Molecular Mechanisms of NAFLD in Metabolic Syndrome
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Guest Editors: Maria João Martins, António Ascensão, José Magalhães, Maria Carmen Collado, and Piero Portincasa
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Metabolic syndrome (MetSyn) and nonalcoholic fatty liver disease (NAFLD) are increasing worldwide, as often mentioned in this special issue. Lifestyles (with diet being one of the relevant factors) play an important role as preventive determinants of MetSyn and NAFLD, conversely being potential inducers of MetSyn and NAFLD. Liver steatosis has been considered not only the hepatic MetSyn manifestation but also one of the earliest MetSyn signs. Liver steatosis represents the initial step of the whole spectrum of NAFLD.

Review and experimental articles including both basic and clinical data, pursuing the cross-talk between obesity/insulin resistance/MetSyn features and diet, lipid metabolism, redox state, oxidative and endoplasmic reticulum stress, mitochondrial dysfunction, inflammatory processes and mediators, glucocorticoid excess, developmental programming, and gut microbiota in NAFLD, are included in this special issue. Possible strategies for intervention against NAFLD are also presented and discussed.

There is growing evidence that the transcription factor nuclear factor erythroid-2 related factor 2 (Nrf2), a key regulator of cellular antioxidant responses, is also implicated in the regulation of hepatic lipid metabolism. The topic is addressed in the article of S. S. Chambel et al. N. Duarte et al. comprehensively reviewed the role of Kupffer cells on inflammation under conditions of hepatic fat deposition. E. Lau et al. analyzed in detail the recent research on the contribution of gut microbiota to NAFLD. A broad and extensive review on the growing evidences regarding the developmental programming of NAFLD by both maternal obesity and undernutrition is presented (M. Li et al.).

E. Maslak et al. showed that rats fed with a MetSyn-inducing diet supplemented with conjugated linoleic acid isomers (c9t11 and t10c12) had (globally) beneficial effects on the hepatic lipid content and glycogen accumulation as well as on the circulating lipid profile; the isomers also modulated the fatty acid composition and decreased lipogenic enzymes mRNA expression in the liver. A. Cordeiro et al. found that, in class III obesity, triglycerides, HDL-cholesterol and insulin levels (as well as insulin resistance index and waist circumference values), independently considered MetSyn features, linked to NAFLD, diagnosed by liver biopsy.

The analyzed topics included in the present special issue allow a better mechanistic understanding of NAFLD pathology in the context of the MetSyn and further contribute to the possible implementation of important countermeasures, either preventive or therapeutic, against the disease. Therefore, we hope that this special issue will be of interest to a wide audience in basic and applied biomedical areas,
especially those that embrace with excitement the search for new hypothesis and approaching challenges regarding NAFLD pathophysiology in the context of the MetSyn.

Maria João Martins
António Ascensão
José Magalhães
Maria Carmen Collado
Piero Portincasa
Review Article

Gut Microbiota: Association with NAFLD and Metabolic Disturbances

E. Lau, D. Carvalho, and P. Freitas

Department of Endocrinology, Diabetes and Metabolism, Centro Hospitalar São João, Institute for Research and Innovation in Health Sciences, Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal

Correspondence should be addressed to E. Lau; evalu.med@gmail.com

Received 3 November 2014; Accepted 7 January 2015

Academic Editor: Maria Carmen Collado

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Nonalcoholic fatty liver disease is the hepatic expression of metabolic syndrome, being frequently associated with obesity, insulin resistance, and dyslipidemia. Recent lines of evidence have demonstrated a role of gut microbiota in insulin resistance, obesity, and associated metabolic disturbances, raising the interest in its relationship with NAFLD pathogenesis. Therefore, intestinal microbiota has emerged as a potential factor involved in NAFLD, through different pathways, including its influence in energy storage, lipid and choline metabolism, ethanol production, immune balance, and inflammation. The main objective of this review is to address the pathogenic association of gut microbiota to NAFLD. This comprehension may allow the development of integrated strategies to modulate intestinal microbiota in order to treat NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a very common disease, ranging from simple hepatic steatosis, characterized by excessive fat deposition in hepatocytes without any inflammation or necrosis to nonalcoholic steatohepatitis (NASH), characterized by steatosis and hepatic inflammation [1]. NAFLD is the hepatic expression of metabolic syndrome, being frequently associated with obesity, insulin resistance, and dyslipidemia [2]. Thus, its prevalence rises in parallel with the worldwide metabolic diseases epidemic, frequently developing on the background of obesity [3].

Although the pathogenesis of NAFLD is not completely understood, considerable progress has been made in recent years in elucidating the mechanisms responsible for liver injury. Initial theories were based on a “2-hit hypothesis” [4]. The “first hit” was characterized by hepatic triglyceride accumulation, which increases susceptibility of the liver to injury mediated by “second hits,” such as inflammatory cytokines/adipokines, mitochondrial dysfunction, and oxidative stress, which in turn lead to steatohepatitis and/or fibrosis [5].

The gastrointestinal tract harbors the largest number of bacteria, representing more than 150-fold their eukaryotic nuclear genome [6]. This “microbial organ” is recognized to perform a variety of physiological functions, from protective functions to metabolic regulation, including an active part on glucose and lipid metabolism [7]. Recent lines of evidence suggest a role of gut microbiota in insulin resistance and obesity [8–11], raising the interest of gut microbiota as an active intervenient on NAFLD (Figure 1). Microbiota seems to induce obesity through several mechanisms: ability of microbial products such as acetate and propionate to signal via intestinal epithelial receptors; increased intestinal permeability with translocation of bacterial products resulting in high level of metabolic inflammation; and caloric salvage by some microbes being able to extract calories from food [12]. There is a close anatomical and functional relationship between gut and liver, through portal circulation, favoring bidirectional influences [13]. Liver receives approximately 70% of its blood supply from the intestine, representing the first line of defense against gut-derived antigens [13]. Thus, gut microbiome may play an important role in the maintenance of gut-liver axis health and in NAFLD pathogenesis.

In the background of obesity and insulin resistance, this systematic review aims to explore the relationship which links microbiota to NAFLD.
Figure 1: Schematic view of how the gut microbiota affects host fat storage and insulin resistance, which may result in NAFLD. The microbiota acts through an increase in the transactivation of lipogenic enzymes by liver carbohydrate response element binding protein (ChREBP) and sterol regulatory element binding protein 1 (SREBP-1), an increase in the uptake of dietary polysaccharides and through Fiaf inhibition with increased LPL activity in adipocytes, thereby promoting increase of hepatic lipogenesis and storage of calories harvested from the diet into fat.

<table>
<thead>
<tr>
<th>NAFLD</th>
<th>Fatty liver [MeSH terms] or NASH or nonalcoholic steatohepatitis or nonalcoholic fatty liver or NAFLD or nonalcoholic fatty liver disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut microbiota</td>
<td>Microbiota [MeSH terms] or gut microbiota</td>
</tr>
<tr>
<td>Exclusion of reviews</td>
<td>Not review [publication type]</td>
</tr>
</tbody>
</table>

38 papers excluded:
- not relative (other diseases or themes)
- other languages than English
- letters or comments

Total of 56 publications initially selected

Inclusion of 18 publications

Total of 51 publications

Selection of 33 addition relevant publications

2. Methods

A literature search was conducted with the aim of finding original experimental, epidemiological, and clinical studies on the association between gut microbiota and NAFLD. The search strategy used in PubMed, including the studies selection, is shown in Figure 2. Additional papers were identified in the reference lists of selected articles that met the inclusion criteria. Inclusion criteria were as follows: clinical studies with participants of any sex or ethnic origin with NAFLD/NASH diagnosed on the basis of radiological/histological evidence of fatty liver and epidemiological or experimental studies, regarding association between NAFLD/NASH and gut microbiota. Exclusion criteria were as follows: other causes of hepatic steatosis, such as alcoholic hepatic steatosis or viral hepatitis, and papers written in other languages than English.

All articles were read in full. Two independent investigators assessed papers for inclusion. Disagreement was resolved by discussion.

3. Results

Experimental and clinical studies have explored the pathogenic association between gut microbiota and NAFLD. A summary of studies, both in animal models and humans, are resumed in Tables 1 and 2, respectively.

3.1. Gut Microbiota Profile and Driven Mechanisms Associated with NAFLD

3.1.1. Experimental Data. Experimental data have addressed the role of gut microbiota in the regulation of immune balance, low-grade inflammation, gut permeability, and lipid metabolism on NAFLD.

Due to its anatomical links to the gut, the liver is constantly exposed to gut-derived bacterial products. Immune cells like Kupffer cells recognize molecular pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors, for example, toll-like receptors (TLR), thereby playing an important role in the protection against systemic bacterial [32].

Dietary fructose intake is associated with NAFLD development [33]. In a fructose-induced NAFLD mice model, hepatic steatosis was associated with a significant induction of TLR1–4 and 6–8 [31]. Fructose-fed animals also had significantly higher number of F4/80 positive cells, a macrophages marker, and lower protein concentration of occludin, a tight junction protein [31]. Furthermore, the activation of TLRs is associated with an increase in levels of endotoxemia, produced by Gram-negative bacteria and lipopolysaccharides (LPS), emphasizing their role in intestinal permeability regulation and bacterial translocation [20]. Basically, LPS and other microbial components, in the intestine, bind to the specific receptor-activating TLRs signaling, triggering the
The exact composition of an inflammasome in myeloid cells and are a component of the innate immune inflammatory cytokines Interleukin 1 variant. The inflammasome promotes the maturation of the some composition whereas asbestos will assemble a different assembly; for example, dsRNA will trigger one inflammasome composition whereas asbestos will assemble a different variant. The inflammasome promotes the maturation of the inflammatory cytokines Interleukin 1β (IL-1β) and Interleukin 18 (IL-18). Thus, the inflammasome is responsible for activation of inflammatory processes and has been shown to induce cell pyroptosis, a process of programmed cell death distinct from the immunologically silent death mechanism that characterizes apoptosis. Pyroptosis is an intriguing inflammasome-mediated host defense mechanism, which prevents intracellular replication of pathogens, by releasing their intracellular content into circulation and therefore targeting the destruction of surviving bacteria by phagocytes and neutrophils. Different animal models reveal that inflammasome deficiency-associated changes in the gut microbiota composition were associated with exacerbated hepatic steatosis and inflammation through the influx of TLR4 and TLR9 agonists into the portal circulation. Subsequently, hepatic TNF-α expression was enhanced, inducing NASH progression [21]. Porphyromonadaceae was found to be increased in inflammasome-deficient mice and associated with exacerbated hepatic steatosis and inflammation [21]. Le Roy et al. had also showed higher concentrations of Porphyromonadaceae in a mouse model of hepatic steatosis [22].

The microbiota also regulates energy and lipid metabolism, directing the host to a rapid increase in body fat content, despite reduced chow consumption, and to increase hepatic production of triglycerides [11]. Conventionalization of germ-free mice promoted absorption of monosaccharides and short chain fatty acids by fermentation and thus increased de novo hepatic lipogenesis and fat storage, by increasing liver carbohydrate response element binding protein (ChREBP) mRNA and regulating lipoprotein lipase activity [11]. Additionally, it seems that saturated fat stimulates hepatic steatosis and affects gut microbiota composition by an enhanced overflow of dietary fat to the distal intestine [23]. A saturated fat diet based on palm oil increased liver fat accumulation, reduced microbial diversity, and increased protein (ChREBP) mRNA and regulating lipoprotein lipase activity [11].

### Table 1: Clinical studies on NAFLD and gut microbiota in humans.

<table>
<thead>
<tr>
<th>Study patients and methodology</th>
<th>Outcomes</th>
<th>Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomized controlled trial of 38 patients, 16 NASH patients (7 supplemented with probiotic versus 9 usual care group) versus 22 controls</td>
<td>NASH patients had lower fecal abundance of <em>Faecalibacterium</em> and <em>Anaeroprobacter</em> but higher abundance of <em>Parabacteroides</em> and <em>Allisonella</em></td>
<td>[14]</td>
</tr>
<tr>
<td>Cross-sectional study of 63 children, 16 controls versus 25 obese versus 22 NASH patients</td>
<td>Proteobacteria/Enterobacteriaceae/<em>Escherichia</em> was similarly represented between healthy and obese microbiomes but was significantly elevated in NASH</td>
<td>[15]</td>
</tr>
<tr>
<td>Cross-sectional study of 60 patients, 30 NAFLD patients versus 30 controls</td>
<td>Lactobacillus and selected members of phylum Firmicutes (<em>Dorea</em>, <em>Robinsonella</em>, and <em>Roseburia</em>) were higher in NAFLD patients; <em>Oscillibacter</em> was underrepresented</td>
<td>[16]</td>
</tr>
<tr>
<td>In-patient study of 15 female subjects placed on well-controlled diets in which choline levels were manipulated</td>
<td>Variations between levels of Gammad proteobacteria and <em>Erysipelotrichi</em> were directly associated with changes in liver fat in each subject during choline depletion</td>
<td>[17]</td>
</tr>
<tr>
<td>Randomized controlled trial of 48 children with NAFLD-22 supplemented with VSL#3 versus 22 placebos</td>
<td>A 4-month supplementation with VSL#3 improved NAFLD in children</td>
<td>[18]</td>
</tr>
<tr>
<td>Randomized controlled trial of 66 patients with NAFLD-34 supplemented with <em>Bifidobacterium longum</em> with fructooligosaccharides (FOS) and lifestyle modification versus 32 lifestyle modifications alone</td>
<td><em>Bifidobacterium longum</em> with FOS and lifestyle modification significantly reduces endotoxin, hepatic steatosis, and NASH activity index</td>
<td>[19]</td>
</tr>
</tbody>
</table>

Inflammasomes are cytoplasmic multiprotein complexes consisting of caspase 1, PYCARD, NALP, and sometimes caspase 5, which act like sensors of endogenous or exogenous PAMPS or damage-associated molecular patterns (DAMPs). They regulate the activation of effector proinflammatory cytokines, such as pro-IL-1β and pro-IL-18, and are expressed in myeloid cells and are a component of the innate immune system [21]. The exact composition of an inflammasome depends on the activator, which initiates inflammasome assembly; for example, dsRNA will trigger one inflammasome composition whereas asbestos will assemble a different variant. The inflammasome promotes the maturation of the inflammatory cytokines Interleukin 1β (IL-1β) and Interleukin 18 (IL-18). Thus, the inflammasome is responsible for activation of inflammatory processes and has been shown to induce cell pyroptosis, a process of programmed cell death distinct from the immunologically silent death mechanism that characterizes apoptosis. Pyroptosis is an intriguing inflammasome-mediated host defense mechanism, which prevents intracellular replication of pathogens, by releasing their intracellular content into circulation and therefore targeting the destruction of surviving bacteria by phagocytes and neutrophils. Different animal models reveal that inflammasome deficiency-associated changes in the gut microbiota composition were associated with exacerbated hepatic steatosis and inflammation through the influx of TLR4 and TLR9 agonists into the portal circulation. Subsequently, hepatic TNF-α expression was enhanced, inducing NASH progression [21]. Porphyromonadaceae was found to be increased in inflammasome-deficient mice and associated with exacerbated hepatic steatosis and inflammation [21]. Le Roy et al. had also showed higher concentrations of Porphyromonadaceae in a mouse model of hepatic steatosis [22].

