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NON ALCOHOLIC FATTY LIVER DISEASE: NON INVASIVE MARKERS OF SEVERITY AND NEW EXPERIMENTAL TREATMENTS

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INTRODUCTION

NON ALCOHOLIC FATTY LIVER DISEASE

DEFINITION

The term "non-alcoholic fatty liver disease" (NAFLD) refers to hepatic steatosis accounting for more than 5% of the total weight of the liver, which is not caused by excessive consumption of alcohol (women ≤20 g/d, men ≤30 g/d) or hepatotropic viruses. NAFLD is one of the most important chronic liver disorders worldwide (1) It covers a wide spectrum of hepatic damage in which simple steatosis (NAFL) can progress to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (2). NAFLD is considered to be the hepatic component of metabolic syndrome as its features are similar to those of metabolic disorders such as obesity, inflammation, insulin resistance, and type 2 diabetes (3, 4) Moreover such as metabolic syndrome, NAFLD has been demonstrated to be associated with increased risk of cardiovascular diseases (5).

EPIDEMIOLOGY

In Europe non-alcoholic fatty liver has become the most common cause of chronic liver disease. The proportion of NAFLD among chronic liver diseases rose from 47% to 75% between 1988 and 2008. The reason for this increase is most probably an increase in metabolic risk factors, also in the context of aging populations (6).

In Europe, the prevalence of NAFLD in the population is estimated to be 20% to 30%. A look at special subgroups in the population reveals a wide range of the observed prevalence rates—from 2% in unselected children to 44% in selected risk groups, such as people with type 2 diabetes, or 90% in obese subjects. No reliable data are available on the prevalence of advanced NAFLD and cirrhosis (7).

A strong increase of NAFLD incidence has recently been observed especially in adolescents and in older people. The prevalence depends not only on the population under study but also on the study method; the differences may be substantial. Studies in potential live liver donors found histologically confirmed NAFLD in 20% and 51% of cases, respectively (8). Sonographically the prevalence rate varied between 17% and 46%, depending on the population under study (7). Worldwide, the assumed prevalence of NAFLD in the general population is between 6% and 33%, with a median of 20%; the estimated prevalence of NASH is notably lower, at 3% to 5% (9).

The prevalence of NAFLD is even higher in patients with type 2, but not type 1, diabetes than in the general population. An ultrasound based study found a prevalence of 69% among patients with type 2 diabetes (10, 11).

Lipid metabolism also seems to have a substantial influence. In many type 2 diabetes patients, raised concentrations of triglycerides and lowered concentrations of HDL cholesterol are also observed. In patients with dyslipidemia who were attending an outpatient clinic, the prevalence of NAFLD was 50% Interestingly, the risk of NAFLD increases independently of the presence of diabetes with each individual metabolic risk factor (12). Factors such as age, sex and ethnicity have also been found to play a role: male sex, older age, and Hispanic origin are associated with a significantly higher risk of developing NAFLD (9, 13).

Patients with NASH are prone to a progressively advanced liver fibrosis and cirrhosis in a rather short time (14-16) but there is no specific symptom of NASH that reflect the advanced condition of the patients (14,17).

NAFLD-cirrhosis or NAFLD-Hepatocellular Carcinoma (HCC) are now the second cause of liver transplantation in the USA (18) and, as a consequence of the new and effective therapies for chronic viral hepatitis, NAFLD-cirrhosis and HCC will become the most common indication for liver transplantation in the near future. HCC can also develop in NASH in the absence of cirrhosis (45% of cases) (18).

The risk of progression to end-stage liver disease is influenced by the severity of underlying liver histology; the majority of patients with NAFLD have simple steatosis, however, up to 30% of patients may have NASH (19) and are at greater risk. Whilst simple steatosis in the absence of significant fibrosis is considered to be a relatively benign condition, the presence of fibrosis predicts both disease progression and liver-related complications over a subsequent 10-year period. 40% of patients with NAFLD related cirrhosis die in 10 years (19).

Several studies with up to 20 years follow-up, have demonstrated that the risk of progression to cirrhosis in patients with simple steatosis is between 0% and 4% (20-22).

Decreased survival in NASH patients is due predominantly to cardiovascular causes, although there is a significant increase in liver-related deaths. NASH also carries an increased risk of hepatocellular carcinoma (HCC and thus the observation of increased incidence of HCC in type 2 diabetes is likely to be due to their high prevalence of NASH. In US, NASH was found to account for at least 13% of overall cases of HCC (14-18-23).

HYSTOLOGICAL   CLASSIFICATION

Figure A: normal liver at histological evaluation.
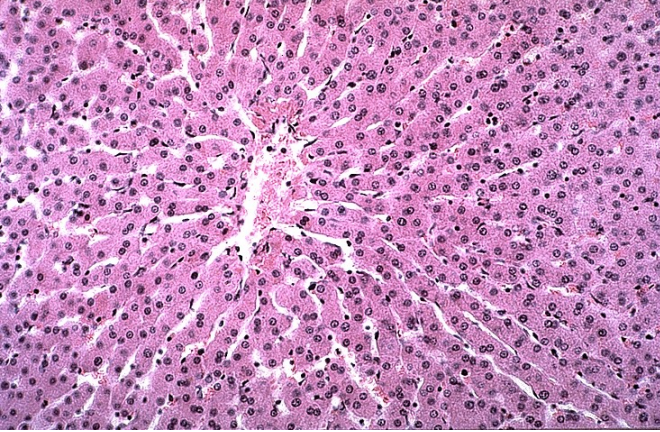
Figure B: liver steatosis at hystological evaluation. Presence of lipid droplets within hepatocytes. Macrovescicular steatosis is characterized by the presence of sa single big lipid droplet (as in the picture). Microvescicular steatosis is characterized by the presence of small lipid vesicles.
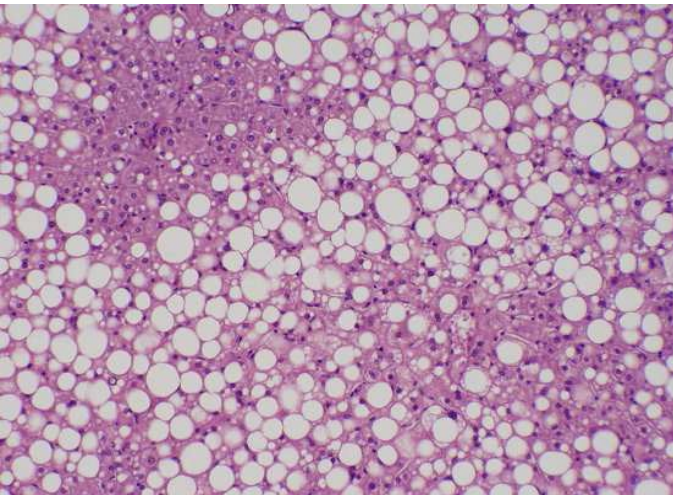
The disorder has a broad histological spectrum from simple NAFL to a progressive hepatic injury condition characterized by steatosis-induced inflammation, results from recruitment and activation of inflammatory cells in the liver (NASH) (24).

The diagnosis of NASH is established by the presence of a characteristic pattern of steatosis, inflammation and hepatocellular ballooning on liver biopsies in the absence of significant alcohol consumption. However, diagnostic criteria for NASH. For this reason a scoring system for nonalcoholic fatty liver disease (NAFLD) was developed and validated the NAFLD Activity Score (NAS) The methodology proposed for feature-based scoring of histologic lesions of NAFLD has been widely utilized, as evidenced by its application in numerous clinical and experimental settings in NAFLD-related studies. The recognized strengths of the

method include the relative ease of understanding and therefore, application of the system; division of lesions of active and potentially reversible injury ("grade") in the NAFLD Activity Score (NAS) and those potentially less reversible and characterized by collagen deposition and architectural alterations that may evolve toward more permanent parenchymal remodeling ("stage"). The proposed NAS also clearly separates the three lesions that comprise grade: steatosis, lobular inflammation, and ballooning. This allows detailed analysis of histologic changes for comparative and correlative studies in therapeutic intervention trials (24).

Item	Score	Extent
Steatosis	0	<5%
	1	5-33%
	2	>33-66%
	3	>66%
Hepatocyte Ballooning	0	None
	1	Few balloon cells
	2	Many cells/prominent ballooning
Lobular Inflammation	0	No foci
	1	<2 foci/200x
	2	2-4 foci/200x
	3	>4 foci/200x

NAFLD activity score is determined based on three components: steatosis, hepatocyte ballooning degeneration, and lobular inflammation. Higher scores indicate a greater severity of NASH.

Macrovescicular and microvescicular steatosis

Steatosis in NAFLD is usually seen as macrovesicular steatosis (large droplet steatosis) in which a single, large vacuole of fat fills up the hepatocyte and displaces the nucleus to the periphery. Often macrovesicular steatosis can be present with both large and small droplets that may be seen to coalesce. Macrovesicular steatosis alone is considered to have a good long-term prognosis with rare progression to fibrosis or cirrhosis. On the other hand, diffuse microvesicular steatosis denotes a separate clinical entity the diseases share severe mitochondrial β-oxidation defects from genetic or acquired causes. Examples include acute fatty liver of pregnancy, Reyes syndrome, drugs or toxins (25,26).

These diseases either resolve, or lead to death if not managed with liver transplant. Histologically, microvesicular steatosis is characterized by distended hepatocytes with foamy appearing cytoplasm; small lipid vesicles (less than 1μm in diameter) may or may not be discernible. The nucleus is typically centrally

located unlike in macrovesicular steatosis where the nucleus is displaced peripherally. Because of the diffuse cytoplasmic alteration, special staining such as oil red O may be required for its diagnosis. Microvesicular steatosis is also commonly present in the same hepatocytes that harbor visualizable mitochondria, known as "megamitochondria". Microvesicular steatosis is related to advanced histological features such as ballooning, inflammation, steatohepatitis, and fibrosis and is often related to a worse prognosis and predisposition to develop liver cirrhosis (27). The worse prognosis of microvescicular steatosis has been related to its aetiology (drugs and toxins) and to the presence of inflammation (25, 26), however microvescicular steatosis is described also in NAFLD ant its significance is still under debate even it is commonly thought to be related to an increased risk of NASH and cirrhosis (27).

PYSIOPATHOLOGY

Few studies are available on its natural history and although the pathogenesis of NAFLD/NASH is not yet fully understood, much progress has been made in recent years in elucidating the mechanisms of progression from steatosis to more advanced liver inflammation and fibrosis.

As previously described NAFLD is the accumulation of more than 5% of fat and specifically triglyceride within hepatocytes. Triglycerides are formed from the esterification of FFA and glycerol within the hepatocyte. FFAs arise in the liver from three distinct sources; lipolysis (the hydrolysis of FFA and glycerol from triglyceride) within adipose tissue, dietary sources, and *de novo* lipogenesis (DNL). In contrast, FFA may be utilized either through β-oxidation, re-esterification to triglycerides and storage as lipid droplets, or packaged and exported as very low density lipoprotein (VLDL). Hence hepatic fat accumulation can occur as a result of increased fat synthesis, increased fat delivery, decreased fat export, and/or decreased fat oxidation (28).

While in healthy subjects <5% of liver triglyceride content derives from DNL, in diabetic patients DNL contributes >25% (29). Triglyceride can also be exported from the liver in VLDL particles, which are formed by the incorporation of triglyceride into apolipoprotein B (apoB). Aberrant alterations of apoB synthesis and secretion have been demonstrated to be involved in the pathogenesis of NAFLD leading to a decreased capacity for lipid export (28).

Some of the mechanisms involved in hepatic triglyceride accumulation are oxidative stress, lipotoxicity, mitochondrial damage, insulin resistance, inflammation, and excessive dietary fat intake, which increase hepatic lipid influx and de novo lipogenesis and impair insulin signaling. Moreover overproduction of proinflammatory adipokines from adipose tissue also affects hepatic metabolic function (19).

At the end of the 90' the two-hit-hypothesis has been proposed to explain the pathogenesis of NASH (30).

The first hit is insulin resistance and excessive fatty acids in the circulation, which lead to simple hepatic steatosis. The second hit involves oxidative stress, lipid peroxidation, and mitochondrial dysfunction.

A further component, has been added to reflect inadequate hepatocyte proliferation. In the healthy liver, cell death stimulates replication of mature hepatocytes which replace the dead cells and reconstitute normal tissue function. Oxidative stress inhibits the replication of mature hepatocytes which results in expansion of the hepatic progenitor cell (oval cell). In chronic liver injury, the development of fibrosis/cirrhosis is dependent on the efficacy of hepatocyte regeneration, and therefore cell death with impaired proliferation of hepatocyte progenitors represents the proposed 'third hit' in NAFLD pathogenesis.

With the identification of more advanced mechanisms, NASH was shown to develop through a multifactorial better than a consequential process the so called "multiple parallel hits hypothesis". According to this hypothesis a number of different processes such insulin resistance, oxidative stress, inflammation, immune system, genetic determinants, nutrition and lifestyle, and changes in the intestinal microbiota (30-31) may contribute to liver injury in an overlapped way.

The role of insulin resistance and NAFLD

Among these multiple factors, insulin resistance is pivotal for the progression of NAFLD (32). 70%–80% of obese and diabetic patients have NAFLD (33, 34). In obese patients with concomitant type 2 diabetes and NAFLD, hyperinsulinemia and dyslipidemia are more severe than in patients without NAFLD (35). Insulin resistance is related to the excess of circulating FFAs and oxidated FFAs derived from lipogenesis and de novo fatty acid synthesis (36).

These FFA either undergo β-oxidation or are esterified with glycerol to form triglycerides, leading to hepatic fat accumulation. There is now substantial evidence that FFA can directly cause toxicity by increasing oxidative stress and by activation of inflammatory pathways.

Adipose tissue is a mediator of systemic lipid storage but can be considered also an endocrine organ that secretes hormones and the group of cytokines known as adipokines, such as adiponectin and leptin (36). Adiponectin regulates fatty acid oxidation and inhibits lipid accumulation, both in adipose tissue and in the liver (37). It also maintains whole-body glucose homeostasis, including hepatic insulin sensitivity (38). Recent studies have shown that serum adiponectin levels are lower in patients with NAFLD (39).

