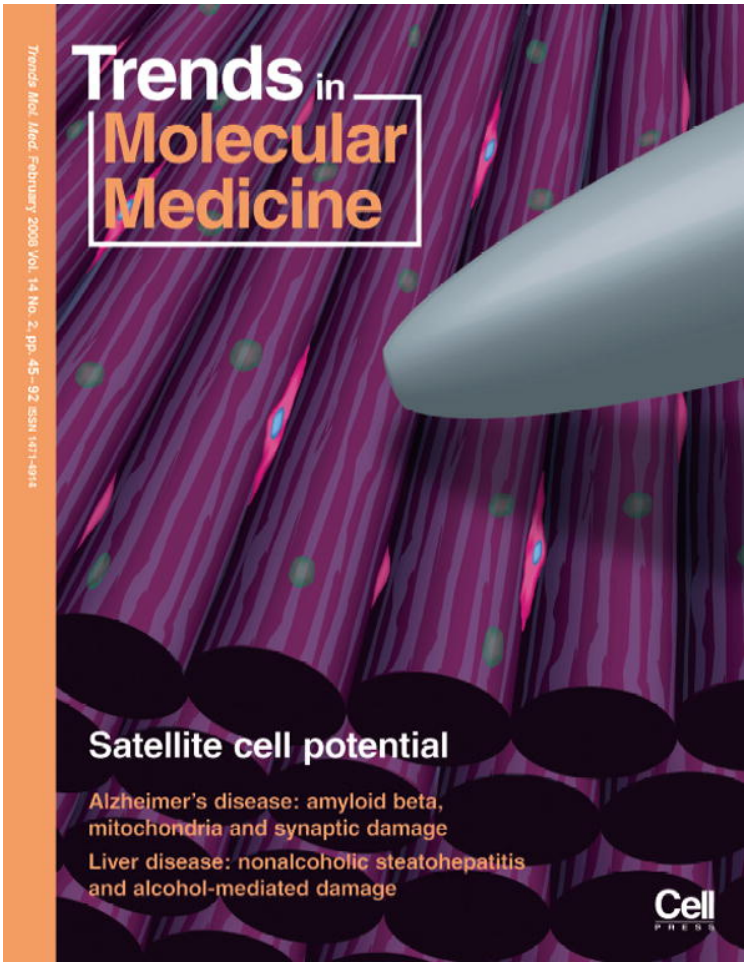

Review

Molecular basis and mechanisms of progression of non-alcoholic steatohepatitis

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Non-alcoholic steatohepatitis (NASH), a cause of cirrhosis and hepatocellular carcinoma, is characterized by fatty infiltration of the liver, inflammation, hepatocellular damage and fibrosis. Progress has been made in understanding the molecular and cellular mechanisms implicated in the pathogenesis of this condition, therefore, we here review recent developments regarding the basic mechanisms of NASH development. Accumulation of triglycerides in the hepatocytes is the result of increased inflow of free fatty acids and *de novo* lipogenesis. Steatosis leads to lipotoxicity, which causes apoptosis, necrosis, generation of oxidative stress and inflammation. The resulting chronic injury activates a fibrogenic response that leads eventually to end-stage liver disease. A better understanding of these mechanisms is crucial for the design of novel diagnostic and therapeutic strategies.

Introduction to the problem

The term non-alcoholic steatohepatitis (NASH) (see Glossary; Box 1) was coined initially by Ludwig to describe histopathological findings typical of alcoholic liver disease in a group of patients without significant alcohol consumption [1]. NASH is observed in a subset of patients with non-alcoholic fatty liver disease (NAFLD), defined as fat accumulation in the liver exceeding 5–10% by weight. The clinical relevance of these conditions is related to the high prevalence of NAFLD in the general population and to the possible evolution of NASH towards end-stage liver disease, including hepatocellular carcinoma, as well as the need for liver transplantation [2].

NAFLD is considered the hepatic manifestation of the metabolic syndrome, a cluster of closely related clinical features linked to visceral obesity and characterized by insulin resistance, dyslipidemia and hypertension [3]. With the rapidly increasing prevalence of the metabolic syndrome in the general population, NAFLD has become the most common cause of liver disease in Western countries [2]. Hepatocellular injury, inflammation and fibrosis are hallmarks of NASH, which are observed in only a fraction of subjects with NAFLD, although the exact mechanisms leading from NAFLD to NASH are still largely unknown. In recent years, a large amount of information on the mechanisms of fat infiltration, damage, inflammation and fibrosis in NASH has become available. This review summarizes recent data that have been generated on this topic. An understanding of these aspects is crucial in this rapidly moving field, in which developments in basic science are translated quickly into diagnostic and/or therapeutic applications. Data discussed herein are derived from studies on clinical pathophysiology, cellular and molecular biology and animal experimentation. The animal experimentation is performed in several different models and the most commonly used, or of greatest relevance to the present review, are reported in Table 1.

Glossary

Adipokines: cytokines produced predominantly at the level of adipose tissue. Their action is both local (autocrine or paracrine) and distant (hormonal). Adipokines produced by visceral fat target the liver primarily through the portal circulation.
***De novo* lipogenesis**: endogenous synthesis of FFAs within the liver.
Hepatic stellate cells: these surround the sinusoids in the normal liver, where they have a 'quiescent' state. After injury, these cells 'activate' and acquire features that are relevant for the development of fibrogenesis.
Non-alcoholic fatty liver disease (NAFLD): fat accumulation in the liver of subjects with absent or low (<20–30 g/day) alcohol consumption.
Non-alcoholic steatohepatitis (NASH): the histological picture observed in a subset of patients with NAFLD. Besides fatty infiltration, variable degrees of hepatocyte damage, inflammation and fibrosis are present.
Peroxisome proliferator-activated receptors (PPARs): molecules belonging to the family of nuclear hormone receptors. Regulate transcription on binding with ligands. Transcriptional activity is modulated by a wide number of co-activators and co-repressors.
Reactive oxygen species (ROS) and reactive nitrogen species (RNS): highly reactive molecules, the levels of which can increase dramatically during different types of stress, resulting in damage to cell structures. This cumulates into a situation defined as oxidative stress.
Thiazolidinediones: a class of antidiabetic drugs that function as PPAR-γ ligands.
Visceral adiposity: fat accumulation inside the abdominal cavity, localized mostly in the omentum. Products of this type of adipose tissue are drained by the portal circulation.

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1471-4914/$ – see front matter doi:10.1016/j.molmed.2007.12.003 Available online 22 January 2008

Box 1. Non-alcoholic steatohepatitis (NASH) in a nutshell

- It is observed in a subset of patients with NAFLD (daily alcohol intake $\leq$20 g/day).
- The metabolic syndrome (central obesity, hyperglycemia, dyslipidemia and hypertension) is a major risk factor for fatty liver and NASH.
- Besides fatty infiltration of the liver, NASH is characterized by histological evidence of inflammation, hepatocellular damage (ballooning, Mallory bodies) and/or fibrosis.
- More severe insulin resistance, susceptibility to oxidative stress, cytokine imbalance favoring inflammation and a 'profibrogenic' phenotype have been proposed as factors involved in the progression from bland steatosis to NASH.
- In the absence of NASH, simple steatosis is considered benign, although it might worsen the course of chronic viral hepatitis or of alcoholic liver disease.
- Most patients with NAFLD are referred to physicians for mild elevations in liver enzymes (aminotransferases and/or γGT) and/or for detection of a 'bright liver' on abdominal ultrasound.
- Magnetic resonance spectroscopy enables a precise measurement of the amount of fat in the liver.
- No imaging technique has the capability to differentiate NASH from simple steatosis; this is only achieved by liver biopsy.
- In a fraction of patients, NASH progresses to severe fibrosis, cirrhosis and liver decompensation. Evolution of NASH is now considered the cause of the majority of cases formerly defined as 'cryptogenic cirrhosis'.
- As in other forms of cirrhosis, the appearance of hepatocellular carcinoma might occur as a consequence of NASH evolution.
- Diet (leading to reduction of body weight) and an increase in physical activity have proven beneficial for patients with NAFLD.
- No drugs are approved currently for the treatment of NASH. Individual components of the metabolic syndrome should be treated according to the existing guidelines.