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The steatosis that first characterizes NAFLD may progress toward steatohepatitis, fibrosis, and cirrhosis [34]. To understand if gut microbiota is associated with liver fibrosis, a microbiota modification was induced by the creation of a bile duct ligation in high-fat mice models, taking into account that the bile acids have antimicrobial properties [20]. This process leads to decreased hepatic triglyceride (TG) content and increased fibrosis, thus allowing the correlation of microbiota...
Table 2: Experimental studies on NAFLD and gut microbiota in mice.

<table>
<thead>
<tr>
<th>Model</th>
<th>Outcome</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-fat diet- (HFD-) fed mice versus controls subjected to bile duct ligation (BDL) or hepatotoxin CCl4</td>
<td>HFD mice subjected to BDL had an increase of Bacteroidetes, Firmicutes, and Proteobacteria</td>
<td>[20]</td>
</tr>
<tr>
<td>Methionine-choline-deficient diet-fed mice versus HFD-fed mice</td>
<td>Inflammasome deficiency-associated changes in gut microbiota were associated with exacerbated hepatic steatosis and inflammation</td>
<td>[21]</td>
</tr>
<tr>
<td>HFD-fed germ-free mice colonized with intestinal microbiota from a responder donor (developed hyperglycaemia and higher proinflammatory cytokines) or a nonresponder</td>
<td>Responder-receiver developed hepatic macrovesicular steatosis and harbour distinct gut microbiota</td>
<td>[22]</td>
</tr>
<tr>
<td>Low-fat diet based on palm oil (LFD-PO) fed mice versus HFD based on palm oil (HFD-PO) versus olive oil (HFD-OO) versus safflower oil (HFD-SO)</td>
<td>The HFD-PO diet induced higher liver triglyceride content, reduced microbial diversity, and increased the Firmicutes-to-Bacteroidetes ratio</td>
<td>[23]</td>
</tr>
<tr>
<td>HFD-fed mice versus low-fat diet-fed mice</td>
<td>Quantitative variation in dietary choline induced an inverse quantitative variation in liver fat content; conversion of choline into methylamines by microbiota in mice on a HFD caused NAFLD</td>
<td>[24]</td>
</tr>
<tr>
<td>HFD-fed mice versus HFD supplemented with chitin-glucan (CG) versus controls</td>
<td>CG treatment significantly decreased hepatic triglyceride accumulation, which was negatively correlated with specific bacteria of clostridial cluster XIVa, that is, <em>Roseburia spp.</em></td>
<td>[25]</td>
</tr>
<tr>
<td>High-fructose diet-fed mice supplemented with <em>Lactobacillus rhamnosus</em> GG (LGC) versus controls</td>
<td>Supplementation with LGC reduced liver fat accumulation and increased intestinal Firmicutes and Bacteroidetes</td>
<td>[26]</td>
</tr>
<tr>
<td>Methionine-choline-deficient-diet-fed mice (MCD) versus MCD-fed mice supplemented with <em>Lactobacillus casei</em> strain Shirota (LcS) versus controls</td>
<td><em>Bifidobacterium</em> and <em>Lactobacillus</em> were markedly reduced by the MCD diet. Administration of LcS increased the <em>L. casei</em> subgroup and other lactic acid bacteria</td>
<td>[27]</td>
</tr>
<tr>
<td>HFD-fed rats supplementation with an herbal formula (HF) versus no supplementation versus controls</td>
<td>Supplementation of HF decreased hepatic steatosis; <em>Escherichia/Shigella</em> were enriched in HFD-fed rats but decreased to control levels after HF treatment</td>
<td>[28]</td>
</tr>
<tr>
<td>HFD-fed mice supplemented with <em>Bacteroides uniformis</em> CECT7771 versus controls</td>
<td>Supplementation with <em>Bacteroides uniformis</em> reduced NAFLD in HFD-mice; HFD resulted in marked changes in gut microbiota, partially restored by the intervention</td>
<td>[29]</td>
</tr>
<tr>
<td>N-3 PUFA-depleted diet-fed mice supplemented with fructooligosaccharides (FOS) versus controls</td>
<td>Supplementation with FOS reverses NAFLD induced by n-3 PUFA-depleted diet; FOS-treated mice exhibited higher caecal <em>Bifidobacterium spp.</em> and lower <em>Roseburia spp.</em> content and reduced hepatic triglyceride accumulation</td>
<td>[30]</td>
</tr>
<tr>
<td>Fructose-fed mice versus controls treated or not with antibiotics</td>
<td>Hepatic fat accumulation was associated with a significant induction of TLR 1–4 and 6–8. The effects of fructose were attenuated in antibiotic-treated mice. No systematic alterations of microbiota were found</td>
<td>[31]</td>
</tr>
</tbody>
</table>

...to fibrosis and not to potential effects of fat liver content [20]. In this model, an increase in percentage of Gram-negative versus Gram-positive bacteria was also observed, a reduced ratio between Bacteroidetes and Firmicutes, as well as a dramatic increase of Gram-negative Proteobacteria. To further support the role of microbiota in liver fibrosis, high-fat-diet (HFD) microbiota was transplanted to control mice, resulting in an increase in liver injury [20].

3.1.2. Clinical Data. Some studies have documented small intestinal bacterial overgrowth (SIBO) in NAFLD and NASH patients, suggesting that increased exposure to intestinal bacterial products may contribute to their pathogenesis [35, 36]. Furthermore, hepatic steatosis has been associated with increased permeability caused by disruption of intercellular tight junctions in the intestine, which was linked to SIBO [36]. Since then, growing lines of evidence have suggested that NAFLD patients are characterized by different gut microbiota composition, termed fecal dysbiosis.

To determine the association between fecal microbiota and hepatic steatosis, Wong et al. have analyzed fecal microbiota from 16 NASH patients and 22 controls [14]. NASH patients had lower fecal abundance of *Faecalibacterium* and *Anaerobacteroides* but higher abundance of *Parabacteroides* and *Allisonella*. However, intrahepatic triglyceride content improvement was generally associated with a reduction in the abundance of Firmicutes and increase in Bacteroidetes, which reflects the contradictory data that still exists regarding the association between gut microbiota profile and hepatic steatosis.
In line, NASH children had unique characters in the composition, ecological diversity, and enterotyping patterns of gut microbiome [15]. However, when comparing NASH children, with healthy subjects and obese patients, fewer differences were observed between obese and NASH microbiomes. Among taxa with greater than 1% representation, Proteobacteria, Enterobacteriaceae, and *Escherichia* were the only phylum, family, and genus showing significant difference between obese and NASH microbiome [15]. *Escherichia*, under anaerobic conditions, is capable of converting sugars to a mixture of products by fermentation, including ethanol under anaerobic conditions, is capable of converting sugars to a mixture of products by fermentation, including ethanol [38], NASH patients had elevated blood alcohol, compared to healthy subjects and obese patients [15]. Taking into account that intestinal microflora is the major source of endogenous alcohol [38], these data have supported the hypothesis that elevated representation of alcohol-producing bacteria in NASH microbiome may cause liver inflammation in NASH by a constant supply of reactive oxygen species to the liver. However, Raman et al. failed to demonstrate an increase in *Escherichia* abundance in NALFD patients and ethanol was identified as a ubiquitous fecal volatile organic component (VOC) in both obese NALFD adults and healthy controls; besides ester VOC were more frequently present in fecal samples from obese NALFD patients [16]. Nevertheless, neither blood nor breath alcohol concentrations were measured and population was limited to obese NALFD and healthy subjects, turning difficult to conclude if the observed differences in ester VOC were a consequence of NAFLD rather than obesity [16].

Low-choline diets have been associated with NAFLD [24]. In this context, it was demonstrated that gut microbiota composition changes according to dietary choline levels. During choline depletion, the levels of Gammaproteobacteria and Erysipelotrichi were directly associated with liver fat content in each subject [17]. Thus, a model was created that accurately predicted the degree to which subjects developed fatty liver on a choline-deficient diet, taking into account these bacteria levels and changes in amount of liver fat and a single nucleotide polymorphism that affects choline. Taking into account that Gammaproteobacteria and Erysipelotrichi are Gram-negative bacteria, containing LPS, which was previously described as an inductor of chronic inflammation that characterize metabolic dysfunction, insulin resistance, and diabetes [39], it can therefore be hypothesized that LPS may contribute to NAFLD development in these patients.

### 3.2. Pre- and Probiotics Intervention and Their Mechanism of Action in NAFLD

#### 3.2.1. Experimental Data

Prebiotics and probiotics are known modulators of gut microbiota. The role of intestinal microbiota in NAFLD has garnered significant attention, by demonstration of the beneficial effects of pre- or probiotics administration in NAFLD models. This finding is sustained at different levels, including gut microbiota profile, gut barrier function, LPS and low-grade inflammation, lipid metabolism, and energy balance.

Supplementation of a HFD with fungal chitin-glucan (CG) decreased hepatic triglyceride accumulation and restored the number of bacteria from clostridial cluster XIVa including *Roseburia spp.*, which were decreased due to HFD [25]. CG treatment also significantly decreased HFD-induced body weight gain, fat mass development, fasting hyperglycemia, glucose intolerance, and hypercholesterolemia, independently of the caloric intake. These beneficial effects were correlated with specific bacteria of clostridial cluster XIVa, that is, *Roseburia spp.*, and did not appear to be mediated by incretin glucagon-like peptide 1 (GLP-1) [25].

*Lactobacillus rhamnosus* GG (LGC) supplementation in high-fructose-induced NAFLD mice had strongly reduced liver fat accumulation [26]. The fat liver content improvement by LGC was associated with manipulation of gut microbiota: LGC have increased the total numbers of Firmicutes and Bacteroidetes; LGC attenuated the expression of the proinflammatory cytokines TNF-α and IL-1β and IL-8R in the liver. To determine whether changes in portal LPS levels and intestinal inflammation were associated with changes in intestinal barrier, the levels of occludin and claudin-1, tight junction proteins, were measured [26]. Occludin and claudin-1 expression was reduced in mice fed high-fructose diet compared to control diet and restored after LGC supplementation. These data support the hypothesis that the associated beneficial effects of increased members of the Firmicutes are due to the fact that they produce butyrate, which is known to regulate gut barrier function [40].

LPS role was further supported by other experimental studies. *Lactobacillus casei strain* (LcS) administration suppressed LPS elevation and protected against methionine-choline-diet-induced NASH development in a mice model [27]. Thus, gut modulation by LcS administration may contribute to the normalization of tight junction proteins, protects against impairment of gut permeability, and subsequently diminishes inflammation and reverse hepatic steatosis. Furthermore, treatment with a Chinese herbal formula (CHF) supplementation ameliorated NAFLD and resulted in a reduction in *Escherichia/Shigella* levels, Gram-negative bacteria containing LPS in their cell walls that may impair the gut barrier and trigger a low-grade chronic inflammation state [28]. CHF supplementation increased *Collinsella*, short chain fatty acid (SCFA) producers [28]. SCFA may also be responsible for the beneficial effects of CHF treatment, as SCFA can stimulate epithelial cell proliferation, which may improve gut barrier integrity [41].

Immune modulation was also observed after oral administration of *Bacteroides uniformis* CECT 7771, which reduced liver steatosis in HFD-fed mice, improved immune defense mechanisms on macrophages and dendritic cells, and reduced the gut inflammatory signals [29].

To support the effects of gut microbiota in modulation of fat storage and host metabolism, the expression of ChREBP, a transcription factor required for glucose-induced expression of the lipogenic genes acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS), as well as that of ACC2 and FAS was found to be significantly increased in mice fed with fructose-rich diet and significantly reduced after LGC [26]. Moreover, in another mice model of NAFLD, fructooligosaccharides (FOS) supplementation reduced hepatic triglyceride accumulation through changes in microbiota composition, thus
leading to an increase in GLP-1, which stimulates fatty acid oxidation by peroxisome proliferator-activated receptor-alpha and lessened cholesterol accumulation by inhibiting sterol regulatory element binding proteins (SREBPs) [30].

3.2.2. Clinical Data. In human, few prospective, randomized, and controlled clinical trials have yet been designed to address the potential role of intestinal microbiota in NAFLD and the potential beneficial effects from modulation of gut microbiota, by pre- or probiotics intervention.

VSL#3 is a mixture of eight probiotic strains (Streptococcus thermophilus, bifidobacteria [B. breve, B. infantis, B. longum], Lactobacillus acidophilus, L. plantarum, L. paracasei, and L. delbrueckii subsp. bulgaricus) [18]. In children, a 4-month supplementation with VSL#3 has improved NAFLD [18]. Therefore, it is conceivable that the effects of VSL#3 in these patients could be dependent on the restoration of normal gut microbiota. Also, circulating levels of GLP-1, both in total and in active form, have significantly increased after the 4-month supplementation, which may have improved fat metabolism. GLP-1 is an incretin secreted by L-cells in the small intestine in response to food intake, whose main roles are stimulation of glucose-dependent insulin secretion, inhibition of postprandial glucagon release, delay of gastric emptying, and induction of pancreatic β-cell proliferation [42]. Besides improving hepatic glucose metabolism, GLP-1 seems to be a novel target against NAFLD, by increasing fatty acid oxidation, decreasing lipogenesis, and improving hepatic glucose metabolism [42], and may also be an active intervenient, establishing the link between NAFLD and gut microbiota.

Treatment with Bifidobacterium longum plus fructooligosaccharides (Fos) reduced HOMA-IR and NASH activity in association with reduced endotoxin, C-reactive protein, and TNF-α levels [19]. These data further support the hypothesis that endotoxin-induced activation of macrophages plays a key role in the pathogenesis of liver injury in NAFLD patients.

4. Discussion

NAFLD is an emerging complex multifactorial disease resulting from the interaction of genetic, environmental, metabolic, and inflammatory factors. Both obesity and diabetes are major risk factors for NAFLD [43]. As it has been previously described, the gut microbiota exerts a profound influence on fat deposition, being a key regulator of energy storage [11, 44].

Germ-free mice colonized with gut microbiota from obese animals showed body fat mass and liver triglyceride content and an insulin resistance increase. Microbiota promoted absorption of monosaccharides from the gut lumen, with resulting induction of de novo hepatic lipogenesis, by increased activity of acetyl-CoA carboxylase and fatty acid synthase [11]. In humans, this relationship is further reinforced by the demonstration of the relative fewer proportion of Bacteroidetes in obese people by comparison with lean people and the shift toward higher relative abundance of Bacteroidetes and decreased number of Firmicutes in obese patients losing weight through low-calorie diets [9]. These named “obese microbiomes” have increased capacity of harvesting energy from food, resulting in fat accumulation. However, the relationship between Bacteroidetes and Firmicutes levels and obesity and associated metabolic disturbances is still controversial.

According to what has been described in Section 3, differences in gut microbiota profile may also have impact on the liver, on the background of obesity and insulin resistance. Most of the available data demonstrating this association is based on association studies, lacking human intervention studies, which would further improve the knowledge of gut microbiota influence on NAFLD.

