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Cholesterol and Inflammation at the Crossroads of Non-Alcoholic Fatty Liver Disease (NAFLD) and Atherogenesis

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a variety of diseases ranging from simple steatosis or fatty liver through non-alcoholic steatohepatisis (NASH) to fibrosis that can eventually lead to irreversible cirrhosis. NASH was first described by Ludwig et al. (Ludwig et al., 1980) in a number of patients who reported no "significant" alcohol intake but whose liver histology resembled that of alcoholic liver disease. Today the term NAFLD is used either when referring to a full spectrum of liver disease or when the aetiology of the disease is unknown, excluding secondary causative factors such as excessive alcohol intake, hepatotoxic drugs, metabolic/genetic and other liver diseases (for instance autoimmune or viral hepatitis) (Treeprasertsuk et al., 2011).

The occurrence of NAFLD has been persistently increasing in parallel with the concerning worldwide epidemic of obesity and diabetes and is expected to rise in the future (Portincasa et al., 2005). In the Western countries NAFLD is already becoming the most common cause of liver disease with the estimates of prevalence being between 17 and 33 % in the general population (McCullough, 2005) and rising as high as 90 % in morbidly obese individuals (Machado et al., 2006). NASH, the most severe and clinically significant form of NAFLD, is less common and is expected to be present between 5.7 and 17 % of the population (McCullough, 2005), again the numbers increase in the morbidly obese (up to 37 %) (Machado et al., 2006). Although NAFLD can occur in lean patients, the majority of the patients are overweight or obese (McCullough, 2005). Of particular concern, especially in the view of the future disease burden, is the presence of NAFLD in children and adolescents. For instance, the prevalence of obesity among US children has tripled in just one decade, rising from 5 % in 1980 to 15 % in 1990 and is currently around 17 %(Centers for Disease Control and Prevention CDC, 2011). The estimates of NAFLD prevalence in childhood have risen accordingly and are already between 2.6 and 9.6 % among the general pediatric population (Pacifico et al., 2010), reaching 68 % in obese children (Fu et al., 2011). Most cases of NAFLD arise in the detrimental environment of various metabolic disorders commonly known as the metabolic syndrome. The disease is strongly associated with insulin resistance, hypertension, glucose intolerance, central obesity and dyslipidemia and is thus recognized as the hepatic manifestation of the metabolic syndrome (Marchesini et al., 2005). Simple steatosis is largely benign and has a good prognosis. Nevertheless, a significant

proportion of steatotic patients will develop steatohepatitis, which is characterized by hepatocyte injury and inflammation with 20–25 % possibility of progression to advanced fibrosis and cirrhosis (Farrell et al., 2005). In patients with cirrhosis liver failure is the most common cause of death warranting a liver transplantation (Grattagliano et al., 2011). NAFLD is also significantly associated with cardiovascular disease (CVD) independently of metabolic syndrome components and the classical risk factors (Nakao & Yoneda, 2009; Targher & Arcaro, 2007). In fact, one of the leading causes of death in NAFLD patients is coronary artery disease (Adams et al., 2005; Matteoni et al., 1999). Recent evidence thus suggests that NAFLD might play an important role in the development of atherosclerosis (see Chapter 4). NASH has also been found to be associated with increased risk of hepatocellular carcinoma (Marrero et al., 2002).

NAFLD is clinically silent. The symptoms usually described by the patients, such as fatigue and vague discomfort over the liver, are quite non-specific (Farrell et al., 2005). The presence of the disease is often suspected upon abnormal results of routinely performed liver tests in the presence of metabolic syndrome risk factors and exclusion of secondary causes. It should be noted, however, that elevated liver enzymes have poor sensitivity since serum transaminases can be normal in up to 80 % of patients with steatosis (Browning et al., 2004). Hepatic imaging, which can be ordered as a part of investigations into abdominal pain or elevated liver enzymes, usually provides the first clues of the presence of steatosis. Hepatic ultrasound, where steatosis is seen as increased echogenicity or "bright liver", has a quite high sensitivity, especially when more than 33 % of hepatocytes are fatty. The same sensitivity has been found with the use of computerized tomography and magnetic resonance imaging, but unfortunately all three methods are unable to detect features of fibrosis and cirrhosis, which present the greatest risk of liver failure (Saadeh et al., 2002). Today liver biopsy is the gold standard for diagnosing NASH, since only histological evaluation of the liver tissue is able to discern simple steatosis from steatosis with inflammation, which represents the hallmark of NASH. It is of vital clinical importance to distinguish advanced liver disease from the more benign states because of its poor outcome. Guidelines on when to perform the liver biopsy are still a matter of discussion since the procedure is costly, invasive, prone to complications such as pain or even death (0.01 % procedural mortality) and inclined to sampling variability (Guha et al., 2006; Poynard et al., 2006). Indication for liver biopsy is currently based upon risk assessment for fibrosis, which takes into consideration obesity, diabetes, age over 45 and aspartate transaminase (AST) to alanine transaminase (ALT) ratio over 1 (Angulo & Lindor, 2002).

