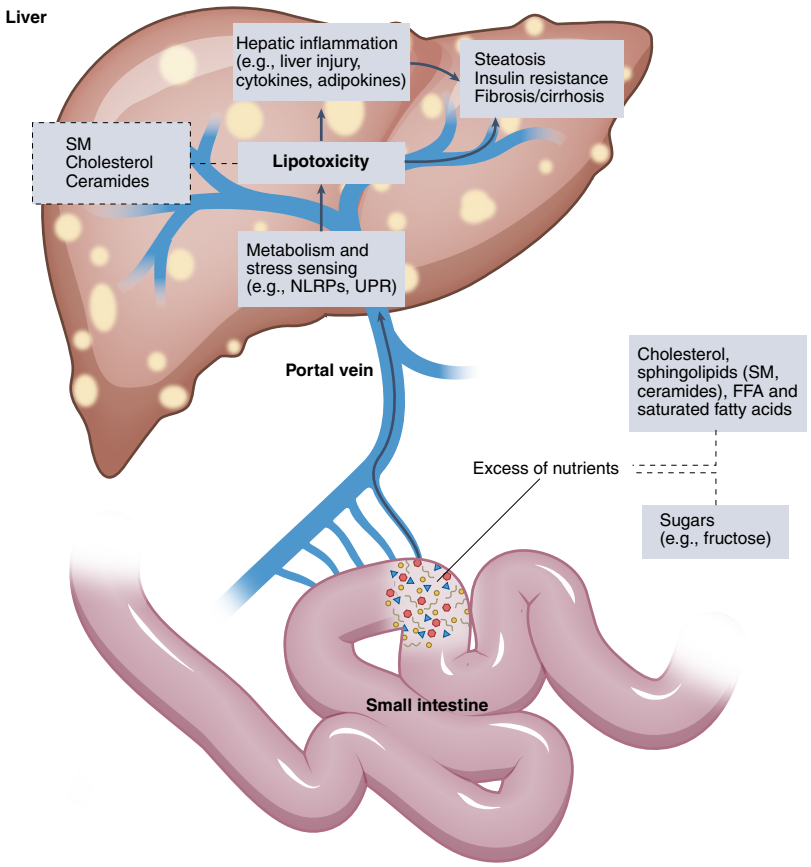


**Fig. 1 | Diet constituents and lipotoxicity as a fuel for NAFLD.** A range of hepatic diseases can cause secondary fatty liver disease (for example, viral infection), while primary cases occur consequent to a Western lifestyle and an excess of nutrients. Metabolism and sensing of dietary excess (for example, sugar and lipids) critically drive the development of NAFLD. For example, excessive intake of fructose fuels lipogenesis and steatosis. Likewise, dietary lipids such as cholesterol or sphingolipids accumulate in the liver, are metabolized and trigger immune responses in hepatocytes and hepatic immune cells (for example, Kupffer macrophages) by activation of pattern recognition receptors and the unfolded protein response (UPR), which mediates lipotoxicity, hepatic inflammation and various aspects of NAFLD.

factor (NF)-κB and c-Jun N-terminal kinase (JNK), which influence liver immune responses and cell death[53]. ER stress is also notable in NAFLD, in which evidence from mice has shown that hepatocytes transmit these stress signals to neighbouring hepatocytes by connexin 43 channels, indicating transmission of stress events across the liver[54].

## Lipids, inflammation and their effects on insulin resistance

Insulin resistance has been recognized as a key feature in NAFLD, although it remains unclear whether hepatic steatosis precedes insulin resistance or insulin resistance drives hepatic steatosis. Hepatic insulin resistance, defined as an impaired ability of insulin to suppress hepatic glucose production[55], is present in most patients with NAFLD, supporting the notion that NAFLD constitutes a systemic metabolic disorder[56,57]. The pathophysiology underlying hepatic insulin resistance remains complex, and various non-inflammatory and inflammatory cues might be involved[16]. FFA were recognized decades ago as causing skeletal muscle insulin resistance[58], and various lipids are key drivers of systemic and tissue-specific insulin resistance, potentially including ceramides, diacylglycerols, long-chain fatty acyl-CoA and acylcarnitines (aside from FFA)[15]. Indeed, a lipid infusion induces hepatic insulin resistance even in lean individuals[59], and NAFLD is characterized by increased hepatic gluconeogenesis and glycogenolysis[60,61]. Moreover, intrahepatic lipids contribute to hepatic insulin resistance and hyperinsulinaemia typically observed in NAFLD[62,63].

**Inflammation and insulin resistance.** Aside from lipids, tissue inflammation also affects insulin resistance[64]. Pro-inflammatory hits involving cytokines (and activation of related transcription factors) have been characterized in the past 2 decades as important regulators of insulin resistance by disruption of insulin signalling[65]. Ablation of TNF or its receptors in mice improved insulin sensitivity in obesity[66]; however, the use of TNF-neutralizing agents to treat chronic inflammatory conditions such as rheumatoid arthritis for more than 2 decades appeared to have no significant effect on insulin resistance. IL-1β is a potent pro-inflammatory cytokine that alters insulin signalling, and, in contrast to anti-TNF therapy, the use of an IL-1 receptor antagonist (anakinra) improved insulin sensitivity in patients with T2DM[67]. As such, this study links production of inflammatory mediators with reduced insulin sensitivity in humans. Accordingly, several transcription factors involved in inflammation, such as NF-κB, the inhibitor of NF-κB (IKKβ) or JNK1, contribute to inflammation-related insulin resistance in experimental animal models[68–71]. However, the sequence of events in the development of insulin resistance remains poorly understood. For example, obesity-induced insulin resistance might precede macrophage accumulation in adipose tissue and subsequent cytokine and chemokine production[72]. Overall, a complex interaction of non-inflammatory and inflammatory components might contribute to hepatic and systemic insulin resistance in NAFLD.