As it has been described, insulin resistance is a common feature of metabolic syndrome and NAFLD. Thus, the decrease of the inhibitory effects of insulin on peripheral lipolysis increases the availability of free fatty acids, playing a critical role in the development of fatty liver [45]. Metabolic endotoxemia triggers insulin resistance, obesity, and diabetes, through LPS, which in combination with CD14 serves as ligand for TLR [46]. LPS and other endotoxins also can activate TLRs, inducing an inflammatory response, linked to hepatic fat accumulation [20, 21, 31, 32]. An interesting finding was the observation that small intestinal bacterial overgrowth predicted severe hepatic steatosis [47]. In fact, bacterial overgrowth may increase intestinal permeability, by disruption of intercellular tight junctions, subsequently exposing liver surface to bacterial products, resulting in hepatic fat deposition [36].

An additional contributor is the modulation of choline metabolism by intestinal microbiota. Choline and methionine-deficient diets have been associated with hepatic steatosis [17, 48]. The gut microbiota catalyzes the conversion of choline to dimethylamine and trimethylamine [49]. A high-fat diet in a mice model susceptible to impaired glucose homeostasis and NAFLD reduces the bioavailability of choline, mimicking the effect of choline-deficient diets [24]. These results establish a possible association between choline bioavailability and hepatic steatosis, through metabolic activity of gut microbiota, which is affected by diet. In addition, Gammad proteobacteria and Erysipelotrichi levels were associated with hepatic steatosis, during choline depletion [17]. As these bacteria are Gram-negative, these data further support the role of LPS as an active player on NAFLD development.

Endogenous production of ethanol by bacteria also seems to mediate hepatic fat accumulation. In an obese mouse model, in the absence of ethanol ingestion, ethanol was detected in exhaled breath [50]. Hence, intestinal production of ethanol may contribute to the genesis of obesity-related fatty liver, triggering inflammatory signals [15].

Interventional studies with pre- and probiotics gave further support to the possible effects of intestinal microbiota modulation on NAFLD pathogenesis. Besides the impact on fat storage and host metabolism, GLP-1 may be an important contributor, linking NAFLD, insulin resistance, and gut microbiota. FOS supplementation in a mice model of hepatic steatosis reduced fatty liver accumulation, through changes in gut microbiota, responsible for GLP-1 increase [30]. GLP-1 stimulated fatty acid oxidation by peroxisome proliferator-activated receptor-alpha and inhibited SREBPs [30]. In children, VSL#3 supplementation improved NAFLD and had
increased GLP-1, supporting the impact of gut microbiota modulation on fat metabolism [18].

Therefore, intestinal microbiota, beyond its capacity to regulate body fat gain and insulin resistance, seems to play a fundamental role on NAFLD, through different pathways (Figure 3), including

(i) increasing energy harvest from diet,
(ii) change in expression of genes involved in de novo lipogenesis,
(iii) regulation of choline metabolism,
(iv) ethanol production,
(v) inflammasome and innate immunity,
(vi) inflammation.

However, the majority of studies were conducted under experimental conditions, namely, under fat rich diets, which limits the demonstration of a definitive role of gut microbiota in hepatic steatosis, especially in NAFLD nonobese patients. Further comprehension of the relationship between gut microbiota and hepatic steatosis will allow the development of new specific targets and integrated strategies to modulate intestinal microbiota, including prebiotics and probiotics, in order to improve or even cure this prevalent metabolic disease.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**References**


[38] T. Sarkola and C. J. P. Eriksson, “Effect of 4-methylpyrazole on endogenous plasma ethanol and methanol levels in humans,”


Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent liver diseases globally. Population studies suggest that the prevalence of NAFLD is up to 30% in different countries studied to date including USA, Italy, China, and Japan [1]. Obesity is strongly associated with NAFLD. The incidence of NAFLD in severely obese populations is approximately 74%, and in developed nations 60% of NAFLD patients are obese [2–4]. With obesity rates increasing worldwide, particularly in developing societies undergoing nutritional transition, the prevalence of NAFLD is set to increase markedly in the near future [5, 6].

NAFLD represents a spectrum of pathological changes from isolated hepatic steatosis (fatty liver) without hepatocellular damage to nonalcoholic steatohepatitis (NASH, fatty liver with inflammation) which is the extreme form of the disease characterised by hepatocellular injury and inflammation with or without fibrosis [7]. The natural progression of NAFLD is not fully understood and long-term outcomes are dependent on pathological subtypes. The majority of isolated steatosis has a relatively benign outcome displaying slow progression over many years. However, 10–20% of cases progress to NASH, which is closely linked to hepatic cirrhosis and hepatocellular carcinoma (HCC), and carries a significantly increased mortality risk [8–13].

Although initially considered as a sequential progression from simple steatosis to accompanied inflammation, it is now widely accepted that the pathogenesis of simple steatosis and NASH is likely to progress via different mechanisms [13, 14]. The development of NASH consists of a number of events whereby steatosis and inflammation and cell damage may occur in parallel rather than in strict sequence [13]. The accumulation of fat in hepatocytes can be achieved via four main mechanisms: (1) increased free fatty acid and lipid uptake, (2) increased de novo lipogenesis, (3) decreased lipid oxidation, and (4) decreased hepatic very low density lipoprotein (VLDL)-triglyceride secretion, all of which have been reviewed in detail by Fabbrini et al. [15]. On the other hand, in the case of NASH, as proposed by Tilg and Moschen, the evolution of steatosis and inflammation may enhance each other under a number of parallel processes [13]. These
Figure 1: An overview of the development of NAFLD in the context of developmental programming. The developmental programming of NAFLD may occur secondarily to programmed obesity and/or via direct programming effects on the liver. Increased lipid accumulation and inflammation in liver can lead to NASH which is the severe form of NAFLD. NASH is associated with hepatic cirrhosis and HCC and carries a significantly increased mortality risk. NAFLD: nonalcoholic fatty liver disease, NASH: nonalcoholic steatohepatitis, HCC: hepatocellular carcinoma.

processes include, but are not limited to, increased mitochondrial dysfunction, oxidative stress, endoplasmic reticulum (ER) stress, and adipose tissue and gut derived signals such as proinflammatory cytokines, decreased adiponectin, and endotoxin release ("leaky" gut).

In addition to the identified risk factors (including age, obesity, and genetic factors), there is growing evidence that exposure to an unfavourable environment before birth or in early infancy may contribute to an individual’s susceptibility and severity of NAFLD through direct effects on the liver and indirect effects via adiposity and metabolic dysfunction. In this review, we focus specifically on the relationships between alterations in the early life developmental environment and potential impact on the occurrence of NAFLD (Figure 1).

2. Early Life Nutrition and Metabolic Disorders in Adulthood: Developmental Origins of Health and Disease (DOHaD) Hypothesis

The nutritional environment during preconception, pregnancy, and early life is critical for optimal offspring development and long-term health. Over 20 years ago, Barker and Osmond reported that infant mortality is related to later life ischemic heart disease, suggesting that poor conditions during childhood increase the risk of adult cardiovascular disease [16]. Studies on famine events such as the Dutch Hunger Winter and the Great Chinese Famine have shown that the associations between poor early life nutrition and later life disease are not only limited to cardiovascular disease but also include obesity and the metabolic syndrome [17–19]. Nevertheless, assessing the risk of developmental programming on later health consequences is specific to different contexts and outcomes. The "thrifty phenotype" [20] or predictive adaptive response (PARS) hypotheses have been proposed to explain this phenomenon [21]. These theories argue that poor fetal nutrition leads to metabolic adaptations which act to maximally utilise limited nutrient availability and therefore increase the chances of survival in continued poor conditions after birth. However these adaptations serve to increase the risk for metabolic disorders when exposed to an enriched postnatal nutrient environment. Interestingly, a number of studies on diabetic pregnancies (gestational diabetes and type 2 diabetes) and maternal obesity suggest that excess calorie intake during early life has similar effects on offspring long-term health outcomes [22–25]. Whilst the "thrifty phenotype" or PARS framework may be appropriate in the setting of relative undernutrition, it does not adequately describe the outcomes in offspring observed in the context of maternal obesity. One would argue that offspring of obese mothers
would be “matched” to an obesogenic postnatal environment, but offspring of obese mothers display metabolic disorders similar in nature to that observed for maternal undernutrition in the absence of further nutritional insults. This may lie in the observation that excessive maternal caloric intake per se may represent a form of fetal malnutrition due to altered placental function and nutrient transport (obesity is commonly associated with micronutrient deficiencies) and thus program the fetus in a way similar to that observed in the setting of direct maternal undernutrition.

The DOHaD hypothesis proposes, from a broad perspective, that alterations in the intrauterine environment can affect the developing fetus in a number of aspects including organogenesis, cell differentiation, and lipid and glucose metabolism, thereby altering risk for development of a range of cardiometabolic disorders in later life [26]. A number of experimental models have provided empirical evidence to support the DOHaD hypothesis including global undernutrition, low protein, and high fat dietary exposures and have provided insights into the physiological and molecular mechanisms linking early life adversity and later disease risk.

3. Developmental Programming of NAFLD

3.1. Maternal Obesogenic Environment and Offspring NAFLD.

In the past few decades there has been mounting evidence to suggest that a maternal obesogenic environment may contribute to offspring obesity and metabolic syndrome [22–25]. However, direct association between maternal obesity and offspring hepatic lipid accumulation in human cohorts was only evidenced in recent years due to the implementation of appropriate diagnostic technologies [27]. It was reported by Modi et al. [28] and Brumbaugh et al. [29] that maternal body mass index (BMI) is directly correlated to neonatal intrahepatocellular lipid content as measured by MRI. In particular, Brumbaugh et al. also showed that the relationship between maternal BMI and neonatal hepatic fat may be independent of neonatal subcutaneous fat leading to speculation that fetal hepatic fat storage may be driven directly by excessive maternal free fatty acid via a pathway distinct from adipose tissue development [29].

A number of experimental animal models using a range of dietary approaches have provided detailed evidence linking a maternal obesogenic environment and the development of NAFLD in offspring. A chronic maternal high fat (HF) diet can lead to a NAFLD phenotype in offspring independent of maternal and offspring obesity [30, 31]. In nonhuman primates (NHP), chronic consumption of a HF diet prior to and during pregnancy, independent of maternal obesity, led to fetal liver steatosis which persisted into the juvenile period [30]. Interestingly, changing the maternal diet to a low fat diet in subsequent pregnancy improved offspring outcome, which highlights that diet during pregnancy has a significant role in the programming of offspring hepatic fat deposition [30]. Similar findings were observed in rodent offspring born to dams chronically consuming a HF diet from preconception to lactation, with a maternal HF diet inducing hepatic steatosis in adult offspring, despite being fed a standard chow diet after weaning [31].

To further investigate whether the severity of NAFLD is influenced by a maternal obesogenic environment, many of the animal models introduced a postweaning HF diet to enhance susceptibility to NAFLD. As expected, when exposed to postweaning HF diet, offspring born to HF diet dams exhibit NASH in early adulthood compared to offspring born to the normal diet dams, which only developed simple steatosis [31–34]. These observations suggest that maternal HF diet increases vulnerability to steatohepatitis rather than simple steatosis in offspring. This is consistent in other dietary models of mixed resource high energy Western and cafeteria diets [35–37]. Kruse et al. demonstrated that offspring exposed to a perinatal HF diet had increased susceptibility to develop NAFLD, despite consuming a normal chow diet for 23 weeks after weaning [38]. This finding emphasises that the pregnancy and lactation period are the critical windows for programming susceptibility to NAFLD. This highlights the irreversibility of such effects in later life, which is consistent with the developmental programming models of other metabolic conditions [39].

In addition to the consumption of a maternal obesogenic diet, preexisting maternal metabolic dysfunction such as insulin resistance also contributes to offspring NAFLD. Thorn et al. compared juvenile NHP born to females chronically exposed to a HF diet with or without development of insulin resistance. Offspring from insulin resistant females, but not insulin sensitive females, developed significant hepatic steatosis despite consuming a healthy diet after weaning and in the absence of obesity [40]. Additionally, an intergenerational study by Li et al. showed that HF feeding through three generations progressively induced severe hepatic steatosis in offspring. Adult offspring from the second generation of HF diet fed animals demonstrated exacerbated NAFLD and increased secretion of the adipokine leptin compared to the previous generation suggesting that programming of NAFLD can accumulate in an intergenerational manner [41].

3.2. Early Life Growth Restriction, Undernutrition, and NAFLD.

Early life growth restriction discussed here refers to decreased body weight compared to normal birth weight peers, which is commonly observed as a consequence of maternal undernutrition or conditions such as preeclampsia and other forms of placental dysfunction whereby sufficient nutrient supply fails to reach the fetus. Several human studies suggest that early life growth restriction may program liver disease in later life. Fraser et al. reported an association between low birth weight and increased liver enzymes alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT) in a random sample of over 2000 women aged 60–79 years, indicating possible hepatic cellular injury in these subjects [42]. A case control study by Nobili et al. showed an association between paediatric NAFLD and intrauterine growth restriction (IUGR), with low birth weight children demonstrating high prevalence of NASH [43]. However, two other studies suggest that the rapid growth pattern following early growth restriction rather than low birth weight per se is strongly associated with the risk of NAFLD.
Subjects with accelerated weight gain in the first 3 months of infancy have a significantly higher risk for NAFLD in early adulthood than subjects with slow catch-up growth [44]. An epidemiological study including over 1500 aged participants from the Helsinki Birth Cohort Study showed that childhood body size was negatively associated with NAFLD outcomes after adjustment for adult BMI. Particularly, individuals who were lean in early life and subsequently obese in adulthood had significantly increased risk for NAFLD [45].

In animal models, macronutrient restriction is one of the most commonly used methods to establish offspring growth restriction. Moderate to severe dietary protein restriction during pregnancy and lactation in rats leads to offspring hepatic steatosis in late adulthood without a paralleled increase in adiposity [46, 47]. In sheep, aged lean female offspring born to mothers that received global nutrient restriction in the first half of gestation showed significantly increased hepatic lipid accumulation [48]. Moreover, a study by Yamada et al. showed that hepatic fat deposition occurs in fetuses exposed to maternal undernutrition, as early as embryonic day 20, prior to the development of offspring adiposity [49]. Therefore, it is possible to speculate that growth restriction induced susceptibility to NAFLD is at least partially independent of development of obesity.

In addition to macronutrient restriction, other animal models have shown that factors leading to early growth restriction can also influence liver development. Prenatal hypoxia-induced IUGR increased susceptibility to hepatic steatosis in adulthood [50]. Offspring born to dams subjected to vitamin B12 and folate deficiency have significantly increased hepatic lipid accumulation [48]. Moreover, a study by Yamada et al. showed that hepatic fat deposition occurs in fetuses exposed to maternal undernutrition, as early as embryonic day 20, prior to the development of offspring adiposity [49]. Therefore, it is possible to speculate that growth restriction induced susceptibility to NAFLD is at least partially independent of development of obesity.

In summary, there is evidence to suggest that obesity, consumption of high fat diets, and undernutrition, during the critical early periods of developmental plasticity, may increase the susceptibility and severity of NAFLD. The programming effect may be partially independent of adiposity. A summary of the related studies is presented in Table 1.