Despite an ever increasing knowledge about the mechanisms of NAFLD pathogenesis (see Chapter 3), an efficient therapy remains elusive. Several key pieces of information for targeted treatment are still missing, such as why only a certain proportion of patients with simple steatosis progress to steatohepatitis and a better understanding of causal relationships of NAFLD and metabolic syndrome components. In fact, the use of term NAFLD itself suggests an unknown aetiology of the disease. Because of this and because of strong association of NAFLD to metabolic syndrome, the therapy is primarily directed towards lifestyle modifications. Patients are encouraged to lose weight through physical exercise improving body mass index (BMI), insulin resistance and diabetic control (Musso et al., 2010). It was shown that the benefits of relatively consistent weight loss are removal of fat from hepatocytes, which can even lead to improved necroinflammation and decreased fibrosis (Hickman et al., 2002; Palmer & Schaffner, 1990; Ueno et al., 1997). It is estimated that a change in nutritional habits in which we would reduce caloric intake by as little as 100

kilocalories per day would prevent epidemic of both obesity and NAFLD (Hill et al., 2003). Pharmacological interventions are indicated for patients with a risk of developing advanced liver disease. Because insulin resistance is the single most prevalent predisposing factor for NAFLD (Bugianesi et al., 2005b), treatment is mainly aimed to improve insulin sensitivity. Insulin sensitizers metformin and thiazolidinediones are gaining acknowledgement as drugs with beneficial effects on NAFLD (Ahmed & Byrne, 2009). Other therapy strategies are directed towards improving dyslipidemia and oxidative stress, which are commonly associated with NAFLD, and drugs such as statins, polyunsaturated fatty acids, vitamins C and E and ursodeoxycholic acid are currently being evaluated in clinical trials (Musso et al., 2010).

At present, NAFLD can best be described as a multi-factorial disorder with no specific diagnostic tests and no approved treatment regimen. Increasing interest in the research of this complex disease is implicating more and more factors that contribute to the pathogenesis of NAFLD. In this chapter we will first describe pathology and pathogenesis of NALFD and move on to establish a link between NAFLD and atherosclerosis. Subsequently we will depict the interplay of cholesterol metabolism and inflammation and their relation to atherosclerosis through NAFLD.

2. Pathology of NAFLD

NAFLD covers a wide range of pathological states and it is often difficult to draw the line where one condition ends and the other begins. First efforts were put into defining histopathological criteria for the diagnosis of NASH. In order to avoid the early confusions, it was proposed that liver biopsies of NASH should closely resemble those of alcoholic steatohepatitis (Lee, 1995). However, many of the more subtle forms of steatosis with inflammation were thus unjustifiably excluded from being designated as NASH. To overcome the diagnostic inconsistencies, Matteoni et al. (Matteoni et al., 1999) divided NAFLD into four categories (Table 1).

Category of NAFLD	Pathology	Clinical correlation
Type 1	Simple fatty liver (steatosis)	Not progressive with a good prognosis
Type 2	Steatosis and lobular inflammation (steatohepatitis)	Probably benign, does not resemble alcoholic steatohepatitis, not diagnosed as NASH
Type 3	Steatosis, lobular inflammation and ballooning degeneration (steatonecrosis)	NASH without fibrosis – may progress to cirrhosis
Type 4	Steatosis, ballooning degeneration and Mallory bodies, and/or fibrosis	NASH with fibrosis – may progress to cirrhosis and liver failure

Table 1. Categories of non-alcoholic fatty liver disease (NAFLD) and their clinical correlation (Farrell et al., 2005; Matteoni et al., 1999).

Steatosis (or fatty liver) is the hallmark of NAFLD and is characterized by the accumulation of fat droplets in hepatocytes. To diagnose steatosis at least 5 % of fatty hepatocytes need to be present (Kleiner et al., 2005), while less liver fat accumulation can be physiological and is transient in nature. At the histological level, fat is seen as a single macrovesicular droplet that displaces the nucleus to the periphery of the cell although a smaller amount of fat accumulation can be microvesicular (smaller vacuoles around the rim of the cell) (Hall & Kirsch, 2005). Steatosis, which is entirely microvesicular, is prompting other aetiology such as excessive alcohol intake or drugs (Hall & Kirsch, 2005). Other types of lipid accumulation that can be present in steatosis are lipogranulomas and fat cysts (Brunt, 2011).

Steatosis with lobular inflammation is considered a type 2 NAFLD. The inflammation infiltrate comprises of neutrophils, lymphocytes, plasma cells and macrophages. It is usually present as scattered clusters of cells across the lobule, but it can also be seen in portal tracts (Yerian, 2011). It is rather difficult to delineate simple steatosis from steatosis with lobular inflammation, because truly simple steatosis is very uncommon. One large study that evaluated 933 adult and pediatric liver biopsies found out that only four were completely void of inflammatory infiltration (Brunt et al., 2009). On the other hand, just one or two focal collections of mononuclear cells in the parenchyma are not enough to diagnose a type 2 NAFLD. Minimum criteria to define any type of hepatitis in fatty liver are yet to be defined (Hall & Kirsch, 2005).

The third prerequisite to diagnose NASH, besides the fatty hepatocytes and inflammatory infiltrate, is the hepatocyte injury present either as reversible hepatic ballooning degeneration or irreversible hepatic necrosis or apoptosis. Ballooned hepatocytes are large in size and have a pale, "cobweb-like" cytoplasm that is a consequence of fluid retention (Yerian, 2011). They are quite difficult to distinguish from fatty hepatocytes with small fat droplets that resemble mildly hidropic cells. It is possible to discern fat from fluid with certain histochemical stainings of liver tissue but these are not routinely performed (Brunt & Tiniakos, 2010). Mallory bodies are often seen in ballooned hepatocytes and they appear as irregularly shaped eosinophilic masses in the cytoplasm. They are composed of cytokeratin polypeptides and can be stained with antibody to ubiquitin (French, 2000). Mallory bodies are not required for the diagnosis of NASH. Apoptotic hepatocytes are observed as deeply eosinophilic cytoplasmic aggregates that may or may not be surrounded by Kupffer cells (liver macrophages), however, apoptosis of hepatocytes in NAFLD is never so prominent as in viral hepatitis (Hall & Kirsch, 2005).

In the progressive forms of liver disease fibrosis and cirrhosis may occur. The former is characterized as aberrant deposition of extracellular matrix (collagen) by activated hepatic stellate cells as part of the injury healing process. Fibrosis is characteristically pericellular in distribution and is first observed in the centrilobular region of the liver. With progression it extends towards portal areas (Brunt, 2011). Cirrhosis or liver scarring is defined as a complete loss of the normal lobular architecture and replacement of liver tissue by fibrosis, scar tissue and regenerative nodules of hepatocytes (Grattagliano et al., 2011). It eventually leads to the loss of liver function.