Factors leading to fat accumulation in the liver

Accumulation of fat, mainly in the form of triglycerides, is the *sine qua non* for the development of NAFLD and NASH. The dynamics of lipid in the hepatocytes is both under control of distantly produced hormones (primarily insulin) and locally generated factors and represents the result of complex interactions among multiple cell types located in different tissues.

Insulin resistance

Insulin resistance (IR) is usually regarded as an impairment in peripheral (i.e. skeletal muscle) glucose metabolism because the muscle is quantitatively the major site of insulin-stimulated glucose uptake. To overcome IR and promote glucose storage, insulin-resistant subjects increase insulin secretion and reduce insulin clearance [4], resulting in metabolic changes in other tissues (Figure 1). Insulin is a potent inhibitor of hepatic endogenous glucose production (HGP), which is impaired in the presence of hepatic insulin resistance [5]. Another important target of IR is adipose tissue, in which the antilipolytic effect of insulin is reduced, resulting in an increased release of free fatty acids (FFAs) into the systemic circulation.

Subjects with NAFLD are highly insulin resistant at the level of: (i) muscle because they exhibit reduced glucose uptake [6]; (ii) liver because they show impaired suppression of HGP [6,7] and (iii) adipose tissue because they exhibit high lipolytic rates and increased circulating FFAs [6,8]. IR in NAFLD is a primary defect independent of obesity and/or diabetes, as shown recently in non-obese, non-diabetic subjects with NAFLD [6]. The presence of diabetes worsens the problem because these subjects tend to accumulate more abdominal fat (both hepatic and visceral), which is correlated strictly to the degree of both peripheral and hepatic IR [9].

Sources and dynamics of hepatic lipids

The mechanism(s) responsible for the increase in hepatic fat accumulation is unclear and little information is available on the time course of the development of hepatic steatosis. Animal models indicate that the liver might accumulate lipids within a few weeks and even a few days [10]. It has been suggested that fatty liver might result from increased delivery of fatty acids, impaired hepatic fatty acid oxidation and/or impaired synthesis or secretion of very low-density lipoprotein (VLDL). Among these factors, the increased flow of FFAs to the liver is considered as the most important. There is general agreement that NAFLD subjects have increased lipolysis and high circulating FFA levels; tracer studies have shown that neither hepatic nor total lipid oxidation is reduced but rather increased [6,8]. Elevated secretion of VLDL is also observed in these subjects [11]. The increased availability of FFAs to the liver might be multifactorial depending on increased release of fatty acids from adipocytes, excess lipid content in the diet or increased *de novo* lipogenesis (DNL) (i.e. endogenous FFA synthesis in the liver) [12] (Figure 1). Animal and human studies indicate that visceral adipose tissue is the major source of the increased FFA flow to the liver. Increased visceral fat has been associated with hepatic IR [7,9] because visceral fat is highly lipolytic and FFAs are released directly into the portal vein. Animals administered with high-fat feeding show both hepatic steatosis and impaired suppression of HGP, whereas, in humans, hypocaloric, low-fat diets decrease hepatocellular lipid levels by 40–80% [13]. Thus, it has been postulated that preferential influx of FFAs through the portal circulation is a relevant determinant of hepatic lipid accumulation. High splanchnic lipid flux is observed not only after high fat meals but also during fasting conditions in subjects with predominantly abdominal obesity [14]. Accordingly, visceral fat accumulation has been associated with NAFLD because a strong correlation exists between visceral and liver fat content [7,9]. DNL might be another important source of hepatic FFAs. Although the contribution of DNL during fasting is rather small ($\sim$5%) in normal subjects, in patients with NAFLD, DNL is elevated, with rates of approximately 25% [15]. DNL requires transcription of lipogenic genes, which is stimulated by insulin through sterol regulatory element binding protein 1 (SREBP-1) and by glucose through carbohydrate-response element-binding protein [16].

Adipokines

The enlargement of adipose tissue and in particular of visceral fat has been associated with a decreased release of insulin-sensitizing and anti-inflammatory cytokines and increased expression of proinflammatory molecules. IR is associated with adipose tissue inflammation, which modifies adipokine secretion. Recent evidence indicates

Table 1. Animal models for the study of NAFLD and NASH[a]

Aspect investigated	Description	Limitations
Thrifty genotype	*Psammomys obesus* (sand rat) is a gerbil that shows normal metabolic parameters when fed a low-calorie diet, whereas, on standard chow, it develops all the parameters of the metabolic syndrome, aminotransferase elevation and macrovesicular steatosis [78]	The possibility of a pathogenetic cascade leading from the thrifty genotype to the metabolic syndrome and to NAFLD or NASH remains to be determined
Diet-induced thermogenesis	A contribution to obesity is provided by abnormalities in energy expenditure, which depends in part on adaptive thermogenesis. Adaptive thermogenesis involves sensing of dietary excesses, leading to an increase in energy expenditure, that is, 'diet-induced thermogenesis'. The sympathetic nervous system is the efferent pathway by which the brain regulates adaptive thermogenesis and mice lacking all three β-adrenergic receptors (β-less mice) have been created [79]. When fed a hypercaloric diet, β-less mice develop massive obesity, due entirely to a defect in diet-induced thermogenesis	The target tissues of the sympathetic nervous system in mediating diet-induced thermogenesis are still unknown. No data on the development of insulin resistance, NAFLD and NASH are available on β-less mice
Models of insulin resistance	Mutations in the *obese* (*ob*) or *diabetes* (*db*) genes prevent the synthesis (*ob/ob* mice) or the effects (*db/db* mice and *fa/fa* rats) of leptin, a satiety hormone that inhibits feeding and increases energy expenditure. These strains share a common phenotype, being hyperphagic, obese, diabetic and hyperlipidemic, with expanded white adipose tissue mass [22]. Increased expression of TNF-α and relatively low levels of adiponectin promote lipolysis and FFA release. Activated sterol regulatory element binding protein (SREBP)-1c promotes *de novo* lipogenesis	These models have been studied extensively, although the *ob* mutation is not prevalent in the human situation. Leptin levels correlate poorly with the development of NASH. Little or no fibrosis is observed in these animals
Models of NASH	Feeding mice or rats with a methionine- and choline-deficient diet (MCD) leads to the development of steatosis, inflammation and fibrosis. MCD impairs VLDL assembly and secretion, reduces mitochondrial β-oxidation and leads to induction of CYP2E1 expression, which induces ROS production, mtDNA damage and apoptosis, with hepatic stellate activation and extracellular matrix deposition [38]. Rats administered a high-calorie diet containing 49% saturated fat develop visceral obesity, increased levels of portal FFA, hepatic insulin resistance and increased ROS production. Steatosis is associated with hepatocyte ballooning, inflammation and apoptosis [80]	The MCD model does not replicate the phenotype or the pathogenetic mechanisms of human NASH because animals are cachectic, have low plasma triglyceride levels and reduced liver weight/body weight ratio and no insulin resistance. Collagen deposition is evident after 6 months of the high-fat diet

[a]Several animal models have been proposed to investigate the different aspects that lead to NASH, although their ability to mirror the genetic or environmental abnormalities observed in human beings might be limited. This table reports some of the aspects that might be investigated in rodent models, relevant for the exploration of mechanisms leading to NASH. For detailed information on animal models of steatosis, please refer to [81].

that the chemokine monocyte chemoattractant protein-1 (MCP-1) is involved in the generation of adipose tissue inflammation via an increased recruitment of macrophages, leading to fatty liver and insulin resistance [17].