4. Potential Mechanisms Involved in the Developmental Programming of NAFLD

4.1. Hepatic Lipid Accumulation. The primary feature of NAFLD is accumulation of lipids. Fatty acid accumulation occurs when fatty acid uptake and synthesis exceed hepatocyte oxidative capacity. A human study by Donnelly et al. demonstrated, using stable isotopes, that the dominant source of fat which accumulated in liver originates from serum free fatty acids; this is closely followed by de novo lipogenesis [52]. Generally, lipolysis in white adipose tissue (WAT) is the major contributor to serum free fatty acid concentrations [15]. However, this is not the case during fetal life as WAT only starts to develop in the middle of the third trimester in human and NHP and after birth in the rodent [53, 54]. It has been shown in NHP that maternal obesogenic diets induce fetal hepatic steatosis and that fetal and maternal plasma glycerol concentrations are strongly correlated [30]. Fat accumulation in fetal liver may thus originate directly from maternal lipid transfer and represents a “very first hit” of lipotoxicity during early life development.

In animal studies, offspring de novo lipogenesis can be increased during early adulthood as a result of a maternal obesogenic diet. These various studies have reported increased expression of hepatic transcription factor sterol regulatory element binding protein 1c (SREBP1c) and its coactivators and downstream lipogenic targets: peroxisome proliferator-activated receptors (PPARs), fatty acid synthase (FASN), stearoyl-CoA desaturase-1 (SCD1), and acetyl-CoA carboxylase (ACCI) in adult offspring exposed to obesogenic diets in utero [33, 36, 37, 40, 41]. The proposed causes for SREBP1c activation include altered offspring insulin signalling and polyunsaturated fatty acids (PUFAs) metabolism [36, 55–57]. Apart from lipogenesis, the role of hepatic fatty acid β-oxidation in the programming of steatosis is not consistent across animal studies. Some find no change in the key enzyme for fatty acid oxidation, carnitine palmitoyltransferase 1 (CPT1) [31, 35], while one study observed persistent decreases in CPT1A gene expression from late gestation to weaning [37]. The synthesis and secretion of VLDL in adult offspring appear to be enhanced by a maternal obesogenic diet [37, 57], which is likely to be a result rather than a cause of hepatic lipid accumulation. Of note, in the maternal obesogenic environment, several genes that are involved in lipid metabolism showed epigenetic modification in adult offspring. Epigenetic modification is considered as a key regulation mechanism in developmental programming [58]. Liver X receptor-α (LXRα), which is an important mediator for SREBP1c [59], displayed decreased histone methylation after three generations of HF diet consumption in rats, providing a possible explanation for the intergenerational programming effects observed [41]. In a mouse model, alterations in DNA CpG methylation in PPARα, FASN, and insulin-induced gene protein (Insig) were observed in NAFLD offspring with perinatal exposure to Western diet [36]. Overall, these findings suggest that maternal lipid dysregulation and de novo lipogenesis have major effects on the developmental programming of offspring hepatic steatosis in the maternal obesogenic setting, with epigenetic modification representing a potential mechanism.

Upon exposure to an undernourished in utero environment, increased activation of de novo lipogenesis is observed in parallel with the occurrence of fatty liver in rat offspring, with upregulation of hepatic carbohydrate-responsive element-binding protein (ChREBP) and SREBP1c expression at both transcriptional and protein levels [47, 49, 60]. Glucocorticoid exposure is proposed to play a role in the programming of offspring lipogenesis in this setting. It has been demonstrated that maternal protein restriction can lead to reduction of 11β-hydroxysteroid dehydrogenase (11β-HSD) in the placenta and subsequently increases fetal exposure to maternal glucocorticoids [61]. Inhibiting glucocorticoid synthesis reversed the suppressive effect of low protein diet on offspring hepatic SREBP-1c expression [62]. The role of glucocorticoid in programming NAFLD is also supported by Drake et al., who showed that prenatal dexamethasone treatment can increase rat offspring susceptibility to fatty liver without promoting adiposity [63]. However, in vitro experiments showed a contrary effect of glucocorticoid on the expression of SREBP1c, suggesting that other factors may be involved in the glucocorticoid effect in vivo [62].
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4.2. Mitochondrial Dysfunction: Oxidative and Endoplasmic Reticulum (ER) Stress. Mitochondria are important organelles which are essential for energy generation and are the primary site for fatty acid β-oxidation [64]. The relationship of mitochondrial dysfunction and NAFLD has been reviewed extensively by others [65, 66]. Briefly, mitochondrial fatty acid oxidation increases to adapt to excessive hepatic fat accumulation [67] and in turn leads to a rise in oxidative products—reactive oxygen species (ROS). Most mitochondrial ROS are detoxified to residual molecules through mitochondrial respiratory chain (MRC) activity. However, increased mitochondrial oxidation can progressively induce a vicious cycle including reduction in MRC activity, overproduction of ROS, and damage to mitochondrial DNA. The imbalanced state that favours ROS production over antioxidant defence is defined as oxidative stress. Oxidative stress with excessive mitochondrial ROS has been shown to participate in cell death, inflammation, and fibrosis [68] and therefore may play a significant role in the progression of NASH. In maternal obeseogenic animal models, mitochondrial dysfunction and oxidative stress are observed in offspring, reflected by reduced MRC key components—mitochondrial electron transport chain complex (ETC) I, II/III, and IV activity [31], uncoupling MCR activity [69], decreased liver mitochondrial DNA copy number [70], and reduced concentrations of antioxidant enzymes [69, 71]. Nevertheless, it is not completely clear how maternal factors elicit these changes in the next generation. It has been shown by Igosheva et al. that diet induced maternal obesity prior to conception is associated with altered mitochondrial function in mouse oocytes and zygotes [72]. Since mitochondria are maternally inherited, it is possible that the mitochondrial dysfunction in offspring is a combination of inheritance of predisposed maternal mitochondria and exposure to suboptimal early life environment.

The ER is an important organelle for lipid and protein synthesis and export. Disturbance in ER homeostasis (ER stress) has been shown to contribute to both steatosis and the progression to NASH [73, 74]. Emerging evidence suggests that ER stress is associated with de novo lipogenesis, mitochondrial dysfunction, oxidative stress, inflammation, and cell death. Details of these interactions have been reviewed elsewhere [75]. In the intergenerational obeseogenic diet study, ER stress markers (binding of immunoglobulin protein (BIP), C/EBP homologous protein (CHOP), ER-associated oxidoreductin 1-α (ERO1-α), and eukaryotic translation initiation factor 2a (eIF2a)) were progressively increased, indicating an intergenerational accumulation of ER stress in these animals [41]. Epigenetic modification on the ERO1-α promoter provides a possible explanation for this observation [41].

4.3. Proinflammatory Cytokines. NAFLD is linked to obesity and type 2 diabetes, conditions which are associated with chronic low-grade inflammation. Obesity results in altered adipose tissue-derived cytokine and adipokine secretion and progressive infiltration of innate immune cells such as macrophages, which contribute to a state of local and systemic inflammation [76, 77]. This altered inflammatory profile can have peripheral effects on the liver [78], and during pregnancy, can result in enhanced placental and fetal inflammation [79]. It is well established that inflammatory mediators have a critical role in the pathogenesis of NAFLD. In particular, expression of the cytokine tumor necrosis factor-α (TNF-α) is correlated with the severity of steatohepatitis [80, 81], and reduction of TNF-α with metformin improves steatosis in ob/ob mice [82]. Free fatty acid accumulation promotes a proinflammatory phenotype through activation of the Toll-like receptor 4 (TLR4) signalling pathway, which culminates in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation [83]. Chronic low-level hepatic activation of NF-κB further contributes to hepatic production of TNFα, interleukin-1β (IL-1β), and IL-6 and local and systemic insulin resistance [84]. Furthermore, NAFLD is linked to oxidative stress, ROS production, and generation of toxic lipid peroxides which can damage DNA. Damaged hepatocytes release damage-associated molecular patterns (DAMPs), promoting proinflammatory processes. DAMPs activate the inflammasome, a multiprotein complex responsible for the cleavage of inactive proforms of the proinflammatory cytokines IL-18 and IL-1β in the cytoplasm to their bioactive forms, which can then be secreted by the cell [85]. When steatosis occurs, the liver is more susceptible to injury from proinflammatory cytokine stimulation, resulting in progression from NAFLD to NASH. Although the mechanisms underpinning this progression remain unclear, NASH is characterized by hepatocellular degeneration and infiltration of immune and inflammatory cells, which can advance to fibrosis and cirrhosis.

In animal models of maternal obesity, offspring of dams fed a HF diet have significantly reduced natural killer T (NKT) cell populations and upregulated expression of proinflammatory cytokines such as IL-1β, IL-6, IL-12, IL-18, and TNF-α [31, 32, 34, 86]. Male offspring from dams that consumed a HF diet present clinical features of metabolic syndrome, liver lipid accumulation, and activation of c-Jun N-terminal kinases (JNK) [86]. Pruis et al. demonstrated in mice that exposure to a maternal western diet during pregnancy and/or lactation primed NAFLD in adult male offspring [36]. Early life exposure to a Western-style diet during pregnancy and lactation resulted in hepatomegaly and hepatic cholesterol/triglyceride accumulation, upregulated de novo lipid synthesis, and increased expression of inflammatory mediators and macrophage markers including TNF-α, transforming growth factor-β (TGF-β), monocyte chemoattractant protein-1 (MCP-1), and cluster of differentiation 11 (CD11). These changes may be mediated by epigenetic alterations in DNA methylation of PPARα, a transcription factor involved in energy metabolism, hepatic steatosis, and inflammatory processes.

Thorn et al. demonstrated in NHP that in utero exposure to HF diet-induced insulin resistance resulted in a programmed increase in hepatic triglycerides and upregulation of hepatic de novo lipid synthesis and inflammatory pathways, despite postweaning consumption of a healthy chow diet [40]. Additionally, even though these offspring did not display obesity or insulin resistance, they had both classical and alternatively activated hepatic macrophages and...
Potential mechanisms that contribute to the developmental lasting adaptations under different early life environments. Dysfunction, and the activation of inflammatory response level, programming of NAFLD are multifactorial. At a molecular level, de novo lipogenesis, primed mitochondrial and ER dysfunction, and the activation of inflammatory response are the main pathways that are most likely to have long lasting adaptations under different early life environments. Potential mechanisms that contribute to the developmental programming of NAFLD are summarised in Figure 2.

5. Sexual Dimorphism in Programming NAFLD

Although initially thought to be more common in females [7], recent evidence shows that the prevalence of NAFLD is higher in males [87–89]. In particular, paediatric NAFLD is more prevalent in boys, with a male to female ratio of 2.5:1 [90, 91]. Although the majority of animal studies only examine male offspring due to the potential confounds of estrus, there is some evidence suggesting that female offspring are likely to be moderately protected from NAFLD in the maternal obesogenic environment. Bayol et al. reported that a maternal junk food diet promotes exacerbated steatosis and hepatocyte ballooning in both male and female offspring. However, increased expression of genes associated with de novo lipogenesis and lipid oxidation were only observed in males [35]. Strakovsky et al. found that feeding a HF diet to an obesity resistant strain of rats during pregnancy led to a significant increase in hepatic triglycerides in male neonates but not females with a sex-specific change in the antioxidative system [92]. In another maternal HF diet model, HF feeding during early life programmed hepatic steatosis and insulin resistance in male offspring chronically exposed to HF diet in adult life, whereas female offspring were protected from the NAFLD phenotype [70]. One of the potential explanations for this disparity is the liver-protective role of estrogens, as well as the potential role of androgens in aggravating NASH [91, 93]. Nevertheless, sexual dimorphism is frequently observed in developmental programming models, with molecular and phenotypic outcomes of adverse in utero conditions often more prominent in male offspring [94].

6. Potential Intervention Strategies during Early Life

Work by Godfrey et al. highlighted that the earlier the intervention during the life course the greater the impact on later life health and well-being of offspring [95]. Intervention strategies to ameliorate the developmental programming of NAFLD need to be introduced in early life during critical windows of developmental plasticity to elicit the most effective benefits. Animal models indicate that programmed effects are highly irreversible after weaning; long-term consumption of a normal chow diet after weaning may not be effective in normalising offspring susceptibility to NAFLD induced by maternal HF diet [35, 38].

Evidence suggests that breastfeeding may confer some protection against the development of NAFLD in humans. A study of 191 children with NAFLD showed that early breast feeding may have a protective effect on the progression to NASH and liver fibrosis independent of the present or neonatal characteristics of the children [96]. It has been shown in other studies that longer duration of breastfeeding can decrease the risk of offspring becoming overweight in later life [97]. Breast milk is a rich source of long-chain PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [98]. It has been reported that PUFAs can suppress de novo lipogenesis via inhibition of SREBP1c [99]. Moreover, DHA can act as a PPAR-agonist reducing experimental liver fibrogenesis in mice [100], hence having a preventive effect on NASH. Breast milk also contains numerous peptides such as insulin and leptin (which are not present in infant formula) that are bioactive and may influence infant growth and body composition [101]. In particular, leptin administration during the neonatal period reverses developmental programming of metabolic disorders in the rat [102]. Although these interventions have not been tested directly in the setting of NAFLD, these factors have the potential to protect against the progression of hepatic steatosis.

Several dietary supplements have been investigated in animal models as intervention strategies to combat adverse developmental programming effects. Fish oil is naturally enriched in PUFAs and has shown antiobesity effects in animal models [103]. Brighenti et al. reported that introducing fish oil to a postweaning diet can reverse maternal low protein diet induced hepatic steatosis in offspring [104]. This effect is likely achieved via a reduction in de novo lipogenesis and enhanced lipid oxidation [104]. The plant extract resveratrol, which is a naturally occurring compound of various fruits such as red grapes, is known to have multiple chemoprotective properties including antioxidant and anti-inflammatory effects [105]. Franco et al. reported that, although given in adulthood, resveratrol reversed early weaning induced adult offspring liver steatosis and dyslipidemia [106]. This may be due to the beneficial effect of resveratrol on mitochondrial oxidative stress [106, 107]. The progress from NAFLD to NASH is critically regulated by proinflammatory cytokines.
As discussed previously, taurine is a sulfonic amino acid with anti-inflammatory properties [108]. A recent study by our group suggested that taurine supplementation during pregnancy and lactation may ameliorate an adverse proinflammatory hepatic profile observed in offspring following a maternal obesogenic diet [109]. This may potentially reduce the susceptibility to NASH by moderating inflammatory responses upon exposure to insults. Even though these supplementalations look promising, further thorough experiments regarding safety profiles are required before implementation as a therapeutic option.

7. Summary

The development of NAFLD is a multifactorial process. In addition to obesity, age, genetic factors, and lifestyle, suboptimal early life nutrition including a maternal obesogenic environment or undernutrition may increase the susceptibility, age of onset, and severity of the disease. The influence of early life nutrition on the development of NAFLD is likely in part independent of adiposity. Animal models representing different maternal nutritional insults provide in-depth views on the mechanisms relating to hepatic lipid accumulation and the progression to liver inflammation. Particularly, maternal lipid dysregulation and later life de novo lipogenesis are the major contributors for offspring hepatic steatosis in the maternal obesogenic setting, while in a growth restricted environment, glucocorticoid alteration is proposed to play an important role in the development of offspring fatty liver. Furthermore, mitochondrial dysfunction, oxidative stress, ER stress, and inflammatory responses are all involved in the progression of the disease in the setting of developmental programming. While breastfeeding shows a possible protective effect, dietary supplements with anti-inflammatory and antioxidant capacity may also have the potential to reduce further increases in NAFLD, partly attributed to poor in utero and early life nutritional programming.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.
References


[34] J. A. Oben, A. Mouralidarane, A.-M. Samuelsson et al., "Maternal obesity during pregnancy and lactation programs
the development of offspring non-alcoholic fatty liver disease in mice,” *Journal of Hepatology*, vol. 52, no. 6, pp. 913–920, 2010.