3. Pathogenesis of NAFLD

3.1 Insulin resistance as a predominant factor for NAFLD

NAFLD is strongly associated with components of the metabolic syndrome and the majority of cases of NAFLD occur in patients with obesity (60–95 %), type 2 diabetes mellitus (28–55

%) and hyperlipidemia (27–92 %) (Marchesini & Bugianesi, 2005). Insulin resistance (IR), being the cardinal feature of the metabolic syndrome, is almost uniformly found in patients with NAFLD (Bugianesi et al., 2005a; Comert et al., 2001; Marchesini et al., 1999; Sanyal et al., 2001). The causes of IR are as of yet unknown and are being researched extensively. IR can be classified as either peripheral IR in which we have reduced insulin-mediated uptake of glucose by skeletal muscle and adypocites or hepatic IR where insulin is unable to suppress glucose production in the liver. There is mounting evidence that the main pathological event leading to peripheral IR is ectopic accumulation of fat (fatty acid metabolites) in skeletal muscle, which causes a defect either in glucose transport or phosphorylation of insulin receptors (Samuel & Shulman, 2005). The same mechanism is believed to cause hepatic IR (Kim et al., 2001). In the presence of peripheral IR, insulin is unable to efficiently exert its antilipolytic effects on adipose tissue resulting in increased free fatty acid (FFA) flux into the bloodstream. Hyperlipidemia thus arising exacerbates IR in the muscle and adipose tissue and causes fat deposition in the liver affecting its function and inducing hepatic IR. It was found out that not only in obese, but also in lean NAFLD patients with normal glucose tolerance and lipid levels lipolysis and lipid oxidation at the basal level were increased and inefficiently inhibited after insulin administration (Marchesini & Bugianesi, 2005). The source of increased FFA flux in NAFLD patients is not clear but it seems that visceral adiposity may play an important role since it is more insulin resistant than subcutaneous adipose tissue (Lefebvre et al., 1998). This notion is supported by the fact that the aforementioned lean NAFLD patients had enlarged waist girth, which is in good correlation with central (visceral) adiposity.
NAFLD could thus possibly stem from a defect in insulin sensitivity causing derangements in glucose metabolism (which explains its link with other metabolic disorders) and in lipid metabolism that predisposes to hepatic steatosis, which is the first step in the pathogenesis of advanced liver disease.

3.2 The two-hit hypothesis and beyond

According to the initial "two-hit" hypothesis NASH develops in two subsequent steps (Day & James, 1998). The "first hit" leads to hepatic accumulation of triglycerides (steatosis) that makes liver susceptible to hepatocyte injury mediated by "second hits" such as inflammatory cytokines, oxidative stress and mitochondrial dysfunction, which in turn promote inflammatory infiltration and fibrosis.

3.2.1 Steatosis

Lipids that accumulate in the liver are mainly triglycerides formed from esterification of glycerol and FFA within the hepatocyte (Figure 1). Three distinct sources of FFA are: lipolysis in the adipose tissue, *de novo* lipogenesis in the liver and dietary sources. Mechanisms for FFA utilization in the liver are: use as energy source in the process of β-oxidation, storage in the form of triglyceride droplets or export in the form of very low-density lipoproteins (VLDL). Hepatic fat accumulation can therefore be a consequence of a malfunction of any of these three mechanisms of FFA utilization or because of an increased fat delivery. In the case of NAFLD the increased influx of FFA from adipose tissue as a consequence of IR and obesity plays the dominant role since 60 % of liver triglycerides derive from FFA overflow from adipose tissue, 25 % from *de novo* lipogenesis and 15 % from the diet (Donnelly et al., 2005). IR also has a more direct effect on the liver.

Hyperinsulinemia, which accompanies IR, inhibits β-oxidation of FFA (Postic & Girard, 2008) and at the same time increases expression of lipogenic genes through up-regulation of transcription factor, sterol regulatory element binding protein-1c (SREBP-1c) (Kohjima et al., 2008). As a consequence, synthesis of lipids is intensified, which further promotes hepatic steatosis. As an answer to the increased synthesis of triglycerides, the production of VLDL is also increased whereas insufficient to adequately remove fat from the liver (Zoltowska et al., 2001).