Overall, the above-discussed principles demonstrate that hepatic steatosis is a first and initial event in the pathogenesis of NAFLD.

It remains a major challenge to understand why lipid accumulation is inert in some NAFLD cases, whereas, by contrast, lipids become 'toxic' and might drive the disease process by causing inflammation and liver injury in a substantial number of patients with NAFLD. The role of other nutrients as drivers of inflammation remains less clear, but, as stated, fructose could reflect another candidate with such potential.

## Gut microbiome and NAFLD

There is growing evidence that the gut microbiome–liver axis plays a key role in NAFLD and especially in progression toward more advanced disease stages[73]. Mainly preclinical reports but also several large clinical studies from past years strongly support the notion that a gut microbiome signature exists in NAFLD.

## Gut microbiome alterations in NAFLD: clinical evidence

Several large human studies have now investigated the gut microbiome in NAFLD. Patients with NASH demonstrated a microbial signature potentially allowing differentiation between early and advanced liver fibrosis, which was characterized by an increase in Proteobacteria and *Escherichia coli* abundance, whereas that of Firmicutes and *Faecalibacterium prausnitzii* was significantly decreased[74]. In a large human study from Rotterdam, 472 of 1,355 participants showed evidence of hepatic steatosis associated with lower microbial diversity and the presence of *Coprococcus* and *Ruminococcus gnavus*[75]. A certain gut microbiome composition distinguished cirrhosis from non-cirrhosis irrespective of disease aetiology and in samples from geographically separated regions[76]. Importantly, the gut microbiota might be of importance in lean patients with NAFLD as suggested by a study from Asia[77]. The abundance of *Ruminococcaceae* and *Veillonellaceae* was mainly correlated with fibrosis in lean individuals accompanied by enhanced faecal bile acids and propionate. Administration of these bacteria to mice instigated features of NAFLD[77]. The role of *Veillonella* is challenged, however, as treatment of NASH with the fibroblast growth factor (FGF)19 analogue aldafermin resulted in a dose-dependent enrichment of this genus[78] (Table 1).

A recent study from Germany demonstrated that long-term gut microbiome instability over a 5-year interval with dominance of *Enterobacteriaceae* and *Escherichia* and *Shigella* was associated with development of NAFLD and T2DM[79]. Collectively, numerous preclinical models and adequately powered human studies have shown an altered microbiome in NAFLD[80] (Fig. 2). Many preclinical studies have demonstrated that hepatic steatosis is linked to the gut microbiome, as, for example, the probiotic VSL#3 abolished hepatic steatosis in *ob*/*ob* mice[81] and both obesity and dietary factors are considered major confounders known to affect the gut microbiome beyond NAFLD[73]. However, causality between microbiome signals and the course of NAFLD (in particular, disease progression) in humans has not been established, and most insights have been generated in experimental models.

## Potential mechanisms of how the gut microbiome affects fatty liver disease

**Metabolites derived from intestinal bacteria.** The first contact between the gut microbiota and the immune system occurs with dendritic cells (DCs) when they phagocytose live commensal organisms in Peyer's patches. These loaded DCs stay within mesenteric lymph nodes, and induction of an immune response against commensals is restricted to the mucosa. In case of an impaired intestinal barrier, as frequently observed in NAFLD[82], commensals and bacterial components might gain access via portal venous blood to the liver[83]. It has been well recognized in recent years that especially microbe-derived but also host-derived metabolites are sensed by immune cells. For example, imidazole propionate, a microbial histidine-derived metabolite, affects insulin signalling, and systemic serum and portal vein concentrations were increased in patients with T2DM[84]. Likewise, *N*,*N*,*N*-trimethyl-5-aminovaleric acid (a metabolite of gut bacteria) serum levels are increased in NAFLD, and, when mice are treated with antibiotics (or kept free of germs), its concentration decreases[85]. This is notable because *N*,*N*,*N*-trimethyl-5-aminovaleric acid worsened experimental hepatic steatosis induced by a high-fat diet[85]. Finally, the bacterial metabolite phenylacetate triggers hepatic steatosis in mice, as did faecal transfer from obese women into mice[86]; however, further studies are needed to prove the relevance of host-derived or microbe-derived dietary metabolites in human NAFLD.

**Bacterial endotoxin as a driver of NAFLD.** Increased circulating endotoxin concentrations were described in alcohol-related liver disease and NAFLD 3 decades ago[87]. Today, collective evidence formed the basis for Christopher Day's second-hit hypothesis of NAFLD[88], which we further developed to the multiple-hit model, suggesting that various inflammatory cues from adipose tissue and the gut contribute to the progression of NAFLD[89]. Patients with NASH indeed exhibit increased circulating endotoxin levels compared to patients with simple steatosis, and hepatocytes in NASH livers stained positive for endotoxin accompanied by an increased number of TLR4$^+$ hepatic macrophages[90]. Several bacteria such as *Enterobacter cloacae* B29, *E. coli* PY102 and *Klebsiella pneumonia* A7, all endotoxin producers, enforce the development of NAFLD in germ-free mice exposed to a high-fat diet[91]. As such, gut-derived endotoxin and related hepatic Toll-like and NOD-like receptor signalling are important drivers of experimental NAFLD and, potentially, of more advanced stages of human NAFLD.