Subjects with NAFLD exhibit decreased adiponectin levels [18], which are correlated negatively with hepatic TG content. The direct role of adiponectin in NAFLD is still unclear, but this hormone is supposed to improve primarily glucose and lipid metabolism. Adiponectin also inhibits the expression of several proinflammatory cytokines [19], including tumor necrosis factor (TNF)-α. Leptin is also involved in the accumulation of hepatic TG through the regulation of fat and its distribution [20] and the modulation of hepatic oxidation [21]. A suggested mechanism for the accumulation of hepatic TG could therefore be a resistance to leptin action independently of IR [20]. Leptin also has a multifunctional role in inflammation, acting as a proinflammatory stimulus [22]. Among other factors released within adipose tissue, TNF-α promotes lipolysis and increases FFAs; both TNF-α and interleukin (IL)-6 are related to mitochondrial dysfunction. The role of resistin is still unclear. Resistin is produced by adipocytes and mononuclear cells in rodents and mainly by stromal cells of adipose tissue in humans [23]. Although plasma-resistin levels correlate with hepatic TG and are decreased during pioglitazone treatment, its circulating levels are not increased with increased body-mass index or with insulin resistance and are not elevated uniformly in Type 2 diabetes [23]. Intrahepatic resistin is increased in patients with NASH and inflammatory cells contribute to its expression [24]. Recently, the proinflammatory action of visfatin, a protein produced preferentially by visceral adipose tissue, has also been reported [25]. In spite of the different molecules involved in the inflammatory cytokines, the role of each of these factors and the interconnection among each other needs still to be elucidated.

Peroxisome proliferator-activated receptors

Three isoforms of peroxisome proliferator-activated receptors [(PPARs); PPARα, PPARγ and PPARβ (or δ)] have been identified and are believed to have a central role in the sensing of nutrient levels and in the regulation of lipid and glucose metabolism [26]. Synthetic ligands for PPARγ (thiazolidinediones) and PPARα (fibrates) are used in the treatment of diabetes and dyslipidemia. PPARγ agonists augment insulin-mediated adipose tissue uptake and storage of FFAs and also inhibit hepatic fatty acid synthesis through the activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) [27]. Thus, local effects of PPARγ agonists in adipose tissue can result in reduced IR in distant sites, leading to a reduction of circulating FFAs, glucose production, visceral fat accumulation and liver steatosis [5,28]. Both PPARα and PPARγ have anti-inflammatory actions [27] and reduce adipose

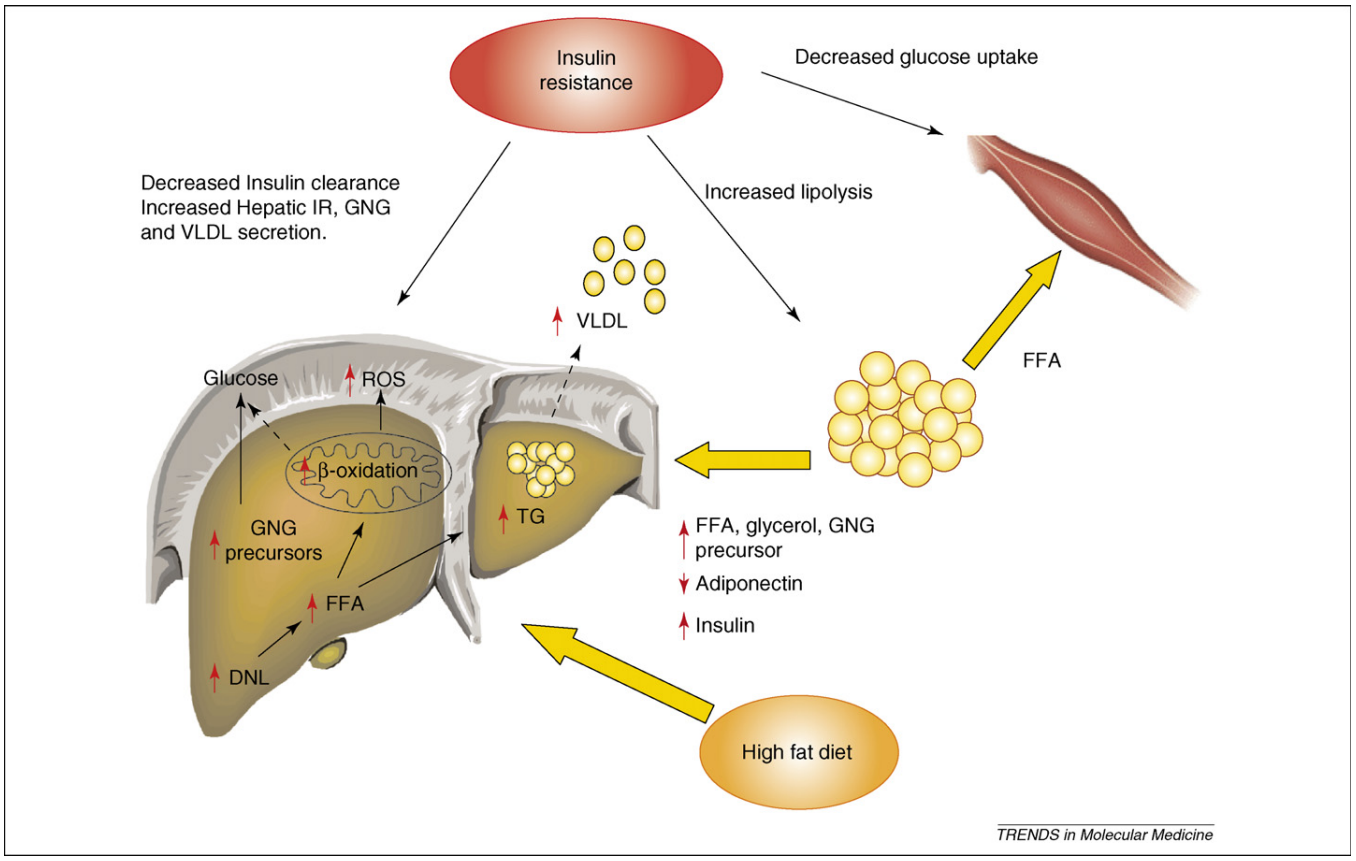

Figure 1. Mechanisms of fat accumulation in non-alcoholic steatohepatitis. Insulin resistance causes an influx of FFAs to the liver, owing to increased lipolysis, especially in the visceral adipose tissue. Increased *de novo* lipogenesis and fat from the diet also contribute to the fatty-acid pool. Both VLDL generation and FFA oxidation are increased and are sufficient to prevent intrahepatic lipid accumulation. Abbreviations: DNL, *de novo* lipogenesis; GNG, gluconeogenesis; ROS, reactive oxygen species; TG, triglycerides; VLDL, very low-density lipoproteins.

tissue-derived circulating factors that could promote IR. In particular, PPARγ agonists increase adiponectin levels and reduce resistin, TNF-α, IL-6 and C-reactive protein levels in Type 2 diabetes patients and in patients with NASH [29,30]. PPARγ-induced increases in plasma adiponectin could also mediate some of the insulin-sensitizing effects of PPARγ agonists. Recently, treatment with thiazolidinediones was found to increase adiponectin levels in NASH patients and this change was correlated with the improvement in hepatic steatosis [30].

Mechanisms of damage in fat-laden hepatocytes

Fat accumulation in the cell might induce cytotoxicity either directly or through sensitization to other agents. Metabolic dysregulation, mitochondrial impairment and oxidative stress have a significant role in determining hepatocyte damage and result in profound changes in gene expression, leading ultimately to apoptosis and contributing to the inflammatory process [31].

