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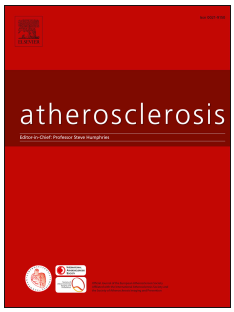

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Review

A concise review of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and the incidence of which is rising rapidly due to the increasing epidemic of obesity in both adults and children. The initial accumulation of fat followed by subsequent inflammation is central to the development of liver damage, and is critically influenced by host factors including age, gender, presence of diabetes, genetic polymorphisms and more recently by the gut microbiome. An increasing body of data suggest that NAFLD is also an independent risk factor of cardiovascular disease, which remains the commonest cause of mortality in such patients. This review focusses on the pathogenesis of NAFLD, and the evolution of new approaches to the management and treatment of NAFLD.

Keywords: *Non-alcoholic fatty liver disease, pathogenesis, dyslipidaemia, insulin resistance, cardiovascular disease*

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is becoming an important public health concern due to the rising incidence of obesity in both children and adults. NAFLD, defined by the presence of hepatic steatosis (the presence of fat in liver parenchyma without inflammation) in the absence of excess alcohol consumption (less than 21 units in men and 14 units in women), is considered to represent the hepatic component of metabolic syndrome [1]. NAFLD represents a spectrum of disease, ranging from simple steatosis to steatohepatitis (the presence of fat in liver parenchyma with inflammation, hepatocyte ballooning and lobular inflammation) through to fibrosis and cirrhosis [2]. Simple steatosis (SS) rarely progresses to advanced disease whereas in approximately 20% of patients with non-alcoholic steatohepatitis (NASH), it progresses to fibrosis and cirrhosis over a 15 year time period [3].

It is strongly associated with insulin resistance and other metabolic risk factors such as diabetes mellitus, central abdominal obesity and dyslipidaemia [4]. NAFLD is an independent risk factor for cardiovascular disease (CVD) and predicts future events, independently of other risk factors such as age, gender, low density lipoprotein (LDL) cholesterol, smoking and other features of metabolic risk factors [5-7]. NAFLD is also associated with increased risk of all-cause mortality, contributed by liver related deaths as well as non-liver related causes such as malignancy, diabetes and coronary artery disease [8].

Epidemiology

The prevalence of NAFLD in normal weight individuals without the presence of metabolic risk factors is reported to be around 16% [9] rising to 43-60% in patients with diabetes [10, 11], 91% in obese patients undergoing bariatric surgery [12], and up to 90% in patients with hyperlipidaemia [4, 13]. The prevalence of NAFLD also increases with age from less than 20% under the age of 20 to more than 40% in over the age of 60 [6] and indeed older age has been shown to be an independent risk factor for hepatic steatosis and progression to fibrosis and cirrhosis [14]. The male gender has been regarded as a risk factor for progression to NASH and fibrosis [14]. Although earlier studies suggested that ethnicity impacts on the prevalence of NAFLD [15], subsequent studies did not confirm this on multi-variant analysis [16]. Intriguingly though the link between insulin resistance (IR) [measured by homeostasis model assessment-estimated insulin resistance (HOMA-IR)] and the risk of NASH is seemingly different between Latino and non-Latino individuals [16].

Mortality is increased in patients with steatohepatitis and advanced fibrosis but not in patients without evidence of steatohepatitis and fibrosis (known as bland steatosis). A long term follow up study of 129 patients with biopsy proven NAFLD showed that mortality was not increased in patients with simple steatosis [17],but increased in patients with NASH. The mortality was related mainly to cardiovascular disease although liver related deaths were more common in patients with NASH cirrhosis [17]. A recent systematic review of 221 patients with biopsy proven NASH showed that patient's age and the degree of inflammation on initial liver biopsy were independent predictors of progression to advanced fibrosis whilst factors such as diabetes, hypertension and obesity were not statistically significant predictors [18]. These findings supported that the presence of advanced fibrosis was associated with increased overall mortality [19] , most likely from cardiovascular events [19].

Hamabe et al demonstrated in a retrospective study over a 10 year period that smoking was an independent risk factor for NAFLD irrespective of the presence of other metabolic risk factors [20]. Notably, the rate of development of NAFLD was similar in those individuals that stopped smoking to those that carried on, perhaps related to subsequent weight gain in individuals after cessation of

smoking [20]. Modest alcohol consumption (one alcoholic beverage per day) had not been demonstrated to increase the prevalence of NAFLD, and in the case of modest wine consumption it would appear to reduce the incidence [21].

Natural history and pathogenesis of NAFLD

The natural history and the pathogenesis of NAFLD is not clearly well described. In NAFLD, simple steatosis is regarded as the presence of fat in <5 % of hepatocytes [22] and in about 20-25% of cases, it progresses to NASH and of these patients with NASH, 20% will develop fibrosis and subsequently cirrhosis [3]. The mechanisms involved in the progression of steatosis to NASH are complex and not completely understood although increased visceral adiposity and insulin resistance (IR) with increased free fatty acids (FFA) release might play a role in the development of liver steatosis [22]. In healthy subjects, insulin stimulates hepatic as well as peripheral glucose uptake and suppress hepatic glucose production [23] whereas in the fasting state, the liver becomes the major site of glucose production as mediated by gluconeogenesis and glycogenolysis [2, 24]. In patients with IR, hepatic auto- regulation is disrupted and therefore, both gluconeogenesis and glycogenolysis are increased resulting in the development of hyperglycaemia [13]. The mechanism of the liver injury in NAFLD is currently thought to be a 'multiple hit process' involving IR, oxidative stress, apoptosis and perturbations of adipokines levels [25].