Innate immune system and NAFLD

The innate immune system, also known as the non-specific immune system or in-born immune system (40) is an important subsystem of the overall immune system that comprises the cells and mechanisms that defend the host from dangerous substances. The cells of the innate system recognize and respond to pathogens in a

similar way, but, unlike the adaptive immune system, the system does not provide long-lasting immunity to the host (41).

The major components of the innate immune system include inflammatory mediators and specific cells activation. The innate leukocytes include: natural killer cells, mast cells, eosinophils, basophils; and the phagocytic cells including macrophages, neutrophils, and dendritic cells, and function within the immune system by identifying and eliminating pathogens that might cause infection (42)

Inflammation is one of the first responses of the immune system to infection or irritation. Inflammation is stimulated by chemical factors released by injured cells and serves to establish a physical barrier against the spread of infection, and to promote healing of any damaged tissue following the clearance of pathogens (43).

The process of acute inflammation is initiated by cells already present in all tissues, mainly resident macrophages, dendritic cells, hystiocytes, Kupffer cells, and mastocytes. These cells present receptors contained on the surface or within the cell, named pattern recognition receptors (PRRs), which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). Chemical factors produced during inflammation (histamine, bradykinin, serotonin, leukotrienes, and prostaglandins) sensitize pain receptors, cause local vasodilation of the blood vessels, and attract phagocytes, especially neutrophils (43). Neutrophils then trigger other parts of the immune system by releasing factors that summon additional leukocytes and lymphocytes. Cytokines produced by macrophages and other cells of the innate immune system mediate the inflammatory response. These cytokines include TNF, HMGB1, and IL-1(44).

- **Innate immune cells and NAFLD**

Overnutrition or insufficient exercise leads to adipose expansion, with the hypertrophic adipocytes secreting TNF, IL-1, and IL-6. These pro-inflammatory cytokines down-regulate hepatic insulin sensitivity via the activation of pro-inflammatory signaling and the inhibition of insulin receptor signaling. The result is the development of liver steatosis and fibrosis (45)

Immune cells, macrophages/Kupffer cells, natural killer cells, and T-cells contribute to the progression of NAFLD to NASH. A pivotal role is played by the innate immune system which include monucleate circulating cells and inflammatory citokines (46).

In particular, hepatic macrophages, which include both resident Kupffer cells and macrophages recruited from the peripheral circulation, are the major immune cells that secrete inflammatory mediators, such as tumor necrosis factor (TNF)α and interleukin (IL)1β, leading to systemic insulin resistance and NASH (47). These cells localize within liver sinusoids, accounting for ~10% of the total number of liver cells (48).

Macrophages can be classified as M1, or "classically activated" pro-inflammatory macrophages, and M2, or

"alternatively activated" non-inflammatory macrophages (49–51). Alternative M2 macrophages sustain insulin sensitivity via the secretion of anti-inflammatory while classical M1 macrophages secrete pro-inflammatory cytokines such as TNFα, IL6, and IL1β, which leads to insulin resistance (50,51). IL-1 is a potent inflammatory cytokine mainly produced by macrophages. IL-1 production requires stimulation with TLR ligands as well as a second signal. IL-1 participates in toxic, ethanol and NASH-induced fibrosis. In HSCs IL-1 mediates upregulation of fibrogenic proteins. Moreover, IL-1 can prolong the survival of HSCs (46).

The dysregulation and polarization of M1 and M2 macrophages are closely related to multiple metabolic disorders, among them, obesity, insulin resistance, and NAFLD. Hepatic lipid accumulation promoted the activation of macrophages/Kupffer cells (52).

Inflammation in the liver is regulated by the balance of pro-inflammatory M1 Kupffer cells and anti-inflammatory M2 Kupffer cells (53). Thus, the exacerbated release of M1 Kupffer-cell-derived mediators contributes to the pathogenesis of liver steatosis, the recruitment of inflammatory immune cells, and the activation of fibrogenesis (53,54]. Inflammatory cytokines, which in addition to TNF include chemokines such as monocyte chemoattractant protein (MCP)-1/C-C chemokine ligand 2 (CCL2) and RANTES/CCL5, are produced by M1 cells and increase hepatic lipid accumulation, which results in the discordant regulation of lipid metabolism and homeostasis (55). IM2 cells promote the caspase-3-dependent apoptosis of classically activated M1 cells and thus provide a protective mechanism against NAFLD (54). Because the M1/M2 ratio is increased during NAFLD progression, the polarization of cells into M2 Kupffer cells might be an important mechanism protecting against fatty liver disease (46).

- **Inflammatory signaling and chemokine production in NAFLD**

The presence of steatosis is tightly associated with chronic hepatic inflammation (56) an effect in part mediated by activation of the Iκκ-β/NF-κB signalling pathway. In murine models of high-fat diet (HFD)-induced steatosis, increased NF-κB activity is associated with elevated hepatic expression of inflammatory cytokines such as TNF-α, interleukin-6 (IL-6) and interleukin 1-beta (IL-1β), and activation of Kupffer cells (56).

Liver-specific NF-κB inhibition prevents HFD-induced inflammatory gene expression, whereas HFD-induced hyperglycaemia and IR can be reproduced by selective over-expression of constitutively active Iκκ-β in hepatocytes (56) The Iκκ-β/NF-κB pathway in hepatocytes can also be activated directly by FFA, providing a further mechanism by which central obesity with consequent increased hepatic FFA supply can contribute to inflammation (57, 58).

Both serum and hepatic levels of TNF-α are elevated in patients with NASH (54) and levels correlate with histological severity (55) In addition to its proinflammatory effects, TNF-α promotes IR. Conversely, inhibition of TNF-α signalling improves IR and histological parameters of NASH (59-61). Similarly, serum

IL-6 levels are also elevated in both animal and human models of IR and NAFLD (62, 63) and levels correlate with increasing liver inflammation and fibrosis (64) The key role of hepatocyte cytokine production in the progression of steatosis to NASH is supported by studies demonstrating that cytokines can replicate all of the histological features associated with NASH, including neutrophil chemotaxis, hepatocyte apoptosis/necrosis, Mallory body formation and stellate cell activation (65) Additionally, data suggests that inflammation and NF-κB activation can promote carcinogenesis(66) and that the chronic inflammatory state associated with hepatic steatosis may also play a key role in HCC development (65).

Chemokines are a family of cytokines that activate leukocyte chemotaxis and play important roles in the progression of systemic inflammation (59,67). By recruiting immune cells to adipose tissue, liver, and skeletal muscle, chemokines lead to acute inflammation and the development of insulin resistance, as well as fatty liver disease (60-62). The chemokine (C-C motif) ligand 2 (CCL2) is a small cytokine primarily secreted by monocytes, macrophages and dendritic cells CCL2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation. CCL2 impairs insulin signaling in skeletal muscle significantly reduced insulin-stimulated glucose uptake in myocytes. CCL2, is up-regulated in obese adipose tissue, secondary to macrophage infiltration (63, 64). By binding to the CCR2 receptor, CCL2 causes the infiltration of bone marrow-derived macrophages into obese adipose tissue or liver. It is also involved in the development of hepatic steatosis and insulin resistance (62-64). Recent studies show how the CCL2/CCLR axis plays a central role in murine model of obesity-induced liver disease. The specific overexpression of CCL2 in the adipose tissue of mice leads to the development of insulin resistance, inflammation, and hepatic steatosis (66). Conversely, mice with a genetic deletion of CCR2 have improved insulin sensitivity and inflammation, without a decrease in body weight, in agreement with the findings obtained with pharmacological antagonists of CCR2 (63, 68). However, there are also studies reporting conflicting results, showing that MCP-1-deficient mice do not exhibit reduced macrophage infiltration or improved insulin sensitivity, which suggests that CCL2-CCR2 signaling is not critical for obesity-induced macrophage recruitment or systemic insulin resistance (69, 70).

The hepatic infiltration of macrophages/Kupffer cells is primarily promoted by CCL2, as these cells express CCR2 (71). CCL2 expression in hepatocytes is increased in animals fed a high-fat diet and leads to the hepatic recruitment of CCR2+ myeloid cells that promote hepatic steatosis (62).

The CCL2-CCR2 pathway is also up-regulated in the livers of animals with NASH and is critical to the development of hepatic steatosis and fibrosis by promoting the activation and migration of hepatic stellate cells (72) Serum and liver CCL2 levels are increased in NASH patients (71), whereas in animal NASH models, the genetic deletion of CCL2 and CCR2 or the inactivation of CCR2 reduces macrophage infiltration, attenuates obesity, and improves both insulin resistance and hepatic

steatosis (63, 64, 72). Thus, collectively, CCL2-CCR2 signaling is central to the progression of hepatic steatosis to NASH.

- **The Inflammasome**

The **inflammasome** is a multiprotein oligomer responsible for the activation of inflammatory responses (73). It is expressed in myeloid cells and is a component of the innate immune system. The inflammasome promotes the maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18.

During an infection, one of the first forms of defense employed by the innate immune response is a group of pattern recognition receptors (PRRs) encoded in the germline to recognize molecular patterns expressed by invading pathogens. These may either be on the membrane surface e.g. Toll-like receptors (TLRs) and C- type Lectin Receptor (CLRs) or inside the cytoplasm e.g. Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs). In 2002, it was first reported by Martinon *et al.* that a subset of NLRs named NLRP1 were able to assemble and oligomerize into a common structure which collectively activated the caspase-1 cascade, thereby leading to the production of pro-inflammatory cytokines especially IL-1B and IL-18. This NLRP1 multi-molecular complex was dubbed the 'inflammasome', which spurred much interest in the following years; since then, several other inflammasomes were discovered, two of which are also NLR subsets—NLRP3 and NLRC4 (74). One of the most recently identified contributor to the cross talk between hepatocytes and inflammatory macrophages is represented by the multiprotein platform complex nucleotide- binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome, whose activation has been suggested to play a crucial role in the progression of NAFLD (75)

In particular, the transition from NAFLD to NASH associates with NLRP3-inflammasome activation and an increased expression of inflammasome-related components, including apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), caspase-1 (CASP-1) and pannexin (76-79).

In addition, inflammasome activation involves not only liver innate immunity cells but also parenchymal cells (78, 79) with studies indicating that saturated fatty acids can specifically activate the inflammasome complex in hepatocytes inducing IL-1β expression and release (75, 79). However, no study has so far investigated whether MVs released from fat-laden hepatocytes may promote NLRP3-inflammasome activation (75).

FIGURE C: Schematic representation of the the progression of NAFLD/NASH: Excess fat intake and obesity lead to hyperglycemia, hyperlipidemia, and the oversecretion of adipocytokines and chemokines such as tumor necrosis factor (TNF), interleukin (IL)-1 β, and monocyte chemoattractant protein (MCP)-1/C-C chemokine ligand 2 (CCL2). These factors further contribute to the development of systemic insulin resistance and hepatic steatosis. The latter causes hepatic inflammation and induces NASH and even cirrhosis. Hepatic inflammation involves the recruitment of macrophages/Kupffer cells and an M1-dominant phenotypic shift in macrophages in the liver, activating hepatic stellate cells and finally leading to liver fibrosis.
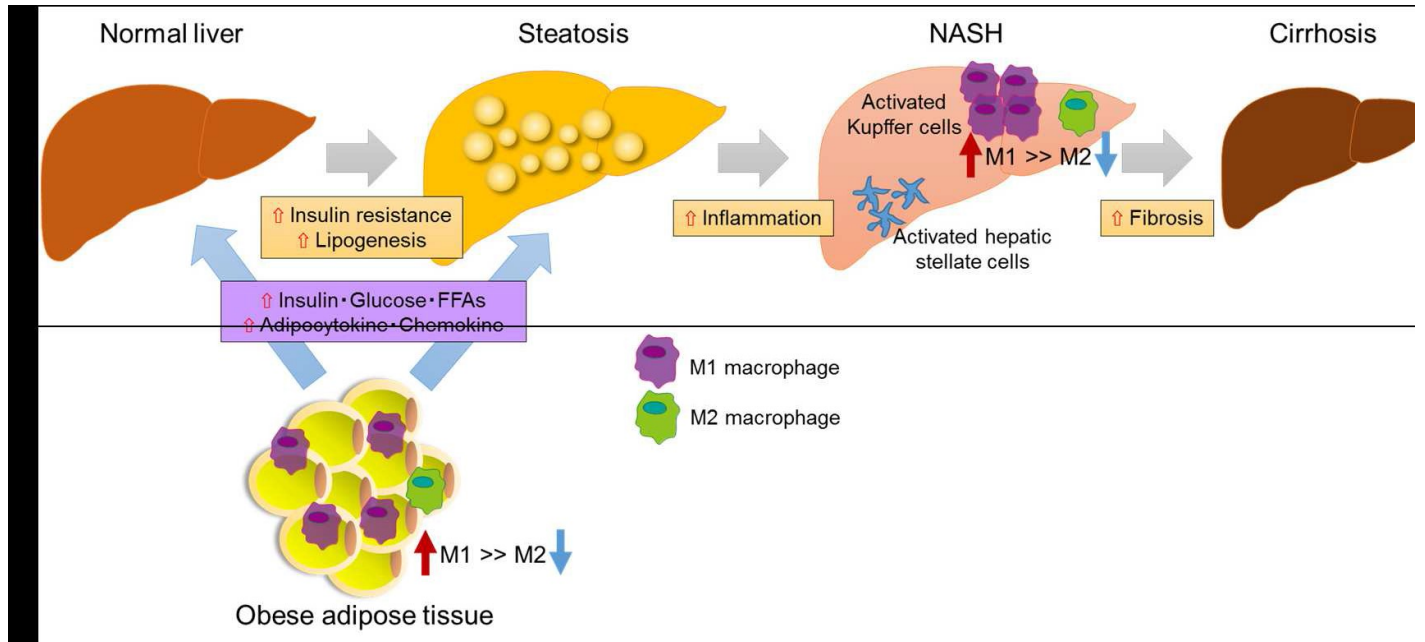
Adapted from "Kitade H, Chen G, Ni Y, Ota T. Nonalcoholic Fatty Liver Disease and Insulin Resistance: New Insights and Potential New Treatments. Nutrients. 2017 Apr; 9(4): 387"

CLINICAL PRESENTATION AND DIAGNOSTIC EVALUATION

The findings in NAFLD tend to be non-specific. Most patients have no symptoms or signs of hepatic disease at the time of diagnosis.