It has been increasingly recognized that, aside from endotoxin, other microbial components and bacterial DNA are detectable in 'sterile' tissues such as the liver, suggesting that tissue microbiome components might reflect a new aspect in human NAFLD (Box 1).

## Bile acids: linking metabolism with the gut microbiota

Bile acids are metabolites of cholesterol metabolism, secreted into the gut via the biliary tree, and they control hepatic and extrahepatic metabolism and specifically energy homoeostasis[92]. Bile acids improve glucose metabolism and obesity by, for example, farnesoid X receptor (FXR) signalling. Importantly, secreted bile acids are processed (that is, conjugated) by the gut microbiota into secondary bile acids and affect the growth of bile acid-metabolizing bacteria. In NAFLD, the role of bile acids is increasingly appreciated[93]. For example, patients with NAFLD exhibit increased fasting and postprandial serum bile acid concentrations, which correlate with more severe liver disease[94] and are associated with insulin resistance but not hepatic inflammation[95]. Clinical evidence for a critical role of bile acids in NAFLD stems from trials demonstrating that obeticholic acid or 24-norursodeoxycholic acid ameliorates aspects of NAFLD[96,97](Table 1). For example, administration of obeticholic acid resulted in a significant improvement in hepatic inflammation and fibrosis and metabolic disturbances[96]. The role of nuclear receptors (for example, FXR) and FGFs in NAFLD is also emerging and has been reviewed elsewhere[98,99].

To summarize, there is increasing evidence that gut dysbiosis plays a role in NAFLD, and this is not only supported by preclinical trials but increasingly by well-designed clinical studies. It remains to be elucidated in the future which bacterial strains (or lack of strains) might drive key features of this disease such as hepatic steatosis, inflammation, liver injury or the evolution of insulin resistance. Furthermore, microbial components might also act on other important features of this disease such as liver fibrosis. Host variables such as alcohol consumption, stool frequency and consistency or genetic factors have a major effect on gut microbiome composition and therefore require attention for careful interpretation

**Table 1 | Emerging therapies targeting metabolism, gut microbiome and immunity in NAFLD**

| Compound | Drugs in class | Mechanism of action |
|---|---|---|
| Metabolism and lipotoxicity | | |
| ACC inhibitors[152] | Cilofexor | Inhibitor of DNL |
| FGF19 analogues[153] | Aldafermin | Reduces lipotoxicity; decreases food intake, gluconeogenesis and bile acid synthesis, increases FGF21 expression |
| FGF21 analogues[154] | Pegbelfermin | Reduces lipotoxicity, increases adipose tissue browning and energy expenditure and/or thermogenesis |
| FXR agonists[96] | Obeticholic acid, cilofexor, tropifexor | Increases energy expenditure and metabolic rate, decreases adiposity and DNL |
| GLP-1 agonists[155] | Liraglutide, semaglutide | Decreases food intake, stimulates secretion of insulin, inhibits secretion of glucagon |
| Norursodeoxycholic acid[97] | | Interaction with bile metabolism |
| PPAR-α–PPAR-δ agonists[156] | Elafibranor | Increases fatty acid oxidation |
| Pan-PPAR agonist[157] | Lanifibranor | Improves lipid, inflammatory and fibrosis biomarkers |
| PPAR-γ agonists[158] | Pioglitazone | Decreases visceral and hepatic fat accumulation |
| SGLT1–SGLT2 inhibitors | Licogliflozin | Decreases renal glucose reabsorption and increases renal glucose excretion |
| Stearoyl-CoA desaturase-1 (SCD-1 modulator) | Aramchol | Decreases hepatic lipogenesis |
| THR-β agonists[159] | Resmetirom | Reduces low-density lipoprotein cholesterol and triglyceride levels |
| **Gut microbiome** | | |
| Probiotics[160] | Pilot studies with various strains including lactobacilli, bifidobacteria, VSL#3 | Decreases hepatic steatosis |
| Faecal microbiota transplantation[161] | FMT | Improves insulin sensitivity |
| **Inflammation and/or immunity** | | |
| C–C motif chemokine receptor 2 and CCR5 antagonist[162] | Cenicriviroc | Anti-inflammatory, anti-fibrotic |
| Galectin-3 receptor agonists | Belapectin | Mainly anti-fibrotic |
| Vitamin E[158] | | Reduces oxidative stress, anti-inflammatory |
| Potential further targets | | TLRs, NLRP3, LPS, CD3, NKT cells, metabolic T cell activation |

ACC, acetyl-CoA carboxylase; CCR, C–C motif chemokine receptor; FMT, fecal microbiota transplantation; GLP-1, glucagon-like peptide 1; PPAR, peroxisome proliferator-activated receptor; SCD-1, stearoyl-CoA desaturase-1; SGLT, sodium–glucose transporter; THR-β, thyroid hormone receptor β. Many drugs listed drugs here are pleiotropic, that is, they affect various pathways including metabolic and inflammatory pathways. Despite the importance of inflammation, fibrosis and immunity in NAFLD, most therapeutic approaches currently focus on metabolic dysfunction.

of microbiome-sequencing results[100,101], while neglect of such confounders leaves room for biased interpretation.