Two hit hypothesis:

The current model of 'two hit hypothesis in NAFD' was proposed in 1998 by Day et al [26]. The first hit reflects the accumulation of triglycerides (TG) and FFA in hepatocytes which is a consequence of IR, enhanced dietary influx and increased hepatic lipogenesis [26]. The second hit involves lipid peroxidation, mitochondrial dysfunction and inflammation which results in hepatocyte damage and development of liver fibrosis [26]. Activation of pro-inflammatory pathway is mediated by cytokine and pattern recognition receptors, including toll like receptors and these pathways emerge on two main intracellular signalling pathways, known as nuclear factor-κβ (NF- κβ) and c-Jun N-terminal kinase (JNK)[27, 28]. NF- κβ activation is reported in NASH and can lead to increased transcription of many pro-inflammatory genes, whereas JNK causes IR via direct phosphorylation and degradation of insulin receptor substrate 1 (IRS1), reducing the intracellular signalling pathway downstream the insulin receptor [27]. Lipid peroxidation can promote stellate cell proliferation contributing to fibrogenesis [29], whereas reactive oxygen species (ROS) induce cytokines release from hepatocytes that lead to the initiation of various immune mediated mechanisms contributing to further liver cell injury. The combination of hyperinsulinaemia, hepatic iron and lipid peroxidation induce oxidative stress [15], which can cause mitochondrial dysfunction in NASH and contribute to TG accumulation and eventually cell necrosis [11].

Insulin resistance

Patients with NAFLD have reduced insulin sensitivity not only in muscle but also in liver and adipose tissue [30], which play a major role in the pathogenesis of NAFLD. Due to IR, the adipose tissue becomes resistant to the anti-lipolytic effect of the insulin and results in peripheral lipolysis which causes an increase delivery of FFA to the liver as well as driving de novo lipogenesis (DNL) [6, 15]. In addition, lipid overload in pancreatic-B cells leads to dysregulated insulin secretion and changes in the expression of peroxisome proliferator-activated receptor-α (PPAR-α), glucokinase, the glucose transporter-2 (GLUT 2), pre-pro-insulin and pancreatix duodenal homeoboc-1 (PDX-1), which can lead to IR as a result of FFA- induced B-cell apoptosis [12].

A study on liver insulin knock out mice (LIKO) showed that on a standard chow diet, the mice have a proatherogenic lipid profile with low high density lipoprotein (HDL) cholesterol and very low density lipoprotein (VLDL) particles that are mainly enriched in cholesterol, although in the case of normal mice, VLDL is mainly composed of TG [31]. Within 12 weeks on an atherogenic diet (15% dairy fat, 1% cholesterol, 0.5% cholic acid), the mice showed marked hypercholesterolemia and all of LIKO mice developed severe atherosclerosis [31]. Therefore, it has been suggested that IR at the level of liver is sufficient enough to produce the dyslipidaemia and increased the risk of atherosclerosis [32].

Free fatty acids

De novo lipogenesis (DNL) is an integrated metabolic pathway consisting of glycolysis (conversion of glucose to acetyl-CoA); biosynthesis of saturated fatty acids followed by desaturation, and the formation of TG and this pathway mainly takes place in adipose tissue and liver. Dysregulation of DNL is observed in patients with obesity, metabolic syndrome or NAFLD. DNL is up-regulated after a high carbohydrate and low fat diet leading to an increase in blood triacylglycerol levels that leads to an increase in the synthesis and secretion of VLDL in the liver and subsequently hepatic hypertriglyceridemia leading to steatosis [33]. Fat accumulates in the liver due to excessive intake of dietary FFAs and increased influx of FFA into the liver due to peripheral IR and increased hepatic DNL [37]. It has been demonstrated that around 26% of FFAs in the liver derived from hepatic DNL in individuals with NAFLD [38] with the remaining TG content being derived from lipolysis of adipose tissue store (59%) and from diet (15%) [38]. Although the exact underlying mechanism is not completely understood, it has been suggested that hepatic IR can be both a cause and/or a consequence of steatosis in the liver. Chronic hyperinsulinaemia, as found in NAFLD, promotes hepatic DNL through an up-regulation of lipogenic transcription factors [39, 40].

A study [33] has demonstrated successfully that both serum concentration of carbomoyl phosphate synthase 1 (CPS1) and glucose regulated protein (GRP78) has decreased gradually in subjects from SS to NASH. GRP78 is immunoglobulin binding protein and is a central regulator of endoplasmic reticulum (ER) function and crucial for cell survival [34]. GRP78 inhibit both insulin dependent and ER-stress dependent sterol regulatory element-binding protein 1c (SREBP-1c) proteocleavage that plays an important role in DNL [34]. SREBP-1c regulates genes required for glucose metabolism, fatty acid and lipid production and its activity is regulated by insulin [35].

Recent rodent studies demonstrated that in obesity, DNL is down regulated in white adipose tissue (WAT) and that its selective restoration in WAT reverts obesity-dependent IR [34, 35]. Human studies demonstrate similar findings; DNL enzymes are markedly suppressed in WAT of obese subjects, as is the glucose transporter type 4 (GLUT-4) [33]. This suppression is closely linked to impaired metabolic control and can be reversed by weight loss through bariatric intervention. This suggests that the level of DNL or its relevant products such as monounsaturated fatty acids in WAT is an important determinant of systemic IR and subsequent metabolic disease [33]. In contrast to WAT, DNL in the liver has been found to be upregulated in rodent and human obesity where it is believed to promote lipotoxicity, IR, atherogenic dyslipidaemia and NAFLD [36]. Based on this association between hepatic DNL and the metabolic syndrome, it is believed that inhibition of DNL may be a viable approach to treating obesity-related disorders such as type 2 diabetes (T2D) [37].

The mechanisms of lipotoxicity as consequence of ectopic fat accumulation in the liver are poorly described. When the energy expenditure is less than energy intake, there is abundance of FFA resulting in overspill which are subsequently stored in muscle and liver as ectopic fat [38]. Lipid overload can also be found in other organs such as heart, muscle, pancreas and arterial wall where it is stored ectopically [13, 39]. Adipose tissues can store large amounts of excess FFA and when the

limited storage is exceeded, cell dysfunction or death will result, known as lipotoxicity [39]. Mechanisms of lipotoxicity involve apoptosis, decreased cardiolipin content, increased membrane permeability and cytochrome release in mitochondria, nuclear factor-kappa beta (NF-κβ) activation and oxidative stress [39, 40]. Previous studies from transgenic mice with over expression of lipoprotein lipase lead to lipid accumulation in the liver and in muscle leading organ specific IR [41, 42]. In NAFLD, the accumulation of hepatic fat is believed to be the consequence of an imbalance between the uptake/production of fat in the liver and its subsequent secretion or metabolism [36].