In terms of laboratory chemistry, pathological values of aspartate-aminotransferase [AST] and alanine-aminotransferase [ALT] may be noted (12)

However, even normal levels for transaminases do not rule out NASH, and raised concentrations are partly re-normalized when cirrhosis develops. The ferritin concentration is raised in about half of patients, and transferrin saturation increased in 6–11%. The liver's iron content is typically within the normal range (12). Furthermore, commercial combined tests and apoptosis markers (cytokeratine-18 fragments) (80) are available however, these have not gained any importance in routine clinical practice (6)

A recently proposed diagnostic tool for NAFLD evaluation is the determination of Lysosomal acid lipase (LAL) activity on dried blood spots (81).

Lysosomal acid lipase (LAL) is a key enzyme in lipid metabolism. LAL Deficiency (is an autosomal recessive disorder caused by a deficiency of the enzyme. It can manifest as a severe infantile and rapidly progressive disorder, Wolman's disease, or the more attenuated form which manifests in childhood or adolescence as a progressive disease. The natural history and the clinical manifestations of the disease in children and adults are less well defined and the diagnosis is often incidental. Lipid abnormalities are common, and patients may present early signs of systemic atherosclerosis. Moreover, hepatomegaly and microvescicular steatosis with liver cell damage and splenomegaly are common features of the disease (81).

LAL is a key enzyme involved in intracellular lipid metabolism and trafficking; it is responsible for the intra-lysosomal hydrolysis of LDL CE and triglycerides into free cholesterol and free fatty acids (82). Therefore, the reduction of LAL activity determines intra-lysosomal lipid accumulation and a consecutive reduction of free cholesterol in cytosol

LAL activity reduction should always be suspected in non-obese patients presenting with NAFLD and/or cryptogenic cirrhosis, unexplained persistently elevated liver transaminases or with elevation in LDL-C and decreased HDL-C.

In vitro, it has been demonstrated that several factors may modulate LAL activity (83).

In particular, enhanced LAL activity was associated with eicosanoids, gonadotropins and glucagon, and reduced activity was correlated with Lp(a), LDL remnants and oxidized LDL concentrations.

Initial reports have suggested a role for a relative acquired LAL deficiency in non-alcoholic fatty liver disease (NAFLD)-however, it is still unclear whether this mechanism is specific for NAFLD. Baratta et al. in 2015 reported for the first time reduced blood LAL activity in adult patients with NAFLD (84, 85).

Tovoli et al recently demonstrated that LAL activity is significantly reduced in NAFLD as compared to that in HCV patients. In their study this finding was particularly evident in the pre-cirrhotic stage of disease. LAL activity was also correlated with platelet and white blood cell count, suggesting an analytic interference of portal-hypertension-induced pancytopenia on dried blood spots -determined LAL activity (86).

Despite the potential usefulness of the described biochemical tests, liver biopsy is still the gold standard in the diagnostic evaluation of NAFLD, because NASH can be formally diagnosed only by means of histological testing. However, liver biopsy is an invasive procedure, which carries a risk of potentially life threatening complications (6). Moreover there is evidence that NASH is erroneously not identified in up to one-third of patients, and that the degree of fibrosis may be subject to overestimation as well as underestimation (87)

Since patients are usually asymptomatic and the laboratory parameters often normal, the question in routine clinical practice is which patients should be investigated for NAFLD. A practical recommendation is urgently needed in this setting. The current US NAFLD guideline advises against general NAFLD screening at this time, owing to the lack of evidence of benefit and the relatively high cost, not even in high-risk groups such as obese patients or patients with diabetes (88). In Germany, the clinical practice guideline on the diagnosis and treatment of hepatocellular carcinoma recommends a general ultrasound follow-up not only in cirrhosis but also in patients with NASH (89) In principle this requires a liver biopsy in all patients with a fatty liver, in order to identify high-risk patients with NASH or higher-grade fibrosis. To date, no clear guidance is given in the literature with regard to defining an indication for liver biopsy in NAFLD

The non-invasive method that is certainly most suitable for detecting hepatic steatosis is ultrasound (sensitivity 60–94%, specificity 66–97%), but this is rather less precise in milder degrees of steatosis (90) According to available study data, the positive predictive value in mild steatosis is only 67% at best (90). Outside the study setting, the positive predictive value is likely to be even lower. The degree of hepatic fibrosis can now be estimated non-invasively by using several techniques of elastography (including FibroScan and acoustic radiation force impulse imaging [ARFI]) (91). The FibroScan investigation enables distinction between fibrosis (F1–F3) and cirrhosis (92), but in morbid obesity it is not equal to the task.

For routine clinical practice, the question remains how high-risk patients can be identified. Recently, a number of simple clinical risk scores has shown excellent consistency with the degree of fibrosis in patients with steatosis (93). The best result was seen for the NAFLD fibrosis score (http://nafldscore.com), which consists of the parameters age, BMI, diabetes, AST, ALT, thrombocytes, and albumin (positive predictive value 82–90%, negative predictive value 88–93%). An increased risk of higher-degree fibrosis was described for patients with a BMI >32 kg/m^2, age >45 years, diabetes, and a ratio of ALT/AST>1 (94). New

genetic markers, such as variants of PNPLA3 (adiponutrin), which indicate an increased risk of progression towards NASH, fibrosis, and HCC, have not yet become established in routine clinical practice (95, 96)

In combination with findings from sonography or elastography, these clinical scores can help identify patients for diagnostic liver biopsy or close clinical and sonographic monitoring of the clinical course (six-monthly in NASH). The high coincidence of type 2 diabetes and NAFLD justifies routine diabetes screening (HbA1c and oral glucose tolerance test, if required) (6).

US and Doppler evaluation in NAFLD

US is a non-invasive, extremely safe, widely available, and inexpensive method used for detecting fatty liver and assessing the stages of NAFLD (97,98). The bright liver, consisting of hyperechogenic liver tissue with fine, tightly packed echoes at US examination, is considered characteristic for fatty liver (99, 100). Studies have documented a sensitivity of 82-89% and a specificity of 93% for B-mode US to identify liver fatty infiltration (100).

US can both estimate the degree of fatty infiltration on gray scale mode and evaluate its effect on the hepatic vascular system through spectral Doppler analysis .

On the basis of the gray scale intensity, our group in Padova developed a quantitative classification. The degree of steatosis is defined by the comparative analysis of grey intensity measured on digital sonographic images of the liver and right kidney. Briefly, US images of both organs were acquired with an ATL 5000 scanner (ATL Ultrasound, City), transferred to a personal computer, and analyzed with dedicated software (HDI-Lab) (11, unpublished data, J Hepatol 2008, abstract 921) The grey-scale intensity of selected regions of interest was measured, and a liver/ kidney (L/K) ratio was calculated, which has been shown to display direct correlation with the degree of steatosis measured by histology.

FIGURE D: sonographic evaluation of L/K ratio *(adapted from Gaiani et al J Hepatol 2008, abstract 921)*
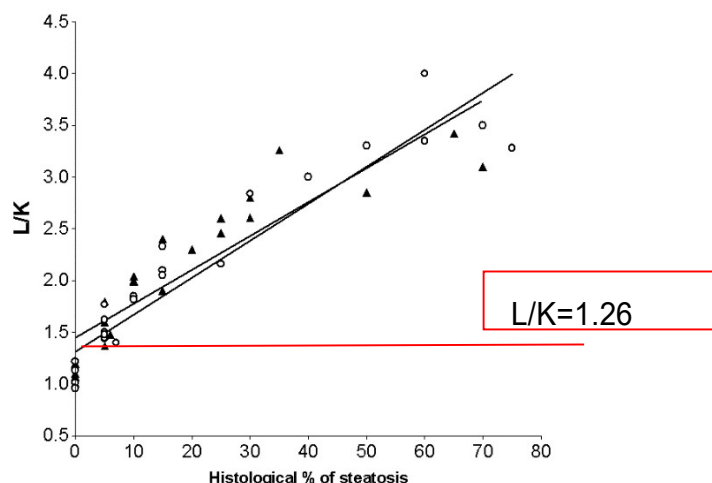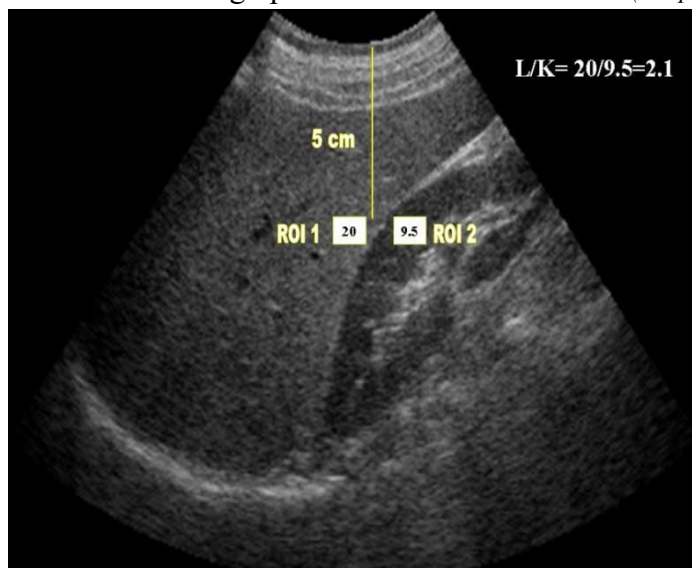
Portal vein blood flow velocity is reduced in patients with fatty liver when compared to the controls and it also correlated with the severity of the fatty liver.

Erdogmus B et al., and Balci et al., and most of the others emphasizes the hypothesis that the fatty infiltration of the liver causes increased resistance to the flow of portal vein thereby reducing the portal blood flow to the liver (104-108).

However, the relative contribution of intrahepatic fat deposition to the hepatic vessels' flow pattern alterations is still controversial (105, 109).

Diffuse fatty infiltration of the liver can alter the also hemodynamics in the hepatic veins as well as the hepatic artery (101, 102).

Recent studies have evaluated hepatic vessel flow abnormalities as an indicator for early diagnosis of fatty liver, and reported a decrease in hepatic artery resistance indexes (RI) in Doppler US in patients (110-112). The resistance in vascular bed can be measured by Doppler indexes such as RI and PI, which are widely used to evaluate arterial vascular resistance and compare systolic and diastolic flow. The normal hepatic arterial system has low resistance flow characteristics reported at 0.60-0.70 in fasting healthy subjects. However, there are not large-scale studies assessing hepatic artery RI so far (113).

According to Mihmanli *et al*. RI decreases gradually as severity of fatty infiltration increases (97). In another study performed in Iran, hepatic artery RI also decreased in subjects with fatty liver (100). Balasubramanian et al found a better correlation of hepatic artery PI than portal vein flow velocity (PVV) with the grading of the severity of NAFLD (114).

Recent studies have documented that waveform pattern of portal vein and PI index may also change in subjects with fatty liver (104,109), whereas a criterion for response to therapy is still under debate (97, 98)

In summary, Doppler indexes including hepatic artery RI might contribute to evaluating treatment efficacy on NAFLD, and might show improvement in NAFLD patients during the course of therapy, which helps to prevent unnecessary health care costs by negating the need for further diagnostic tests and interventions (115)

TREATMENTS

Despite an increasing understanding of the mechanisms of NAFLD pathogenesis, there is not well established therapy for the disease. Current treatments are primarily directed towards improving the metabolic parameters which contribute to disease pathogenesis, such as weight loss and exercise, reducing IR and improving diabetic control.

Therapeutic options in NAFLD and NASH are currently limited mainly to interventions in terms of diet and lifestyle In addition to lifestyle changes, current therapies utilized for patients with NAFLD include insulin sensitizers, e.g. metformin and the thiazolidinediones, vitamin E and consideration of bariatric surgery for morbidly obese patients. In recent years, different drug based approaches have been investigated in randomized, placebo controlled studies.

When such measures fail, liver transplantation remains the only option for patients with end-stage cirrhosis (28).

A medication with long-term effectiveness that would beneficially affect the course of fibrosis ~~does~~ currently does not exist.

Diet and lifestyle

The most effective treatment consists of weight reduction and intensive lifestyle modification with an increase in physical activity/exercise, which has been confirmed to be able to improve histological results (88).

In a randomized controlled trial, an increase in physical activity of moderate intensity to some 200 minutes per week resulted in weight loss of 9% and significant improvements of steatosis and necroinflammation on hepatic histology over 48 weeks (116). In general, a reduction in weight of at least 3–5% seems required to positively affect the steatosis; with regard to necroinflammation, a weight loss of at least 9% is required (88)

Metformin

Several studies investigated the effect of metformin on aminotransferases and liver histology in patients with NASH. Early small, open-label, studies demonstrated a reduction in insulin resistance and aminotransferases but no significant improvement in liver histology (117, 118).

Aminotransferases improved more with metformin than with vitamin E or diet alone. However, there was only a modest improvement in hepatic steatosis and inflammation.

However, a recent meta-analysis has shown that treatment with metformin for 6–12 months, combined with a lifestyle intervention, does not improve neither transaminases nor liver histology compared with a lifestyle intervention alone (9). Another meta-analysis and a case–control study in combination with an in-vitro study indicated, however, that metformin may have a positive effect in terms of the incidence of HCC (6). However, according to AASLD guideline, Metformin has no significant effect on liver histology and is

not recommended as a specific treatment for liver disease in adults with NASH. (Strength – 1, Evidence - A (14)

Thiazolidinediones

Thiazolidinediones or TZDs act by activating peroxisome proliferator-activated receptors (PPARs), PPARs are free fatty acids-activated transcription factors and belong to the nuclear receptor superfamily. PPARs regulate transcription of target genes. The 3 PPAR isotypes, PPARα, PPARγ, and PPARδ play a key role in the regulation of lipid and glucose metabolism (119,120).