Mitochondrial dysfunction and oxidative stress

Mitochondria are involved in both FFA β-oxidation and reactive oxygen species (ROS) generation. Patients with NASH are characterized by abnormal mitochondria from both a functional and a morphological point of view. Accumulating evidence indicates that respiratory-chain defects are a key determinant of mitochondrial dysfunction [32]. Mitochondrial dysfunction is mainly the result of IR and the excess of FFAs (lipotoxicity). Mitochondrial impairment causes enhanced ROS production, which, in turn, initiates a self-sustaining loop that leads to chronic organelle damage. In fact, malondialdehyde and 4-hydroxynonenal, resulting from cellular lipid peroxidation, are able to inhibit cytochrome *c* oxidase of mitochondrial complex IV and ROS *per se* might damage both mitochondrial DNA (mtDNA) and iron–sulphur cluster enzymes of the respiratory chain [33]. Inflammatory cytokines, including TNF-α, also contribute to mitochondrial dysfunction by interfering with the mitochondrial respiratory chain and by forming superoxide anion [34]. An indirect effect of TNF-α in promoting mitochondrial dysfunction is the increased production of reactive nitrogen species (RNS) as a consequence of nitric oxide synthase 2 (NOS2) induction. RNS might inactivate proteins of the mitochondrial respiratory chain functionally through nitration of tyrosine residues or the intermediate formation of S-nitrosocysteine or S-nitroso-glutathione [35] and might also induce DNA damage [36]. The relevance of RNS for mitochondrial dysfunction is confirmed by data obtained in *ob*/*ob* mice, in which administration of uric acid, which reacts with peroxynitrite to form inactive nitrogenous urates, reduced cytochrome *c* nitro-tyrosine formation and lipoperoxide content [37].

The excess FFA oxidation in mitochondria, peroxysomes and microsomes as well as the activation of Kupffer cells

are also relevant sources of ROS, contributing to the condition of oxidative stress typical of NASH. The increased availability of FFAs determines the activation of microsomal cytochrome P450 isoforms CYP2E1 and CYP4A10/4A14, both involved primarily in FFA ω-oxidation, leading to an increased ROS production and uncoupling mitochondrial respiration [32,38]. Accumulation of cholesterol within mitochondria has been identified recently as an additional factor linking fatty infiltration with the development of NASH [39].

Peroxisomal and microsomal β-oxidation cooperate in limiting mitochondrial overload in the presence of excess FFAs and it has been proposed that polymorphisms affecting these pathways might contribute to the pathogenesis of NASH [40]. Of notice is the finding that a non-mitochondrial source of ROS, such as the NADPH-oxidase system of Kupffer cells, is also activated in NASH models, probably as a consequence of lipoperoxide or endotoxin phagocytosis [41]. This pathway is also present in activated hepatic stellate cells (HSCs), possibly contributing to the general oxidative stress condition. Nonetheless, inactivation of NADPH oxidase by genetic deletion of gp91phox was shown recently to be ineffective in blocking cytokine production and fibrogenesis in dietary steatohepatitis [42].

Effects of oxidative stress

Chemical modification of essential biomolecules by ROS and RNS causes their functional inactivation and leads to either cell death or to an adaptive cellular response, for example, activation of redox-sensitive transcription factors [43,44] (nuclear factor (NF)-κB, Nrf-1 and Sp-1), which contributes to the production of proinflammatory and fibrogenic mediators by Kupffer cells and HSCs. Interestingly, and potentially relevant for the pathogenesis of NASH, Houstis showed a causal connection recently between disequilibrium in ROS production and IR [45]. The well established primary role of oxidative stress in the pathogenesis of NASH is demonstrated by: (i) a significant increase of markers of oxidative damage for lipids (i.e. thiobarbituric acid reactive substances, malondialdehyde, 4-hydroxynonenal) [46,47], proteins (i.e. nitro-tyrosine protein content) [8] and DNA (i.e. 8-OH-dG) [46], as well as an increase in thioredoxin, a plasmatic oxidative stress marker [48]; and (ii) a concomitant reduction of antioxidant factors, including coenzyme Q10, CuZn-superoxide dismutase, catalase activity, glutathione and glutathione S-transferase [47]. Thus, as described earlier, consequences of oxidative stress are associated with promotion and maintenance of cellular damage, involving the mitochondrial 'vicious cycle' both acting at local and systemic levels (Box 2).

Hepatocyte cell death in NASH

In spite of their powerful antioxidant resources, hepatocytes suffer from the cytotoxic effect of oxidative stress, leading to cell death by necrosis and apoptosis, as well as triggering inflammation [49,50]. Both the intrinsic (triggered by cellular stress) and the extrinsic (induced by death receptors) pathways are involved in the pathogenesis of NASH [50]. The finding that the anti-apoptotic Bcl-2 is significantly elevated in NASH patients compared with controls [51] is suggestive of a major involvement of the extrinsic pathway, in which Bcl-2 action is secondary. Inhibition of apoptosis induced by death receptors and evaluation of plasmatic content of caspase-activity products (i.e. cytokeratin-18 fragments, as in [50]) are potentially promising targets for non-invasive diagnosis and therapy of NASH.

Box 2. Mechanisms leading to hepatocyte damage in NASH

- Excessive FFA oxidation can cause oxidative stress through mitochondria, peroxisomes and microsomes.
- Plasmatic markers of oxidative stress are increased in NASH patients.
- Increase in FFA (i) determines hyperactivation of microsomal CYP450 enzyme isoforms CYP2E1 and CYP4A leading to an increase in ROS production and (ii) acts in uncoupling mitochondrial respiration.
- NASH is characterized by functional and morphological mitochondrial abnormalities.
- Impairment of oxidative phosphorylation inhibits the respiratory chain and β-oxidation, ultimately causing a marked impairment of mitochondrial fatty acid oxidation and leading to ROS production.
- TNF-α secreted by adipose tissue, hepatocytes and Kuppfer cells contributes significantly to mitochondrial dysfunction by promoting ROS and RNS production by iNOS.
- Hepatocyte apoptosis (both by the intrinsic and the extrinsic pathway) is involved in the pathogenesis of NASH.
- A deep alteration in gene expression profiles occurs during NASH pathogenesis. The transcription factors Sp1 and Sp3, C/EBPβ, Nrf-1 and NF-κB are actively involved in controlling inflammation and fibrogenesis.

Gene expression control and NASH

The complex changes in hepatocyte biology imply a relevant alteration in the gene expression profile. In patients with NASH, several cellular responses coexist, involving apoptotic genes, lipid metabolism, chaperone activity, extracellular matrix remodeling, mitochondrial activity and nucleic acid metabolism, as well as genes involved in the adaptive response to oxidative stress [52]. The transcription factors Sp1 and Sp3 have been identified as potential new factors in the development of NASH, being responsible for the early activation of genes, such as *type I procollagen*, which is involved in active fibrogenesis [31,53], or *CCAAT-enhancer-binding protein β (C/EBP β)*, which is involved in lipid synthesis, the inflammatory response and endoplasmic reticulum stress [54]. Of interest is the recent finding of a role for the transcription factor Nrf-1 [44], a pivotal player in controlling gene expression of antioxidant genes through the binding to AU-rich element sequences, and the upregulation in NASH biopsies [31] of apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE/Ref-1), a central coordinator of the adaptive cellular response to oxidative stress. Interestingly, both Nrf-1 and APE/Ref-1 are overexpressed in liver cancer [44]. A new system emerging from gene-deletion studies is represented by the phosphatidylinositol 3-kinase/Akt/phosphatase and tensin homolog (PTEN) pathway [55] because PTEN-deficient mice display features characteristic of NASH as well as hepatocellular carcinoma. Despite the enormous amount of data provided by platforms for gene-expression analysis, little information on proteins, both in terms of expression levels and post-translational modifications, or on potential circulating biomarkers for the diagnosis of NASH is available currently. Future work in this

direction is needed to fully understand whether some kind of specificity can be ascribed to the onset of NASH with respect to other etiologies and to provide the reliable availability of serum biomarkers for non-invasive diagnosis for this pathology.