Kupffer cells (KC) constitute the largest cellular component of the reticuloendothelial system, representing 80%–90% of all tissue macrophages in the body [43] and about 10% of the resting total liver cell population. They are located in the liver sinusoids and can migrate into the space of Disse where they orchestrate cross-talk between various resident and recruited cells of the liver [44]. Hepatic steatosis and inflammation is also mediated by KC through the Toll-like receptors (TLR), mainly TLR-3 and TLR-4 as well as scavenger receptor pathway, mediating production of cytokines such as TNF-α and Interleukins-15 (IL-15) and IL-1 β [45, 46]. Animal study showed that the effect of KC on liver TG are medicated partially by IL-1 β which suppresses PPAR-α which is a gene involved in fatty acid oxidation [46]. It has also been shown that KC in NAFLD have a defective phagocytic function which may result in an impaired clearance of toxic substances, such as endotoxin, leading to hepatocyte damage [47], as supported by a study showing that the numbers of hepatic CD68+KC correlate with histological severity in patients with NAFLD [48].

Adipose tissue

Adipose tissue is the major source of FFA and responsible for 60% of TG accumulation. Previous studies had suggested that obese individuals often have enlarged adipocytes due to lipid overload. Excess lipid contents spill over from the incompetent and dysfunctional adipose tissue lead to ectopic lipid deposition in organs such as liver and muscle [48]. Moreover, patients with central obesity have large amounts of visceral adipose tissue (VAT) which is defined as intra-abdominal fat bounded by parietal peritoneum or transversalis fascia and it is a major source of FFA, hormones, cytokines such as IL-6, tumour necrosis factor (TNF)-α, plasminogen activator inhibitor-1 (PAI-1), leptin, and adipokines [49]. These factors are secreted into the circulation and frequently taken up by the liver, with the peripheral FFA flux derived from VAT being the most notable example [39] . This flux in particular is a major determinant of accumulation of hepatic and lipoprotein fat in NAFLD [39]. Waist circumference is highly correlated with VAT in both genders and is used as a clinical marker for abdominal obesity and found to be better predictor than body mass index (BMI) [25].

Adiponectin is a protein that acts a protective adipokine by inhibiting liver gluconeogenesis and suppressing lipogenesis [25], an effect mediated mainly through activation of AMP mediated protein kinase (AMPK) and PPAR-α, thus stimulating fatty acid oxidation in liver and muscle [13]. Adiponectin levels also correlate inversely with plasma TG and positively with HDL-cholesterol and low LDL- cholesterol levels [13]. Patients with NAFLD have lower adiponectin concentration than normal subjects despite higher lipolysis and fatty acid concentrations [13]. Therefore, low adiponectin in NAFLD enhances FFA overload and lipid oxidation and consequently play a role in progression from steatosis to NASH [7].

Death and repair

Hepatocyte apoptosis plays a critical role in liver injury and the development of NASH [50]. Soluble Fas, a death receptor membrane of the TNF family appears to have an important role in hepatocyte apoptosis. The accumulation of FFA in hepatocytes triggered upregulation of Fas ligands and

activation of Fas receptors which results in apoptosis [51]. In addition, effector caspases (mainly caspase-3) cleave various substrates inside the cell including cytokeratin 18 (CK-18), which is a major intermediate filament protein in the liver, resulting in apoptosis [52]. Fragments of CK-18 are detectable in the bloodstream by ELISA and it has a promising aspect as a diagnostic tool in near future.

Hedgehog (Hh) signalling has been implicated in hepatic tissue repair resulting in its study in NAFLD to establish whether aberrant or prolonged signalling may impact on disease progression in NAFLD [14]. Notably, the level of Hh activity seems to be proportional to the severity and duration of liver disease in both rodents and humans [14]. Macrophage infiltration is also seen in patient with NASH [24], and monocyte chemoattractant protein (MCP)-1 is also an important factor in NASH as it can potentially mediates the progression of disease by causing persistent inflammation as a result of infiltration of leucocytes into the liver [24].

Genetic polymorphisms

Two independent genome-wide association studies linked the common rs738409 polymorphism of palatin-like phospholipase domain-containing 3 gene (PNPLA3) with hepatic fat content and liver enzyme levels [53, 54]. This single variant (rs738409) substitutes cytosine to guanine that changes codon 148 of the protein from isoleucine to methionine [55]. The protein encoding PNPLA3, known as adiponutrin is particularly associated with increased hepatocyte fat content [14]. In humans, PNPLA3 is mainly expressed in intracellular membrane fractions in hepatocytes, and is induced in the liver after feeding and during IR [53]; the G allele has been shown to be associated with increased risk of hepatic TG accumulation ad hence NAFLD [14]. The normal genotype is CC allele and the most common found in NAFLD is CG allele and GG allele which have the worse outcome with rapid progression to fibrosis and cirrhosis [56, 57]. Since the discovery of the association between the *PNPLA3* mutation with steatosis and steatohepatitis, several additional single nucleotide polymorphisms (SNPs) have been identified in individuals with NASH. Genome wide studies looking at non-Hispanic, Caucasian women with biopsy proven NAFLD [58] showed that non-alcoholic steatosis (NAS) was significantly associated with rs2645424 on chromosome 8 in the farnesyl diphosphate farnesyl transferase-1 gene (FDFT-1), which is a key regulator of cholesterol biosynthesis. This pilot study also showed that the degree of fibrosis was strongly correlated with SNP rs343062 on chromosome 7 although the exact function of this SNP is unknown. Three SNPs were associated with the lobular inflammation phenotype: SNP rs1227756 on chromosome 10 in the *COL13A1* (and collagen, type XIII, α 1) gene, rs6591182 on chromosome 11, and rs887304 on chromosome 12 in the EF-hand calcium binding domain 4B(*EFCAB4B)* gene.