Targeting PPAR isotypes for the treatment of fatty liver disease has been extensively studied, mainly in the case of PPARα However, although the beneficial effect of activating PPARα for NAFLD has been proven in several mouse models fibrates, which are PPARα agonists, do not correct NAFLD in humans (120, 14)

Pioglitazone and Rosiglitazone are ligands for the transcription factor (PPAR) γ (119)

The endogenous ligands for these receptors are free fatty acids (FFAs) and eicosanoids. When activated, the receptor binds to DNA in complex with the retinoid X receptor (RXR), another nuclear receptor, increasing transcription of a number of specific genes and decreasing transcription of others. The main effect of expression and repression of specific genes is an increase in the storage of fatty acids in adipocytes, thereby decreasing the amount of fatty acids present in circulation. As a result, cells become more dependent on the oxidation of carbohydrates, more specifically glucose, in order to yield energy for other cellular processes. By this mechanism TZDs are able to improve both hepatic and peripheral insulin sensitivity (119-121).

Several studies investigated the effect of TZDs on NAFLD and NASH. Pioglitazone seems to have a positive effect, which in several studies improved the steatosis as well as the inflammation (122) In an an early uncontrolled open label study (123) in 22 subjects with biopsy-proven NASH, rosiglitazone improved aminotransferases and hepatic steatosis, ballooning and inflammation scores, but not fibrosis.. This result is

confirmed by a meta analysis (9, which. showed that pioglitazone significantly improves steatosis and inflammation, but not fibrosis.

Data on the long-term effects and safety are lacking (9, 88). Moreover, due to increased risk of coronary events, rosiglitazone is no longer marketed in Europe and its use is highly restricted in the United States. according to AASLD guideline Pioglitazone can be used to treat steatohepatitis in patients with

biopsy-proven NASH. However, it should be noted that majority of the patients who participated in clinical

trials that investigated pioglitazone for NASH were non-diabetic and that long term safety and efficacy of pioglitazone in patients with NASH is not established. (Strength – 1, Evidence - B) (14).

Vitamin E

Oxidative stress is considered to be a key mechanism of hepatocellular injury and disease progression in subjects with NASH. Vitamin E is an anti-oxidant and has been investigated to treat NASH (124) The use of vitamin E is associated with a decrease in aminotransferases in subjects with NASH, studies where

histologic endpoints were evaluated indicate that vitamin E causes improvement in steatosis, inflammation, and ballooning and resolution of steatohepatitis in adults with NASH, and vitamin E has no effect on hepatic fibrosis.

In the randomized, placebo controlled PIVENS study in non-diabetic patients, vitamin E lowered transaminases and histologically improved steatosis and inflammation after two years of treatment, but did not affect the degree of fibrosis (125- 127) One concern with vitamin E is the controversial issue of whether it increases all-cause mortality. Some meta-analyses have reported an increase in all-cause mortality with high dose vitamin E (128) but others failed to confirm such an association (129). The US guideline therefore does not recommend vitamin E in non-diabetic patients with histologically confirmed NASH, but it does recommend against its use in diabetes patients, in the absence of histological results, and in case of NASH cirrhosis or cryptogenic cirrhosis, until robust data are available (88)

Other treatmens: PUFAs, such as omega-3 and omega-6 fatty acids, are therapeutic targets for CVD and metabolic disorders because they can regulate inflammation. PUFAs are essential fatty acids; they are only available from dietary sources. Their regulatory role in inflammation was first identified when high dietary PUFA intake was correlated with low risk for CVD in humans. They have been proposed as therapeutic compound for NAFLD, initial reports demonstrate their effectiveness and large clinical studies are actually ongoing (130)

Bariatric Surgery

As the majority of patients undergoing bariatric surgery have associated fatty liver disease, there has been an interest in foregut bariatric surgery as a potential treatment option for NASH. There are no RCTs that evaluated any type of foregut bariatric surgical procedure to specifically treat NAFLD or NASH. However, there are several retrospective and prospective cohort studies The study by Mathurin *et al.*(131) prospectively correlated clinical and metabolic data with liver histology before and 1 and 5 years after bariatric surgery in 381 adult patients with severe obesity. Compared to baseline, there was a significant improvement in the prevalence and severity of steatosis and ballooning at 1 and 5 years following bariatric surgery. Two meta-analyses (132, 133) evaluated the effect of bariatric surgery on the liver histology in patients with NAFLD. The meta-analysis by Mummadi *et al.*, (132) showed that steatosis, steatohepatitis,

and fibrosis appear to improve or completely resolve after bariatric surgery. However, a Cochrane review (133) concluded that lack of randomized clinical trials or quasi-randomized clinical studies prevents definitive assessment of benefits and harms of bariatric surgery as a therapeutic approach for patients with NASH.

New potentially therapeuthic compounds:

As no one of the described compounds meets the ideal characteristics of a specific drug for NAFLD the urgent need for specific treatments for NAFLD has rendered this a critical area of research, with particular focus on developing treatments which can reverse or prevent the more advanced and clinically relevant stages of NASH.

The main phisyopathological targets for NAFLD therapies are lipid metabolism and insulin sensitivity but also inflammation and in particular early inflammatory mediators that can be involved in the early phases of NAFL evolution to NASH.

ANIMAL MODELS OF NAFLD

An ideal animal model of NAFLD/NASH should reflect hepatic histopathology and pathophysiology of human NAFLD/NASH. Accordingly, the liver of the animal model of NASH should show steatosis, intralobular inflammation, hepatocellular ballooning, and, ideally, perisinusoidal fibrosis in zone 3 and susceptibility to liver tumors. Furthermore, the animal should show metabolic abnormalities such as obesity, insulin resistance, fasting hyperglycemia, dyslipidemia, and altered adipokine profile. It is questionable whether the results of a study using an animal model that does not completely fulfill these

conditions can be extrapolated to human disease. Animal models of NAFLD/NASH are classified into genetic models, nutritional models, and combination models of genetic and nutritional factors (134)

Rodents genetic models refers almost exclusively to mice. Mice are easily to be genetically manipulated but due to their small size the amount of blood and tissues from each animal is restrained, moreover the phisiophatology of the disease is often invalidated by the lack or the overexpression of key proteins.
The most commonly used transgenic mice models of NAFLD are:

- SREBP-1c transgenic mice In the fat tissue of these mice, SREBP-1c, a lipogenic transcription factor, is overexpressed. This creates a model of congenital lipodystrophy in which severe insulin resistance and diabetes develop secondary to impaired adipose differentiation (135).
- Ob/ob mice Ob/ob mice possess a spontaneous mutation in the leptin gene (leptin-deficient). Leptin is an adipokine produced by white adipose tissue and operates on the hypothalamic ventral median nucleus exerting a marked anorexic effect (136)

- Db/db mice Db/db mice possess a natural mutation in the leptin receptor (Ob-Rb) gene (137) and, therefore, show normal or elevated levels of leptin but are resistant to the effects of leptin.

On the other hand dietary models of NAFLD can be applied both to mice and rats. The most commonly used models of dietary induced NAFLD are:

- **Methionine and choline deficiency (MCD)** The MCD diet contains high sucrose and fat (40% sucrose, 10% fat) but lacks methionine and choline, which are essential for hepatic β-oxidation and production of VLDL (136). In addition, choline deficiency impairs hepatic VLDL secretion (138). As a result, lipid is deposited in the liver. Furthermore, oxidative stress (139, 140) and changes in cytokines and adipocytokines (141) occur, contributing to the liver injury. Steatohepatitis occurs at day 10 (142, 143), and perisinusoidal fibrosis is observed by 8-10 wk in mice (139, 143)

- **HF diet** Lieber *et al* (144) reported a diet model of NASH by using an HF diet (71% of energy from fat, 11% from carbohydrates, and 18% from proteins). Rats fed this diet *ad libitum* for 3 wk showed elevated plasma insulin levels reflecting insulin resistance. Rats fed the HF diet developed marked panlobular steatosis, and the hepatic lipid concentrations of these rats were approximately twice those of control rats fed the standard Lieber-DeCarli diet (35% fat, 47% carbohydrates, and 18% protein). Like human NASH, the rats fed the HF diet developed oxidative damage in the liver. An HF diet is widely used to cause hepatic steatosis and NASH in experimental animals. It is probably the model that better reproduces the physiopathology of human NAFLD. However, it seems that the HF diet model produces variable results with regard to the degree of steatosis, inflammation, and fibrosis, and the results depend on rodent species and strain, the fat content in the diet, the composition of dietary fat,and the duration of treatment. On the other hand, it was reported that long term high saturated fat feeding did not induce hepatic steatosis and NASH in Wistar rats (145).

- **Cholesterol and cholate** Matsuzawa *et al* (146) fed mice an atherogenic diet containing 1.25% cholesterol and 0.5% cholate and observed the progressive formation of steatosis, inflammation, and fibrosis in a time-dependent manner over 6-24 wk. In the model, hepatocellular ballooning, characteristic of human NASH, was observed at 24 wk. When 60% fat (cocoa butter) was added to the diet, development of these histopathological features was accelerated, and hepatocellular ballooning was observed at 12 wk. Furthermore, the atherogenic diet induced oxidative stress. Thus, it is conceivable that a combination diet of HF, cholesterol, and cholate in animals would cause histological features reminiscent of human NASH. However, the mice fed this diet were systematically insulin sensitive, albeit they showed hepatic insulin resistance. In fact, the mice lost 9% body weight during the experiment and low plasma triglyceride levels compared with those in control mice. Therefore, this model appears to differ from human NASH in metabolic status.

- **Fructose** Experimental animals fed a fructose enriched diet are recognized as good models of metabolic syndrome. Wistar rats fed a highfructose (70%) diet for 5 wk develop significantly higher macrovesicular steatosis and intralobular inflammation grades, liver:body weight ratios, and hepatic

triglyceride concentrations than those in control rats (147). Rats fed a high-fructose diet show significantly higher expressions of interleukin (IL)-6 protein and TNF-α protein in the liver compared with those in control rats Armutcu *et al* (148) reported that male Wistar albino rats provided with drinking water containing 10% fructose for 10 d developed macrovesicular and microvesicular steatosis but did not develop inflammation in the liver.

AIMS

As described in the introduction NAFLD is a worldwide increasing disease but still many questions about its evolution the need of a screening and the availability of effective specific treatments are open.

Aims of my PhD project were

1) the evaluation of NAFLD natural history in a subgroup of NAFLD affected diabetic patients enrolled during the daily clinical activity of our splenohepatology ecoDoppler laboratory (DIMED, university of Padova) in order to identify, if present, predictive factors of "evolutive NAFLD";

2) the experimental evaluation, in HFD fed rats, of the potential therapeutic effect of 3 molecules targeting respectively:

   a) lipid metabolism (Apolipoprotein A analogue compound -L4F),

   b) insulin sensitivity (peroxisome proliferator activated receptor delta agonist –PPARd agonist)

   c) endothelial function (EET Analog).

We developed two studies: a clinical observational study and an experimental study.

CLINICAL STUDY: NATURAL HISTORY OF NAFLD IN TYPE 2 NON OBESE DIABETIC PATIENTS: A 6 YEARS FOLLOW UP

Thirty % of adults and 70-90% of diabetic patients have NAFLD. (13). The disorder has a broad histological spectrum from simple fatty liver (NAFL) to a progressive hepatic injury condition, called non-alcoholic steatohepatitis NASH. Those who suffer of NASH are prone to a progressively advanced liver fibrosis and cirrhosis in a rather short time (14, 16).

Though histopathology is still considered the gold standard for the diagnosis of NAFLD, it is not possible in clinical practice to do biopsy in the huge population of NAFLD patients, considering its invasiveness. Hence, imaging modalities such as sonography are now considered a reasonable and acceptable option for diagnosing NAFLD (149)

Recent reports suggest that NAFL may not be as benign as previously thought, with evidence of progression to advanced fibrosis, challenging the idea that the risk of fibrosis progression is dichotomized according to the presence or absence of NASH (12, 16, 150). Moreover Wong *et al.* (151) reported in a prospective study of paired liver biopsies taken a median three years apart, that 28% of patients with histological NAFLD activity score (NAS) <3 (*i.e.*, non-NASH) had fibrosis progression at three years. This confirms the idea that histological evaluation is not sufficient to identify among patients with NAFLD those who will develop NASH.

Collectively histological prospective and retrospective studies (1-3, 5-7, 9-12) suggest that overall NAFL has a more indolent rate of progression than NASH; however, there is considerable heterogeneity, with one quarter of NAFL patients developing bridging fibrosis over a relatively short time period. Currently, reliable histological and clinical predictors of disease progression are lacking. The majority of studies on NAFLD focus on which are the main factors that influence fibrosis development in NASH, in fact fibrosis development is considered the pathogenic step that leds from NASH to cirrhosis (28-30).

Few studies focus on the causative element of NAFL progression to NASH (28) and fibrosis although the early identification of patients with NAFL who are at higher risk of NASH development could be the real preventive goal of NAFLD related cirrhosis development.

Aims of this study were the evaluation of NAFLD "natural history" in non-obese diabetic patients without any specific intervention and the identification of non-invasive early markers of potentially evolutive disease.

We chose this specific subpopulation of NAFLD patients because of the high prevalence of disease and to reduce aetiology variability. Moreover a recent study demonstrated that although patients with non obese NAFLD have lower stages of fibrosis, they are at higher risk for development of severe liver disease compared to patients with NAFLD and a higher BMI (152).

Design of the study and materials and methods:

100 patients with type 2 diabetes (60 male and 40 female) were evaluated as far as steatosis is concerned. Among them, 80 had sonographic signs of steatosis. There was no difference in the prevalence between male and female patients. Twenty-one type 2 diabetic patients with liver steatosis were reevaluated after 6 years.

These 21 patients were our study population as we had basal and "after 6 years" data for each patient.

All patients signed an informed consent according to our Ethical Committee indication. According to AASLD guidelines (153) the diagnosis of NAFLD requires that (a) there is hepatic steatosis by imaging or histology, (b) there is no significant alcohol consumption, (c) there are no competing etiologies for hepatic steatosis, and (d) there are no co-existing causes for chronic liver disease.