It should be underscored that recent data indicate that other factors, besides the overall amount of fat, might be crucial for the induction of metabolic abnormalities, cell damage and fibrogenic progression of NASH. Disruption of the gene encoding the elongase Elovl6 in mice prevents the formation of very-long-chain fatty acids [56]. When administered a high-fat, high-sugar diet, the Elovl6-deficient mice had a comparable degree of steatosis when compared with wild-type mice but their sensitivity to insulin was much higher. Recent reports indicate that the lipid accumulation in the form of triglyceride might even be protective for the development of liver fibrosis. Inhibition of diacylglycerol acyltransferase 2 in mice by the use of antisense oligonucleotides was effective in limiting triglyceride accumulation, although oxidative stress, lobular necroinflammation and fibrosis were more severe [57]. These changes were associated with higher intrahepatic levels of FFAs, which are likely to have a major detrimental role in the process of lipotoxicity.

NASH: inflammation and fibrosis

Together with hepatocyte damage, inflammation and fibrosis are the key features denoting the progression from simple steatosis to steatohepatitis. The molecular mechanisms of inflammation are largely cross-talking with those responsible for hepatocellular damage and fibrosis. In a large series of patients, hepatic inflammation or alanine aminotransferase (ALT) levels, as a proxy of necroinflammatory activity, are independent predictors of the fibrogenic progression of the disease. Finally, liver inflammation contributes to insulin resistance and possibly to cardiovascular risk through systemic inflammation [58].

Signaling pathways leading to hepatic inflammation

Among different components of the inflammatory infiltrate, neutrophils are an important source of oxidative stress-related molecules [59], although different types of mononuclear cells are also present. Inflammation in NASH is the result of a cross-talk between parenchymal and non-parenchymal cells through biologically active soluble mediators. Liver cells are also the target of factors generated by the visceral adipose tissue, especially when infiltrated by inflammatory cells, as in obesity. Activated Kupffer cells and hepatic stellate cells also contribute significantly to cytokine expression during steatohepatitis [60]. Finally, sinusoidal endothelial cells are important for the interaction with circulating leukocytes, through the expression of adhesion molecules (Figure 2). Hepatocyte damage and oxidative stress are initial triggers to inflammation, although additional factors, including endoplasmic reticulum stress, contribute to the generation of inflammatory signals and IR [61]. These upstream events converge on the activation of proinflammatory transcription factors that ultimately cause the recruitment of leukocytes. NF-κB activation is crucial for inflammation,

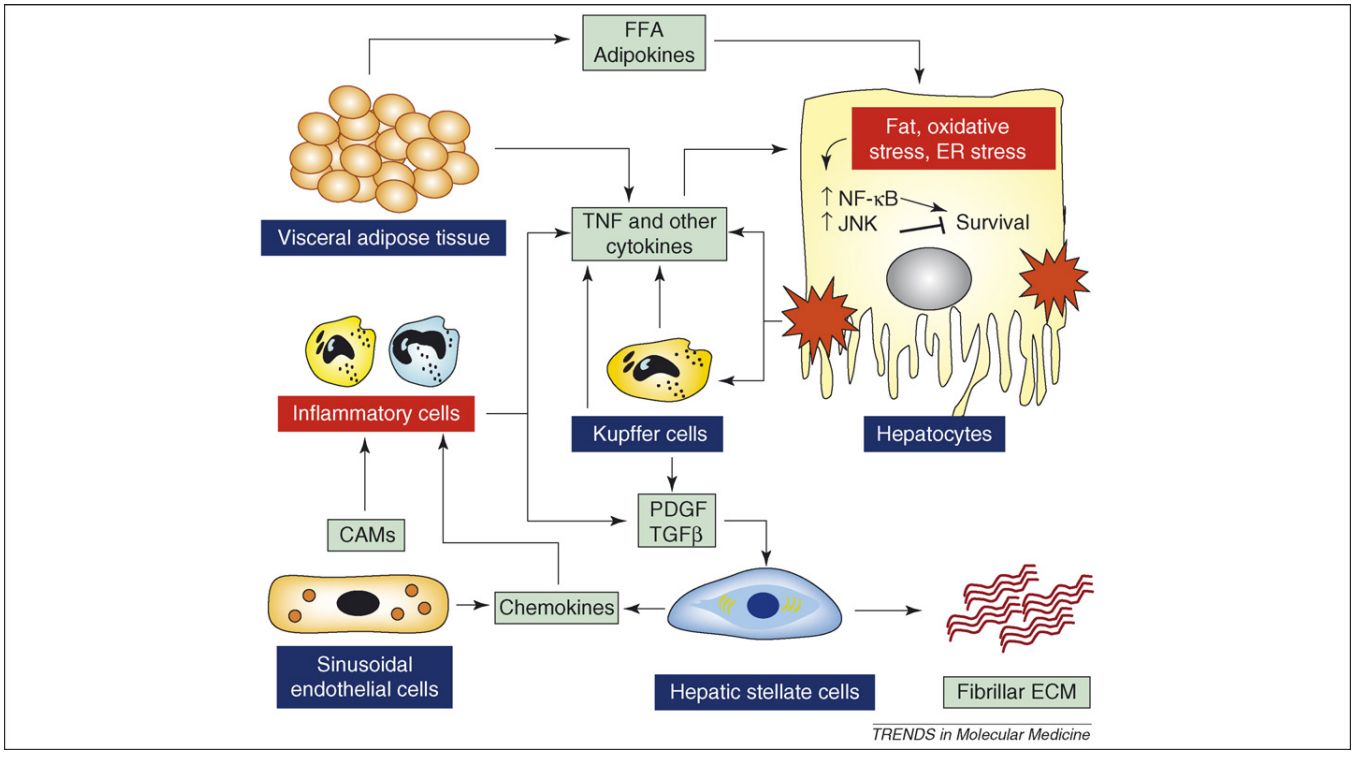

Figure 2. Mechanisms of inflammation in NASH. Damage to hepatocytes caused by fat loading and resulting lipotoxicity leads to the activation of intracellular signaling pathways, which leads to the expression of several cytokines that are responsible for the recruitment of inflammatory cells. Hepatic damage also affects the biology of other liver cells, such as Kupffer cells, which become activated and contribute to cytokine secretion. Recruitment of inflammatory cells is also conditioned by factors produced by activated hepatic stellate cells and sinusoidal endothelial cells. Most liver-resident cells are targeted by adipokines secreted by visceral adipose tissue These events lead to a vicious circle that causes worsening of liver damage, further inflammation, maintenance of steatosis, disease progression and insulin resistance. Abbreviations: CAM, cell adhesion molecule; ECM, extracellular matrix; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase, PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β.

although it is also involved in the maintenance of cell survival [43]. The NF-κB pathway is upregulated in rodent models and in patients with NASH [62] (Figure 2). Conditional activation of NF-κB within hepatocytes is sufficient to trigger low-grade liver inflammation, steatosis and IR [60]; deletion of the upstream kinase IκB kinase β (IKKβ) preserves the sensitivity to insulin when mice are placed on a high-fat diet [63]. Recent data indicate a much more complex role of this transcription factor because neutralization in liver parenchymal cells of NF-κB essential modulator (NEMO or IKKγ), which is required for NF-κB activation, causes steatohepatitis, fibrosis and the development of liver cancer in mice [64]. This apparent discrepancy might be explained by the fact that severe deficiency of NF-κB activation, such as in NEMO conditional-knockout mice, leads to enhanced apoptosis, triggering compensatory hepatocyte proliferation, oxidative stress, inflammation and fibrosis. It could be speculated that strategies that are extremely effective in neutralizing NF-κB activation might be detrimental ultimately for the evolution of steatohepatitis and might even favor the development of cancer.