Another common gene variant, glucokinase regulatory protein (GCKR) has been studied extensively. GCKR regulates glucokinase, a phosphorylating enzyme that responsible for hepatic glucose metabolism and activates hepatic lipogenesis [59]. Polymorphism of this particular gene (rs780094 and rs1260326) was associated with increased risk of type 2 diabetes, especially in Asian population [60].

Gut-Liver axis and the microbiome

The human gastrointestinal tract predominantly consists of anaerobic bacteria and harbors three dominating bacterial phyla: the gram-positive Firmicutes and Actinobacteria, and the gram-negative Bacteroidetes [61]. In adults, almost 60%–80% of the gut microbiota consists of Firmicutes and approximately 20%–40% are Bacteroidetes [62]. In healthy host, the gut microbiota plays many important roles by the secretion of bioactive metabolites, protect against pathogens by maintaining

immunity, perform digestion of complex carbohydrates, synthesize vitamins and store fat. The gut epithelium acts as natural barrier and keeps bacteria, their byproducts and other harmful elements at bay due to the presence of tight junctions and specialised intracellular structures.

The intestinal mucosa serves as a defence barrier that helps prevent the entrance and the systemic spread of bacteria and endotoxins, most of which are lipopolysaccharides (LPS) from the cell walls of gram-negative bacteria [63]. However, under certain conditions, this intestinal barrier fails, resulting in bacterial and endotoxin invasion into the gastrointestinal (GI) tract, after which the pathogens reach systemic organs and tissues; this process is termed bacterial translocation [63]. These microbial products exert pro-inflammatory actions mediated through TLRs [63]. TLRs detect the pathogens enabling the host to regulate immune responses evident on the surfaces of hepatocytes, KC and hepatic stellate cells [63]. As explained by Baldwin, the activation of TLR4 by LPS triggers an essential intracellular inflammatory cascade, includes stress-activated and mitogen-activated protein kinases, c-Jun-N- terminal kinase, p38 and the NFκβ pathway [64]. Therefore, TLR4 has a prominent role in promoting inflammation and injury in conditions such as alcoholic liver disease and NASH [63]. Increasing data have underlined the potentially critical role of the microbiome in the pathogenesis of NAFLD [65]. Different animal mouse models showed that inflammasome-deficiency-associated changes in the configuration of the gut microbiota are associated with increased hepatic steatosis and inflammation through the influx of TLR-4 and TLR-9 agonists into the portal circulation, leading to the increased expression of TNF-α, which drives NASH progression [65]. Defective NLR related protein (NLPR)-2 and NLPR-6 inflammasomes altered the interactions between the host and the gut microbiota and they negatively regulate NAFLD/NASH progression as well as the many aspects of metabolic syndrome via modulation of the gut microbiota [65].

The liver continuously receives blood from the gut through the portal system and therefore, subjected to translocation of bacteria, bacterial products, endotoxin or secreted cytokines present in the gut. Normally, endotoxaemia in the portal circulation is rapidly cleared by the liver's endothelial system, mainly KC [63]. However, in the case of liver disease, the endotoxaemic burden is higher and the mechanisms to clear these endotoxins are poorer resulting in ongoing inflammation. Miele et al demonstrated that patients with NAFLD have increased gut permeability, possibility related to small bacterial overgrowth (SIBO) resulting in tight junction disruption, which may impact on NAFLD progression [31]. Most of these studies were performed on animal models and hence, more research is needed in this area of field to understand how such mechanisms apply in the clinical setting.

A recently published prospective, cross-sectional study identified important differences in intestinal microbiota (IM) in patients with biopsy proven NAFLD (simple steatosis/SS or NASH) and healthy controls [66]. Patients with NASH had a lower percentage of Bacteroidetes and a higher percentage of Coccoides compared to both SS and healthy controls with no differences seen in the remaining microorganisms. Differences in levels of faecal Bacteroidetes still remained after adjusting for BMI and dietary fat intake between the groups suggesting that IM may play a role in the pathogenesis of NAFLD.

Recent study [67] determined the effect of gut microflora alterations in a murine model of high-fat-diet (HFD) induced NAFLD . The study showed that when HFD-fed mice were treated with antibiotics, there was a significant increase in conjugated bile acid metabolites, which inhibited intestinal farnesoid X receptor (FXR) signalling and as a result, there was a reduction of hepatic TG accumulation. This was noted to be due to modulation of gut microflora by reduction of ceramide levels in the serum and ileum resulting in down regulation of hepatic SREPBP1C and decreased DNL.

This particular study suggested that there might be a potential therapeutic target for NAFLD treatment by inhibiting intestinal FXR.

NAFLD and cardiovascular risk

The pathophysiological mechanisms that link NAFLD with coronary heart disease (CHD), myocardial dysfunction/hypertrophy, aortic valve sclerosis (AVS) and cardiac arrhythmias are incompletely understood [68]. The complex interactions including IR and visceral obesity make it extremely difficult to dissect out the precise causal relationships responsible for the increased risk of CHD and other cardiac and arrhythmic complications observed in patients with NAFLD [68]. The concept so far was that in patients with NASH, there was an increase in systemic and hepatic IR which in turn caused the accumulation of atherogenic dyslipidameia, characterised by high TG, low HDL and high VLDL. In NASH, there seemed to be increased production of many pro-inflammatory markers such as uric acid and C-reactive protein (CRP) [69], IL-6, TNF-α as well as pro fibrogenic markers such as tumor growth factor-β, endothelin 1 and insulin like growth factor-1 which can lead to CHD [70]. A observational study showed that high CRP levels are higher in biopsy proven NASH compared to SS although CRP levels do not reflect the degree between inflammation and fibrosis [71].