Sonographic and Doppler evaluation

All patients underwent abdominal sonographic evaluation at baseline and after 6 years. Sonographic evaluation was performed after a night fasting. The presence of hepatic steatosis was sonographically evaluated. As described by Saverymuttu, the sonographic criterion for the diagnosis of liver steatosis is the fall in echo amplitude with depth (rate of posterior beam attenuation), increasing discrepancy of echo amplitude between liver and kidney and loss of echoes from thewalls of the portal veins (154).

In our laboratory we developed a sonographic tool for liver steatosis quantification based on the ratio between the numeric values of the gray intensity of liver and right kidney evaluated by a computerized imaging analysis: the liver/kidney ratio (L/R) (11) This method has 100% sensitivity and specificity in the identification of more than 5% liver steatosis (steatosis=L/K>1.26).

The severity of steatosis was therefore sonographically evaluated by the liver/kidney ratio (steatosis=L/K>1.26)

We also evaluated the following sonographic and Doppler hepatosplenic parameters: right liver size,spleen diameter, portal vein diameter (PV) and flow velocity (PVV), hepatic (PI-L) and splenic arterial pulsatility index (PI-S), hepatic vein flow profile and a and s wave velocities.

Biochemical tests:

All patients underwent the following biochemical tests at baseline and after 6 years: ferritin, cholesterol, triglycerides, AST, ALT, HbA1c, and insulin sensitivity evaluation by the homeostatic model assessment (HOMA) test. The HOMA test is a method used to quantify insulin resistance and beta-cell function using a mathematical equation: HOMA-IR= fasting plasma glucose x insulin/22.5 (mmol/l).

Statistical analysis:

Results are shown as mean ± SD. Differences among groups were analyzed by Anova and paired and unpaired Student's *t*-test. Statistical significance was set at $P < 0.05$.

Results:

Mean age of evaluated patients at baseline was 63±8 years, 13 male, 8 female. BMI was 27.3±1.9.

The prevalence of steatosis according to L/K ratio (L/K ratio > 1.26) was 100% at baseline and 80% after 6 years.

Mean L/K in NAFLD patients decreased from 2.33±0.53 to 2.02±0.9 after 6yr (p< 0.001) (Fig 1a).

BMI was similar after 6 years (26.9±1.8).

AST and ALT were reduced (AST: from 26±11 to 20±6 UI/l; ALT from 31±21 to 23±10 UI/l; p<0.05); the same was for cholesterol (from 191,5±43,7 to 156±33 mg/dl, p< 0.001) and ferritin (from 209.4±171 to 117±112 ng/l, p<0.01). No significant changes were found in triglycerides, HbA1c and HOMA test. EcoDoppler evaluation: PVV increased from 29.9±5.9 cm/sec to 35.6±6.9 cm/sec (p<0.001); hepatic a wave velocity increased from 1.4±11 cm/sec to 6.5±6 cm/sec (p<0.05) while PI-L and PI-S did not change (Fig 1 a and b).

After 6 years in 6 patients (28%) sonographic steatosis degree worsened while in 15 it improved (Fig 2)

We divided patients in two groups: the 6 patients with increased steatosis after 6 years (IS) and the 15 patients with decreased steatosis (DS), then we evaluated if at baseline IS group had different biohumoral or ecoDoppler parameters as compared to DS group in order to identify if there were some parameters useful to identify those patients who will increase liver steatosis. (Fig. 2)

The result has been that patients with increased steatosis were those with significantly higher basal values of ferritin (297±116 vs 171±140 ng/l, p<0.05) , AST (33±16 vs 23±9 UI/l, p<0.05) and, surprisingly, with lower PI-L (1.2±0.3 vs 1.9±0.3, p< 0.01).(Fig. 3)

Discussion

Our results ~~seem to co~~nfirm that liver steatosis of non-obese type-2 diabetic patients is a benign disease which doesn't worsen with time in the majority of them in absence of any specific treatment. In 20% of patients liver steatosis recovered after 6 years. Our echographic finding, based on L/K evaluation, are similar to those described by Bertot and Adams based on the review of more than 10 studies with histological evaluation of NAFL evolution (22).

Also mean values of biochemical tests improved with time: in particular AST, ALT, ferritin and cholesterol.

Epidemiological and clinical studies describe frequent association between increased liver fat content and transaminases elevation. Transaminases and specifically serum alanine aminotransferase ALT has been proposed as a surrogate marker for NAFLD. Nevertheless, only a small proportion of patients with NAFLD have elevated serum ALT (155, 156).

In our population mean basal transaminases levels were in the range of normality however the reduction after 6 years was statistically significant. Given that serum ALT values within the current "normal" range have been associated with NAFLD and a higher risk of cardiometabolic disorders, persons with these conditions are included in the apparently healthy sample population that is considered "normal." Therefore, it has been suggested that the upper "normal" limit for serum ALT should be re-evaluated to facilitate the identification of individuals with NAFLD (156-158).

Concerning cholesterol levels, extensive dysregulation of cholesterol homeostasis has been documented in nonalcoholic fatty liver disease (NAFLD), causing both increased synthesis and uptake of cholesterol as well as decreased removal (159) thus, a decrease of serum cholesterol levels reflect an improvement of liver metabolism of cholesterol.

Triglycerides levels were not significantly different after 6 years. This data is not in contrast with the finding of reduced cholesterol levels because while cholesterol levels are mainly dependent on hepatic methbolic function and homeostasis, serum triglycerides are related to adipose tissue (160) and in our study BMI was similar after 6 years.

Even if Tarantino et al didn't find any difference in serum ferritin levels between histologically identified NAFL and NASH patients (161), it has been recently demonstrated that ferritin levels are correlated with liver stiffness evaluated by transient elastography (162). A serum ferritin >1.5 × ULN was associated with hepatic iron deposition, a diagnosis of NASH, and worsened histologic activity and was an independent predictor of advanced hepatic fibrosis among patients with NAFLD. Furthermore, elevated SF was independently associated with higher NAS, even among patients without hepatic iron deposition (163). Thus, the finding of a decrease of ferritin may reflect an improvement of liver fibrosis.

All these results agree with our hepatic hemodynamic finding of a mean increase of PVV. Our findings are similar to the studies by Balci (105) and most of the others emphasize the hypothesis that the fatty infiltration of the liver causes increased resistance to the flow of portal vein thereby reducing the portal blood flow to the liver (105-107). Also the mean increase of A wave can be read as due to an improvement of liver steatosis and in particular of adipose related liver stiffness because of the effect of the reduction of liver fatty infiltration on hepatic wave retrograde flow due to atrial contraction. It is known that normal hepatic venous waveform shows a triphasic pattern and the "triphasic" pattern is due to atrial contraction

related retrograde wave, and the loss of A wave leeds to a biphasic (or monophasic) pattern. Loss of tripasic pattern in cirrhotics is commonly related to the loss of compliance of liver due to fibrosis (108,.164)

Surprisingly we didn't find any difference in HbA1c and HOMA test. HOMA IR has been recently demonstrated to be independently associated with the presence of NAFLD in adult diabetic patients and a cut-off value of HOMA-IR of 4.5 was proposed as an optimal threshold for discriminating NAFLD from non-NAFLD cases (165).

In our study basal mean HOMA-IR was 5.2 ± 0.4 and 5.3 ± 0.5 after 6 years. We can hypothesize that in our populations insulin resistance was too high to be useful and probably HOMA IR above value of 5 is not enough sensitive to reflect an improvement of liver steatosis.

However the most interesting finding of this study was that patients with increased steatosis after 6 years were those with significantly higher basal values of ferritin (297 ± 116 vs 171 ± 140 ng/l, $p<0.05$) , AST (33 ± 16 vs 23 ± 9 UI/l, $p<0.05$) and with lower PI-L (1.2 ± 0.3 vs 1.9 ± 0.3, $p< 0.01$) (Fig 3).

As already discussed, transaminases levels have been proposed as a surrogate marker for NAFLD because an increase of serum AST and ALT is related to an increased fat content (156) however transaminase are known as a marker of liver injury thus we can hypothesize that increased levels of transaminases are a marker of fat related hepatic injury that is not necessary directly related to hepatocytes fat content but to hepatocytes fat related damage (58).

Moreover our findings support the idea that AST and ALT upper limits should be reduced in diabetic population to better identify patients with higher risk of evolutive steatosis.

Higher ferritin levels could reflect an increased stiffness related to fibrosis as described by (162) but ferritin is also a marker of inflammation (166), thus higher levels of ferritin could be related to an inflammatory state that can play a pathogenic role in NAFLD worsening. Also in this case large studies are needed to identify a reliable cut-off.

The finding of lower PI-L values in patients with "increased steatosis" is more controversial (108) however Balasubramanian et al recently reported the same finding and moreover an inverse even not strong inverse correlation between PI-L values and liver steatosis (114).

The decrease of PI-L suggests an increased hepatic artery blood flow which could represent the compensatory mechanism to a reduced portal flow but patient with increased steatosis had not lower basal values of PVV, thus it is hard to explain this hypothesis pathophysiologically by the "buffer theory". It is more likely to hypothesize an imbalance of vasoactive factors related to endothelial dysfunction.

Emerging evidence highlighted that increased portal pressure occurs in NAFLD, also in the absence of fibrosis, and that both dynamic factors with marked endothelial dysfunction and overproduction of vasoactive mediators, and morphological factors with pronounced architectural derangement of sinusoidal anatomy are implicated in its pathogenesis (167, 168).

These factors may be involved also in hepatic arterial flow regulation but more specific studies are needed to confirm this hypothesis.

In conclusion this study shows that, without any specific treatment, in a follow up of 6 years, liver steatosis increases only in less than 1/3 of non-obese diabetic patients and demonstrates that in the majority of them sonographic degree of steatosis improves or recovers concurrently with biohumoral parameters. The presence of increased levels of serum AST and ferritin and lower PI-L seems to be correlated to a worse prognosis and may be used to identify those patients who deserve a higher surveillance. These combined biohumoral and Doppler parameters might become important for the prognosis of patients with fatty liver but larger studies are needed to identify cutoff value for any of these parameters alone or in combination (composite model with a good sensitivity and specificity.

Limits of the study

The major limitations of this study are the small number of patients and the lack of liver histology and NAS score to confirm the diagnosis of fatty liver and to grade the severity of the condition. However the long follow up, even without intermediate evaluations, offsets this limit and strengthens our observation.

EXPERIMENTAL STUDY : EVALUATION OF THE EFFECT OF PPRd ANALOG, L4F and EETA IN NAFLD PROGRESSION IN HFD FED RAT

It is very likely that the importance of NAFLD will continue to increase in the future, when the new therapies and prevention programs for hepatitis C and B will further reduce the size of viral infections of the liver. As previously described, despite the disease severity and prevalence, there are still no approved pharmaceutical specific treatments for NAFLD. beyond management of comorbidity and weight loss. Unfortunately, weight loss has a poor long-term success rate, which emphasizes the need of alternative approaches (28, 88, 116).

The urgent need for specific drugs which can reverse or prevent the more advanced and clinically relevant stages of NAFLD has rendered this a critical area of research.

Most recent theories (40-44; 50-52, 167) show how innate immune system is involved in NAFLD development and progression, and it is likely involved also in the first phases of the disease. As circulating monucleate cells (peripheral blood mononucleate cells PBMCs) include circulating monocytes which are the circulating precursors of resident macrophages, we assumed that PBMCs "activation" could be representative of macrophages/Kupffer cells activation. This idea could led to the development of new non invasive blood tests to evaluate NAFLD. In this study we tested the effect of three new potentially usefull therapies for NAFLD and we also aimed to evaluate the role of innate immune system in the early phases of the disease and the effect of these drugs on plasmatic citokines and chemokines levels and PBMCs activation.

We tested the following drugs:

a) Apolipoprotein A analogue compound: L4F,

b) peroxisome proliferator activated receptor delta agonist: PPARdelta agonist

c) EET Analog

RATIONALE FOR THE COICE OF THE DRUGS

Apolipoprotein A analogue compound: L4F

L4F is a synthetic ApoA1 analog commercially available. ApoA1 is the major protein component of high density lipoproteins. ApoA1 as a component of HDL is involved in fat molecules efflux from cells to target organs including back to the liver for excretion. It helps to clear fat (triglycerides and cholesterol) from white blood cells within artery walls. Deficiency in apolipoprotein A-I (ApoA-I) sensitizes mice to diet-induced obesity, glucose intolerance and NAFLD (169).

Recent studies suggested that patients with non-alcoholic fatty liver disease (NAFLD), including individuals with non-alcoholic steatohepatitis (NASH), have smaller HDL particles when compared to individuals

without liver pathologies. The pilot data from this study suggest that changes in the HDL proteome may impact the functionality of HDL particles in NAFLD and NASH patients (170)

These proteome changes may alter cardio-protective properties of HDL, potentially contributing to the increased cardiovascular disease risk in affected individualsIt thus ApoA1 evaluation is often used as a biomarker for prediction of cardiovascular disease. (171, 172). No data are available on the use of this compound in NAFLD therapy.

PPAR delta agonist

PPARs are free fatty acids-activated transcription factors and belong to the nuclear receptor superfamily.
The 3 PPAR isotypes, PPARα, PPARγ, and PPARδ play a key role in the regulation of lipid and glucose metabolism and thazolidinedione are PPARγ agonists (173).

PPARδ is a ligand-activated transcription factor involved in the regulation of glucose and lipid homeostasis. PPARδ is widely expressed and plays a critical role in mitochondrial function, fatty acid oxidation and it has been proposed as a therapeutic target for the treatment of metabolic syndrome. Genetic manipulation of PPARδ as well as its activation by agonists attenuate dyslipidemia and hyperglycemia, improve whole-body insulin sensitivity, and prevent diet-induced obesity. "Long-term activation of PPARδ can improve hepatic steatosis by activating fatty acid oxidation in different mouse models (173-175).

PPARδ regulates hepatic expression of very low-density lipoprotein receptor VLDLR and VLDLR plays an important role in the development of hepatic steatosis (174).

In humans, 2-week clinical studies in healthy volunteers and moderately overweight subjects demonstrated that the synthetic PPARδ agonist GW501516 improves dyslipidemia (reducing plasma triglycerides and increasing HDL cholesterol) and glucose metabolism (decreasing plasma insulin), whereas liver fat content was reduced (175, 176).