## Immunity and NAFLD

Low-grade systemic inflammation in NAFLD might be especially relevant in patients with progressive disease such as NASH and advanced liver disease[102]. Whereas certain extrahepatic sites such as adipose tissue have evolved as a major source of inflammatory mediators in obesity-related disorders, evidence is accumulating that the gastrointestinal tract might also be critically involved in the pathogenesis of low-grade inflammation[89].

## Innate immunity driving metaflammation

**NAFLD: a disorder characterized by metaflammation and caused by multiple parallel hits.** Innate immunity is of crucial relevance in NAFLD. Many immunometabolic disorders such as NAFLD are characterized by intermittent chronic low-grade inflammation (termed 'metaflammation')[13,102]. Liver inflammation (that is, NASH) and systemic inflammation appear in 10–30% of all patients with NAFLD and are commonly followed by liver damage and consequently liver fibrosis. Inflammation and especially fibrosis have emerged as key prognostic parameters of this disease, being drivers of hepatic complications, such as liver cirrhosis or hepatocellular carcinoma, or extrahepatic complications, such as cardiovascular complications and extrahepatic malignancies[3,103,104]. Whereas certain biomarkers such as circulating cytokines, for example, IL-1 receptor antagonist, might reflect the inflammatory nature of NASH, liver biopsy still defines the gold standard to detect liver inflammation.

A matter of major discussion is where chronic inflammation develops and how it is initiated. We proposed more than a decade ago that liver inflammation might be initiated by various signals derived from the gastrointestinal tract (derived from microbes and/or diet) and signals from adipose tissue (lipids, cytokines)[89]. Indeed, evidence is accumulating that the degree of physical activity, gut microbiome stability, dietary factors and certain lipid concentrations or combinations, as outlined in this review, might be responsible factors that orchestrate chronic inflammation in NAFLD[13,105]. In particular, adipose tissue has emerged in obesity and obesity-related disorders as a major source of circulating pro-inflammatory cytokines (for example, 30% of circulating IL-6), thereby affecting the extent of liver inflammation[106]. A key role for inflammation of SAT (as a major sink for pro-inflammatory cytokines) stems from our laboratory, where we demonstrated substantial IL-1β and IL-6 expression in SAT of morbidly obese patients[107,108]. Microbial

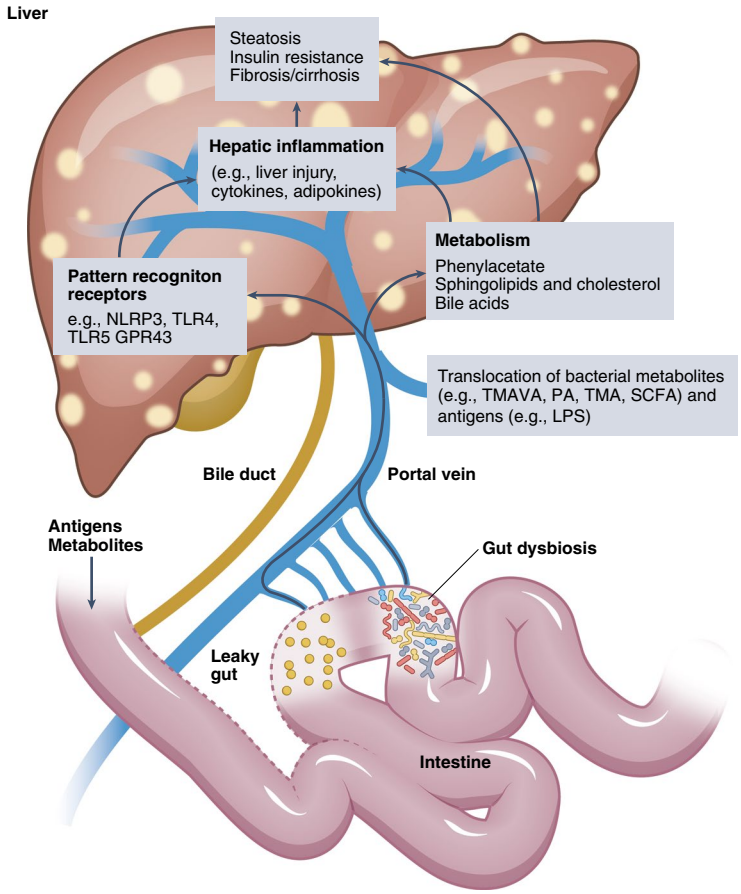


**Fig. 2 | Gut dysbiosis and bile acid metabolism in NAFLD.** NAFLD is associated with alterations in gut microbial composition and bile acid metabolism. Additionally, a 'leaky gut' promotes translocation of bacterial metabolites or antigens derived from the gut microbiota. These impact metabolism and stress sensing by pattern recognition receptors in the liver, leading to hepatic inflammation, featuring aspects of NAFLD (GPR43, G protein-coupled receptor 43; LPS, lipopolysaccharide; PA, phenylacetate; SCFA, short-chain fatty acid; TMA, trimethylamine; TMAVA, *N*,*N*,*N*-trimethyl-5-aminovaleric acid).

components, lipids and diet-derived signals all contribute substantially to a so-called gut–liver axis, which also seems of crucial relevance to the pathophysiology of this disease.