JNK (c-Jun N-terminal kinase), a mediator of TNF-induced cell death, is another signaling pathway associated with both inflammation and IR. Genetic deletion of both JNK-1 and JNK-2 in hepatocytes is accompanied by a reduction of inflammation and IR in mice [65]. However, although JNK-1-deficient mice are protected markedly from the development of inflammation and liver injury, the phenotype of JNK-2-knockout mice is similar to that of wild-type animals [66]. These data point to JNK, and particularly the JNK-1 isoform, as an attractive target to limiting the development of inflammation, injury and apoptosis associated with lipid accumulation in the hepatocyte.

Cytokines as effectors of proinflammatory signals

Intrahepatic gene expression and/or plasma levels of TNF-α and IL-6 are increased in fatty liver and in NASH in humans [67,68]. These factors are produced under the control of NF-κB, JNK or $p38^{MAPK}$. Modulation of TNF-α expression by genetic deletion or other means results in amelioration of steatosis, inflammation and hepatocyte damage in *ob*/*ob* mice and in dietary models of steatohepatitis [69], suggesting a pivotal role for this cytokine in NASH. Contrary to this conclusion, it was reported recently that, in dietary steatohepatitis, lipid peroxidation and hepatocellular damage were similar in wild-type mice or in animals with targeted deletion of TNF-α or its receptor, TNFR1 [62]. By contrast, interference with NF-κB activation protected significantly from the development of steatohepatitis and reduced the expression of TNF-α and intercellular adhesion molecule-1 [62]. These observations

Table 2. Molecular mechanisms of hepatic fibrosis in NASH[a]

Mechanism	Evidence	Potential clinical relevance
Inflammation	Inflammatory cells release fibrogenic factors, such as platelet-derived growth factor and TGF-β1, which stimulate the profibrogenic actions of HSCs. Depletion of natural killer cells worsens matrix accumulation, inducing programmed cell death of activated HSCs	Anti-inflammatory drugs or cytokines might block fibrosis. Chemokine antagonists are in development
Oxidative stress	Oxidative stress-related molecules mediate progression of liver fibrosis and increase procollagen expression in human HSCs. Reactive aldehydes induce nuclear translocation of JNK in HSCs and induce expression of TGF-β and MCP-1 [82]	Potential usefulness of antioxidant approaches, although their efficacy has not been proven convincingly in humans
Apoptosis	Generation of apoptotic hepatocytes stimulates fibrogenesis *in vivo* and *in vitro* [83]. Phagocytosis of apoptotic bodies by HSCs amplifies the process, causing NADPH-mediated production of ROS	IDN-6556, a general caspase inhibitor, is being tested currently in clinical trials
Altered glucose metabolism	Diabetes is a potent predictor of fibrosis. Elevated glucose levels or insulin upregulate TGF-β and connective tissue growth factor and also affect HSC biology [84]. Receptors for advanced glycation end-products are expressed by HSCs and have a role in migration of activated HSCs [85]	Aggressive management of hyperglycemia might slow fibrosis progression
Nuclear hormone receptors	PPARγ is expressed in quiescent HSCs and decreases with the activation process. PPARγ ligands revert most features of the activated phenotype of HSCs and inhibit fibrosis *in vivo* [86]	Experimental work indicates that the antifibrotic response occurs when animals are treated early. Clinical trials suggest a beneficial action of thiazolidinediones on fibrosis during NASH
Endogenous cannabinoid system	Cannabinoids act through CB1 and CB2 receptors. CB2 mediates antifibrogenic actions whereas CB1 modulates fibrosis positively. Inactivation of CB1 receptors decreases fibrogenesis by lowering hepatic TGF-β and reducing accumulation of fibrogenic cells in the liver [87]	Availability of rimonabant, a CB1 receptor antagonist, which reduces obesity and ameliorates parameters of the metabolic syndrome
Renin–angiotensin system	Angiotensin II induces profibrogenic actions in HSCs, which express all components of the renin–angiotensin system. Interference with the renin–angiotensin system attenuate fibrosis development in different experimental models of chronic liver injury [88,89]	Patients with the metabolic syndrome and NASH often require pharmacological treatment of hypertension. Clinical trials are underway
Adipokines	Leptin is a potent profibrogenic factor, acting directly on HSCs and potentially involved in the development of cancer [22,90,91]. Adiponectin ameliorates liver damage in models of steatohepatitis and blocks fibrogenesis after toxic liver damage [92,93]	Adiponectin receptors have been characterized and are probably a target for future studies in NASH

[a]Information is derived from studies in animal models of steatohepatitis and in cultures of fibrogenic cells, primarily human or rodent stellate cells.

suggest that TNF-α is only one of the multiple effectors of steatohepatitis, under the control of NF-κB, especially in the dietary model.

Serum levels of IL-6, another NF-κB target, correlate with parameters of chronic low-grade inflammation [68]. The role of IL-6 is somewhat controversial because this cytokine has stimulatory effects on liver regeneration and a possible beneficial effect on fatty liver in mice has also been reported [70]. Nonetheless, chronic exposure to IL-6 is actually characterized by sensitization to liver damage and several lines of evidence identify IL-6 as a mediator of inflammation and IR [71]. IL-6 signals through activation of the Jak–STAT pathway, which is regulated negatively by the suppressors of cytokine signaling (SOCS) family [72]. SOCS1 and 3 mediate insulin resistance in response to cytokines, blocking insulin-receptor signaling at different levels [72]. SOCS also interfere with leptin-receptor signaling, suggesting their contribution to the induction of leptin resistance.

Other proinflammatory mechanisms

The pattern-recognition receptor (PRR) family has also been suggested as contributing to the proinflammatory responses in fatty liver, especially in the later stages of the disease, when bacterial-derived products are increasingly absorbed from the gut and target non-parenchymal liver cells, including Kupffer cells. PRRs comprise Toll-like receptors (TLRs) and other receptors that recognize endotoxin and other bacterial products [73,74]. TLR activation triggers multiple intracellular signaling pathways, including NF-κB, and could be important for the amplification and maintenance of inflammatory signals and fibrosis [74,75]. Chemokines are a subfamily of cytokines that regulates inflammation through receptor-mediated recruitment of different leukocyte subclasses. IL-8 and MCP-1, which recruit neutrophils and mononuclear cells, respectively, are under the control of NF-κB and their expression is increased in patients with steatohepatitis and in animals models thereof [68,76].

Molecular mechanisms of hepatic fibrosis in NASH

Fibrosis and cirrhosis are the final outcomes of all chronic liver disease, however, some morphological and biological differences distinguish fibrosis due to NASH from the one secondary to other causes of liver damage. NASH-related fibrosis develops primarily in the pericentral areas, where thin bundles of fibrotic tissue surround groups of hepatocytes and thicken the space of Disse, in a 'chicken wire' fashion. The main cell type responsible for extracellular matrix deposition is represented by HSCs, which undergo activation in conditions of liver injury, acquiring a phenotype that enables them to participate in the liver wound-healing process (reviewed in [77]). The profibrogenic mechanisms operating in NASH are partly in common with those observed in other chronic liver diseases. However, the increase in circulating adipokines, oxidative stress generated by accumulation of fat in hepatocytes and the hormonal profile associated with the metabolic syndrome might have a specific role for the induction of fibrogenesis in this condition. The molecular mechanisms of fibrogenesis in NASH are summarized in Table 2.

Box 3. Outstanding questions

- Is the recruitment of different subsets of leukocytes relevant to the pathogenesis of NASH?
- What are the molecular effectors of lipotoxicity? Could triglyceride accumulation be protective, at least in some instances? What is the role of different FFAs?
- What are the basic mechanisms underlying the lack of effects of antioxidants in clinical trials?
- Can we identify the molecular and genetic mechanisms of transition from simple steatosis to NASH?
- Is leptin resistance relevant to NASH? Are all liver cells leptin resistant?
- Reversibility of NASH: is the return to steatosis without inflammation possible?
- Is the role of locally generated, intrahepatic molecules more important than that of fat-derived or circulating factors?
- Can we dissociate the molecular factors associated with increased cardiovascular risk from those leading to advanced liver disease?
- Are we getting closer to obtaining animal models that resemble human NASH thoroughly?
- Is the development of fibrosis more linked to inflammation or to hepatocellular damage?