EETA analog

Arachidonic acid metabolites, also known as eicosanoids, represent a large biological class of lipids that have important activities in maintaining cellular homeostasis. Epoxyeicosatrienoic acids (EETs) are arachidonic acid metabolites produced by Cytochrome P450 (CYP450) monooxygenases/epoxygenases (177)

EETs exert potent anti-inflammatory properties in the vasculature by inhibiting NF-κB activation in endothelial cells (177). Vasodilatory, anti-inflammatory and anti-apoptotic actions of EETs are well established. Moreover EETs play a central role in insulin resistance induced endothelial dysfunction.

EETs are very important for renal function: renal damage in cardiometabolic syndrome has been attributed, at least in part, to impaired eicosanoids metabolism. EET agonists prevent both vascular dysfunction and adiposity in vitro and in mice fed high-fat diets (HFD). Moreover EETs seem to be able to modulate insulin sensitivity by acting as endogenous ligands of the PPAR family (177, 178).

Liu et al. had recently demonstrated that sEH inhibition could alleviate high-fat diet-induced hepatic steatosis, which might involve its anti-inflammatory effect in adipose tissue and direct inhibition in liver(179).

CYP450 system expression and EETs levels are diminished within both the diabetic and obese states, while in non-obese diabetic rats epoxide hydrolase, the enzyme responsible for the metabolism of EETs to DHETs, is increased (180, 181). DHET has been shown to be involved in lipid metabolism. Both 14,15 EET and 14,15 DHET activate and bind to PPAR alpha and gamma which regulate the beta-oxidation of fatty acids (182). Though these particular effects of EETs and DHETS are poorly understood, these findings suggest that EETs and DHETs play a role in both lipid metabolism and inflammation and therefore may play a critical role in signaling processes involved in diabetes and obesity (183). Insulin sensitivity and, thus, "healthy adipogenesis", is mediated by the expression and activation of the PPAR gamma. PPAR gamma can be activated by both endogenous and exogenous synthetic ligands such as thiazolidinediones (a drug of great importance in diabetes treatment). EETs have been demonstrated to be potential ligands for PPARs (184). In diabetic and obese animal models the presence of big and inflammed adipocytes producing TNF alfa, MCP-1 and IL6 instead of adiponectin have been described and there is evidence that insulin resistance is a consequence of this "pathological" adipogenesis. (185, 186).

Taken together all these data suggest a potentially therapeutic role of EETs in NAFLD. EETA is an EET analog that can be solved in the drinking water thus it could be an easily available compound. No data are available on its use in dietary model NAFLD.

DESIGN OF THE STUDY

We used a rat model of diet induced liver steatosis (High fat diet model) in order to reproduce human physiopathology of early phases of NAFLD.

Thirty male Wistar rats (4-5 weeks old, 150 grams body weight) were purchased from Charles River Laboratories. 24 rats have been fed with high fat diet (HFD, 50-60% of calories from fat) for 8 weeks. After 8 weeks of diet (and a bioptic diagnosis of liver steatosis -more than 5% of fat within hepatocytes- in 3/3 rats) animals have been divided in 4 groups: 7 untreated (HFD); 7 treated with ApoA1 analog L4F (L4F), 7 treated with PPARd agonist GW501516, 10 mg/kg every 2 days, intraperitoneally, for 6 weeks (PPARd) and 3 treated with EET Analog 10 mg/Kg per day in drinking water for 6 weeks (EET). Treatments lasted 6 weeks. Cardiac and abdominal ecoDoppler evaluation has been performed before treatment and before

sacrifice. Results have been compared with those obtained from 6 control rats fed with standard diet. Histological evaluation of liver steatosis has been performed in all animals at sacrifice.
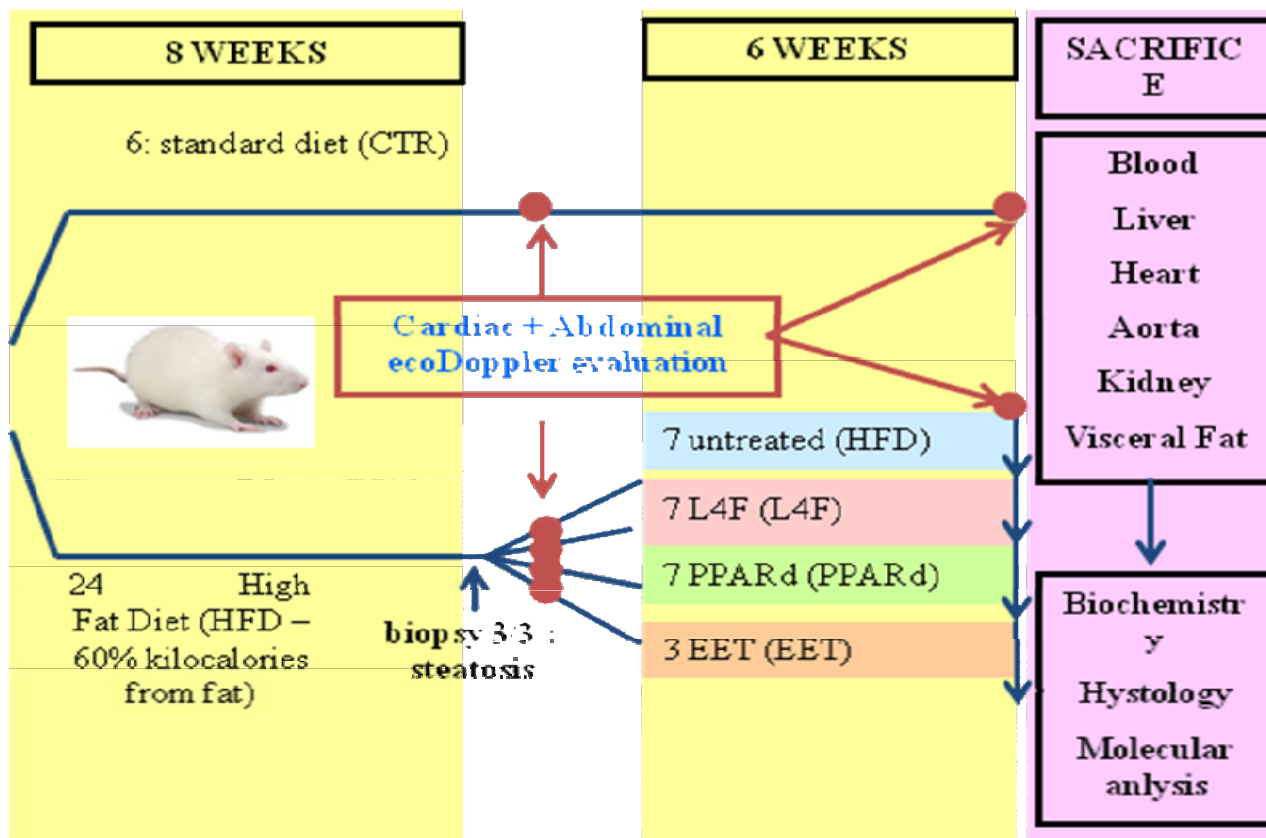
Material and Methods

All described procedures were conducted in accordance with all institutional and Italian guidelines for the care and use of laboratory animals and were approved by the local animal care and use committee (CEASA) and by the Italian Ministry of Health.

EcoDoppler Evaluation

All animals underwent renal EchoDoppler evaluation and echocardiography performed using the Vevo high-resolution in vivo micro-imaging system (VEVO 2100 Visualsonics) at 8 weeks and before sacrifice (14weeks).

Echocardiographic evaluation: transthoracic echocardiography was performed using a 30 Mhz probe. Animals were chest shaved and anesthetized with 3% isoflurane, and temperature controlled anesthesia was maintained with 1.5% isoflurane. Two-dimensional cine loops and M-mode cine loops of a long-axis view and a short-axis view of the LV were recorded. M-mode cine loop of aortic valve was recorded and Doppler analysis of LV flow was performed from the long axis B-mode image placing the sample volume in the left ventricle, below the mitral annulus.

End diastolic area (EDA) and end systolic area (ESA) were measured from the long axis B-mode images. Inter ventricular septum (IVS) and left ventricular posterior wall (LVPW) thicknesses, left ventricle end systolic and diastolic diameter (LVID) and maximal left ventricular length (LV length) were measured in systole and in diastole (s,d) from the long axis and short axis M-mode images, according to standard procedures. Cardiac contractility, measured as ejection fraction (EF) were determined using the following simplified formula: end diastolic volume (EDV) – end systolic volume (ESV)/ EDV (where EDV = (3.14/6)* LVIDd * LVIDd *LV length d and ESV= (3.14/6)* LVIDs * LVIDs *LV length s.

Isovolumetric contraction and relaxation times (IVCT, IVRT), and ejection time (ET) were measured from transmitral Doppler analysis and myocardial performance index (MPI) was calculated using the following formula MPI= IVCT+IVRT/ET.

Hepatic and renal echoDoppler evaluation: immediately after echocardiographic evaluation, abdomens of the mice were shaved, renal B-mode cine loops of a transversal section of liver and both kidneys were recorded. Color Doppler was used to identify: portal vein, hepatic artery and renal interlobar arteries, then Doppler analysis of portal vein flow and identified arteries' flow was performed. Portal vein was measured (PV Vel) and arterial Peak Velocity (Vmax), End Diastolic Velocity (Vmin) and Mean Velocity (VM) were measured and Pulstility Index (PI) was calculated using the following formula PI= (Vmax-Vmin)/Vmean. The mean value of three different measurements was calculated as described. Resistive Index (PI) was calculated using the following formula RI= (Vmax-Vmin)/Vmin. The mean value of three different measurements was calculated as described (L= liver, for hepatic artery; K= kidney, for renal interlobar arteries).

Qualitative assessment of liver steatosis evaluation liver brightness

Qualitative assessment of liver steatosis with echographic evaluation is not described in rats, however we evaluated the echographic brightness of the liver as compared to right kidney and the operator gave a numeric evaluation from 0 to 2 to liver brightness: 0 = less bright than kidney; 1 = mildly less bright than kidney; 2 = as bright as kidney.

All rats were imaged by a single operator.

Hystological evaluation:

At the end of the study rats were euthanized by using carbon dioxide and after sacrifice liver slices were fixed in paraformaldehyde for the histological analysis of liver steatosis.

Triglyceride content of rat liver tissue

Triglyceride content of rat liver tissue was assessed using a commercially available colorimetric assay kit according to manifacturer's instructions.

LAL hepatic levels

Lysosomal acid lipase (LAL) in rat liver tissue was evaluated by western blot analysis using specific commercially available antibodies. LAL expression was compared with actin expression. We also compared LAL expression to liver triglycerides content and measured the hepatic LAL/triglycerides ratio.

Plasmatic cytokines

The levels of IL-1β, and CCL2 were determined in serum samples ELISA test according to manifacturer's instructions (eBioscience; Raybiotech).

CCL2 and IL-1 b production in cultured PBMCs

PBMCs were isolated from peripheral blood samples by centrifugation and then cultured. PBMCs from each rat were dived into two samples: one sample was cultured without stimuli, the second one stimulated with the specific activator of innate immunity receptors lipopolysaccharide (LPS) and the inflammatory response (CCL2 and IL1b production) was measured in the supernatant using ELISA kit according to manifacturer's instructions (eBioscience; Raybiotech).

Statistical analysis: the data are presented as mean ± standard error (SEM). For comparison between treatment groups, the null hypothesis was tested by unpaired t-test. Statistical significance ($p < 0.05$) between the experimental groups was determined by the Fisher method of analysis for multiple comparisons. Correlation between two variables was evaluated with linear regression.

RESULTS

Effects of HFD on liver triglycerides content and LAL expression, cardiac and abdominal ecoDoppler evaluation and cytokines production.

100% of rats fed with HFD developed obesity and mild liver steatosis (Fig 4). In 60% of animals liver steatosis was microvescicular, without fibrosis and without inflammatory infiltration (Fig 5). Liver content of triglycerides: 16±4 mmol/g protein vs 5±1 mmol/g protein of CTR (Fig 5). Liver steatosis was associated with a significant reduction of portal vein velocity (PV vel) as compared to CTR (173±40 vs 354±87 mm/sec $p<0.05$) (Tab 1). Portal vein velocity was inversly correlated with liver steatosis (Fig 6a). No rats fed with standard diet developed steatosis. No significant differences were noticed in hepatic arterial resistance but hepatic artery resistance had a direct correlation with liver steatosis (Fig 6b). Renal resistances (K- RI) were significantly increased as compared to control rats (0.63±0.03 HFD vs 0.55±0.02 CTR, $p<0.01$).

Liver brightness in HFD rats was 2 in all animals and 0 in all CTRs.

Rats fed with HFD developed a significant cardiac diastolic dysfunction (Myocardial Performance Index -MPI- 0.92±0.1 vs 0.76 ±0.1 of CTR; p<0.05).

Hepatic LAL expression was increased in HFD group (Fig 7) but LAL/triglycerides ratio was significantly reduced (Fig 8).

Plasma levels of CCL2 were significantly increased in HFD rats (Fig 10) while IL1 β was not detectable (data not shown). PBMCs isolated from HFD showed only a minimum increase in CCL2 production but the stimulation with LPS increased an average of 5 fold its production (the average increase in LPS stimulated CTR PBMCs was less than 2 fold) (Fig 11 a and b). Il1 β was produced only by stimulated PBMC isolated from HFD rats. (Fig 9).

Effect of L4F

Treatment with L4F reduced the percentage of rats with steatosis (72% of animals) and medium triglycerides liver content (11±2 mmol/g protein, p< 0.05 vs HFD) (Fig 5) and improved PV velocity (326 ± 80 mm/sec; p<0.05 vs HFD) although body weight was not different from HFD group (Tab 1). Only 25% of L4F treated steatosic rats had microvescicular steatosis (Fig 5).

Hepatic and renal arterial resistances were no significantly different from HFD group, the same was for MPI although its value was lower than HFD rats and not significantly different from CTR rats.

Liver brightness: L4F=0,58 (p<0.01 vs HFD)

Hepatic LAL expression was reduced but not significantly different from HFD rat, the same was for LAL/triglycerides ratio (Fig 7, 8).