Review Article

The Dual Role of Nrf2 in Nonalcoholic Fatty Liver Disease: Regulation of Antioxidant Defenses and Hepatic Lipid Metabolism

Silvia S. Chambel,1,2 Andreia Santos-Gonçalves,1,2 and Tiago L. Duarte1,2

1Basic and Clinical Research on Iron Biology Group, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre 823, 4150180 Porto, Portugal
2Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal

Correspondence should be addressed to Tiago L. Duarte; tduarte@ibmc.up.pt

Received 10 October 2014; Revised 16 January 2015; Accepted 19 January 2015

Academic Editor: Maria Carmen Collado

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Nonalcoholic fatty liver disease (NAFLD) is a progressive liver disease with ever-growing incidence in the industrialized world. It starts with the simple accumulation of lipids in the hepatocyte and can progress to the more severe nonalcoholic steatohepatitis (NASH), which is associated with inflammation, fibrosis, and cirrhosis. There is increasing awareness that reactive oxygen species and electrophiles are implicated in the pathogenesis of NASH. Transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a positive regulator of the expression of a battery of genes involved in the protection against oxidative/electrophilic stress. In rodents, Nrf2 is also known to participate in hepatic fatty acid metabolism, as a negative regulator of genes that promote hepatosteatosis. We review relevant evidence in the literature that these two mechanisms may contribute to the protective role of Nrf2 in the development of hepatic steatosis and in the progression to steatohepatitis, particularly in young animals. We propose that age may be a key to explain contradictory findings in the literature. In summary, Nrf2 mediates the crosstalk between lipid metabolism and antioxidant defense mechanisms in experimental models of NAFLD, and the nutritional or pharmacological induction of Nrf2 represents a promising potential new strategy for its prevention and treatment.

1. Hepatic Lipid Metabolism

The liver plays a key role in the processing of lipids, including the synthesis and degradation/oxidation of fatty acids (FA), and the metabolism of cholesterol and phospholipids. Hepatocytes convert the excess dietary glucose into FAs (lipogenesis), which can be stored as triglycerides (TGs) in lipid droplets or used in the generation of phospholipids [1, 2]. Lipogenesis involves acetyl-CoA precursors and is an insulin-dependent process. Under normal conditions, TGs along with cholesterol and phospholipids are assembled into very low density lipoprotein (VLDL) particles that can be secreted into the bloodstream for storage in other tissues in the form of lipid droplets, thus preventing TG accumulation in the cytoplasm of hepatocytes [1, 3]. On the other hand, when the available glucose cannot meet the energy demands, hepatocytes break down TGs and cholesterol stored in lipid droplets through a lysosomal degradative pathway of macroautophagy designated lipophagy. The breakdown of TGs by lipophagy supplies free fatty acids (FFAs) required to sustain rates of mitochondrial \( \beta \)-oxidation for the generation of ATP [4]. FA degradation occurs also in peroxisomes (\( \beta \)-oxidation) and in the endoplasmic reticulum (ER) (\( \omega \)-oxidation).

When fatty acid input exceeds the capacity of \( \beta \)-oxidation, accumulating acyl-CoA is drained by triglyceride synthesis, leading to the supraphysiological accumulation of fat in hepatocytes (hepatosteatosis), which is a hallmark of both alcoholic and nonalcoholic fatty liver disease (NAFLD) [5]. Hepatic steatosis increases the oxidation of FFAs and the rate of the tricarboxylic acid cycle. Increased FFA \( \beta \)-oxidation results in increased rates of electron leakage from the mitochondrial respiratory chain [6], resulting in higher free radical formation, and increases hydrogen peroxide production in the peroxisomes. A decrease in the mitochondrial quinone
pool and the associated reduction of mitochondrial oxidation metabolism were also proposed to account for the increased mitochondrial reactive oxygen species (ROS) production under high-fat diet (HFD) [7]. Microsomal oxidation also contributes to oxidative stress via the activity of cytochromes P450 2E1 and P450 4A. The incorporation of free saturated fatty acids, the first products of de novo lipogenesis, into membrane phospholipids is detrimental to the ER and leads to Ca\(^{2+}\) release, which has the potential to injure adjacent mitochondria and promote apoptotic cell death (lipoapoptosis) [2, 8].

2. NAFLD: Prevalence and Etiology

The increase in obesity has become an alarming public health trend in the industrialized world, with a growing burden on health care costs [9, 10]. Obesity leads to an increased propensity for the development of a complex array of factors that increase the risk to develop cardiovascular disease or type 2 diabetes, which have been termed the metabolic syndrome (abdominal obesity, atherogenic dyslipidemia, hypertension, and insulin resistance) [11]. NAFLD is the hepatic manifestation of the metabolic syndrome. In western countries, NAFLD is estimated to affect 20–30% of the general population, although the incidence may be even higher in obese individuals. The disease is highly correlated to obesity. It starts with the relatively benign accumulation of lipids in the hepatocyte. In up to one-third of patients, steatosis can progress to the more severe nonalcoholic steatohepatitis (NASH), which is associated with inflammation, fibrosis, and impaired liver function (cirrhosis) [12].

The detailed pathological mechanisms leading to the transition from lipid deposition to necroinflammation and cytotoxicity remain unclear, but a “two-hit” theory is widely advocated in the literature [14]. The first hit corresponds to “simple” hepatocellular lipid accumulation (steatosis) resulting from increased inflow of FFAs derived from insulin resistant adipose tissue, increased hepatic de novo lipogenesis, or impaired lipid export from hepatocytes. The second hit may be a consequence of increased ROS production in the liver resulting from the increased metabolism of FFAs, resulting in mitochondrial dysfunction, upregulation of proinflammatory cytokines, and activation of hepatic stellate cells, which begin to proliferate and increase production of collagen, causing fibrosis. There is nevertheless growing evidence that the development of NAFLD and the progression to NASH are more complex processes than initially predicted by the two-hit theory. For instance, while hepatic TG formation is an early indicator of liver metabolic stress and disease, it is unlikely to be the initiating step in NASH. Instead, TGs may represent harmless storage compartments that divert FFAs away from potentially toxic pathways (e.g., microsomal oxidation), thus protecting hepatocytes from lipoapoptosis [5, 8, 15]. Moreover, multiple mechanisms/factors may contribute to the progression of steatosis to NASH (second hit), including increased ROS production in the liver (via activation of inflammatory cells, mitochondrial dysfunction, or the uncoupling of cytochrome P450 2E1 and 4A isoenzymes), the failure of hepatocytes to synthesize sufficient endogenous antioxidants, ER stress, liver and adipose tissue inflammation, decreased autophagic function, and/or the mild hepatic iron overload that is frequently seen in NAFLD patients [8, 16, 17].

3. Nrf2 Activation by Hepatic Oxidants/Electrophiles

The liver has relatively high metabolic activity and is the main organ responsible for the biotransformation and subsequent detoxification of xenobiotics. These properties place the organ at an increased risk for exposure to ROS [2] and electrophiles [30] which, in turn, are increasingly implicated in the pathogenesis of NAFLD and other chronic liver diseases. In the hepatocyte, the major sites of ROS production are the mitochondria and the cytochrome P450 system. Electrophiles are produced by oxidation and nitration of unsaturated FFAs, resulting in a series of reactive species, including α,β-unsaturated aldehydes such as 4-hydroxynonenal (4-HNE) [30]. Hepatocytes are equipped with multiple defense systems that ensure protection against the toxic effects of endogenous and exogenous oxidants and electrophiles to which they are exposed, including (a) phase 1 enzymes that introduce functional groups onto largely hydrophobic organic molecules, such as cytochrome P450 enzymes, whose activity may produce highly reactive products that are toxic to the cell; (b) phase 2 enzymes like glutathione S-transferases and UDP glucuronosyl transferases, which conjugate the products of phase 1 enzymes with hydrophilic groups in order to facilitate their excretion, and antioxidant enzymes like superoxide dismutases, glutathione peroxidase, and catalase, which inactivate ROS; (c) phase 3 efflux transporters that export toxic metabolites acting synergistically with phase 2 enzymes to provide protection against electrophiles and carcinogens; and (d) thiol containing molecules such as glutathione and thioredoxin that function to maintain reducing conditions within the cell and inactivate electrophilic compounds [31]. Importantly, many of these cytoprotective enzymes are encoded by genes containing antioxidant response elements (AREs) in their promoter regions. AREs were initially identified as 5′(G/A)TGA(G/C)nnnCG(G/A)3 [32] and subsequently expanded to 5′TMAnnRTGAYnnnGCwwww3′, where M = A or C, R = A or G, Y = C or T, W = A or T, and S = G or C [33].

The ARE is a cis-acting enhancer sequence that mediates transcriptional activation of genes in response to changes in the cellular redox status, such as during increased production of free radical species, or to prooxidant xenobiotics that are thiol reactive and mimic an oxidative insult [34]. Transcription factor nuclear factor-erythroid 2-related factor 2 (Nfe2L2/Nrf2) is a basic leucine zipper transcription factor that regulates transcriptional induction of ARE-containing genes encoding antioxidant enzymes, electrophile-conjugating enzymes, ubiquitin/proteasomes, and chaperone and heat-shock proteins in response to cellular stresses including ROS [35, 36]. Under normal conditions,
**Figure 1**: Activation of the Keap1-Nrf2-ARE pathway by oxidants/electrophiles. Under homeostatic conditions, Nrf2 is mainly localized in the cytoplasm through an interaction with Keap1 and the actin cytoskeleton. Keap1 is a five-domain protein consisting of an N-terminal broad complex, Tramtrack and Bric-à-brac (BTB) domain, an intervening region with cysteine (Cys) residues, a C-terminal Kelch domain with double glycine repeats (DGR), and the C-terminal domain. Keap1 homodimerizes at the BTB domain, which is also a binding site for Cullin 3 (Cul3). The Keap1 homodimer binds to a single Nrf2 molecule through the ETGE and DLG motifs of Nrf2, each binding to a DGR domain in Keap1. According to the proposed hinge and latch model [13], ETGE is a high-affinity motif ("hinge") whereas DLG is a low-affinity one ("latch"). Keap1 functions as an adaptor protein in the Cul3-based E3 ligase complex, which results in the polyubiquitination (Ub) of the lysine residues situated between the DLG and ETGE motifs, and subsequent proteasomal degradation of Nrf2. Under stressed conditions, the modification of critical cysteine residues of Keap1 destabilizes its binding to the DLG motif of Nrf2, which blocks ubiquitination/proteasomal degradation and allows Nrf2 to escape Keap1 control and translocate into the nucleus. In the nucleus, Nrf2 heterodimerizes with small Maf proteins and promotes the expression of ARE-containing genes involved in cell stress response, drug metabolism, detoxification, and transport. Nrf2 may also be phosphorylated (P) by stress-activated kinases.

Nrf2 is mainly localized in the cytoplasm through an interaction with Kelch ECH associating protein 1 (Keap1) and the actin cytoskeleton. Despite the fact that Nrf2 mRNA is constitutively expressed, Keap1 targets Nrf2 for polyubiquitination and degradation, resulting in a short protein half-life. The binding to and regulation of Nrf2 by Keap1 have been explained by a “hinge and latch model” [13], as described in Figure 1. During exposure to electrophiles or oxidative stress, Keap1 becomes oxidized at critical cysteine residues. As a result, Nrf2 escapes Keap1 control and translocates to the nucleus, where it dimerizes with small musculoaponeurotic fibrosarcoma (Maf) proteins and promotes the expression of ARE-containing genes [36–38]. In recent years, it has become apparent that Nrf2 activity is not controlled exclusively through Keap1-mediated proteasomal degradation. In addition to Keap1, Nrf2 protein stability is regulated by another E3 ubiquitin ligase adaptor, β-transducin repeat-containing protein (β-TrCP). Nrf2 degradation independent of Keap1 is promoted by glycogen synthase kinase 3 (GSK-3), which phosphorylates specific serine residues in the Neh6 domain of Nrf2 corresponding to the β-TrCP recognition motif [39]. Alternatively, Nrf2 activation may occur as a result of Nrf2 phosphorylation by mitogen activated protein kinase cascade, phosphatidylinositol 3-kinase, protein kinase C, and protein kinase RNA-like endoplasmic reticulum kinase (Perk) [36, 40].

In addition to the modulation of protein stability, Nrf2 activity is also regulated at the transcriptional level. Polycyclic aromatic hydrocarbons activate Nrf2 transcription through binding of the heterodimer formed by the aryl hydrocarbon receptor (AhR) and the AhR nuclear translocator to xenobiotic response element-like sequences in the Nrf2 promoter [41]. In certain tumor cells, activation of Nrf2 transcription by Kras oncogene is responsible for increased chemoresistance [42]. Moreover, basal Nrf2 activity seems to be regulated by epigenetic mechanisms and miRNA species, as reviewed elsewhere [43].

**4. Nrf2 Protection against Liver Injury**

As discussed, the Keap1-Nrf2-ARE pathway is activated in response to oxidative/electrophilic stress and regulates the basal and inducible expression levels of a battery of proteins involved in the detoxification of reactive molecules in the cytosol, mitochondria, and ER [30]. Overall, this transcriptional response protects cells against a series of insults and favors cell adaptation/survival [40, 44]. Cellular survival is also mediated by the UPR, which restores ER homeostasis.
and by the autophagy-lysosomal pathway, which promotes the degradation of proteins and dysfunctional organelles. There is increasing evidence that hepatic steatosis and ER stress are interconnected. This is not surprising since several enzymatic lipogenic pathways are located in the ER, including fatty acid elongation, cholesterol biosynthesis, complex lipid biosynthesis, and assembly of VLDL particles. In fact, abrogation of the UPR results in ER stress-induced development of steatosis [45]. During the UPR, Perk-dependent phosphorylation may also lead to Nrf2 nuclear translocation and increased transcription of Nrf2 target genes [40]. Nrf2 is also activated during autophagy, via interaction of the selective autophagy substrate p62 with the Nrf2 binding site on Keap1 [46]. Accumulation of p62 (as a consequence of a deficiency in autophagy) results in stabilization of Nrf2 and transcriptional activation of Nrf2 target genes [46]. A study by Kwon et al. demonstrated that high steady-state expression of NAD(P)H dehydrogenase, quinone 1, sustained by p62-induced basal Nrf2 activation, is required to maintain mitochondrial integrity [47]. Other studies have reported that Nrf2 deficiency results in mitochondrial depolarization, reduced ATP production, and decreased rate of oxygen consumption (mitochondrial respiration) [48], as well as less efficient mitochondrial fatty acid oxidation [49].

Several studies have demonstrated that Nrf2−/− mice are more susceptible to chemical-induced oxidative/electrophilic stress in the liver than wild-type mice [50–52]. Nrf2 protects mice from 2,3,7,8-tetrachlorodibenzo-p-dioxin- (TCDD-) induced oxidative damage and steatohepatitis [53] and from hepatic fibrosis caused by chronic treatment with the hepatotoxin carbon tetrachloride [54]. Nrf2-deficient hepatocytes are also more susceptible to the toxicity of excessive iron accumulation [55].