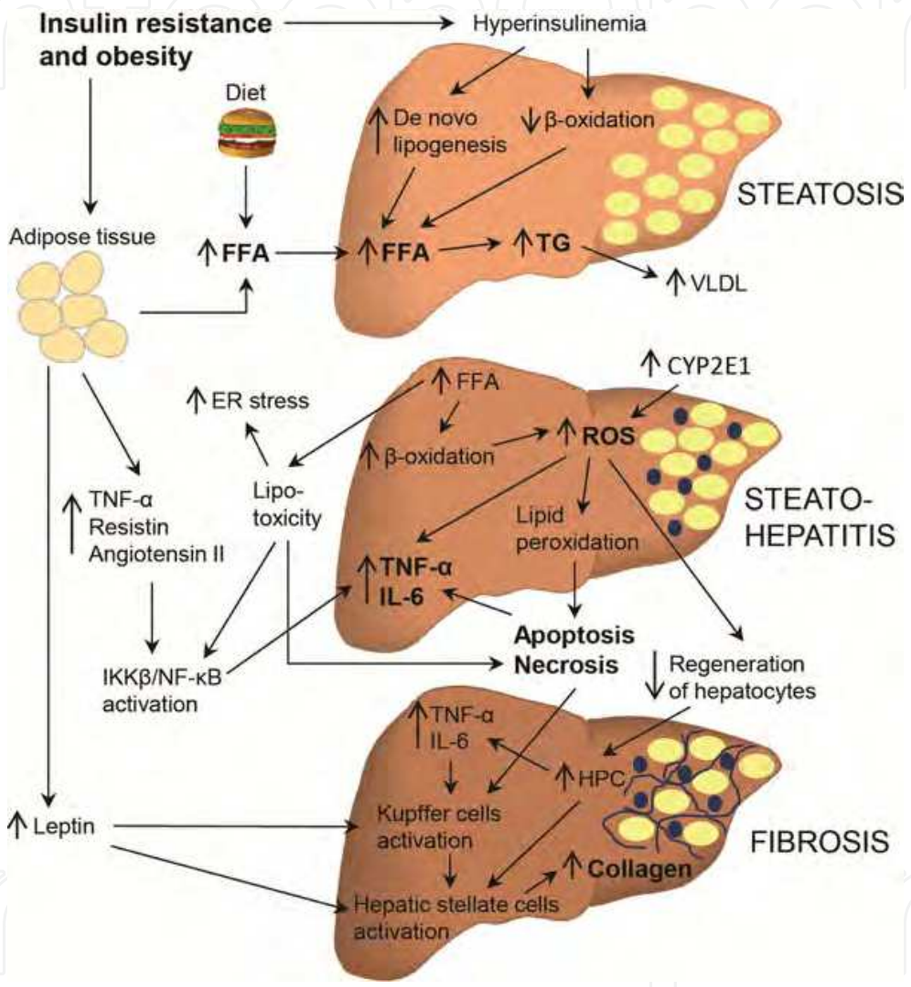

Fig. 1. Pathogenesis of NAFLD. Prominent feature of liver steatosis is the accumulation of excess FFA from the adipose tissue in the form of triglycerides. Gradual increase of FFA β-oxidation due to increased hepatic insulin resistance generates ROS, which activate inflammatory pathways and initiate lipid peroxidation. As a result, apoptosis and necrosis of hepatocytes, which are further aggravated by FFA lipotoxicity, activate Kupffer cells, which in turn stimulate hepatic stellate cells to produce excess amounts of collagen. CYP2E1: cytochrome P450 2E1; ER: endoplasmic reticulum; FFA: free fatty acids; HPC: hepatic progenitor cells; IKKβ/NF-κB: inhibitor kappa kinase beta/nuclear factor kappa B; IL-6: interleukin-6; TG: triglycerides; ROS: reactive oxygen species; TNF-α: tumor necrosis factor α; VLDL: very low-density lipoproteins.

3.2.2 Steatohepatitis and fibrosis

As was already stated, steatosis is tightly associated with hepatic inflammation. Again, visceral adipose tissue (insulin resistant) plays an important role in the transition from simple steatotic to inflamed liver, since it is not solely a site of energy storage, but also actively secreting endocrine organ. Most adipocyte-derived adipokines, such as tumor necrosis factor-α (TNF-α), resistin and angiotensin II, induce both IR and low-grade inflammation through activation of c-Jun NH2-terminal kinase-1 (JNK-1) and inhibitor kappa kinase beta (IKKβ)/nuclear factor kappa B (NF-κB) pathway in the liver. Induction of the latter pathway leads to feed forward loop of increased expression of pro-inflammatory cytokines like interleukin-6 (IL-6), interleukin 1-beta (IL-1β), TNF-α and activation of Kupffer cells (Cai et al., 2005; Wieckowska et al., 2008). Patients with NAFLD have elevated serum and hepatic levels of TNF-α (Haukeland et al., 2006; Hui et al., 2004) as well as IL-6 (Wieckowska et al., 2008). Concentrations of both correlate well with histological severity of NAFLD (Crespo et al., 2001; van der Poorten et al., 2008). Two additional well studied adipokines that have been implicated in NAFLD development are leptin and adiponectin. Leptin has an important role in the regulation of energy intake (Mantzoros, 1999) but also in the immune system (Lord, 2002) and in the promotion of fibrogenesis (Saxena et al., 2002). Together with TNF-α and IL-6 it activates hepatic stellate cells to deposit excessive amounts of collagen either directly (with binding to the receptors) or through stimulating secretion of transforming growth factor-β (TGF- β) by Kuppfer cells (Nieto, 2006; Shoelson et al., 2007). The obese and patients with NAFLD have higher levels of leptin and are considered to be leptin resistant (Uygun et al., 2000). In contrast to other adipokines, adiponectin protects the liver from inflammation and fibrogenesis. It antagonizes actions of TNF-α, inhibits lipogenesis (it down-regulates SREBP-1c) and increases insulin sensitivity (Polyzos et al., 2010; Whitehead et al., 2006). Circulatory levels of adiponection were found to be reduced in NAFLD patients (Bugianesi et al., 2005c) and it was shown that administration of recombinant adiponectin can substantially improve NASH in *ob/ob* mice, one of the animal models of NAFLD (Musso et al., 2010).

Oxidative stress and mitochondrial dysfunction are closely related and are both contributing to the liver injury. Mitochondria are the major site of free radical formation in the hepatocytes. Reactive oxygen species (ROS) are physiological by-products of energy production from glucose breakdown and FFA β-oxidation and are more or less successfully controlled by endogenous radical scavenging system. However, in the IR (NAFLD) state there is an increase of FFA delivery to the hepatocytes and gradual loss of suppression of fatty acid oxidation by insulin, generating levels of ROS that are beyond control of endogenous antioxidants (Sanyal et al., 2001). Mitochondria are the first to be exposed to the effects of ROS, which cause uncoupling of oxidation and phosphorylation process of forming adenosine triphosphate, and positive feedback loop of ROS production is thus created. ROS then activate inflammatory pathways through stimulated production of TNF-α and initiate lipid peroxidation, which is detrimental to mitochondria and which causes cell death either by signaling apoptosis or promoting necrosis (Mylonas & Kouretas, 1999; Tang et al., 2002). Hepatocyte apoptosis caused by peroxidation of excess triglycerides seems to be the critical step in progression from simple steatosis to NASH and cirrhosis (Syn et al., 2009). Apoptotic hepatocytes are phagocytized by adjacent Kupffer cells, leading to hepatic stellate cell activation and fibrogenesis. The role of apoptosis in the progression of liver injury was confirmed in a recent study on animal model of NASH where hepatic fibrosis was

significantly improved upon inhibition of caspase signaling (Witek et al., 2009). Another potent source of ROS in NAFLD patients is overexpression of cytochrome P450 2E1 (CYP2E1), a microsomal fatty acid oxidizing enzyme (Weltman et al., 1998).