**Cytokines and TLRs and their role in NAFLD.** Cytokines and related mediators mainly derived from immune cells play a major role in any inflammatory condition[109], and pro-inflammatory cytokines have been demonstrated to be highly expressed, especially in NASH. TNF is one of the first investigated cytokines, the expression of which is correlated with the degree of inflammation in the NASH liver, while its neutralisation in animals was followed by abolishment of hepatic steatosis[81,110]. Also, IL-1 family members have been shown to affect insulin–glucose metabolism and regulate metabolic dysfunction in NAFLD. Indeed, *Il1a*$^{-/-}$ mice exhibit lower insulin levels and improved insulin sensitivity, and levels of both IL-1α and IL-1β increase after a high-fat diet in mice[111]. Both *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice were protected from liver inflammation after diet-induced steatosis with a decrease in fibrosis-related gene expression. IL-6, another potent pro-inflammatory cytokine, is also expressed at increased concentrations both in liver and adipose tissue in patients with NAFLD, but its exact role in metabolic dysfunction remains unclear[112]. Blockade of the pro-inflammatory cytokine IL-11, however, attenuates hepatic steatosis and liver inflammation and fibrosis development in animal models of NAFLD[113]. It currently remains unclear to what extent these pro-inflammatory cytokines contribute to progression of liver disease in humans, as respective anti-cytokine clinical trials have not been performed thus far.

Hackstein et al. found that, during severe liver damage, microbial translocation from the gut to the liver via the portal vein causes tonic IFN signalling in liver myeloid cells[114]. In such IFN-stimulated macrophages, infections with intracellular bacterial pathogens such as *Listeria* or *Salmonella* trigger an overshooting second IFN response that in turn leads to production of the inhibitory mediator IL-10, revealing a potent negative feedback loop in liver damage in response to bacterial infection. Overshooting IL-10 responses incapacitate anti-microbial defence by myeloid cells[114]. Such IFN–IL-10-mediated paralysis of immune responses may explain the development of often fatal bacterial infections in patients with advanced liver disease including NAFLD[115]. Together, these observations illustrate that inflammation-promoting mechanisms in NAFLD are presumably the result of defined molecular processes rather than of nonspecific activation by cytokines.

Inflammasomes and TLRs have been carefully investigated in NAFLD in many studies. The NLRP3 inflammasome as a critical sensor of various lipid signals has been discussed above. Mice deficient in NLRP3 were protected against obesity-related insulin resistance, accompanied by decreased expression of monocyte chemoattractant protein 1 and macrophage infiltration in adipose tissue[116]. Certain TLRs are activated especially by saturated FFA[117], and other TLRs have been found to interact with lipid signals[118]. These TLRs are prototypic sensors for bacterial signals such as endotoxin derived from the gastrointestinal tract in NAFLD[119]. Mice deficient in TLR5 exhibit dysbiosis, low-grade inflammation, impaired insulin signalling and various features of metabolic syndrome[120]. Mice

**Box 1 | Tissue microbiome: a new relevant feature in NAFLD pathogenesis?**

It is increasingly appreciated that microbial components and bacterial DNA are detectable in various tissues that are considered sterile[163]. The liver is exposed to gut bacteria especially in diseases associated with an impaired intestinal barrier. For example, bacterial DNA was detectable in the liver of patients with NAFLD, especially if they were obese[164]. Proteobacterial abundance was increased in the liver of patients with severe obesity, while Gammaproteobacteria and Alphaproteobacteria as well as Deinococcus–Thermus dominated in moderately obese patients[164]. Likewise, plasma, adipose and liver tissue microbiomes were detectable in patients with T2DM[165], especially in VAT, which was characterised instead by inflammation-enhancing *Enterobacteriaceae*[165,166].

Currently, it is unknown whether bacterial DNA detected in metabolic tissues is inert, reflects live bacteria and/or regulates biological processes (for example, lipogenesis or inflammation). However, tissue microbiome abundance in VAT has been associated with immune cell infiltration and inflammatory parameters, suggesting that detectable signals may affect immune responses[167]. Alternatively, impaired degradation of phagocytosed bacteria by liver macrophages, as observed in liver damage, may contribute to abundance of microbiota in the liver[114]. In the liver, such microbiome signals appear to localize to specific zones in the micro-architecture that are defined by liver-resident sinusoidal endothelial cells[168]. Whether these findings are relevant to human NAFLD remains to be determined, and they could shed light on intricate host–microbe commensalism in the liver.

deficient in CD14, a co-receptor for TLR4 that binds endotoxin, and the pattern recognition receptor NOD1 did not develop glucose intolerance or gain fat mass after a high-fat diet[121]. Therefore, NLRP3 and various TLRs affect critical pathways involved in NAFLD including inflammatory infiltration in the liver and adipose tissue and regulation of insulin sensitivity. In fact, modulation of the gut microbiome through the activity of certain TLRs or inflammatory mediators may lead to change in microbiome-associated inflammation, which in turn may aggravate liver inflammation and NAFLD. Thus, the combination of innate immunity, changes in microbiome composition, increased gut microbial translocation and cell-intrinsic immune sensing pathways may all contribute to establish chronic inflammation in the liver.

## Adaptive immunity in liver damage during NAFLD

Adaptive immunity is instrumental to control pathogen infection or transformed cells in cancer, which is mainly achieved through execution of effector functions from specific $CD8^+$ T cells killing their target cells. Understanding the regulation of adaptive immunity in the context of NAFLD has received increasing attention. Here, we review our current understanding of how adaptive immunity and NAFLD are linked to each other.