5. Nrf2 in Liver Regeneration and Aging

The liver is the only organ in the human body capable of completely regenerating itself after injury. In the regenerating liver, hepatocytes accumulate a significant amount of lipids within lipid droplets, which are mainly composed of triacylglycerol and cholesteryl esters [56–58]. There is growing evidence that Nrf2 is required for effective tissue repair. Cell regeneration is diminished in hepatectomized Nrf2-null mice, which is associated with increased oxidative stress, reduced insulin/insulin growth factor-1 signaling [59], and reduced expression of the gene encoding a hepatotropic factor, augmenter of liver regeneration [60]. Importantly, the ability of the liver to regenerate after hepatectomy or chemical injury declines with old age [61]. Aging is also associated with increased lipid accumulation in the liver, which may ultimately result in lipotoxicity. Aging has been reported to increase the prevalence of the metabolic syndrome and of NAFLD in the human population and to enhance the progression to NASH and fibrosis [61]. Nrf2 plays an important role in the hepatic aging process. With age, there is a substantial reduction in glutathione (GSH) levels and in the expression and activity of glutamate cysteine ligase, the rate-controlling enzyme in GSH synthesis. This is accompanied by lower levels of Nrf2 protein and a reduction in Nrf2/ARE binding [62], as well as increased markers of protein and lipid oxidation [63]. Conversely, the liver of aged Nrf2-null mice shows lower free radical reducing activity [64] and GSH synthesis. The reason why aging organisms gradually lose the ability to activate Nrf2 is currently not understood [43], but a decline in Nrf2 signaling is presumed to contribute to the age-related hepatic oxidative stress. Whether it also contributes to the increased progression from NAFLD to NASH and fibrosis in the elderly is a subject that warrants further investigation.

6. Nrf2 Regulation of Hepatic Lipid Metabolism

Besides activating antioxidant and detoxification genes in response to electrophilic or oxidative stress, there is increasing evidence that Nrf2 participates in hepatic fatty acid metabolism (Table 1). A microarray study by Yates et al. [19] showed that the genetic or pharmacological activation of Nrf2 represses the expression of key enzymes involved in fatty acid synthesis, with concomitant reduction in the levels of hepatic lipids. A global analysis of constitutive hepatic protein expression in Nrf2-null and wild-type mice subsequently identified two main groups of Nrf2-regulated proteins. One group comprised proteins involved in phase II drug metabolism and antioxidant defense, for which expression was enhanced in the Nrf2 wild-type animals. The other group corresponded to proteins involved in the synthesis and metabolism of fatty acids and other lipids, and unlike proteins involved in the cellular defense, these proteins were expressed to a higher level in the Nrf2−/− animals [20]. It is worth noting that both studies were performed with young adult mice (9–10 weeks of age). Another study performed with mice at 8 weeks of age has reported that, under control diet, mRNAs of sterol regulatory element-binding protein-1c and fatty acid synthase were more expressed in Nrf2-null animals than in the wild-type [18]. However, studies employing older mice (12–25 weeks of age) of the same genetic background (C57BL/6) showed that Nrf2 has little effect in hepatic fatty acid metabolism in animals fed control diets [21–23]. Studies in which mice received HFD have also reported that hepatic lipogenesis is negatively regulated by Nrf2 [21, 24]. Once again, studies using older mice (at approximately 6 months of age) either failed to detect an effect [23] or identified Nrf2 as an activator of genes involved in lipid synthesis and uptake (e.g., sterol regulatory element-binding protein, fatty acid synthase, stearoyl-CoA desaturase-1, and peroxisome proliferator-activated receptor-γ) via the induction of nuclear receptor small heterodimer partner (Shp/Nrob2) gene transcription and a downmodulator of genes regulating fatty acid oxidation (e.g., peroxisome proliferator-activated receptor-α, acetyl-CoA oxidase, and carnitine palmityltransferase lα) [25]. It is worth noting that while all studies utilized mice with the C57BL/6 genetic background, the backcross to C57BL/6 was incomplete in the latter study (only 4 backcrosses) [25].

In summary, Nrf2 appears to protect the liver against steatosis by inhibiting lipogenesis and promoting fatty acid oxidation. This may be explained by the activation of
reported that old Ldlr with age. This is supported by a work of Collins et al., who of lipids and/or the attenuation of the antioxidant defenses be a consequence of the progressive hepatic accumulation dependence of the Nrf2 regulation of hepatic lipogenesis may the literature (Table 1). It is tempting to speculate that the age-dependence of the Nrf2 regulation of hepatic lipogenesis may be a consequence of the progressive hepatic accumulation of lipids and/or the attenuation of the antioxidant defenses with age. This is supported by a work of Collins et al., who reported that old Ldlr−/− mice (a model that mimics human NASH and atherosclerosis) suffer increased hepatocyte damage when fed a HFD. When compared with young animals, aged Ldlr−/− mice on HFD showed a decline in the expression of antioxidant genes, which was directly related with a decrease in Nrf2 expression [65]. In addition, these animals displayed more severe hepatic steatosis, along with inflammation and fibrosis (NASH), while their younger counterparts simply developed fatty livers [66]. Studies of Nrf2 activation and/or deficiency in aged mice (>50 weeks of age) would be informative, especially since metabolic disorders are known to have a strong age-dependence.

### Table 1: The effect of Nrf2 activation/deficiency on hepatic lipid accumulation in C57BL/6 mice.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age (weeks)</th>
<th>Hepatic lipid accumulation</th>
<th>Expression of FA synthesis genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Standard diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>Increased steatosis in Nrf2−/−. No difference in TGs</td>
<td>Higher in Nrf2−/−</td>
<td>[18]</td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>Reduced by Nrf2 activation</td>
<td>Reduced by Nrf2 activation</td>
<td>[19]</td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>Not reported</td>
<td>Higher in Nrf2−/−</td>
<td>[20]</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>No difference in TGs</td>
<td>No difference</td>
<td>[21]</td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>Tendency for increased TGs in Nrf2−/−</td>
<td>Tendency for higher levels in Nrf2−/−</td>
<td>[22]</td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>No difference in TGs</td>
<td>No difference</td>
<td>[23]</td>
</tr>
<tr>
<td>(b) High-fat diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>Increased FFAs. No difference in TGs</td>
<td>Higher in Nrf2−/−</td>
<td>[24]</td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>Tendency for increased TGs in Nrf2−/−</td>
<td>No difference</td>
<td>[22]</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>Reduced lipid content by Nrf2 activation</td>
<td>Reduced by Nrf2 activation</td>
<td>[24]</td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>No difference in TGs</td>
<td>No difference</td>
<td>[23]</td>
</tr>
<tr>
<td>Male</td>
<td>26</td>
<td>Reduced total lipid and TGs in Nrf2−/−</td>
<td>Lower in Nrf2−/−</td>
<td>[25]</td>
</tr>
</tbody>
</table>

FFA, free fatty acids; TG, triglyceride.

ARE-containing transcription factors that regulate adipocyte differentiation and adipogenesis (e.g., CCAAT/enhancer-binding protein β, peroxisome proliferator-activated receptor-γ, aryl hydrocarbon receptor, and retinoid X receptor-α) and by the protection against redox-dependent inactivation of metabolic enzymes (e.g., 3-hydroxy-3-methylglutaryl-CoA reductase) [43], as well as by other mechanisms that remain unidentified. Future studies aimed at elucidating the molecular basis of these observations are warranted and authors need to take into account the interference of potential confounding factors. As reviewed herein, the role of Nrf2 on hepatic lipid processing in mice appears to be greatly dependent on the age of the animals, whereas factors such as mouse genetic background or gender do not appear to explain most of the contradictory findings in the literature (Table 1). It is tempting to speculate that the age-dependence of the Nrf2 regulation of hepatic lipogenesis may be a consequence of the progressive hepatic accumulation of lipids and/or the attenuation of the antioxidant defenses with age. This is supported by a work of Collins et al., who reported that old Ldlr−/− mice (a model that mimics human NASH and atherosclerosis) suffer increased hepatocyte damage when fed a HFD. When compared with young animals, aged Ldlr−/− mice on HFD showed a decline in the expression of antioxidant genes, which was directly related with a decrease in Nrf2 expression [65]. In addition, these animals displayed more severe hepatic steatosis, along with inflammation and fibrosis (NASH), while their younger counterparts simply developed fatty livers [66]. Studies of Nrf2 activation and/or deficiency in aged mice (>50 weeks of age) would be informative, especially since metabolic disorders are known to have a strong age-dependence.

### 7. Nrf2: A Therapeutic Target in NAFLD/NASH?

Despite its high prevalence in the industrialized world, there is currently no approved pharmacological treatment for NAFLD. It is crucial to develop and establish new options for the prevention and treatment of NAFLD/NASH. While there is still limited evidence in the literature that Nrf2 is activated in human subjects with NASH [67], Nrf2 deficiency has been repeatedly reported to favor the development of steatohepatitis and fibrosis in rodent models of NASH. In these studies, Nrf2-null animals developed many features of NASH when fed methionine- and choline-deficient (MCD) diet [18, 68, 69], high-fat diet [70], or high-fat and high-cholesterol diet [71]. These studies suggest that Nrf2 plays a key role in limiting the progression of NASH, which could be ascribed to the activation of antioxidative stress response genes but also to the modulation of fatty acid metabolism in hepatocytes. It would be important to assess the efficacy of Nrf2-activating compounds in preventing or treating NAFLD/NASH.

A great variety of thiol-reactive, electrophilic compounds isolated from dietary sources or plants are capable of activating Nrf2/ARE-dependent gene expression through inhibition of Keap1-mediated degradation [72–74]. Nrf2 inducing compounds can be grouped into different categories: (1) pharmacological antioxidants (cafeic acid, epigallocatechin-3-gallate, and butylated hydroxyanisol); (2) dithiolethiones (oltipraz, 3H-1,2-dithiol-3-thione); (3) isothiocyanates (sulforaphane); and (4) triterpenoids (oleanolic acid). The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its derivative 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) are also potent inducers of Nrf2/ARE signaling [19, 75]. Studies demonstrating the in vivo chemopreventive and/or neuroprotective properties of oltipraz [74, 76], sulforaphane [77], and CDDO-Im [78] indicate a potential therapeutic usefulness for these Nrf2-activating molecules. In addition, new pharmacological Nrf2 activators have been synthesized in recent years, some of which have already entered the clinical trial stage [37, 79], including Protandim, dimethyl fumarate (BG-12), and CDDO-Me (bardoxolone methyl). Protandim (LifeVantage) is a dietary supplement that consists of five low-dose natural Nrf2 activators that activate Nrf2 through multiple
kinase pathways [80]. BG-12/Tecfidera, an oral therapeutic agent containing dimethyl fumarate (Biogen Idec), has been recently approved for the treatment of multiple sclerosis [81]. Bardoxolone methyl (methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl) (Reata Pharmaceuticals) was shown to restore kidney function in patients with chronic kidney disease [82], although a recent phase III trial had to be terminated due to adverse effects (http://www.reatapharma.com/).

Whilst there have not been any clinical trials specifically on the effects of Nrf2 activation on liver disease, a number of studies have investigated the effects of Nrf2 activators in rodent models of NAFLD and NASH (Table 2). Oral administration of CDDO-Im, one of the most potent activators of Nrf2 in mouse liver known to date [83], prevented high-fat diet induced obesity and hepatic lipid accumulation in wild-type mice but not in Nrf2-null mice [19]. Likewise, oral CDDO-Me administration reduced hepatic lipid accumulation, lipogenic gene expression, and proinflammatory cytokine expression and ameliorated type 2 diabetes in mice fed HFD [26].

Sulforaphane [(-)-1-isothiocyanato-(4R)-methylsulfinylbutane], a natural-occurring isothiocyanate from cruciferous vegetables, strongly induces Nrf2 and ARE-mediated transcription activation through inhibiting Keap1-mediated Nrf2 degradation [84]. Treatment of rats with sulforaphane increases liver mitochondrial antioxidant defenses and protects from prooxidant-induced opening of the mitochondrial permeability transition pore [85]. A study by Oh et al. demonstrated that sulforaphane suppressed transforming growth factor-β-inducible expression of α-smooth muscle actin and profibrogenic genes in an immortalized human hepatic stellate cell line and attenuated bile duct ligation-induced liver fibrosis in mice [27]. In a study by Okada et al., long-term supplementation with sulforaphane suppressed the oxidative stress, inflammation, and hepatic fibrosis induced by MCD diet in mice [28]. Recently, Shimozono et al. [29] used two chemically distinct types of Nrf2 activator, namely, the dithiolethione oltipraz and a novel biaryl urea compound, termed NK-252 (1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea). The administration of both agents significantly reduced hepatic fibrosis in rats on a choline-deficient L-amino acid-defined diet. Whilst these studies suggest that Nrf2 activation presents new opportunities for treatment of NASH patients with hepatic fibrosis, it is important to bear in mind that NASH is closely related to overnutrition, insulin resistance, and obesity and not to a deficiency of amino acids such as methionine and choline [86]. Ideally, the use of Nrf2-activating compounds should be tested in animal models of NASH associated with obesity, insulin resistance, or dyslipidemia.

### 8. Conclusions

A schematic overview of the proposed protective roles of Nrf2 in NAFLD is depicted in Figure 2. Rodent studies suggest that Nrf2 has a dual protective role in the progression from hepatic steatosis to steatohepatitis: (i) the negative regulation of genes that promote lipid accumulation in hepatocytes (“first hit”), likely by a combination of mechanisms that remain poorly understood; (ii) the activation of genes that promote the elimination of ROS and electrophiles derived from lipid peroxidation, thus preventing hepatocellular oxidative stress and mitochondrial dysfunction (“second hit”). Apparently, these protective mechanisms become less efficient with aging, which is expected to contribute to disease progression. The nutritional/pharmacological induction of Nrf2 signaling represents as a promising potential new strategy for the prevention and treatment of NAFLD/NASH.

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>CDDO-Im</td>
<td>1-[(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl)imidazole</td>
</tr>
<tr>
<td>CDDO-Me</td>
<td>1-[(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl)methyl</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>MCD</td>
<td>Methionine- and choline-deficient</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Nonalcoholic steatohepatitis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
</table>

### Table 2: The effects of Nrf2 activators in rodent models of NAFLD or NASH.

<table>
<thead>
<tr>
<th>Nrf2 activator</th>
<th>Species</th>
<th>Administration route</th>
<th>Reported effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDO-Im</td>
<td>Mouse</td>
<td>Oral</td>
<td>Prevented HFD-induced increases in body weight, adipose mass, and hepatic lipid accumulation</td>
<td>[24]</td>
</tr>
<tr>
<td>CDDO-Me</td>
<td>Mouse</td>
<td>Oral</td>
<td>Reduced hepatic lipid accumulation, proinflammatory cytokine expression, and lipogenic gene expression in mice fed HFD</td>
<td>[26]</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>Mouse</td>
<td>Intraperitoneal</td>
<td>Attenuated hepatic fibrosis induced by bile duct ligation</td>
<td>[27]</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>Mouse</td>
<td>Oral</td>
<td>Suppressed oxidative stress and hepatic fibrosis induced by MCD diet</td>
<td>[28]</td>
</tr>
<tr>
<td>Oltipraz, NK-252</td>
<td>Rat</td>
<td>Oral</td>
<td>Attenuated hepatic fibrosis induced by CDAA diet</td>
<td>[29]</td>
</tr>
</tbody>
</table>

CDAA, choline-deficient L-amino acid-defined diet; CDDO-Im, 1-[(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl)imidazole; CDDO-Me, 1-[(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl)methyl; HFD, high-fat diet; MCD, methionine- and choline-deficient diet.
Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by FEDER funds through the COMPETE (Operational Competitiveness Programme) and national funds through FCT (Fundação para a Ciência e a Tecnologia) under the project FCOMP-01-0124-FEDER-028447 (PTDC/BIM-MET/0739/2012).