3.2.3 FFA lipotoxicity

Recently, new evidence has come into light that urge the "two-hit" hypothesis to be revised and modified. There is an increasing appreciation about a more direct role of FFA in promoting liver injury. It has been shown that accumulation of triglycerides in hepatocytes can actually be beneficial in preventing development of NAFLD. Inhibition of triglyceride synthesis in obese mice with NASH has improved steatosis, but worsened liver injury, inflammation and fibrosis (Yamaguchi et al., 2007). In the same mouse model the pathology of NAFLD has worsened in parallel with the FFA burden. It seems that hepatic triglyceride accumulation acts as a buffering system disabling FFA to exert their toxic effects on the liver. FFA directly induces lipotoxicity in the liver in the following manners:

1. Detergent effects at high concentrations, inhibition of ion pumps, ion channels and calcium ionophor activity (Bass & Merriman, 2005; Nguyen et al., 2000; Schonfeld et al., 2000).
2. Mediation of hepatocyte apoptosis through JNK-1 pathway. Saturated fatty acids seem to be more toxic than unsaturated fatty acids in this respect (Malhi et al., 2006).
3. Saturated fatty acids promote endoplasmic reticulum stress that is initially aimed at compensating for cell damage but can trigger hepatocyte apoptosis when dysregulated or activated for longer time like in the case of NAFLD (Wang et al., 2006). Endoplasmic reticulum stress can also be triggered by other biological stresses such as hyperinsulinemia and hyperlipidemia (Ron, 2002).
4. Adipokine-independent hepatocyte IKKβ/NF-κB pathway activation that leads to increased expression of pro-inflammatory cytokines (Boden, 2005).

3.2.4 The third hit

Recently, a "third-hit" was proposed to be involved in pathogenesis of NAFLD (Dowman et al., 2010; Syn et al., 2009). In the healthy liver, ability of mature hepatocytes to replicate in order to replace dead tissue is a core feature of liver's remarkable capability to regenerate itself after various injuries. Oxidative stress, especially excessive and prolonged production of H_2O_2 by mitochondria, results in impaired replication of mature hepatocytes, which in turn leads to expansion of the hepatic progenitor cells (HPCs). These cells reside in the Canal of Hering near the portal veins (periportal area) of the liver and upon activation first proliferate to intermediate hepatocyte-like cells that finally evolve into either cholangiocytes (epithelial cells of the bile duct) or mature hepatocytes, both contributing to liver repair. It seems that this type of response is an important factor in the development of late stage liver disease since numbers of HPCs and intermediate hepatocytes strongly correlate with the fibrosis stage (Roskams et al., 2003). Possible mechanisms for stimulating a progressive periportal fibrosis that is seen in advanced types of NAFLD include secretion of profibrogenic cytokines (IL-6, IL-8, TGF-β) by HPCs and intermediate hepatocytes (Svegliati-Baroni et al., 2008), as well as a possible transition of cholangiocytes to myofibroblasts (Xia et al., 2006). On the other hand, evidence exists that fibrosis preceds expansion of HPCs, suggesting a more complex interaction between both phenomena (Van Hul et al., 2009).

Why only a small portion of patients with simple steatosis progress to NASH and end-stage liver disease is still poorly explained. There is a rationale that genetic factors in concert with environmental factors might represent the missing link. A number of genes involved in oxidative stress, lipid metabolism and fibrosis are differentially up- or down-regulated in patients with NASH compared to patients with simple steatosis (Younossi et al., 2005). Several candidate genes are currently being investigated for their roles in the development of NAFLD (Day & Daly, 2005).

In respect to the overwhelming new knowledge about pathogenesis of NAFLD, it is perhaps reasonable to gradually abandon the notion of "two hits" acting one after the other in favor of multiple interactive pathogenic networks that centrally converge towards factors promoting liver fat accumulation in a fashion capable of causing liver injury. The underlying origin of these changes is probably a combination of genetic and environmental factors (Farrell & Larter, 2006).

4. NAFLD and atherogenesis

4.1 Associations of NAFLD and atherogenesis

A link between cardiovascular disease (CVD) and metabolic syndrome components has already been established (Dekker et al., 2005). Given the recent recognition of NAFLD as being a hepatic manifestation of the metabolic syndrome, patients with NAFLD would be expected to have an increased risk of CVD development and events. Moreover, NAFLD has actually been established as an independent risk factor of CVD regardless of other confounding metabolic disorders. Last but not least, a possibility exists that NAFLD is not solely a risk marker, but an early mediator of CVD development as well (Targher & Arcaro, 2007).

NAFLD has been shown to be associated with circulatory endothelial dysfunction, one of the early atherosclerosis markers. NAFLD patients in comparison with non-steatotic controls had a significant decrease in the brachial artery flow-mediated vasodilation that was related to the histological severity of NAFLD. In addition, these patients had an increased 10-year probability of CVD events (Villanova et al., 2005). Another reliable marker of subclinical atherosclerosis is an increase in the carotid artery intima-media thickness (IMT) (O'Leary & Polak, 2002). Again, NAFLD histology predicted the carotid IMT independently of age, sex, BMI and other traditional risk factors such as IR and features of the metabolic syndrome (Targher et al., 2006a). IMT has also been found to strongly correlate with elevated liver enzymes alanine aminotransferase and gamma-glutamyl transpeptidase, surrogate markers of NAFLD (Sookoian & Pirola, 2008).