**Antigen-dependent activation of adaptive immunity.** Sterile inflammation during NAFLD is considered to be triggered by the innate immune system as compared to chronic infectious liver inflammation, in which adaptive immunity targeting virus-infected hepatocytes during chronic hepatitis B, C or D destroys liver tissue[122]. Consistent with a role of adaptive immunity in NAFLD, increased numbers of antigen-presenting cells such as DCs or monocytes were found to be associated with the severity of NASH[123,124], and several studies characterized transcriptional profiles at the single-cell level[125,126]. Notwithstanding this increase in understanding of liver and immune cell heterogeneity in NASH, immunogenic auto-antigens that could be targets for specific T cell-based destruction of liver tissue have not been defined in NAFLD[127]. Nevertheless, antibodies specific for oxidative stress-derived epitopes are found in 40% of adult patients with NAFLD or NASH and target the cyclic malondialdehyde acetaldehyde adduct methyl-1,4-dihydroxypyridine-3,5-dicarbaldehyde[128], but no experimental evidence from preclinical models or human in vitro studies for antibody-driven liver damage in NAFLD exists. Instead, liver pathology in NAFLD is exacerbated in mice lacking conventional type 1 DCs or $CD11c^+$ DCs, instead supporting an anti-inflammatory role of DCs in the pathogenesis of NAFLD[124,129].

Similarly, it is unknown whether auto-reactive B cells contribute to the initiation of liver pathology in NAFLD. However, levels of B-cell activating factor, a TNF superfamily member that is secreted by adipocytes and regulates the development of B cells[130], are increased in patients with liver steatosis compared to those in control individuals[131]. Deletion of *B-cell activating factor* in preclinical NAFLD models ameliorates hepatic fat accumulation and inhibits inflammation in VAT, but a direct liver-damaging function of B cells in NAFLD has not been identified[132].

Given the disturbance of lipid metabolism in livers of patients with NASH, natural killer T (NKT) cells were considered to play a role in NAFLD. These innate-like T cells recognize lipid antigens presented by the major histocompatibility complex-like molecule CD1d and are categorized in type I and II NKT cells based on their different expression of T cell receptor (TCR)-αβ chains[133]. Activated NKT cells secrete LIGHT, a member of the TNF super family, which promotes lipid uptake by hepatocytes and is upregulated in patients with NASH[134]. Furthermore, hepatic CD1d expression and increased numbers of $CD3^+CD56^+$ cells in patients with NASH were found, suggesting a potentially important role of NKT cells in this disease[135,136]. Again, however, formal experimental evidence for antigen-specific activation of NKT cells as a mechanism of liver damage in NAFLD has not been obtained.

**Metabolic activation of T cells in NAFLD.** Despite accumulating data that the adaptive immune system plays a role in NASH, it has remained unclear how T cells could cause liver damage in NAFLD. Short-chain fatty acids, which include acetate, butyrate and propionate, are bacterial metabolites produced from the digestion of dietary fibre and are known to have immunomodulatory effects on different T cell subsets, for example, inducing regulatory T cells or IL-22 production in innate lymphoid cells and $CD4^+$ T cells in the gut[137–139]. Bile acids are dysregulated in patients with NAFLD[140], and increased concentrations of two bile acid derivatives of lithocholic acid, 3-oxolithocholic acid and isoallolithocholic acid, favour differentiation of regulatory T cells over the $T_H17$ subset of helper T cells[141] and might thereby provoke tissue damage. Whether mechanisms similar to those in the gut take place in the liver during NAFLD needs to be addressed in the future.

The lack of development of NASH in experimental models after depletion of $CD8^+$ T cells or in $B2m^{-/-}$ mice fed a choline-deficient high-fat diet demonstrated a critical role of T cells in causing liver damage[134,142], but the mechanisms causing T cell activation and how this mediated liver cell damage were not resolved. Recently, it was shown that liver-resident $CXCR6^+CD8^+$ T cells caused liver damage after metabolic activation through the purinergic type 2 receptor P2RX7 in the absence of conventional antigen presentation by hepatocytes[143]. This highlights a new concept of T cell activation that occurs in the absence of canonical T cell receptor activation by peptide-loaded major histocompatibility complex molecules. This metabolic activation of T cells that leads to antigen-independent destruction of target cells such as hepatocytes has been termed