References


Z. Tariq, C. J. Green, and L. Hodson, “Are oxidative stress mechanisms the common denominator in the progression from hepatic steatosis towards non-alcoholic steatohepatitis (NASH)?” *Liver International*, vol. 34, no. 7, pp. e180–e190, 2014.


P. Rada, A. I. Rojo, S. Chowdhry, M. McMahon, J. D. Hayes, and A. Cuadrado, “SCF/β-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner,” *Molecular and Cellular Biology*, vol. 31, no. 6, pp. 1121–1133, 2011.


W. Miao, L. Hu, P. J. Scrivens, and G. Batist, “Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by


**Review Article**

**How Inflammation Impinges on NAFLD: A Role for Kupffer Cells**

Nádia Duarte,1,2 Inês C. Coelho,1,2 Rita S. Patarrão,1,2 Joana I. Almeida,2 Carlos Penha-Gonçalves,2,3 and M. Paula Macedo1,3

1CEDOC, NOVA Medical School, Faculdade de Ciências Médicas (NMS/FCM), Universidade Nova de Lisboa, Edifício CEDOC II, Rua Câmara Pestana, Nos. 6, 6A, 6B, Laboratório 3.8, Piso 3, 1150-082 Lisboa, Portugal
2Instituto Gulbenkian de Ciência (IGC), Rua da Quinta Grande 6, 2780-156 Oeiras, Portugal
3APDP-ERC Portuguese Diabetes Association Education and Research Center, Rua do Salitre, No. 118-120, 1250-203 Lisbon, Portugal

Correspondence should be addressed to M. Paula Macedo; paula.macedo@fcm.unl.pt

Received 7 November 2014; Accepted 5 March 2015

Academic Editor: Maria Carmen Collado

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Nonalcoholic fatty liver disease (NAFLD) is rapidly becoming the most prevalent cause of liver disease worldwide and afflicts adults and children as currently associated with obesity and insulin resistance. Even though lately some advances have been made to elucidate the mechanism and causes of the disease much remains unknown about NAFLD. The aim of this paper is to discuss the present knowledge regarding the pathogenesis of the disease aiming at the initial steps of NAFLD development, when inflammation impinges on fat liver deposition. At this stage, the Kupffer cells attain a prominent role. This knowledge becomes subsequently relevant for the development of future diagnostic, prevention, and therapeutic options for the management of NAFLD.

1. Introduction

We are facing a rampant epidemic of nonalcoholic fatty liver disease (NAFLD) which afflicts adults and children and is frequently associated with obesity and insulin resistance. In Europe and in the United States, it is estimated that 3 out of 10 adults developed NAFLD [1, 2]. As NAFLD is mostly asymptomatic, such estimates have a high degree of uncertainty. For example, European data suggest that NAFLD prevalence can fall in a wide range, between 2 and 44% in the general population, and is present in 42.6–69.5% of people with type 2 diabetes [3]. NAFLD prevalence in obese children was found to reach values as high as 36–44% regardless of the diagnostic criteria. A study that evaluate children in the Japanese population observed that fatty liver may occur in children as young as 6 years of age and is directly related to the degree of obesity and specifically to the abdominal subcutaneous fat thickness [4]. Remarkably, women with history of gestational diabetes have a greater prevalence of NAFLD and prospective studies estimate that these women are at a higher risk of developing diabetes [5]. Another group that presents high prevalence of fatty liver is the elderly representing a public health concern for developed countries with aged populations. The Rotterdam study describes that NAFLD was present in more than one-third of the assessed elderly with a sturdy association with dysglycemia, dyslipidemia, and abdominal fat [6]. More interestingly, the subjects with advanced age show a decrease incidence of NAFLD suggesting the possibility of positive selection of elderly without NAFLD [6].

The estimation of NAFLD impact at population level poses two questions. (1) Realistically, should we assess NAFLD recurrently in the population? (2) Is there a good, easy, and not costly way of doing it? At a first glance, NAFLD could be seen as a response mechanism to deal with increased amounts of lipids intake, representing a protective mechanism. To identify the disease in early stages, we would need liver biopsies, the recognized gold standard for NAFLD diagnosis. Obviously, the invasive nature of such procedure excludes its widespread use. The more widely used ultrasound, however, will not inform us of the grade of liver inflammation. Magnetic resonance imaging and computed
tomography even though more sensitive are expensive and cannot distinguish between simple steatosis and the severe form of NAFLD termed nonalcoholic steatohepatitis (NASH) [7]. Alternatively, several indexes composed of physical and biochemical parameters such as the fatty liver index (FLI) have been proposed to evaluate NAFLD at the population level [8]. Undesirably, such methods do not detail the degree of fat neither the grade of inflammation in the liver. In this scenario, NAFLD evaluation calls for novel biomarkers that reflect relevant NAFLD physiopathology mechanisms providing a solid basis for diagnosis and possible innovative therapeutic approaches.

2. Ectopic Accumulation of Triglycerides, Hepatic Steatosis

Triglycerides (TG) accumulation is an efficient energy storage mechanism. When compared to carbohydrates (4.5 kcal/g) or proteins (4 kcal/g), TG provides higher caloric intake (9 kcal/g) [9]. As so, it is advantageous for the organism to convert carbohydrates and amino acids into TG to be stored in adipose tissue, in order to be used in times of fasting or prolonged exercise [10].

Physiologically, TG are a way of balanced energy storage. However, over the last decades, the excessive consumption of fat and sugars, associated with excessive caloric intake, has led to alterations in lipids and glucose metabolism. The current recommendations for adults are that 20% to 35% of the daily calories ingested are derived from dietary lipids. Nevertheless, this value is estimated to be around 40% in western diets [11]. The imbalance in lipids metabolism is tightly associated with several diseases such as obesity, diabetes, and NAFLD [12], representing an emerging health concern.

Overconsumption of fat and sugar ends up in ectopic lipids accumulation, with the liver being one of the prime targets in this process. Hepatic accumulation results from a disparity between lipids availability (from circulating lipid targets in this process. Hepatic accumulation results from a disparity between lipids availability (from circulating lipid targets to the liver [32]. In contrast to hepatic glucose, fructose is other liver diseases or healthy control individuals [30,31].

Increased oxidative stress was observed in NASH patients and in animal model in association with accumulation of oxidized lipids [26]. Taken together, evidences show that SFA have a huge impact on hepatocytes viability. High fat and high carbohydrate diets have a vast SFA component, with higher SFA/MUFA ratio, which is critical to NAFLD progression.

Highlighting the role of diet composition, it was shown that NAFLD and NASH patients had a dietary intake richer in SFA and cholesterol and poorer in polyunsaturated FFA and fibers [27]. A lower intake of proteins and zinc was also shown in these patients [28].

3. The Role of Carbohydrates

Evidence suggests that high sugar intake combined with lipid oversupply exacerbates liver pathology. Fructose is a popular monosaccharide used industrially in the form of high-fructose corn syrup (45% glucose; 55% fructose) to sweeten foods and beverages [29]. Consumption of fructose has markedly increased and is estimated to be 2-3 times greater in patients with NAFLD, compared with patients with other liver diseases or healthy control individuals [30,31].

In humans, all fructose that is ingested is metabolized by the liver [32]. In contrast to hepatic glucose, fructose is
neither converted into glycogen nor stimulates postprandial ghrelin or insulin secretion, failing to initiate the central satiety response [33]. Moreover, this carbohydrate was reported to stimulate hepatic DNL through increasing the expression of two transcription factors, sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP), that regulate the activity of lipogenic enzymes [34]. Comparison of fructose and glucose intake in human studies revealed that the first is more lipogenic than the later (10% versus 2% increase in DNL) [35]. A recent study suggested that fructose consumption may specifically promote lipid deposition in visceral adipose tissue, particularly in men, whereas glucose consumption appears to favor subcutaneous adiposity [36].

Fructose per se plays a role in triggering inflammation, possibly through the disruption in gut microbiota and increased mucosal permeability. Spruss et al. [37] showed that mice exposed to fructose exhibited markedly intestinal bacterial overgrowth and increased intestinal permeability, leading to an endotoxin-dependent activation of hepatic Kupffer cells (KCs). Increased plasma concentration of tumor necrosis factor (TNF)-α, portal lipopolysaccharide (LPS), and myeloid differentiation primary response gene (MyD)88 have observed these mice [37].

Taken together, these results show fructose as an important dietary contributor to NAFLD pathogenesis and severity [38], being more detrimental than other carbohydrates.

4. Nonalcoholic Steatohepatitis and the Multiple Hit Hypothesis

The severe form of NAFLD disease is termed as NASH and is characterized by the presence of hepatocellular injury, lobular and/or portal inflammation, and frequently, deposition of collagen fibers [39]. Although patients with steatosis are at increased risk of progressing to severe forms of liver disease, NASH may develop in absence or in presence of very little steatosis [39]. This indicates that steatosis and inflammation may not always be sequential events. The initial view that considered steatosis as a primary hit and gut-derived endotoxin as the second hit determining progression of the disease is now followed by a more complex model where many hits may act in parallel [40]. Dysregulation in lipid metabolism associated with diet-induced changes in microbiota and increased proinflammatory signaling to the liver together with the liver local inflammatory response is amongst the hits involved in NASH [40]. In light of this model, hepatic inflammation may be either the cause or consequence of steatosis, but it is always determinant in tipping the balance towards NASH and worse prognosis.

5. The Gut/Adipose Tissue/Liver Axis

Recent evidences place the liver and its local response to metabolic and immune mediated disturbances in the gut-adipose tissue axis [41]. Understanding how this interplay is disturbed in NASH constitutes the current challenge. Certain dietary factors may promote lipotoxicity and/or induce alterations in the gut microbiota that may result in abnormal activation of the gut immune response and bacterial product translocation with systemic impact on immune system modulation [42]. It was observed that mice deficient in inflammasomes, which are cytoplasmic multiprotein complexes that sense endogenous or exogenous pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), showed perturbations in intestinal microbiota. When placed under methionine-choline-deficient (MCD) diet for 4 weeks, known to cause similar features of human NASH, the inflammasome deficient mice developed severe symptoms like a marked impairment in glucose homeostasis, increased weight gain, and NASH development that could be transferred to wild-type cohoused mice. These experiments clearly associate changes in microbiota with NAFLD development and obesity [41]. In support, high levels of endotoxin in circulation have been associated with NAFLD development in both human and mouse studies [43]. Disturbances in nutrient absorption are other suggested potential adverse effects of gut microbiota imbalances [40, 44]. Complex carbohydrates can be fermented by certain gut bacteria and converted into short-chain fatty acids that stimulate DNL [43]. Adipose tissue dysfunction in its turn is highly linked to the development of hepatic steatosis. Excessive adiposity leads to insulin resistance, augmented lipolysis, increase FFA circulation, and secretion of inflammatory mediators that promote liver steatosis and organ inflammation [10, 45].

6. Main Inflammatory Mediators

6.1. The Innate Immune Receptors. The innate immune system recognizes immunogenic signals through pattern recognition receptors (PRRs). Bacterial products such as LPS, lipoproteins, flagellins, and peptidoglycans are amongst the PAMPs recognized by these receptors. Endogenous DAMPs like heat shock proteins, high mobility group box 1 (HMGB1), and breakdown products of extracellular matrix liberated from tissue damage and cell death also signal through PRRs [46]. PRRs have been implicated in the pathophysiology underlying NASH. TLRs and in particular TLR4 that recognizes LPS from gram-negative bacteria and TLR9 that recognizes bacteria-derived CpG-containing DNA have been proven critical for several aspects of disease [47, 48]. Nucleotide oligomerization domain- (NOD-) like receptors (NLRs) are intracellular PRRs that are part of the inflammasomes briefly mentioned above. Inflammasomes are multiprotein complexes that through NLRs sense intracellular danger signals and initiate an activation cascade of events that culminate with autoactivation of caspase 1 and cleavage of prointerleukin- (IL-1β and proIL-18 into mature forms [42]. By controlling the release of these important inflammatory cytokines, Inflammasomes play an important role in the inflammatory process underlying NASH.

TLR signaling and inflammatory cytokines like IL-1 regulate the activation of the transcription factor NF-κB, a critical modulator of liver immune responses [49].
6.2. TLR4, TLR9, and Inflammasomes. TLRs are found in several cellular components like plasma membrane and endosomes and the majority associates with a common adaptor molecule, MyD88 through Toll/IL-1 receptor (TIR) domain. Trafficking and location of TLRs within the cell are important for ligand binding and downstream signaling transduction. TLR4 is located mainly in the plasma membrane and associates with the LPS binding protein CD14 and myeloid differentiation- (MD)-2 molecule to recognize LPS. TLR4 can also bind to TIR-domain containing adapter inducing interferon-β (TRIF) to induce type 1 interferon (IFN) through Myd88 independent pathway [50]. Endosomal TLR4 seems to recruit preferentially TRIF while plasma membrane TLR4 recruits MyD88 and engages MAP kinases and NF-κB signaling [51]. Amelioration of steatosis and NASH achieved by TLR4 deficiency in several diet-induced mouse models of NAFLD has placed this molecule at the center of inflammation driven pathology [37, 43, 48, 52, 53]. Expression of proinflammatory cytokines is suppressed in contributing to leukocyte infiltration and fibrogenesis [50]. Increased LPStranslocation into portal vein is thought to activate the liver resident macrophages, the KCs, inducing the production of inflammatory cytokines and type 1 IFN, activates the liver resident macrophages, the KCs, inducing interferon-α expression in the liver of NASH patients [58]. Augmented levels of these molecules in adipose tissue are also correlated with type 2 diabetes in obese patients [59]. It is important to note that inflammasome activation might lead to different outcomes according to the tissue or cell-type that receives the stimuli [42].

6.3. IL-1, TNFα, and IL-6. High fat diets in mice lead to increased hepatic expression of NF-κB, hepatic steatosis, and rise in IL-6, IL-1β, and TNFα gene expression [39]. Supporting a role for inflammasome in NASH, IL-1 receptor (IL-1R), IL-1β, and IL-1α knockout mice showed attenuation of liver pathology induced by high fat diets [42]. Activation of IL-1R by IL-1 resulted in the activation of the transcription factor NF-κB [49]. IL-1 forms an autoregulatory loop as it induces both the expression of its own precursors and inflammasome components, suggesting that small increases in this cytokine might have significant biological effects [42].