Concluding remarks

An impressive amount of information has been accumulated in the past 5 years on all aspects of pathophysiology, molecular and cellular biology of NASH. Fatty infiltration of the liver is closely linked to IR and is considered a component of the metabolic syndrome. Ectopic fat infiltration might also lead to hepatocyte damage, a process defined 'lipotoxicity'. Lipotoxicity and cell death are associated with activation of proinflammatory pathways, leading to leukocyte infiltration, damage amplification and fibrosis. Despite these advances, several unanswered questions remain (Box 3). Particular attention should be directed to the factors that cause progressive liver damage, interplay among IR, inflammation and fibrosis, and the relationships between fatty liver, NASH and cardiovascular risk. In addition, the molecular factors of lipotoxicity need to be identified more precisely, unraveling the role of different FFAs in this context. It will be crucial to generate animal models that more accurately reflect the metabolic changes characteristic of NASH, to reliably test novel therapeutic approaches because effective treatments for this condition are still lacking.

Conflicts of interest

The authors have no conflicts of interest to disclose in relation to the present paper.

Acknowledgements

Work in F.M.'s laboratory was supported by grants from the Italian Ministry of University and Research and from the University of Florence. C.T. was supported in part by a grant from Fondo Ricerca Stategica (FIST 2004), Fondo Studi Fegato-Onlus and Fondazione CRT. G.T. was supported in part by a grant from Progetto Ricerca Interesse Nazionale (PRIN) and from FIST (04). The authors gratefully acknowledge the contribution of the other members of the Fatty Liver Italian Network (FLIN): Stefano Bellentani, Giorgio Bedogni, Elisabetta Bugianesi, Lory S. Crocè, Giulio Marchesini and Gianluca Perseghin.

References

1 Ludwig, J. *et al.* (1980) Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin. Proc.* 55, 434–438

2 Angulo, P. (2002) Nonalcoholic fatty liver disease. *N. Engl. J. Med.* 346, 1221–1231
3 Marchesini, G. *et al.* (2003) Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 37, 917–923
4 Utzschneider, K.M. and Kahn, S.E. (2006) Review: The role of insulin resistance in nonalcoholic fatty liver disease. *J. Clin. Endocrinol. Metab.* 91, 4753–4761
5 Gastaldelli, A. *et al.* (2006) The effect of rosiglitazone on the liver: decreased gluconeogenesis in patients with type 2 diabetes. *J. Clin. Endocrinol. Metab.* 91, 806–812
6 Bugianesi, E. *et al.* (2005) Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* 48, 634–642
7 Seppala-Lindroos, A. *et al.* (2002) Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J. Clin. Endocrinol. Metab.* 87, 3023–3028
8 Sanyal, A.J. *et al.* (2001) Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 120, 1183–1192
9 Gastaldelli, A. *et al.* (2007) Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and Type 2 diabetic subjects. *Gastroenterology* 133, 496–506
10 Samuel, V.T. *et al.* (2004) Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J. Biol. Chem.* 279, 32345–32353
11 Musso, G. *et al.* (2005) Adipokines in NASH: postprandial lipid metabolism as a link between adiponectin and liver disease. *Hepatology* 42, 1175–1183
12 Carpentier, A. *et al.* (2002) Sensitivity to acute insulin-mediated suppression of plasma free fatty acids is not a determinant of fasting VLDL triglyceride secretion in healthy humans. *Diabetes* 51, 1867–1875
13 Petersen, K.F. *et al.* (2005) Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with Type 2 diabetes. *Diabetes* 54, 603–608
14 Nielsen, S. *et al.* (2004) Splanchnic lipolysis in human obesity. *J. Clin. Invest.* 113, 1582–1588
15 Donnelly, K.L. *et al.* (2005) Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.* 115, 1343–1351
16 Tamura, S. and Shimomura, I. (2005) Contribution of adipose tissue and *de novo* lipogenesis to nonalcoholic fatty liver disease. *J. Clin. Invest.* 115, 1139–1142
17 Kanda, H. *et al.* (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* 116, 1494–1505
18 Bugianesi, E. *et al.* (2005) Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. *J. Clin. Endocrinol. Metab.* 90, 3498–3504
19 Ouchi, N. *et al.* (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100, 2473–2476
20 Fishman, S. *et al.* (2007) Resistance to leptin action is the major determinant of hepatic triglyceride accumulation *in vivo*. *FASEB J.* 21, 53–60
21 Huang, W. *et al.* (2007) Hepatic steatosis and plasma dyslipidemia induced by a high-sucrose diet are corrected by an acute leptin infusion. *J. Appl. Physiol.* 102, 2260–2265
22 Marra, F. (2007) Leptin and liver tissue repair: do rodent models provide the answers? *J. Hepatol.* 46, 12–18
23 Arner, P. (2005) Resistin: yet another adipokine tells us that men are not mice. *Diabetologia* 48, 2203–2205
24 Bertolani, C. *et al.* (2006) Resistin as an intrahepatic cytokine: overexpression during chronic injury and induction of proinflammatory actions in hepatic stellate cells. *Am. J. Pathol.* 169, 2042–2053
25 Moschen, A.R. *et al.* (2007) Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *J. Immunol.* 178, 1748–1758
26 Gervois, P. *et al.* (2007) Drug insight: mechanisms of action and therapeutic applications for agonists of peroxisome proliferator-activated receptors. *Nat. Clin. Pract. Endocrinol. Metab.* 3, 145–156
27 Browning, J.D. and Horton, J.D. (2004) Molecular mediators of hepatic steatosis and liver injury. *J. Clin. Invest.* 114, 147–152
28 Gastaldelli, A. *et al.* (2006) The effect of pioglitazone on the liver: role of adiponectin. *Diabetes Care* 29, 2275–2281
29 Bajaj, M. *et al.* (2004) Plasma resistin concentration, hepatic fat content, and hepatic and peripheral insulin resistance in pioglitazone-treated type II diabetic patients. *Int. J. Obes. Relat. Metab. Disord.* 28, 783–789
30 Belfort, R. *et al.* (2006) A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N. Engl. J. Med.* 355, 2297–2307
31 Rubio, A. *et al.* (2007) Identification of a gene-pathway associated with non-alcoholic steatohepatitis. *J. Hepatol.* 46, 708–718
32 Begriche, K. *et al.* (2006) Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* 6, 1–28
33 Paradies, G. *et al.* (1992) The effect of aging and acetyl-L-carnitine on the activity of the phosphate carrier and on the phospholipid composition in rat heart mitochondria. *Biochim. Biophys. Acta* 1103, 324–326
34 Sanchez-Alcazar, J.A. *et al.* (2000) Tumor necrosis factor-alpha increases the steady-state reduction of Cytochrome *b* of the mitochondrial respiratory chain in metabolically inhibited L929 cells. *J. Biol. Chem.* 275, 13353–13361
35 Gow, A.J. and Stamler, J.S. (1998) Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* 391, 169–173
36 Szabo, C. *et al.* (1996) DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc. Natl. Acad. Sci. U. S. A.* 93, 1753–1758
37 Garcia-Ruiz, I. *et al.* (2006) Uric acid and anti-TNF antibody improve mitochondrial dysfunction in ob/ob mice. *Hepatology* 44, 581–591
38 Leclercq, I.A. *et al.* (2000) CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. *J. Clin. Invest.* 105, 1067–1075
39 Mari, M. *et al.* (2006) Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Metab.* 4, 185–198
40 Fan, C.Y. *et al.* (1998) Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. *J. Biol. Chem.* 273, 15639–15645
41 De Minicis, S. *et al.* (2006) NADPH oxidase in the liver: defensive, offensive, or fibrogenic? *Gastroenterology* 131, 272–275
42 dela Pena, A. *et al.* (2007) NADPH oxidase is not an essential mediator of oxidative stress or liver injury in murine MCD diet-induced steatohepatitis. *J. Hepatol.* 46, 304–313
43 Schwabe, R.F. and Brenner, D.A. (2007) Nuclear factor-κB in the liver: friend or foe? *Gastroenterology* 132, 2601–2604
44 Xu, Z. *et al.* (2005) Liver-specific inactivation of the *Nrf1* gene in adult mouse leads to nonalcoholic steatohepatitis and hepatic neoplasia. *Proc. Natl. Acad. Sci. U. S. A.* 102, 4120–4125
45 Houstis, N. *et al.* (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 440, 944–948
46 Seki, S. *et al.* (2002) In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *J. Hepatol.* 37, 56–62
47 Yesilova, Z. *et al.* (2005) Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic fatty liver disease. *Am. J. Gastroenterol.* 100, 850–855
48 Sumida, Y. *et al.* (2003) Serum thioredoxin levels as a predictor of steatohepatitis in patients with nonalcoholic fatty liver disease. *J. Hepatol.* 38, 32–38
49 Feldstein, A.E. *et al.* (2003) Hepatocyte apoptosis and Fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 125, 437–443
50 Wieckowska, A. *et al.* (2006) *In vivo* assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 44, 27–33
51 Ramalho, R.M. *et al.* (2006) Apoptosis and Bcl-2 expression in the livers of patients with steatohepatitis. *Eur. J. Gastroenterol. Hepatol.* 18, 21–29
52 Younossi, Z.M. *et al.* (2005) A genomic and proteomic study of the spectrum of nonalcoholic fatty liver disease. *Hepatology* 42, 665–674