Notably, there have been numerous reports about associations of NAFLD and increased CVD prevalence. In a study with a large sample of middle-aged male workers they found that people with NAFLD were more likely to have CVD than those without it, even more, the association was independent of obesity and other prognostic factors (Lin et al., 2005). Another study showed that the occurrence of NAFLD was significantly higher in subjects with acute myocardial infarction and also, that the severity of coronary artery disease was greater in these individuals independent of age, sex and BMI (Kessler et al., 2005).

Mortality rate among NAFLD patients is increased in comparison to the general population (Adams & Angulo, 2005). In one study, 132 patients with NAFLD were followed for approximately 18 years and it was reported that CVD was the second most common cause of death, right after cancer and in the same numbers as liver-related mortality (Matteoni et

al., 1999). Another study with greater number of patients (420), but with mean follow-up of only 7.6 years, also found CVD and malignancy to have the highest rates of mortality that was overall higher in comparison to the people without NAFLD (Adams et al., 2005). It is questionable whether findings of these studies are applicable to the broader population since the number of patients was relatively low and the data originated from non-NAFLD oriented institutions (Targher & Arcaro, 2007). However, as in the case of subclinical atherosclerosis, elevated liver enzymes have been found to have a strong correlation not just with CVD risk, but also with increased CVD-related deaths in studies with a much higher number of participants (Lee et al., 2007; Schindhelm et al., 2007; Wannamethee et al., 1995). Practically in all of these studies NAFLD has been shown to be a risk factor of CVD independently of traditional risk factors or components of metabolic syndrome.

It has recently been suggested that childhood NAFLD could be an early contributing factor for development of atherosclerosis (Schwimmer et al., 2005). The authors of the study proposed that the process of atherosclerosis could already start in childhood and progress to symptomatic CVD in adulthood. Namely, in a sample of 817 children, atherosclerosis was twice as prevalent in children with fatty liver as in those without fatty liver. It is therefore reasonable to assume that steatosis is a potential early mediator of atherosclerosis, and this notion predicts an even greater epidemic of CVD in the future in parallel with increasing prevalence of NAFLD in pediatric population.

It seems that NAFLD brings with it an increased risk of developing atherosclerosis independently of other predictive factors. Whether this is true will need to be confirmed in subsequent studies with a larger number of patients and well defined and uniform diagnostic criteria for NAFLD. Nevertheless, a body of evidence clearly points in the direction that NAFLD is not just an innocent by-stander, but a pathological state that is actively contributing to atherosclerosis. From the clinician point of view these findings suggest that when NAFLD is detected either by ultrasound imaging or routinely performed liver enzyme tests, attention should be directed towards establishing whether other underlying CVD risk factors are also present. Similar if not greater efforts than for the treatment of NAFLD should then be considered to prevent development of atherosclerosis, since many NAFLD patients will die because of a major CVD event before the end-stage liver disease actually develops (Targher, 2007).

4.2 Mechanisms linking NAFLD and atherogenesis

Many studies clearly established a strong association of NAFLD and CVD, however, the mechanistic links between both diseases are still inadequately understood. One of the main problems in inferring causal relationships between NAFLD and accelerated atherosclerosis is their almost uniform coexistence with IR and other components of the metabolic syndrome. In this respect we will need to harness the aid of different NAFLD animal models that provide unique and to a certain extent controllable (patho)physiological conditions, which enable us to study these intertwined mechanisms separately from one another or in various combinations (Larter & Yeh, 2008). What are the possible routes of interaction between fatty liver disease and atherosclerosis?

4.2.1 Inflammation

One of the principal features of NAFLD, especially NASH, is increased oxidative stress and chronic, subclinical inflammation. The key pro-inflammatory cytokine that plays a vital role

in mediating inflammation is believed to be TNF-α (Figure 2). Expanded and insulin resistant abdominal adipose tissue is secreting increased amounts of TNF-α into the bloodstream that activates hepatic production of TNF-α through activation of IKKβ/NF-κB pathway. Activation of Kupffer cells by cytokines or hepatocyte damage further increases expression of TNF-α and IL-6 elevating both liver and systemic levels of pro-inflammatory cytokines. These enhance the inflammation and IR of the adipose tissue as well as the liver, and the vicious circle is closed. Another reinforcing route for inflammation starts with the inability of the insulin to suppress lipolysis in the adipose tissue that causes excess FFA flux to the liver where FFA are subjected to increased hepatic β-oxidation. Elevated levels of ROS cause oxidative damage that further intensifies the activation of inflammatory pathways in the liver (see Chapter 3). TNF-α promotes expression of IL-6 and IL-6 is the main hepatic stimulus for the production of C-reactive protein (CRP) (Heinrich et al., 1990). It has been known for a long time that inflammatory processes mediate the initiation and development of atherosclerotic lesions (Ross, 1999). Levels of TNF-α and IL-6 have been commonly associated with increased risk of coronary events (Ridker et al., 2000; Ridker et al., 2000). Until recently, CRP was thought to be an inactive downstream marker of the inflammation process. However, it has been shown that it can actively contribute to the development of atherosclerosis. CRP causes expression of cell adhesion molecules, activation of complement as well as mediating low-density lipoprotein (LDL) uptake by macrophages (Blake & Ridker, 2002). In NAFLD patients the levels of CRP were expectedly found to be increased compared to controls (Brea et al., 2005).