Epidemiological studies performed in United States and Japan showed that NAFLD is associated with increased risk of cardiovascular disease (CVD) and is a predictor of CVD independent of the presence of other metabolic syndrome risk factors such as hypertension, diabetes, dyslipidaemia, obesity and IR [21, 22]. The RISC (Relationship between Insulin Sensitivity and Cardiovascular disease) study showed that patients with NAFLD had an increased 10 year coronary heart disease risk score even when only considering those at perceived low risk patients (i.e. without diabetes or hypertension) [23]. The study also showed that subjects with NAFLD are more prone to early carotid atherosclerosis in the absence of co-existing metabolic syndrome risk factors [23].

Recent phase II trials assessing coronary atherosclerotic plaque in patients with NAFLD have showed that such patients are more likely to have advanced high risk coronary plaque, independent of traditional cardiovascular risk factors as compared with patients without NAFLD [72]. In a recent large cohort study of biopsy proven NAFLD such patients had an increased risk of death from cardiovascular and liver related causes [73]. The worst prognosis was seen in patients with stage 3 or 4 fibrosis at baseline, although surprisingly, in patients with high NAS score (between grade 5 and 8) with no fibrosis did not have increased mortality rate compared to the reference population [73]. This observation requires further confirmation in larger cohorts to exclude the possibility of a type 2 error. In another study of patients with a Framingham cardiovascular risk score ≥20%, the presence of advanced fibrosis was predictive of cardiovascular events [19]. A recent study suggests that progression of NAFLD and risk of cardiovascular disease may not be a shared pathway since the carriers of gene TM6SF2 E167K variant have lower serum VLDL and lower risk of cardiovascular disease compared to non-carriers. However, they have more advanced fatty liver disease with severe steatosis, necro-inflammation, ballooning and fibrosis [74].

Previous studies had shown that carotid-artery intimal medial thickness is related to the severity of NAFLD histology, independently of other cardio metabolic risk factors [75, 76]. A retrospective study showed that NAFLD, assessed by either CT or ultrasonography, was significantly associated with increased coronary artery calcification (CAC) score (*i.e.*, CAC score > 100), independently of traditional CVD risk factors [77]. The concurrent finding of abdominal obesity which is significantly

correlated with left ventricular dysfunction also increases all-cause mortality [14]. The hepatic production of PAI-1 is also elevated in NAFLD patients, which is a known risk factor for CVD [2].
A range of other pro-inflammatory molecules which are secreted by the liver (uric acid, CRP and homocysteine concentration) in response to oxidative stress may further impact on the associated cardiovascular risk in NAFLD [11]. Increased CRP promotes inflammation and accelerates atherosclerosis by increasing expression of PAI-1 and adhesion molecules in endothelial cells, inhibiting nitric oxide formation and increasing LDL uptake into macrophages [5]. IL-6 and TNFα are the major stimuli responsible for increased hepatic production of CRP, fibrinogen and other acute-phase proteins [2, 10]. Uric acid, as result of increase in urea, was regulated by decrease in CPS 1 (a ligase enzyme located in the mitochondria) and it has been shown to erect pro-inflammatory and pro-oxidant effect both on adipose tissue and vascular smooth muscles [78, 79]. Uric acid may also contribute to IR by inducing local adipose tissue inflammation, which may cause reduction in production of adiponectin [80].

Dyslipidaemia when associated with NAFLD tend to be pro-atherogenic in nature [81]. The characteristic findings are increased plasma concentrations of VLDL, TG and decreased HDL cholesterol [7], is found in up to 80% of cases of NAFLD [13]. Lipoprotein is important part of underlying mechanistic action in dyslipidaemia associated with NAFLD. Lipoprotein helped with delivery of cholesterol and TG from the liver and intestine to muscle and fat tissue and that action is mediated either by chylomicron and VLDL particles that contain apolipoprotein (apo) B48 and apoB100 or in the case of cholesterol, it is indirectly by conversion of intermediate density lipoproteins (IDL) to LDL. The second function of lipoprotein is the transport of excess cholesterol from extra-hepatic tissue to the liver for the elimination via bile, which is mediated primarily by HDL particles. Both functions are altered in NAFLD. Individuals with NAFLD have reduced hepatic insulin sensitivity and glucose clearance despite having high circulating insulin levels which can contribute to dyslipidemia [13, 82]. Apolipoproteins are proteins that transport lipids in blood circulation. A recent study showed that Apo B/Apo A1 ratio are associated with the prevalence of NAFLD and was independent of obesity and other metabolic components and therefore, the ratio might be useful as a predictive marker for cardiovascular risk in NAFLD [83].

IR has been regarded as one of the factors responsible for atherogenic dyslipidaemia, as it increases release of non-esterified fatty acids (NEFA) or FFA which stimulates hepatic TG output [7]. IR and increased lipid availability within the liver also inhibit apolipoprotein B degradation, stabilising the formation of more VLDL which contains highest amount of TG [7, 23]. Individuals with type 2 diabetes and NAFLD have increased TG accumulation independent of their BMI [13], resulting in cytotoxicity due to increased ROS generation and mitochondrial dysfunction [84]. Subjects with NAFLD, despite high circulating insulin levels, have reduced hepatic insulin sensitivity, poor postprandial glucose clearance, increased FFA and TG concentrations. The treatment of dyslipidaemia plays a critical role in the overall management of these patients.