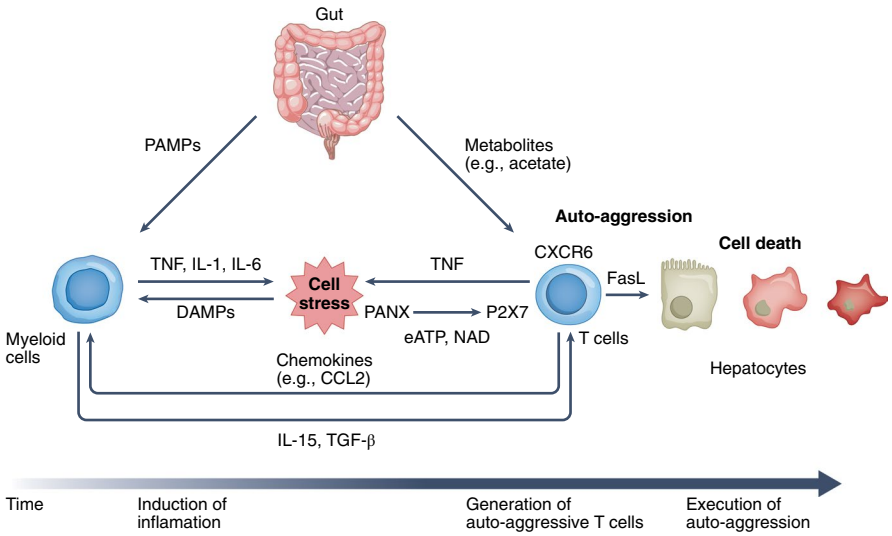


**Fig. 3 | Innate and adaptive immunity pathways driving inflammation in NAFLD.** Progression of inflammation in NAFLD is triggered by the secretion of pro-inflammatory cytokines such as TNF, IL-6 and IL-1β from myeloid cells that are locally activated in the liver as a result of innate immune activation. In addition, local production of IL-15 or transforming growth factor (TGF)-β supports increased generation of liver-resident CXCR6$^+$CD8$^+$ T cells that express the purinergic receptor P2X7. Pathogen-associated molecular patterns (PAMPs) and metabolites such as acetate, which are derived from gut microbiota and reach the liver via the portal vein from the gut into the liver, can modulate the function of liver-resident T cells. Acetate-sensitized liver-resident CXCR6$^+$CD8$^+$ T cells are in close contact with stressed hepatocytes and are activated by DAMPs such as ATP or nicotinamide adenine dinucleotide (NAD), which are released in a pannexin 1 (PANX1)-dependent fashion by hepatocytes. Once activated through P2X7, CXCR6$^+$CD8$^+$ T cells execute a FasL-dominated killing programme, eliminating hepatocytes in NAFLD. It is likely that CXCR6$^+$P2X7$^+$CD8$^+$ T cells perpetuate liver inflammation during NAFLD by attracting myeloid cells that further produce IL-15 to provoke generation of more auto-aggressive T cells (CCL2, C–C chemokine ligand 2; CXCR6, C–X–C chemokine receptor 6; eATP, extracellular ATP).

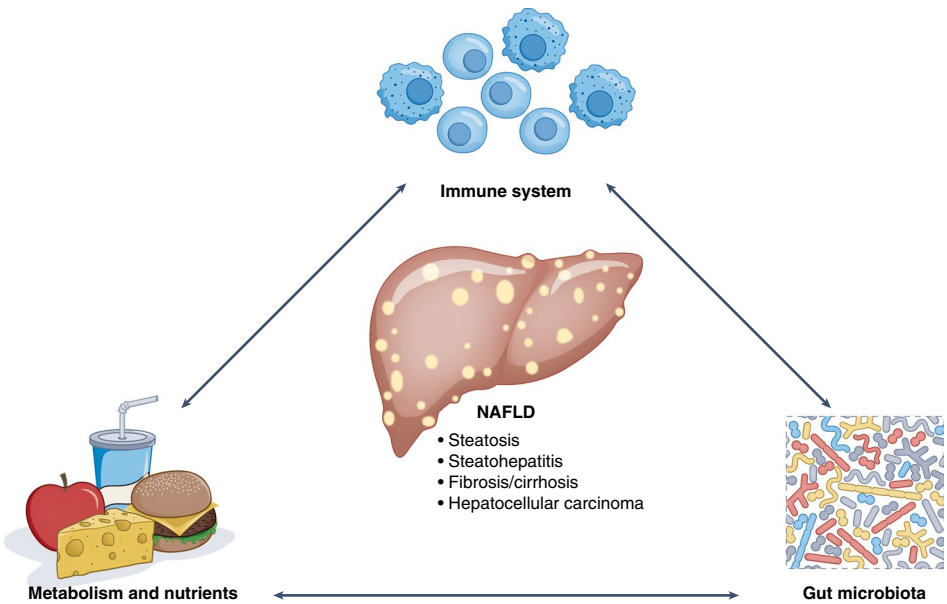


**Fig. 4 | Crosstalk between metabolism, immunity and the gut microbiota in NAFLD.** NAFLD arises from a disturbed interplay between host metabolism, immune responses and the gut microbiota, which are all affected by host genetics. For example, excess of specific dietary constituents associated with a Westernized diet are metabolized in the liver, where they potently elicit immune responses and mediate injury and inflammation. Western dietary habits also affect gut microbiota composition and function, which can modulate hepatic immune responses. This interactive triangle characterizes hepatic health, which spirals out of control during the evolution of NAFLD, with hepatic inflammation being a critical disease driver.

auto-aggression. Auto-aggression develops as a sequential process in which initial IL-15 activation is critical for the generation of high numbers of liver-resident CXCR6$^+$CD8$^+$ T cells, followed by increased effector function triggered by metabolites that are abundant in the liver during NAFLD, in particular, the short-chain fatty acid acetate and ATP[143].

The separate effects of IL-15 and metabolites such as ATP on T cells have already been investigated. IL-15 is induced during

**Box 2 | Outstanding questions arising from the interaction of metabolism, the gut microbiome and immunity in NAFLD**

What are the crucial dietary components driving NAFLD? How do they alter the gut microbiome and/or innate and adaptive immune functions (to provide a better rationale for dietary intervention in NAFLD)? What are the effects of pro-inflammatory diets in this disease? Could dietary components also promote liver fibrosis directly?

Can we identify crucial metabolites, either derived from microbes or not, affecting key features of NAFLD such as insulin resistance?

Which commensals are metabolically detrimental or beneficial? Are we able to design in the future a smart beneficial probiotic for this disease?

Bariatric surgery has proven highly effective in improving NASH and fibrosis. Is weight loss alone sufficient to improve other sequelae of this disease such as liver inflammation and fibrosis? If yes, this would suggest that activation of innate and adaptive immunity are rather consequences of metabolic dysfunction.

Are any therapeutic concepts effective in NAFLD (without causing relevant weight loss), that is, can anti-inflammatory therapies, for example, improve this disease substantially and sustainably by targeting cytokines and/or chemokines?