Plasma levels of CCL2 were similar to those of CTR rats and IL1 β was not detectable. Isolated PBMCs showed a significantly reduced production of CCL2 in basal conditions and also after LPS stimulation (Fig 11) even if the average increase after LPS stimulation was only 3 fold (Fig 11 b).

Effect of PPARδ agonist

Treatment with PPARδ reduced hepatic mean content of triglycerides (9±2 mmol/g protein, p< 0.05 vs HFD) (Fig 5) even if 100% still had histologically detectable liver steatosis after treatment. 28% of PPARδ treated steatosic rats had microvescicular steatosis. Body weight was similar to HFD rats.

PV velocity was increased as compared to HFD rats (260±86 mm/sec; p<0.05 vs HFD) but significantly reduced as compared to CTR rats (Tab 1).

Hepatic and renal arterial resistances were not different from HFD group, the same was for MPI although its value was lower than HFD rats and no significantly different from CTR rats (Tab 1).

Liver brightness: PPAR delta 0.78 (p<0.01 vs CTR e vs HFD)

Hepatic LAL expression was reduced as compared to HFD while LAL/triglycerides ratio was increased even not significantly (Fig 7, 8).

Plasma levels of CCL2 were similar to those of CTR rats and IL1 β was not detectable. Isolated PBMCs showed a significantly reduced production of CCL2 in basal conditions and also after LPS stimulation (Fig 11)

Effect of EET A

All EET analog treated rats developed microvescicular steatosis and triglycerides levels were similar to those of untreated rats (16±4 mmol/g protein, p<0.05 vs CTR) (Fig 5). PV velocity was lower as compared to HFD rats (114±17 mm/sec; p< 0.05 vs HFD). Body weight was not significantly higher than CTR rats (non obese phenotype) Tab 1.

MPI was similar to HFD rats and left ventricle diastolic volume was increased as compared to HFD rats (tab 1).

No differences were observed in arterial resistances although hepatic artery RI and PI were similar to those of CTR mice and lower than HFD, PPARd and L4F rats.

Liver brightness: EETA=1,85 (p<0.01 vs CTR).

Hepatic LAL expression was reduced but not significantly different from HFD rat while LAL/triglycerides was significantly lower than HFD group (Fig 7,8).

Plasma levels of CCL2 were higher as compared to those of CTR rats but lower than those of HFD rats. IL1 β was not detectable. Isolated PBMCs showed a significantly reduced production of CCL2 in basal conditions and LPS stimulation increased CCL2 expression less than 2 fold as in CTR mice (Fig11).

DISCUSSION

Characterization of the model:

This is the first study which characterized the sonographic and hemodynamic alterations of HFD induced NAFLD in rats. Our study shows that HFD related liver steatosis in rat obese-IR phenotype (136) is associated to a systemic activation of innate immunity (increase in plasmatic CCL2 levels) and enhanced

response to pro-inflammatory stimuli such as LPS (increase in plasmatic IL1 β levels). The role of the activation of innate immune system is well described in steatohepatitis (187, 188).

Interestingly in this study we demonstrate that the activation of innate immunity is present even in the absence of liver inflammatory infiltration and fibrosis. This observation is in line with some recent studies which demonstrated that lipids are able to regulate immune cells activation and macrophages phenotype (189).

Altered lipid homeostasis underlies the etiology of some of the most common chronic diseases — obesity, cardiovascular disease (CVD) and liver disease. In 2007, Lumeng *et al.* (190). demonstrated that in lean adipose tissue macrophages display an M2 phenotype while there are more M1 macrophages under obese condition. One explanation is that saturated fatty acids can bind TLRs to activate NFκB pathways, inducing the up-regulation of CCL2, IL-1β and TNF-α. Scavenging activity by macrophages can lead to the intracellular accumulation of saturated fatty acids (SFAs) and other lipids such as diacylglycerols (DAGs). The accumulation of SFAs and DAGs can be toxic by inducing endoplasmic reticulum stress, which also drives the inflammatory M1 phenotype in macrophages (191). Monocyte-derived macrophages are the primary immune cell to accumulate in atherosclerotic plaques (192, 193).

PBMCs are representative of circulating monocytes that are precursor of resident macrophages and in this study we demonstrate that in HFD fed animals PBMCs are involved in CCL2 increased production and moreover PBMCs are significantly more sensitive to LPS stimulation (further increase of CCL2 production: 5 fold as compared to less than 2 fold in CTR). Thus HFD induce a "hyper reactive" state in PBMCs.

As resident macrophages and Kupffer cells are sensitive to the same mechanisms, we can hypothesize a similar activation of Kupffer cells. Kupfer cells activation is involved in liver steatosis development as proposed by Hubler et al who described how molecular pathways driven by lipid accumulation tend to promote a proinflammatory cellular phenotype in immune cells (194)

Moreover, it has been described that LPS activated PBMCs can activate HSC and promote liver fibrosis (195). Thus, a pro inflammatory state of PBMCs can be considered a predisposition to liver fibrosis.

Concerning splanchnic hemodynamics alterations, this rat model amplifies what happens in humans: we found a significant reduction of PVV even in mild degrees of liver steatosis (104-107).

Despite normal cardiac systolic function and normal LV wall thicknesses, HFD rats showed a significant diastolic disfunction (increased MPI) and this is what is described also in the obese phenotype (196). Unfortunately, we lack the values of blood pressure but hypertension is described in HFD rat models (144, 145) and the idea that these rats were probably hypertensive is supported by the finding of increased K- RI.

Contrary to what was described by Baratta (84) in this rat model of NAFLD we found a significantly increased hepatic LAL activity; however the LAL/triglycerides ratio was reduced as compared to control rats. The increase in liver triglycerides content seems to induce LAL activity but not enough to be adequate to the increase of lipids. Thus, the effect is a relative deficiency of LAL. LAL expression in this model is the physiological consequence of the increased lipid influx in cells as the autophagy of lipid droplets is the cellular response to decreased lipids content. As described by Ouimet et al for macrophage foam cells (197), LAL activity is functional to destroy cellular lipid droplets, thus a relative deficiency results in the intracellular accumulation lipids.

Effects of the treatments:

L4F and PPARd improved liver steatosis and reduced plasma and PBMCs derived CCL2 production. Both treatments have a "metabolic effect", the first one particularly on lipid metabolism and HDL related cholesterol flux (169-172) the second on FFAs activated transcriptional pathways protecting cells from cholesterol overload with a particular effect on VLDL related cholesterol flux (173-176).

Both treatments improved liver steatosis that was mainly macrovescicular and portal vein velocity that was higher as compared to HFD untreated mice. Portal vein velocity was correlated with the percentage of liver steatosis.

Their effect on liver triglycerides content is easily comprehensible and, as portal vein velocity, was related to liver fat content (104-107). Also the improvement of portal vein velocity has a direct explication. Less immediate is the understanding of their effect on CCL2 production. While PPARd agonist, as a transcriptional activator, may cause the activation of anti-inflammatory molecular pathways with a reduction of chemokine and cytokine production (173, 176, 198), L4F should have an effect only on cholesterol flux. These data support the idea that immune system activation is strictly related to lipid metabolism (189, 194) thus, improving lipid metabolism reduces the lipid related pro-inflammatory activation of PBMCs.

However these argumentations let infer that the improvement of liver steatosis is the hepatic effect of the systemic improvement of lipid metabolism and immune system activation, except that we observed a clear change in liver steatosis histology: while in HFD group liver steatosis was microvescicular in 60% of animals, in PPARd and L4F groups it was microvescicular respectively 30% and 25% of animals and, (27) as described by Fromenty et al, microvescicular steatosis is related to mithochondrial dysfunction and impaired lipids beta-oxidation- (25, 26). Impaired beta oxidation is a pathogenetic characteristic of liver steatosis and as recently reported by Kim et al the improvement of hepatic steatosis by novel PPARalfa agonist is due to its effect on the improvement of hepatic beta oxidation (199). The effect on this histological feature is a specific hepatic effect of these drugs and is probably related to the pro inflammatory state of hepatocytes environment due to Kupffer cells activation.

An interesting observation is that both PPARd and L4F reduced CCL2 production in PBMCs but preserved the ability of PBMCs to react to LPS stimulation (average 3 fold increase of CCL2 production after LPS stimulation, Fig 11), this is the main difference of these two treatments as compared to EETA effect. In EETA treated rats LPS stimulation didn't increased CCL2 production. This observation sustains the idea that an excessive immunosuppressive effect is not beneficial for liver steatosis as described in some studies about de-novo NAFLD occurrence after liver transplantation in which tacrolimus and steroids are described as causative element of NAFLD development (200, 201)

The unexpected finding of this study was the effect of EETA compound. As described by Spector AA (177) in HFD mice, also in our HFD fed rats the EET agonists reduced adiposity (significant decrease of body weight) and, as widely described (186), EETA exerted an anti-inflammatory effect on PBMCs but didn't improve liver steatosis that histologically was microvescicular in 100% of animals.

Even though the systemic anti-inflammatory activity of this compound was expected (186), the specific reduction of PBMCs related CCL2 production and the reduced response of PBMCS to LPS stimulation is a new finding. A specific relationship between EETs and CCL2 production has already been described by Kundu et al. who demonstrated that sEH inhibition blocks monocyte chemotaxis and particulary sEH derived products, dihydroxyeicosatrienoic acids (DHETs), are able to restore CCL2 dependent chemotaxis, concluding that DHETEs are involved in CCL2 dependent chemotaxis (202). Taken together with our finding we can speculate that probably it is the balance between EETs and DHETs that is involved in CCL2 dependent chemotaxis.

Concerning splanchnic hemodynamics, EETA further decreased portal vein velocity and we found also a decrease of L-PI. This finding reproduces what is described in clinical studies (104-107) in which the decrease of liver PI suggests an increased hepatic artery blood flow which could represent the compensatory mechanism to a reduced portal flow even if, as the well described effect of EETs as vasoactive compounds (186), it is more likely to hypothesize an imbalance of vasoactive factors related to endothelial dysfunction. We have previously shown how EETs, which are potent arterial vasodilators, cause vasonstriction in the portal circulation (203)

Recently Pereira et al described hepatic microvascular dysfunction as a component of non-alcoholic fatty liver disease (204). Thus, the reduced hepatic arterial resistance in EETA treated rats could cause an increase in sinusoidal permeability, and an increased sinusoidal permeability could be one pathogenetic element of NAFLD.

Many recent papers sustain the idea that the gut microbiota might control the severity of NAFLD by increasing production of ethanol, activating TLR signaling and TNF production in the liver, or altering the bile acid profile (205-207)

An increased sinusoidal permeability (but even intestinal as EETA compound was administered by drinking water and should have a systemic effect) increases the flux of bowel derived substances to the liver and this, in conjunction with the "immunosuppressive" effect of the compound could explain the worsening of liver steatosis in HFD fed and EETA treated rats.

CONCLUSIONS

Wistar rat model of HFD induced NAFLD reproduces splanchnic haemodynamic alteration of liver steatosis in humans and shows an activation of innate immune system also at early degree of steatosis without hepatic inflammation and fibrosis. The activation of innate immune system can be evaluated by the analysis of LPS stimulated/unstimulated CCL2 production in cultured PBMCs.

PPARd agonist and L4F improved HFD induced liver steatosis and reduced CCL2 production in PBMCs but preserved the ability of PBMCs to react to LPS stimulation

EETA administration didn't improved liver steatosis and further decreased portal vein velocity and reduced the ability of PBMCs to react to LPS stimulation.

On the basis of these results we recently got the Ministerial approval for an extended study to better understand the effect of EETs related compound on HFD fed mice.

Limits of the study

We chose this rat model of NAFLD because we aimed at reproducing the pathophysiology of NASH. HFD fed rats are perhaps the best model of this disease because of mild progression to NASH as described in epidemiological studies. However, to clarify the real role of innate immune system in NAFLD, a specific innate immune system targeted model may be needed. Unfortunately, even a selective innate immune deficient mice model is not simple to establish. Probably, the best way to improve and demonstrate our hypothesis is in in-vitro models of cellular interaction such as a coculture of fatty liver derived Kupffer cells and/or PBMCs and healthy hepatocytes and vice versa. We need to identify the molecular pathway of interaction between innate immune system and liver. Further studies which look into the exact pathophysiology of the described haemodynamic changes are also needed.

TABLE 1: EcoDoppler evaluation of cardiac and abdominal parameters; body weigt at sacrifice.

	CTR	HFD	L4F	PPARd	EET
LV Vol d (ul)	295 ±75	242± 24	261 ±58	273±67	312 ± 23^
EF (%)	74 ±10	81±6	72 ±10	78± 8	72± 9
MPI	0.76 ±0.1§	0.92±0.1*	0.86 ±0.2	0.85 ±0.2	0.91 ±0.2
PV d (mm)	1.8 ±0.3	1.8 ±0.1	1.9 ±0.4	1.9± 0.4	2.2 ±0.4
PV vel (mm/sec)	354±87§§	173±40 **	326 ± 80§§	260±86§*	114±17§**
L RI	0.58 ±0.07	0.64 ±0.06	0.65 ±0.1	0.66± 0.1	0.60± 0.08
K RI	0.55±0.0	0.63±0.03*	0.55± 0.08	0.59 ±0.03	0.59 ±0.03
Liver Brightness	0	2**	0.58§§	0.75§§**	1.83**
Body weight (g)	530±60	690±70*	650±60*	670±50*	640±90

LV Vol d: left ventricle diastolic volume; EF : ejection fraction; MPI: Myocardial Performance Index; PVd: portal vein diameter; PV vel portal vein velocity; L RI:liver resistance index (hepatic artery); K RI: kidney resi stance index * $p < 0.05$ vs CTR; ** $p < 0.01$ vs CTR; §$p < 0.05$ vs HFD; §§ $p < 0.01$ vs HFD

FIGURES

CLINICAL STUDY:

Figure 1 a: mean values of EchoDoppler measurements at baseline and after 6 years.

Figure 1 b: mean values of biochemical tests at baseline and after 6 years

Figure 2: graphic representation of L/K ratio variation in each patient at baseline and after 6 years. Each line represent a single patient. In red patients in which liver steatosis was increased after 6 years, in light blue patients in which liver steatosis was reduced after 6 years (according to the sonographic evaluation of L/K)

Figure 3: mean values of the parameters that were significantly different at basal evaluation in those patients with an increased steatosis after 6 years: ferritin (a) (increased, $p<0.005$), aspartate-aminotransferase- AST- (b) (increased, $p< 0.05$) and liver pulsatility index- PI-L (c) (decreased, $p<0.01$).