Diagnosis

NAFLD is commonly asymptomatic disease and often identified incidentally [85]. Clinicians should consider a diagnosis of NAFLD in patients with abnormal liver tests and the presence of one or more metabolic risk factors; indeed the likelihood of NAFLD increases proportionately with the number of metabolic syndrome factors present [86]. Whilst serum aspartate transaminase (AST) and alanine transaminase (ALT) levels may be abnormal in the setting of NAFLD, they are often not, even when using more stringent cut-offs for the upper limit of normal [87]. Several studies had demonstrated that

ALT is a poor marker to predict advance fibrosis in NAFLD patients [88]. Ultrasound scan will show an echobright liver in patients with at least 30% steatosis with a sensitivity of 64% and sensitivity of 85% rising to 90 to 100% [6]. Other modalities of identifying hepatic steatosis include the controlled attenuation parameter (CAP software on the Fibroscan system [89, 90] and magnetic resonance spectroscopy (MRS) which are left to be more sensitive and can also provide a quantitative assessment of statuses [91, 92]. For the non-invasive assessment of fibrosis in NAFLD, there are several scoring systems described which have broadly similar ability to identify those patients with little or significant liver fibrosis[93]. Variables common to many of them include the ratio of AST: ALT, age, BMI and impaired glucose tolerance. Other non-invasive tests for determination of liver fibrosis include serum European Liver Fibrosis (ELF) panel (combination of 3 serum markers: Hyaluronic acid, Pro-collagen III amino terminal peptide and Tissue inhibitor of metalloproteinase 1) and Fibro-test which have been reviewed in details in the literature [94]. Transient elastography such as the Fibroscan has been validated in NAFLD and is often used as an adjunctive non-invasive test to assess liver fibrosis in the outpatient setting. Currently, liver biopsy still represents the gold standard for the diagnosis and staging of NAFLD/NASH and is required as the primary end-point for later stage clinical trials [7, 95].

Treatment

Lifestyle management

Lifestyle intervention with diet and exercise is still the mainstay in the management of patient with NAFLD. Although lifestyle intervention when achieved is effective, it can be difficult to implement, and thus the aim should be gradual but consistent weight loss over 6 to 12 months [96]. Commonly individuals are recommended to restrict caloric intake by approximately 500-1000 kcal/day in conjunction with regular interactions with a dietician [97, 98]. Simple carbohydrates in the diet, in particular fructose, have been linked to NAFLD. Carbohydrate consumption affects glucose homeostasis and free fatty acids metabolism in the liver and hence carbohydrate-restricted diet also has been studied previously [99]. Several studies indicate that a reduction of between 7 to 10% of body weight is associated with a reduction in inflammation in the setting of NAFLD, and thus is set as a target [100]. Whilst exercise in isolation has not been proven to be effective, as part of dietary changes moderate intensity exercise such as brisk walking of 30-45 minutes per day can improve biochemical and histological aspects of NAFLD [100]. Recent retrospective data from 169 obese, middle-aged men who were enrolled in a 12-week weight reduction program through lifestyle modification consisting of dietary restriction plus aerobic exercise showed that moderate to vigorous physical activity (MVPA) $\geq$ 250 minutes per week led to significant decrease in VAT severity, lipid peroxidation and a significant increase in adiponectin levels compared to those who exercise less [101].

Weight loss therapy using pharmacological agents

Orlistat produces moderate weight loss by reducing the absorption of fat by 30 % by inhibition of gastric and pancreatic lipases [96]. However, recent studies indicate the effects of Orlistat in patients with NAFLD are modest and restricted only to those patients that achieved 9% weight loss with lifestyle intervention [102].

Sibutramine is a serotonin and noradrenaline reuptake inhibitor that increases satiety and can lead to modest weight loss [103, 104] and in a 6 month treatment study of obese patients with NASH, it reduced body weight by 10%, improved insulin resistance and decreased transaminase levels [105]. However, sibutramine use was suspended due to its risk of cardiac related mortality [106].

Rimonabant, a cannabinoid receptor antagonist, was a promising agent that induced significant weight loss in association with improvements in insulin resistance and serum lipid and adiponectin levels, however it has been taken off the market in 2008 due to severe psychiatric side effects [107-110].

Bariatric surgery

Bariatric surgery, as indicated for weight loss, has been shown to reduce most of the histological features of NAFLD in several studies [111] as well as obesity associated T2DM and IR. NAFLD is not, as yet, a standalone indication for bariatric surgery although it is commonly factored into the decision making in the management of overweight patients. Bariatric surgery increases insulin sensitivity in the liver, muscle and fat due to weight loss and hence, will improve overall metabolic health. Weight loss after bariatric surgery increases insulin sensitivity in liver, muscle and fat. Thus, it is well recognized that bariatric surgery can improve overall metabolic health, but it remains unclear how it improves insulin sensitivity.

Pharmacological management

Insulin sensitizers

Given the important of IR in the pathogenesis of NAFLD, insulin sensitizers such as metformin and thiazolidinedione (TZDs), have been extensively studied in the treatment of NAFLD [4].

Metformin: Metformin is a biguanide insulin sensitizer widely used in treatment of type 2 diabetes, whose major action is mediated through activation of AMPK [112]. This improves peripheral glucose intake, reduces hepatic gluconeogenesis and lipogenesis and also increases beta oxidation of FFA [113] . However, clinical studies in NAFLD have not demonstrated a consistent benefit in patients with NAFLD and thus its use is reserved for the management of patients with NAFLD and concomitant type 2 diabetes [114, 115].

Thiazolidinediones (TZDs): TZDs are agonists for PPAR-gamma and their actions in NAFLD are probably indirect and in large part, relate to their effects on adipose tissue. Moreover, TZDs can also upregulate adiponectin and/or sitrulin in hepatocytes which modulate central hepatic metabolic regulators such as AMPK, Foxo1 (Forkhead box O1), LKB1 (Liver Kinase B1), NAD (Nicotinamide adenine dinucleotide), NADH (Reduced nicotinamide adenine dinucleotide), PGC-1α and PPAR-1α. These result in increased fatty acid oxidation and subsequently lead to reduction in hepatic fat accumulation [4], and indeed these therapeutic agents have been demonstrated to have potent benefits in pre-clinical models of NAFLD. However, clinical concerns remain about their effect on reducing renal excretion of sodium and intestinal ion transport, which results in raised plasma volume/fluid retention [116] and exacerbations of pre-existing heart failure [117]. TZDs also reduce hepatic steatosis and improve insulin sensitivity in muscle and liver by enhancing adipocyte insulin sensitivity and shifting ectopic lipid from muscle and the liver to subcutaneous adipose tissue [38].