Will reduction of hepatic fat by certain drugs also improve inflammation and/or fibrosis?

Are changes in metabolism, the gut microbiome and immunity causal or merely correlative in human NAFLD?

inflammation and may therefore be considered a consequence of innate immunity. IL-15 belongs to the IL-2 cytokine family, binding to common gamma chain receptors. IL-15 signalling in T cells is mediated through trans-presentation of IL-15 together with its receptor CD122 by epithelial cells, DCs or macrophages[144]. Although IL-15, similar to IL-12 or IL-18, is known to be important for the survival and activation of NKT cells, the role of these cells in NAFLD remains controversial[145]. IL-15 is a powerful signal for human $CD8^+$ T cells to upregulate effector function and has been shown to be involved in NAFLD[144,146].

Extracellular ATP, a known DAMP signal, is released from stressed or dying cells, causing activation of antigen-presenting cells that in turn boost local T cell effector functions[147]. Sensing ATP in the tissue is important for survival and for effector function of T cells by upregulating expression of Fas ligand (FasL) on the surface[143,148], but the exact role of ATP-sensing receptors such as P2RX7 in liver-resident $CD8^+$ T cells, especially during NAFLD progression, remains unknown. The report by Dudek et al.[143] has now demonstrated that IL-15 prepares the stage for subsequent metabolic triggers to induce execution of T cell auto-aggression not only in the liver but also at other sites with a predisposing metabolic environment such as adipose tissue. Thus, combinatorial signalling resulting from the simultaneous presence of innate immunity and changes in metabolism creates a stage of T cell differentiation that shows distinct functional properties compared to those of conventional T cells.

The accumulation of $CXCR6^+CD8^+$ T cells observed in the livers of patients with NAFLD[143,149] suggests that the discovery of T cell auto-aggression as a liver damage-inducing mechanism will lead the way to develop targeted immune therapies in the future that aim to prevent metabolic T cell activation and auto-aggression. Moreover, T cell auto-aggression not only accounts for liver damage but is also responsible for driving development of liver cancer, as shown in a recent study by Pfister et al.[150]. Thus, employing standard checkpoint inhibition therapy to trigger anti-cancer immunity that relieves inhibition of T cells that already induce liver damage carries the danger of increasing the pathogenic potential of auto-aggressive T cells. Indeed, Pfister et al. demonstrated that checkpoint inhibition of patients with NASH and liver cancer failed to show a therapeutic effect and accelerated tumour growth in preclinical NASH models[150]. Separating T cell auto-aggression from anti-cancer immunity of T cells to design immune therapies will therefore be an important issue to address in the future. In sum, while there is little evidence for recognition of auto-antigens or T cell auto-immunity, it has now become evident that metabolic activation of liver-resident T cells is a key factor of tissue pathology in NASH (Fig. 3).

The studies discussed here support the concept that not only innate immunity but also adaptive immunity is increasingly recognized to have major relevance in metabolic disorders such as NAFLD. What remains to be defined is whether antigens (for example, those derived from microbes) are especially relevant in driving innate immunity, and there is a need to elucidate how adaptive immunity contributes to other key features of this disease such as liver fibrogenesis.

## Conclusions

Collective evidence highlights multiple interactions between metabolic pathways, the gut microbiome and immunity in experimental and human NAFLD. None of these factors can be portrayed in isolation. For example, metabolism is critically intertwined with the gut microbiome and the immune system in many layers, such that a single (initial) culprit for this disease may not be identified in the future. From a clinical perspective, many cases of NAFLD can be considered a sequelae of obesity and/or T2DM, and therefore metabolic dysregulation appears rather to be an early event in the pathophysiology of NAFLD. Hepatic insulin resistance reflects another 'sine qua non' condition in NAFLD. Genetic studies thus far mainly linked lipid metabolism but rarely immunity or host–microbe interactions with human NAFLD[151]. Likewise, lipotoxicity emerges as a detrimental driver of liver inflammation and fibrosis in NAFLD, which also underlies metabolic perturbation.

Collectively, metabolic dysregulation, dietary factors, obesity and T2DM have the capability to alter the gut microbiota, which serves as a pool of metabolites and inflammatory signals in patients with or without a leaky gut. Such gut microbiota-derived signals direct innate and adaptive immune responses by exploiting pattern recognition receptors and metabolic reprogramming, challenging the sterile nature of liver diseases. This complexity can be depicted in a 'triangle of NAFLD' (Fig. 4), underpinning the idea that advanced disease stages may be driven by immune dysregulation, gut dysbiosis and metabolic perturbation, which collectively fuel hepatic inflammation and other aspects of NAFLD.

The identification of auto-aggressive T cells, activation of which is distinct from that of T cells protecting us against infection and cancer, as drivers of liver damage and liver cancer in NAFLD will help to identify molecular targets for intervention at the level of immune-mediated liver damage. A major challenge in the field is to translate these mechanistic insights into new therapeutic approaches, which are currently under extensive clinical investigation in controlled trials (Table 1), disentangling the often correlative nature of many human NAFLD studies. This may also be facilitated by studies that will delineate remaining aspects in the conundrum of NAFLD, which are discussed in Box 2. Today, this ever growing field already challenges the clinical perception of NAFLD, which gives hope to overcome the NAFLD pandemic.

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## Author contributions

H.T., T.E.A., M.D. and P.K. researched data for the article and wrote, reviewed and edited the manuscript.

## Competing interests

The authors declare no competing interests.

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