EXPERIMENTAL STUDY:

Figure 4: a) macroscopic aspect of the liver at sacrifice in one rat of the HFD group. B) liver histology of a control rat. c) liver histology of high fat diet fed rat: in this animal liver steatosis was mainly macrovescicular. No signs of inflammatory infiltrate or liver fibrosis were present.

Figure 5: Liver triglycerides content express as mmol of triglycerides / gram protein in homogenized liver samples (method: colorimetric assay). In blue the percentage of animals with mainly microvescicular steatosis for each group. $p < 0.05$ vs CTR; ** $p<0.01$ vs CTR; §$p < 0.05$ s HFD; § § $p<0.01$ vs HFD;

Figure 6: a) correlation between portal vein velocity (PV Vel) and percentage of liver steatosis; b) correlation between hepatic artery pulsatility index and percentage of liver steatosis.

Figure 7: hepatic expression of LAL (a) evaluated by Western Blot (b) * $p < 0.05$ vs CTR; §$p < 0.05$ s HFD.

Figure 8: hepatic expression of LAL compared to liver triglycerides content. * $p < 0.05$ vs CTR; § $p < 0.05$ s HFD.

Figure 9: Production of IL1 β by LPS stimulated PBMCs: IL1 β production was relievable only HFD group.

Figure 10: plasmatic levels of CCL2. Blood samples were collected at sacrifice and CCL2 levels were evaluated by ELISA test. * $p < 0.05$ vs CTR.

Figure 11: a) CCL2 production in cultured PBMCs with (black) and without (brown) LPS stimulation. b) graphic representation of CCL2 fold increase after LPS stimulation: CCL2 in LPS stimulated PBMCs/CCL2 in unstimulated PBMCs.

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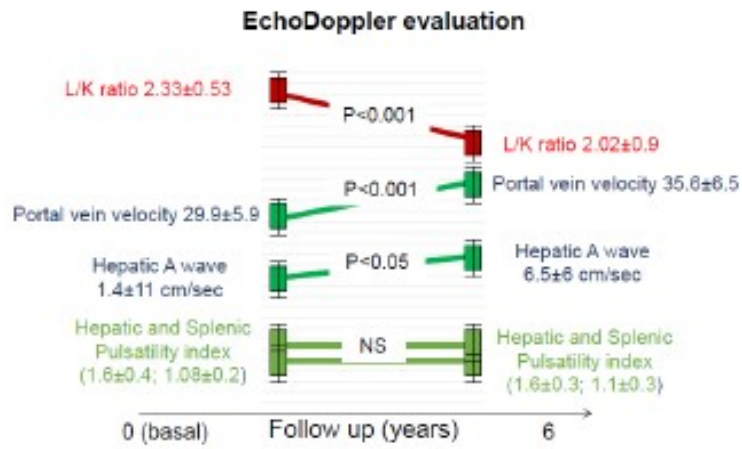
Figure 1a

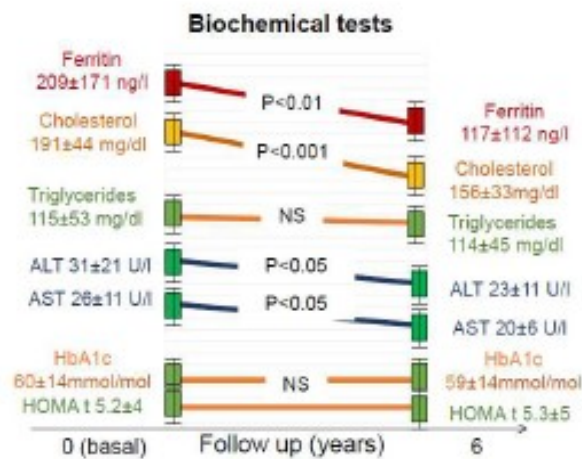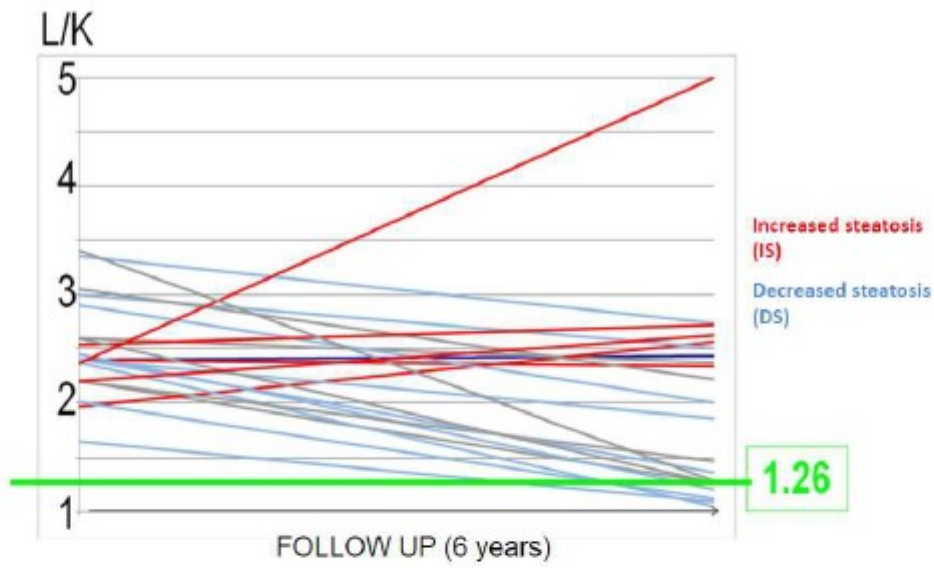**EchoDoppler evaluation**

L/K ratio 2.33±0.53

P<0.001

L/K ratio 2.02±0.9

P<0.001

Portal vein velocity 35.6±6.5

Portal vein velocity 29.9±5.9

Hepatic A wave
1.4±11 cm/sec

P<0.05

Hepatic A wave
6.5±6 cm/sec

Hepatic and Splenic
Pulsatility index
(1.6±0.4; 1.08±0.2)

NS

Hepatic and Splenic
Pulsatility index
(1.6±0.3; 1.1±0.3)

0 (basal)   Follow up (years)   6

Figure 1b

Biochemical tests

Ferritin
209±171 ng/l

P<0.01

Ferritin
117±112 ng/l

Cholesterol
191±44 mg/dl

P<0.001

Cholesterol
156±33mg/dl

Triglycerides
115±53 mg/dl

NS

Triglycerides
114±45 mg/dl

ALT 31±21 U/l

P<0.05

ALT 23±11 U/l

AST 26±11 U/l

P<0.05

AST 20±6 U/l

HbA1c
60±14mmol/mol

NS

HbA1c
59±14mmol/mol

HOMA t 5.2±4

HOMA t 5.3±5

0 (basal)   Follow up (years)   6

Figure 2

L/K

Increased steatosis (IS)

Decreased steatosis (DS)

1.26

FOLLOW UP (6 years)

Figure 3

a) Ferritin
ng/l

P<0.05

IS   DS

b) AST
UI/l

P<0.05

IS   DS

c) PI-L

P<0.01

IS   DS

Figure 4

a) HFD group: macroscopic aspect of
the liver at sacrifice

b)

CTR group: normal liver

c)

HFD group: moderate liver steatosis
(macrovescicular) without inflammation and
fibrosis

Figure 5

Liver triglycerides content

mmol/g protein

CTR   HFD   PPAR δ   L4F   EET

% of rats with
microvescicular
steatosis:

HFD : 60%
PPAR δ: 30%
L4F: 25%
EET: 100%

* p < 0.05 vs CTR; §p < 0.05 s HFD

Figure 6

PV velocity (mm/sec)

L-PI

% of liver steatosis

% of liver steatosis

Figure 7

Lysosomal acid lipase/actin

a) LAL/actina

b)

CTR HFD L4F PPARd EET

LAL 52KD
Actina 42kD

LAL 52KD
Actina 42kD

LAL 52KD
Actina 42kD

• p < 0.05 vs CTR; §p < 0.05 s HFD.

Figure 8

Lysosomal acid lipase/triglicerides content

* p < 0.05 vs CTR; §p < 0.05 s HFD;

Figure 9

IL1β (stimulated PBMCs)

Figure 10

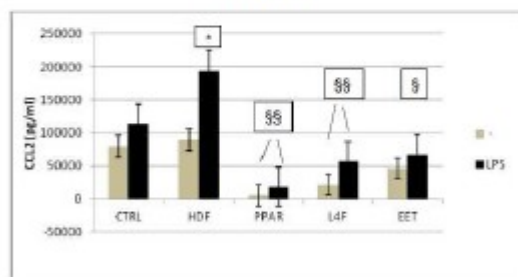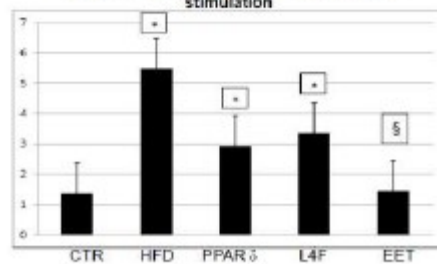## Plasmatic levels of CCL2
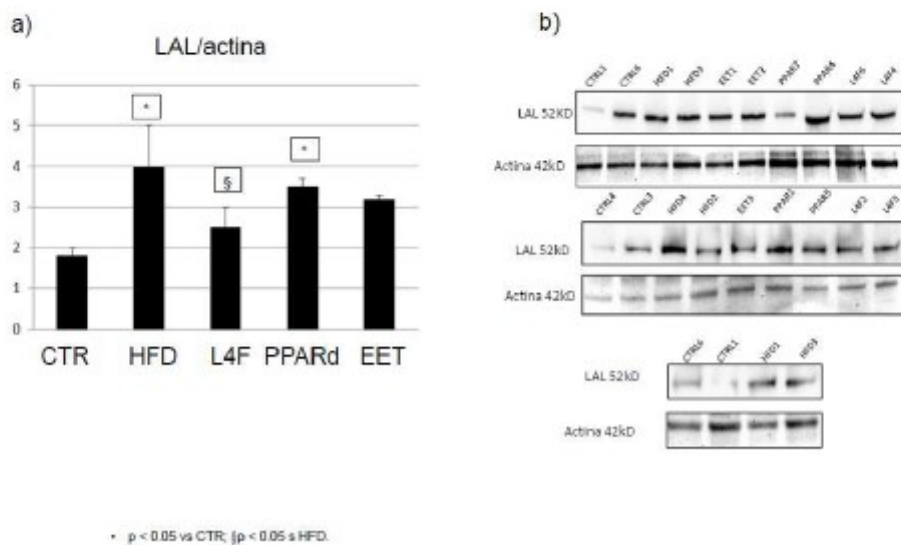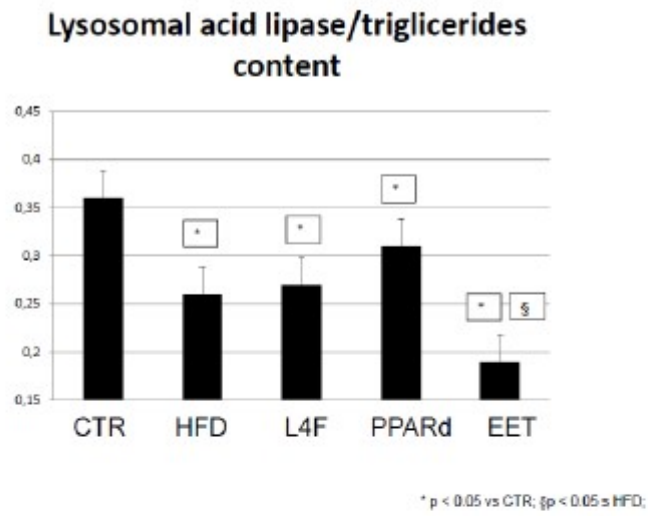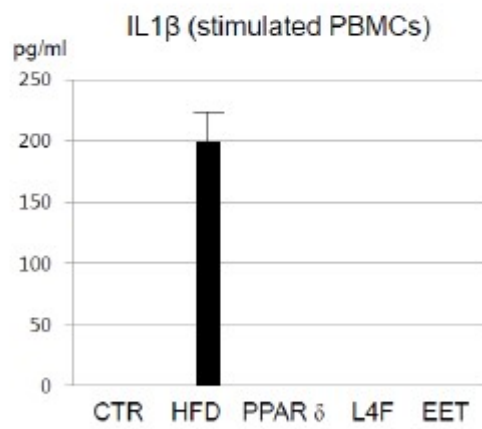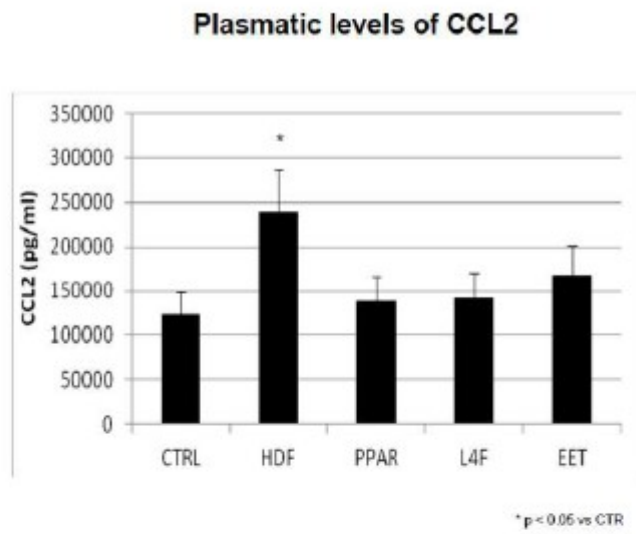
*p < 0.05 vs CTR

Figure 11

CCL2 production in cultured PBMCs with and without LPS stimulation

fold increase in CCL2 production after LPS stimulation

* p < 0.05 vs CTR; ** p<0.01 vs CTR; §p < 0.05 s HFD; § § p<0.01 vs HFD;