On the other side of the inflammation spectrum adiponectin acts as an anti-inflammatory mediator, mainly by antagonizing effects of TNF-α (Whitehead et al., 2006). Adiponectin has also been found to have antithrombotic effects, inhibiting thrombus formation and platelet aggregation (Kato et al., 2006). In NAFLD patients adiponectin levels are decreased (Hui et al., 2004; Targher et al., 2006b), thus lacking its protective effects against vascular diseases (Dekker et al., 2008).

In addition to direct predisposition of cytokines to atherosclerosis, the reinforcing inflammatory response through liver-adipose tissue axis exerts its effect indirectly as well. It has been shown that TNF-α interferes with intracellular insulin signaling cascade in the liver, adding to both hepatic and peripheral IR (Pandey et al., 2009). One of the dominant features of IR is atherogenic dyslipidemia, which will be discussed in the next subchapter. In corroboration of the effects of inflammatory cytokines on lipid status it has been shown that administration of recombinant TNF-α to treat cancer patients resulted in increased concentrations of VLDL and triglycerides and decreased high-density lipoprotein (HDL) particles (Sherman et al., 1988).

4.2.2 Dyslipidemia and aberrant cholesterol metabolism

Patients with metabolic syndrome usually have a state of dyslipidemia characterized by high triglycerides, low HDL-cholesterol, increased small dense LDL particles and increased apolipoprotein B100 concentration (Targher et al., 2008), which have all been recognized as atherogenic with strong associations to increased CVD risk (Heine & Dekker, 2002). In healthy people insulin suppresses hepatic production of VLDL in order to maintain normal hepatic lipid homeostasis (Sparks & Sparks, 1990). However, in the state of hepatic IR that has been further aggravated by steatosis, the secretion of VLDL is increased in conjunction with excessive triglyceride production (Adiels et al., 2006). After VLDL enters the blood,

triglycerides are gradually removed by the action of lipoprotein lipase resulting in small, dense LDL (the most atherogenic subclass of LDL) (Fon Tacer & Rozman, 2011). Concentrations of triglycerides, VLDL and small dense LDL particles have been increased in patients with fatty liver (Adiels et al., 2006; Cali et al., 2007; Sugino et al., 2011). Additionally, it has been shown that hepatic steatosis is a better predictor of the composition and severity of dyslipidemia than hyperglycemia or IR in type-2 diabetic patients (Toledo et al., 2006). In patients with NAFLD, HDL or "the good cholesterol" has been found to be decreased as a secondary abnormality due to increased VLDL and LDL (Cali et al., 2007). Contrary to the simple fatty liver, VLDL synthesis and secretion are impaired in the state of steatohepatitis, contributing further to the accumulation of triglycerides in the liver and thus promoting lipid oxidative damage (Fujita et al., 2009).

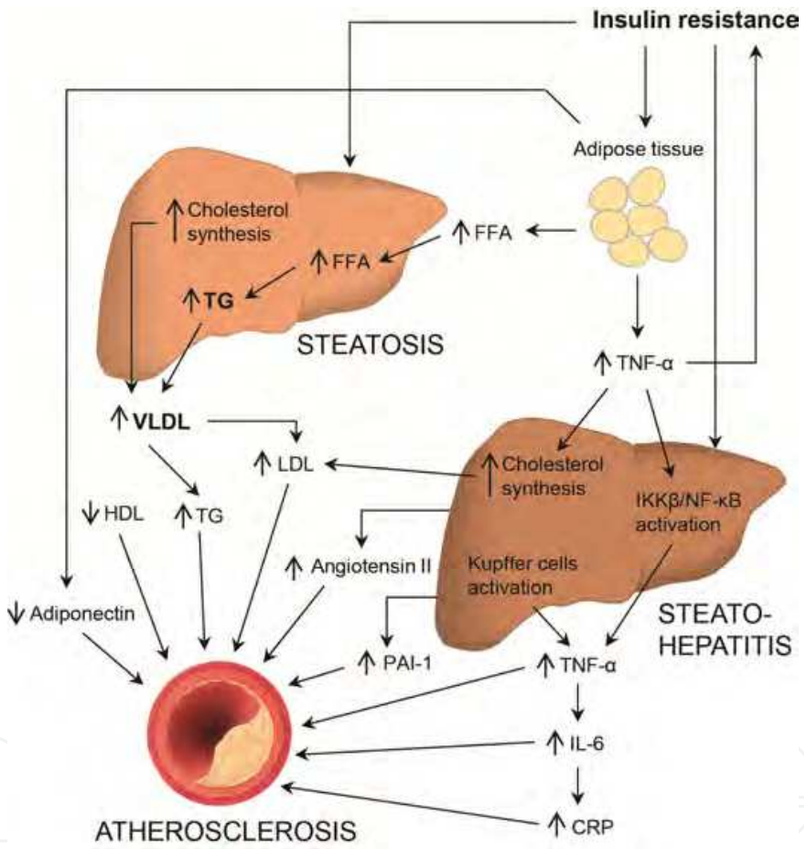

Fig. 2. Mechanisms linking NAFLD and atherosclerosis. Two principal atherogenic features of NAFLD are dyslipidemia and chronic subclinical inflammation. Increased cholesterol and triglyceride synthesis are the driving forces of increased secretion of VLDL and consequentially increased concentrations of LDL and TG. As a secondary abnormality HDL is decreased. Inflammed liver are producing excessive amounts of TNF-α, IL-6, CRP, PA-1, angiotensin II and cholesterol, which all stimulate the process of atherosclerosis. CRP: C-reactive protein; HDL: high-density lipoprotein FFA: free fatty acids; IKKβ/NF-κB: inhibitor kappa kinase beta/nuclear factor kappa B; IL-6: interleukin-6; LDL: low-density lipoprotein; PAI-1: plasminogen activator inhibitor -1; TG: triglycerides; TNF-α: tumor necrosis factor α; VLDL: very low-density lipoproteins.