53 Garcia-Ruiz, I. *et al.* (2002) Sp1 and Sp3 transcription factors mediate malondialdehyde-induced collagen alpha 1(I) gene expression in cultured hepatic stellate cells. *J. Biol. Chem.* 277, 30551–30558
54 Rahman, S.M. *et al.* (2007) CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. *Hepatology* 45, 1108–1117
55 Watanabe, S. *et al.* (2005) Hepatocyte-specific *Pten*-deficient mice as a novel model for nonalcoholic steatohepatitis and hepatocellular carcinoma. *Hepatol. Res.* 33, 161–166
56 Matsuzaka, T. *et al.* (2007) Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. *Nat. Med.* 13, 1193–1202
57 Yamaguchi, K. *et al.* (2007) Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* 45, 1366–1374
58 Van Gaal, L.F. *et al.* (2006) Mechanisms linking obesity with cardiovascular disease. *Nature* 444, 875–880
59 Ikura, Y. *et al.* (2006) Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. *Hepatology* 43, 506–514
60 Cai, D. *et al.* (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK-β and NF-κB. *Nat. Med.* 11, 183–190
61 Marciniak, S.J. and Ron, D. (2006) Endoplasmic reticulum stress signaling in disease. *Physiol. Rev.* 86, 1133–1149
62 Dela Pena, A. *et al.* (2005) NF-κB activation, rather than TNF, mediates hepatic inflammation in a murine dietary model of steatohepatitis. *Gastroenterology* 129, 1663–1674
63 Arkan, M.C. *et al.* (2005) IKK-β links inflammation to obesity-induced insulin resistance. *Nat. Med.* 11, 191–198
64 Luedde, T. *et al.* (2007) Deletion of NEMO/IKKγ in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 11, 119–132
65 Hirosumi, J. *et al.* (2002) A central role for JNK in obesity and insulin resistance. *Nature* 420, 333–336
66 Schattenberg, J.M. *et al.* (2006) JNK1 but not JNK2 promotes the development of steatohepatitis in mice. *Hepatology* 43, 163–172
67 Crespo, J. *et al.* (2001) Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 34, 1158–1163
68 Haukeland, J.W. *et al.* (2006) Systemic inflammation in nonalcoholic fatty liver disease is characterized by elevated levels of CCL2. *J. Hepatol.* 44, 1167–1174
69 Li, Z. *et al.* (2003) Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 37, 343–350
70 Hong, F. *et al.* (2004) Interleukin 6 alleviates hepatic steatosis and ischemia/reperfusion injury in mice with fatty liver disease. *Hepatology* 40, 933–941
71 Klover, P.J. *et al.* (2005) Interleukin-6 depletion selectively improves hepatic insulin action in obesity. *Endocrinology* 146, 3417–3427
72 Howard, J.K. and Flier, J.S. (2006) Attenuation of leptin and insulin signaling by SOCS proteins. *Trends Endocrinol. Metab.* 17, 365–371
73 Brun, P. *et al.* (2005) Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 289, G571–G578
74 Schwabe, R.F. *et al.* (2006) Toll-like receptor signaling in the liver. *Gastroenterology* 130, 1886–1900
75 Seki, E. *et al.* (2007) TLR4 enhances TGF-β signaling and hepatic fibrosis. *Nat. Med.* 13, 1324–1332
76 Leclercq, I.A. *et al.* (2004) Curcumin inhibits NF-κB activation and reduces the severity of experimental steatohepatitis in mice. *J. Hepatol.* 41, 926–934
77 Bataller, R. and Brenner, D. (2005) Liver fibrosis. *J. Clin. Invest.* 115, 209–218
78 Zoltowska, M. *et al.* (2001) Circulating lipoproteins and hepatic sterol metabolism in *Psammomys obesus* prone to obesity, hyperglycemia and hyperinsulinemia. *Atherosclerosis* 157, 85–96
79 Bachman, E.S. *et al.* (2002) βAR signaling required for diet-induced thermogenesis and obesity resistance. *Science* 297, 843–845
80 Svegliati-Baroni, G. *et al.* (2006) A model of insulin resistance and nonalcoholic steatohepatitis in rats: role of peroxisome proliferator-activated receptor-alpha and n-3 polyunsaturated fatty acid treatment on liver injury. *Am. J. Pathol.* 169, 846–860
81 Koteish, A. and Diehl, A.M. (2001) Animal models of steatosis. *Semin. Liver Dis.* 21, 89–104
82 Parola, M. and Robino, G. (2001) Oxidative stress-related molecules and liver fibrosis. *J. Hepatol.* 35, 297–306
83 Canbay, A. *et al.* (2004) Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 39, 273–278
84 Svegliati-Baroni, G. *et al.* (1999) Insulin and insulin-like growth factor-1 stimulate proliferation and type I collagen accumulation by human hepatic stellate cells: differential effects on signal transduction pathways. *Hepatology* 29, 1743–1751
85 Fehrenbach, H. *et al.* (2001) Up-regulated expression of the receptor for advanced glycation end products in cultured rat hepatic stellate cells during transdifferentiation to myofibroblasts. *Hepatology* 34, 943–952
86 Marra, F. (2006) Thiazolidinediones and hepatic fibrosis: don't wait too long. *Gut* 55, 917–919
87 Teixeira-Clerc, F. *et al.* (2006) CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat. Med.* 12, 671–676
88 Bataller, R. *et al.* (2003) NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J. Clin. Invest.* 112, 1383–1394
89 Caligiuri, A. *et al.* (2003) Antifibrogenic effects of canrenone, an antialdosteronic drug, on human hepatic stellate cells. *Gastroenterology* 124, 504–520
90 Aleffi, S. *et al.* (2005) Upregulation of proinflammatory and proangiogenic cytokines by leptin in human hepatic stellate cells. *Hepatology* 42, 1339–1348
91 Saxena, N.K. *et al.* (2007) Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Res.* 67, 2497–2507
92 Kamada, Y. *et al.* (2003) Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. *Gastroenterology* 125, 1796–1807
93 Xu, A. *et al.* (2003) The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *J. Clin. Invest.* 112, 91–100