A study looking at a regimen of hypo-caloric diet (500kcal per day) plus pioglitazone versus hypo-caloric diet and placebo in patients with T2DM and NAFLD showed that pioglitazone led to metabolic and histologic improvement in subjects with non-alcoholic steatohepatitis, although the sample size was small [118]. Whilst pioglitazone did not meet the primary end point in a large, multi-centre randomised controlled trial (PIVENS - Pioglitazone or Vitamin E for NASH study), it did improve insulin sensitivity and clearance of steatohepatitis compared to placebo [119]. Indeed, meta-analysis of clinical trials with Pioglitazone does demonstrate clinical benefit in NAFLD although the associated weight gain, peripheral oedema and other cautions have limited its use in clinical practice

[115]. Overall, the effects of TZDs on NAFLD are indirect and perhaps predominantly a result of reduction of fat deposition in the liver.

Incretin based therapies: Incretins, a gut derived neuro endocrine hormones, are produced by the intestinal tract in response to food ingestion and they stimulate glucose dependent insulin release, decrease glucagon release [120] and prolong gastric emptying [121]. Consequently these drugs are licensed for the management of type 2 diabetes where they improve glycaemic control, increased insulin sensitivity and cause significant weight loss. Circulating glucagon like peptide-1 (GLP-1) has a half-life of about 1-2 minutes due to rapid degradation by the enzyme dipeptdyl peptidase-4 (DPP-4) and thus, GLP-1 receptor agonists with increased DPP-4 resistance (e.g., Liraglutide, Exenatide) [122-125]and DPP-4 inhibitors (e.g., Sitagliptin, Saxagliptin) [126, 127] have been developed as therapies. These agents offer promise in NAFLD and on the basis of encouraging open-label studies controlled trials in NAFLD are underway [128].

Lipid lowering drugs

Statins, fibrates and omega-3 polyunsaturated fatty acids (PUFAs) are used to manage dyslipidaemia [4], which is commonly found in patients with NAFLD. There are no studies to support direct benefit of those drugs in NAFLD, but they will be important in reducing the concomitant cardiovascular risk.

Hypertension

Angiotensin II receptor blockers (AIIRB): AIIRB inhibit the proliferation of stellate cells, reducing inflammation and fibrosis, and although early clinical studies with Losartan and Telmisartan have suggested improvements in transaminases and histology in patients with NASH, larger trials have not yet been performed. [105].

Anti-oxidants and cytoprotective therapies

Vitamin E: Vitamin E, a fat soluble vitamin, has an anti-oxidant property and two recently published, large, randomized controlled trials, PIVENS and TONIC (Treatment of non-alcoholic fatty liver disease in children), assessed its effect on adult and paediatric NAFLD populations, respectively [119, 129]. Vitamin E treatment met the primary end- point in both trials [119, 129] however, there remain concerns about the long term safety at the high dose used (400 IU/day) since it has been shown to increase all-cause mortality.

Betaine: Betaine is a naturally occurring metabolite of choline and has been shown to increase S-adenosylmethionine levels and reduce oxidative stress [130]. Unfortunately, when compared with placebo in a randomized controlled trial, Betaine failed to improve steatosis or other histological outcomes [131].

Ursodeoxycholic acid (UDCA): UDCA is a hydrophilic bile acid with cytoprotective and antioxidant properties [105] . Two well-designed RCTs examining UDCA failed to show any significant histological improvements with UDCA alone in patients with biopsy-proven NASH [132, 133].

Pentoxyphylline: Pentoxyphylline is a tumour necrosis factor alpha (TNFα) inhibitor which has been evaluated in NASH for its anti-inflammatory properties [105]. A meta-analysis of randomized, double-blinded, placebo-controlled trials showed that Pentoxyphylline could reduce the

aminotransferase activities and improve the histological parameters in NAFLD patients [134], but the results needs to be confirmed in larger studies.

Microbiomes

Probiotics have attracted interest given their possible ability to alter the composition of Intestinal microbiota (IM) and the subsequent interaction with the immune system and gut epithelium [135]. Commercial probiotics commonly include lactic acid bacteria (*Clostridium/Bacillus* gram-positive bacteria and *Actinomycetes* gram-positive *Bifidobacteria*) and spore-forming bacteria (*Clostridium-Bacillus* gram-positive bacteria) [135]. Several probiotic stain, mainly lactobacillus species, produce lactic acid and being acid resistant, they persist in the stomach longer than other bacteria which protect them against gastric non immunological barriers such as acidity and mucosal barriers.

A recent meta-analysis of 134 NAFLD/NASH patients from 14 randomised controlled trials showed probiotic therapy significantly decreased liver aminotransferases, total-cholesterol level, TNF-α and improve insulin resistance in NAFLD patients but did not show any changes in BMI, glucose or LDL levels [136]. Of the four RCTs included in this meta-analysis, the studied probiotics included lactobacillus, bifidobacterium and streptococcus [136]. Further data, including histological studies are needed with probiotics. A recent double blind randomised controlled trial looking at effect of VSL#3 in obese children with biopsy-proven NAFLD [137] concluded there was an improvement in the USS appearances of the liver although there was no difference in TG levels, HOMA scores and serum ALT levels. A study which tried to evaluate the effects of Bifidobacterium longum with fructo-oligosaccharides (Fos) with lifestyle modification vs lifestyle modification alone in the treatment of NASH in adults [138] showed that the treated group demonstrated a significant improvement in serum AST levels, reduction of TNF-α, CRP, HOMA-IR, serum endotoxin, steatosis, and the NASH activity index [138].

Liver transplantation

For those patients with end-stage liver disease due to NAFLD, liver transplantation (LT) is the only definitive treatment [139]. Outcomes post-transplant for such patient are comparable to that seen with other indications, although this likely reflects stringent case selection. Recurrence of NASH is common post-transplant due to the presence of existing metabolic risk factors as well as the use of immunosuppression such as corticosteroid, although it rarely results in allograft loss.