Alongside aberrant lipoprotein metabolism driven mainly by IR, disturbances of cholesterol metabolism have also been found in patients with NAFLD. Detrimental effects of hypercholesterolemia on the progression of atherosclerosis have long been established. Cholesterol is capable of accumulating within the vascular wall, causing alterations in vascular structure and interfering with endothelial function leading to lesions, plaques, occlusions and emboli. Excess cholesterol concentration has also been associated with endothelial cell dysfunction, elevated oxidative stress and strong pro-inflammatory state in microcirculation (Stapleton et al., 2010). In humans cholesterol originates either from *de novo* synthesis or from the diet and is excreted mainly in the form of bile acids. Elevated concentrations of cholesterol have been found in the fatty liver of obese hyperlipidemic individuals compared to those without fatty liver (Reunanen et al., 1969). Obesity, metabolic syndrome, type 2-diabetes and IR have all been associated with increased cholesterol synthesis and lowered absorption of dietary cholesterol (Gylling et al., 2007; Miettinen & Gylling, 2000; Pihlajamaki et al., 2004; Simonen et al., 2002). Recent study has shown that the same happens in NAFLD patients (Simonen et al., 2011), where an increase in expression of cholesterogenic genes has been observed (Nakamuta et al., 2009). It seems that excess cholesterol synthesis, along with increased synthesis of triglycerides, presents one of the driving factors for increased production of VLDL particles in hepatic steatosis (Fon Tacer & Rozman, 2011).

Emerging evidence has started to place cholesterol in the "second hit" group that mediates the transition from simple steatosis to steatohepatitis. It has been shown that mitochondrial loading of free cholesterol, rather than FFA or triglycerides, sensitizes the liver to TNF-α induced steatohepatitis (Mari et al., 2006). In hyperlipidemic mouse models of NASH, dietary cholesterol, and not steatosis, predisposed to hepatic inflammation (Wouters et al., 2008). TNF-α also has a direct influence on lipid homeostasis since it activates cholesterol synthesis and inhibits formation of bile acids, thus increasing LDL-cholesterol and decreasing HDL-cholesterol (Fon Tacer et al., 2007; Fon Tacer et al., 2010). In contrast to its role in atherosclerosis, cholesterols implication in NAFLD has been poorly investigated. Nevertheless, limited research in this area is indicating an important role of cholesterol in pathogenesis of NAFLD as well as a novel link between disrupted cholesterol metabolism in NAFLD and atherosclerosis.

4.2.3 Other factors connecting fatty liver and the process of atherosclerosis

Apart from many positive feedback loops between fatty liver and IR, visceral adipose tissue, hyperglycemia and dyslipidemia, several other factors are mediating a cross-talk between atherosclerosis and NAFLD. Many coagulation factors are synthesized by hepatocytes. In NAFLD patients several are overproduced, and of particular concern is plasminogen activator inhibitor-1, which has direct atherogenic effects (Bansilal et al., 2007). Hepatocytes also produce angiotensinogen, a precursor of angiotensin II, and hepatic stellate cells are even capable of synthesizing and secreting mature form of angiotensin II upon activation by hepatocyte damage (Bataller et al., 2003). Angiotensin II is a pro-atherogenic vasoconstrictive peptide that predisposes to elevated blood pressure (Bataller et al., 2003; Massiera et al., 2001) and possibly also to CVD (Silventoinen et al., 2008).

Many other factors that contribute to both NAFLD and increased risk of atherosclerosis, like disturbances in endocrine system, hypoxia and ectopic fat deposition, are reviewed elsewhere (Loria et al., 2008).

5. Conclusion – The intertwined roles of cholesterol and inflammation in NAFLD and atherogenesis

In affluent economies NAFLD is already the most common cause of liver-related diseases and a major cause of morbidity and mortality. Sedentary life style and increased caloric intake, a hallmark of modern societies, are of particular concern in this respect, since NAFLD is found exclusively in obese individuals with insulin resistance. One of the emerging problems is also the increasing prevalence of fatty liver in children and adolescents, forecasting an even bigger social and economic impact of the disease in the future. Even though simple steatosis is benign, if not averted, it could predispose to more severe forms of liver disease with poor outcome. Despite the fact that the first description of NAFLD was made as many as 30 years ago, the decisive factor tipping the scales towards NASH is largely unknown. Genetic factors might play an important role here. Because of poorly understood mechanisms linking different stages of NAFLD, therapy is directed mainly towards improving the metabolic disorders without liver-specific drugs yet available. Surprisingly, cardiovascular events are among the leading causes of death in NAFLD patients, and recent research has enthroned NAFLD as an independent risk factor for CVD. Many direct as well as indirect links between fatty liver and atherosclerotic vessels have been established in the recent years. Inflammation and dyslipidemia are the most important factors contributing to both diseases with disrupted cholesterol metabolism gaining recognition, especially in the pathogenesis of NAFLD. Unfortunately, information about the role of cholesterol in NAFLD is limited, and hopefully new and emerging animal models (Horvat et al., 2011; Keber et al., 2011) of disturbed cholesterol metabolism will provide the missing insights. Better understanding of these mechanisms is important since inhibition of cholesterol synthesis by statins has proven beneficial in NAFLD patients (Ekstedt et al., 2007). However, the use of statins presents a risk of developing adverse drug reactions such as elevation of liver enzymes or hepatotoxicity due to drug interactions (Rozman & Monostory, 2010).

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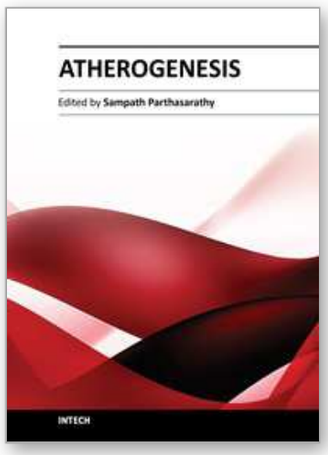

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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