Conclusion

The incidence of NAFLD is increasing rapidly due to rising rates of obesity in both children and adults, and can vary from simple steatosis to inflammation all the way through to fibrosis and cirrhosis. Hepatic steatosis is recognised to be the consequence of a complex interplay between diet, environment and the liver and adipose tissues, although a full understanding of its pathogenesis has not yet been elucidated [4]. The presence of fibrosis is important in the prognosis of the disease since it confers an increased overall mortality from cardiovascular, liver, and also malignancy. Whilst there are no licensed drug therapies at present, there are many late phase clinical trials in progress so this will likely change in the near future. Given that cardiovascular disease accounts for the majority of deaths in NAFLD, the challenge will be managing these two components simultaneously.

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Figure legends:

Figure 1: Clinical progression of non-alcoholic fatty liver disease

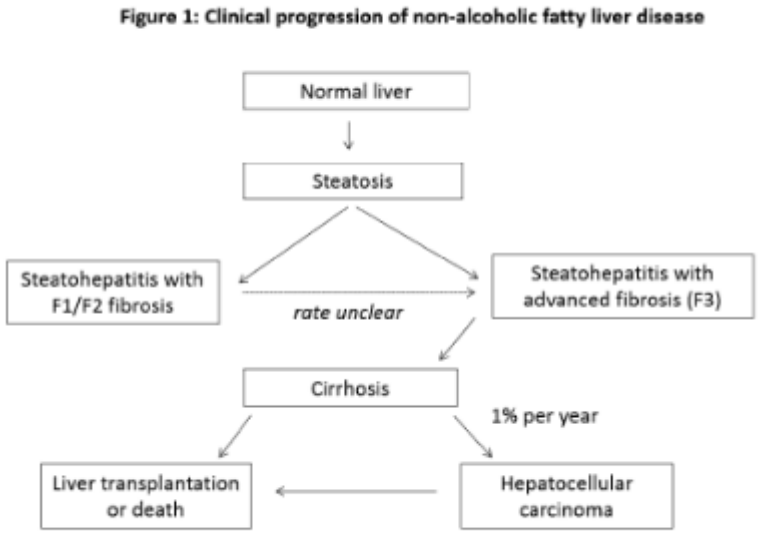
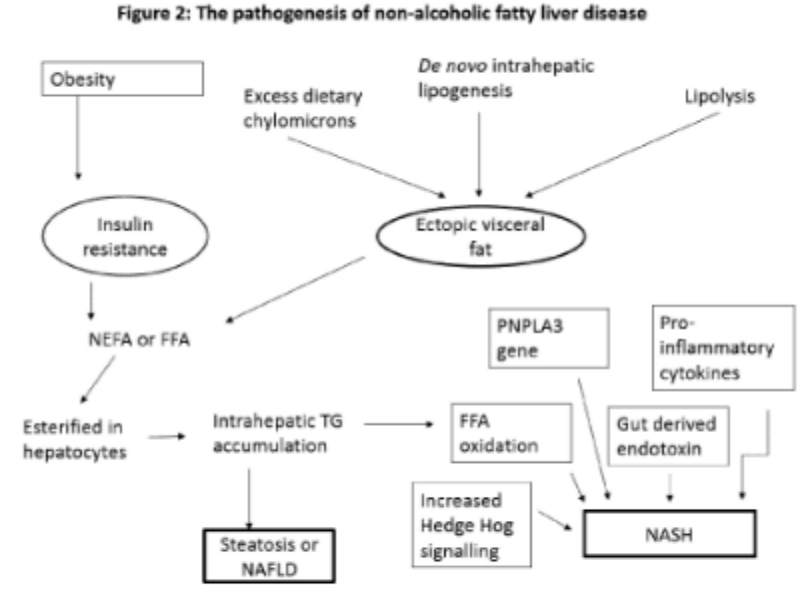


Figure 2: Pathogenesis of non-alcoholic fatty liver disease



NEFA: Non esterified fatty acids, FFA: Free fatty acid, PNPLA: Patatin-like phospholipase domain-containing protein 3, NAFLD: Non-alcoholic fatty liver disease, NASH: Non-alcoholic steatohepatitis

Table 1: Summary of treatments studied for patients with Non-alcoholic fatty liver disease

Lifestyle interventions	Reduction in caloric intake Exercise
Weight loss therapies	Orlistat Bariatric surgery
Insulin sensitisers	Metformin Thiazolidinedione Incretin based therapies (Liraglutide, Exenatide) DDP4 inhibitors (Sitagliptin)
Hypertension	Angiotensin II receptor blockers
Dyslipidaemia	Statins Fibrates Ezetemibe
Antioxidants/cytoprotective therapies	Vitamin E Pentoxyphylline Betaine Ursodeoxycholic acid (UDCA)
Others	Probiotics Polyunsaturated fatty acids (Both drugs have shown little beneficial effects in patients with NAFLD [1]

[1] Sanyal, A.J., et al., *No significant effects of ethyl-eicosapentanoic acid on histologic features of nonalcoholic steatohepatitis in a phase 2 trial.* Gastroenterology, 2014. **147**(2): p. 377-84.e1.

Highlights for review

- Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome and an independent risk factor for cardiovascular disease irrespective of other risk factors such as age, gender, obesity, diabetes mellitus and dyslipidaemia.

- NAFLD is considered to be complex and multifactorial disease phenotype as a result of environmental factors action on an individual with susceptible polygenic background.

- NAFLD ranges from simple steatosis (SS), steatohepatitis (SH), fibrosis, cirrhosis to hepatocellular carcinoma. Unfortunately, the mechanism behind the progression from SS to SH was not fully understood.

- The underlying mechanism for NAFLD pathogenesis is complex, however insulin resistance, accumulation of triglycerides and free fatty acids are thought to play an essential role in the pathogenesis.

- The mainstay of management is lifestyle modification and in addition, other underlying metabolic risk factors should be addressed and managed effectively.

Conflict of interest

No conflict of interest.