TNFα overexpression is viewed as the hallmark of inflammation in obesity and NAFLD pathology and a major link to insulin resistance. Upon binding to its receptor, TNFα can activate proapoptotic or antiapoptotic signaling cascades leading to NF-κB activation, thus regulating cell viability, inflammation, metabolism, and other cytokine productions [39]. It is overproduced in adipose and muscle tissues of obese humans and in rodent models of obesity. TNFα or TNFα receptor knockout obese mice have improved insulin sensitivity compared to wild-type controls [60]. TNFα, through NF-κB both promotes and is activated by insulin resistance and is involved in liver inflammatory and metabolic alterations [39]. Additionally, TNFα antagonizes the anti-inflammatory cytokine adiponectin. Adiponectin is produced by adipocytes and sensitizes cells to insulin. It also promotes fatty acid oxidation with marked anti-inflammatory and antilipogenic effects in the liver [39]. Increased levels of circulating TNFα, on the other hand, correlate with NAFLD disease activity as measured by histological parameters in NAFLD patients. Likewise, TNFα and TNFα receptor gene expression was increased in hepatic and adipose tissues in NASH patients [50]. Experimental models of insulin resistance demonstrated amelioration of inflammation, increased insulin sensitivity, and improved steatosis upon treatment with infliximab, a potent TNFα neutralizing monoclonal antibody. However, highlighting the complexity of inflammatory signaling, infliximab treatment did not improve insulin sensitivity in human obese insulin resistant individuals. Furthermore, pentoxifylline, a phosphodiesterase inhibitor, prevented TNFα production with only modest amelioration of insulin resistance in small studies of NASH patients [50].
Visceral adipose tissue of obese individuals secretes increased amounts of IL-6 when compared to subcutaneous fat. Increased levels of plasma IL-6 were associated with augmented inflammation and fibrosis in NAFLD patients [39]. Adipocyte-derived IL-6 was shown to regulate hepatic insulin resistance via upregulation of suppressor of cytokine signaling 3 (SOCS3) which in turn induces an increase in SREBP-1c and DNL [50].

Interestingly, IL-6 and TNFα deficient mice displayed reduced hepatocarcinogenesis after high fat diet and upon carcinogenesis induction by diethylnitrosamine (DEN) treatment [61]. On the other hand, IL-6 deficiency was associated with increased hepatocyte injury and apoptosis in a mouse model of liver fibrosis [62]. In this model, IL-6 production by nonparenchymal cells was protective possibly by downregulating HSC activation [62].

7. Kupffer Cells

The liver is structurally and functionally heterogeneous. Parenchymal cells, that is, hepatocytes, are the most numerous and comprise 60% of the total liver cells and 80% of the volume of liver. Nonparenchymal cells (NPCs) represent around 20% of the liver cells and include sinusoidal endothelial cells (20% of liver cells), KCs (approximately 15% of liver cells), and hepatic stellate cells (5-8% of liver cells). Other immune cell populations mostly of natural killer T cells (NKTs) comprise a minor fraction of NPCs. Other immune cell populations. Other immune cell populations.

KCs are not only responsive to inflammatory signals but also to metabolic fluctuations (Figure 1). As referred previously, lipids per se and high-energy diets can be harmful to the liver. Evidences show that overload of lipids and cholesterol derivatives activate KCs in models of fatty liver disease and steatohepatitis [68]. A different study also showed that, under a high fat diet or upon FFA treatment, KCs are activated producing high levels of proinflammatory cytokines such as TNFα and IFNγ [69] (Figure 1). In this same study, increased levels of TLR4 were found in KCs. These results are in accordance with another study [70] where depletion of KCs protected against the development of high fat or high sucrose-induced steatosis. Furthermore, in a mouse model of steatohepatitis, mice with KCs derived from MyD88−/− bone marrow donors had improved inflammation and steatosis phenotype [55]. Interestingly, KCs from mice exposed to high fat diet were shown to accumulate increased amount of free cholesterol and diacylglycerol. In vitro assays demonstrated that these fat-laden KCs were more responsive to LPS induced activation when compared to KCs from lean mice [68]. Accordingly, in vitro stimulation of mouse KCs with the SFA palmitic acid was shown to upregulate expression of TLR receptors [71]. Thus, FFA sensing by KCs may condition its responsiveness to proinflammatory triggers.

Insulin resistance is a predominant feature in NAFLD patients. Insulin signaling plays a critical role in modulating both glucose and lipid metabolism. Inflammatory mediators such as TNFα and IL-6 are highly associated with the development of insulin resistance in the setting of obesity. In mice, inactivation of NF-κB mediated signaling, through specific deletion of ikkβ in macrophages, was shown to impair the development of systemic insulin resistance under high fat diet. Nevertheless, adiposity was similar in macrophage ikkβ deficient mice and wild-type controls under high fat diet [72]. Similar observations were obtained in bone marrow chimeric...
mice where JNK1 signaling was disrupted in hematopoietic cells [73].

7.2. Kupffer Cells and Hepatic Stellate Cells: The Path to Fibrosis. Liver fibrosis arises from dysregulation in the wound healing process elicited to dampen hepatocyte damage and is characterized by excessive matrix synthesis and altered matrix degradation. Fibrosis occurs as progressive liver accumulation of proteoglycans, glycoproteins, and collagens with predominance of types I and III fibrillar collagens and failure of physiological mechanisms of matrix turnover. Ultimately, the fibrotic process culminates in cirrhosis with distorted hepatic architecture associated with regenerating hepatocyte nodules surrounded by fibrotic septa and marked impairments in hepatic vascularity [74]. Development of hepatocellular carcinoma occurs in about one-third of individuals with cirrhosis [75]. Nevertheless, reversion of advanced fibrosis and even cirrhosis is possible and has been documented [74].

Myofibroblast that migrate and accumulate at sites of liver injury in response to autocrine and paracrine signals produced by neighboring cells constitute the cellular source of fibrosis during chronic liver diseases [74]. Although they are heterogeneous in composition a great part of myofibroblasts develop from liver resident HSC. HSCs are mesenchymal cells that comprise 5–8% of total liver cells. Their prime location is in the space of Disse between endothelial cells and hepatic epithelial cells. In quiescent state, HSCs store large amounts of Vitamin A in lipid droplets and can be identified by expression of desmin and glial fibrillary acidic protein (GFAP) [76]. Upon liver injury, HSCs sense hepatocyte damage and immune cell signaling and respond by transdifferentiation into active myofibroblast-like cells that express alpha-smooth muscle actin (α-SMA) and migrate.
Figure 2: Role of Kupffer cells in fibrosis regression. Initial activation of Kupffer cells contributes to liver injury and hepatocyte cell death through the release of inflammatory cytokines (1). Phagocytosis of hepatocyte debris, however, may trigger an antifibrogenic phenotype in Kupffer cells by inducing the expression of metalloproteinases that degrade collagen fibers (2). Phagocytosis may also increase Wnt signaling in KC and promote the differentiation of hepatic progenitor cells into new hepatocytes (3). A reduced phagocytic capacity of Kupffer cells may underlie impairments in antifibrogenic responses and contribute to the setting of fibrosis in NASH.

to sites of injury. Besides secreting extracellular matrix proteins, HSCs secrete cytokines, growth factors that promote regeneration of hepatic epithelial cells, and angiogenic factors that modulate endothelial cell and hepatocyte proliferation. Prolonged activation of HSC causes fibrosis and during fibrosis regression the number of these cells is greatly reduced by induction of cellular senescence and apoptosis and return to quiescent state [76].

Genes regulating hepatocellular apoptosis and/or necrosis, genes regulating the inflammatory response to injury (TLR4, TNFα, IL-1β, and IL-6), genes mediating ROS generation, and genes coding for fibrogenic growth factors (TGFβ1), vasoactive substances, and adipokines (Leptin) were shown to be critically involved in liver fibrogenesis [47, 77]. In particular TGFβ1 polymorphism may confer susceptibility to NASH progression to fibrosis [77]. TGFβ signaling in HSC stimulates activation, synthesis of extracellular matrix protein, and inhibition of its degradation [74]. KCs secrete TGFβ and platelet derived growth factor (PDGF) that constitutes a potent mitogenic factor for HSC [47, 77] (Figure 1). Additionally, KCs through the secretion of IL-1 and TNFα lead to activation of NF-κB signaling pathway in HSC promoting cell survival [78]. Interestingly, contact-independent coculture of KCs with HSC influenced gene expression in HSC turning the overall mRNA expression pattern more similar to what was observed in HSC isolated from in vivo mouse models of fibrosis. Among these genes were the NF-κB-regulated genes IL-6, and tissue inhibitor of metalloproteinase 1 (timp1) [78]. Timp1 inhibits metalloproteinases (Mmps) that are able to cut collagen fibers and together modulate matrix degradation. Expression of both Timps and Mmps is tightly regulated according to activation of HSC in regular response to liver injury [74].

Macrophages can produce Mmps and contribute in a later stage of the wound healing process to fibrosis regression [79] (Figure 2). In a well described carbon tetrachloride model of reversible murine hepatic fibrosis, macrophage depletion during fibrosis acute phase leads to impaired activation of HSC and reduced scaring, while depletion during recovery phase reduced matrix degradation and fibrosis resolution [80]. In a similar mouse model of reversible fibrosis, monocytes were recruited into the liver where they were highly proinflammatory in initial stages, but upon stimuli of local microenvironment they differentiated into metalloproteinases producing macrophages necessary for fibrosis resolution [81]. Interestingly, phagocytosis of cellular debris was shown to trigger the phenotype switch, inducing expression of matrix degradation associated genes (Mmp9, Mmp12, and insulin growth factor 1) [81] (Figure 2).

Autophagy is a catabolic mechanism whereby unnecessary or dysfunctional cellular components are degraded in the lysosome. Autophagy-related (ATG) genes are necessary for the formation of the phagophore and autophagosomes, the initial steps in autophagy [82]. Autophagy in macrophages is critical for phagocytic functions. In a mouse model of fibrosis, inhibition of the autophagic gene Atg5 specifically in myeloid cells resulted in increased susceptibility to liver inflammation and liver injury, increased secretion of IL-1,
enhanced monocyte recruitment, and increased hepatocyte apoptosis [82].

Phagocytosis of hepatocyte debris by liver macrophages/KCs was also shown to promote Wnt signaling upregulation. Wnt/β-catenin signalling is crucial for hepatic progenitor cell (HPC) differentiation and engagement into hepatocellular fate. Macrophage depletion during hepatocyte regeneration was shown to remove the stimuli for hepatocyte differentiation and HPC differentiated preferentially into cholangiocyte, forming biliary structures [83].

Macrophage plasticity may differ according to the nature of the stimuli leading to pathology. It is unknown whether impairments on macrophage phagocytosis and differentiation into matrix degrading prone-macrophages underlie impairments on macrophage phagocytosis and differentiation triggered by NASH (Figure 2). Of note, impairment in KCs phagocytosis has been observed in high fat and high cholesterol diet-induced mouse models of NAFLD [84].

8. Conclusions

NAFLD is a rising concern that goes together with the increasing prevalence and incidence of obesity being a current epidemic problem. NAFLD pathogenesis is linked with insulin resistance, adipose tissue distribution, and dietary and genetic factors, which are risk factors not only for NASH but also for diabetes and associated pathologies.

From this review, it is clear that much remains to be understood regarding the mechanism of the disease. The lack of knowledge in relation to this pathogenesis becomes a hurdle in the path towards novel approaches for the prevention and treatment of the disease. Up to our days, prevention can only be based on calorie restriction and favorable dietary composition as well as exercise. More effective lifestyles/therapeutic methods should be addressed not only to prevent fat deposition, but primarily to avoid subclinical inflammation, where KCs play a prominent role. How the interdependent effects of diet microbiota inflammation directly impact liver dysmetabolism is an issue that remains to be elucidated. Nevertheless, from an interventional standpoint, this vicious cycle needs to be broken. If this hypothesis is proven to be correct, we should start to integrate the impact on intestinal microbiota composition and subsequent inflammatory responses in the nutritional value of foods, as a proxy for predicting the potential to evoke dysmetabolic states that are determinants of NAFLD development.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported by Fundação para a Ciência e Tecnologia (FCT) Grants PTDC/DTP-EPI/0207/2012 and PTDC/BIM-MET/0486/2012 and by the Portuguese Diabetes Society (SPD).

References


Individual CLA Isomers, c9t11 and t10c12, Prevent Excess Liver Glycogen Storage and Inhibit Lipogenic Genes Expression Induced by High-Fructose Diet in Rats

Edyta Maslak, 1,2 Elzbieta Buczek, 2 Antoni Szumny, 3 Wojciech Szczepnski, 4 Magdalena Franczyk-Zarow, 1 Aneta Kopec, 1 Stefan Chlopicki, 2,5 Teresa Leszczynska, 1 and Renata B. Kostogrys 1

1 Department of Human Nutrition, Faculty of Food Technology, Agricultural University of Krakow, Balicka 122, 30-149 Krakow, Poland
2 Jagiellonian Centre for Experimental Therapeutics (JCET), Jagiellonian University, Bobrzynskiego 14, 30-348 Krakow, Poland
3 Department of Chemistry, Faculty of Food Science, Wroclaw University of Environmental and Life Sciences, C. K. Norwida 25, 50-375 Wroclaw, Poland
4 Department of Clinical and Experimental Pathomorphology, Jagiellonian University Medical College, Grzegorecka 16, 31-531 Krakow, Poland
5 Department of Experimental Pharmacology, Jagiellonian University Medical College, Grzegorecka 16, 31-531 Krakow, Poland

Correspondence should be addressed to Edyta Maslak; edyta.maslak@gmail.com

Received 10 October 2014; Revised 12 January 2015; Accepted 26 February 2015

Academic Editor: Maria J. Martins

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This study assessed the effects of individual conjugated linoleic acid isomers, c9t11-CLA and t10c12-CLA, on nonalcoholic fatty liver disease (NAFLD) and systemic endothelial dysfunction in rats fed for four weeks with control or high-fructose diet. The high-fructose diet hampered body weight gain (without influencing food intake), increased liver weight and glycogen storage in hepatocytes, upregulated expression of fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1), and increased saturated fatty acid (SFA) content in the liver. Both CLA isomers prevented excessive accumulation of glycogen in the liver. Specifically, t10c12-CLA decreased concentration of serum triacylglycerols and LDL+VLDL cholesterol, increased HDL cholesterol, and affected liver lipid content and fatty acid composition by downregulation of liver SCD-1 and FAS expression. In turn, the c9t11-CLA decreased LDL+VLDL cholesterol in the control group and downregulated liver expression of FAS without significant effects on liver weight, lipid content, and fatty acid composition. In summary, feeding rats with a high-fructose diet resulted in increased liver glycogen storage, indicating the induction of gluconeogenesis despite simultaneous upregulation of genes involved in de novo lipogenesis. Although both CLA isomers (c9t11 and t10c12) display hepatoprotective activity, the hypolipemic action of the t10c12-CLA isomer proved to be more pronounced than that of c9t11-CLA.

1. Introduction

The metabolic syndrome (MS) is a cluster of interrelated risk factors that promote the development of cardiovascular disease (CVD). Recently, MS was also found to be a strong predictor of NAFLD, which is widely accepted to be the hepatic manifestation of MS [1, 2]. To date, increased fat consumption is considered the major pathogenic factor of MS and NAFLD. However, recent data show that elevated consumption of fructose may also contribute to the development of those diseases. Studies in humans and experimental animals demonstrate that increased intake of fructose can result in hypertriglyceridemia, insulin resistance, and liver steatosis [3–5]. Moreover, the existence of cross talk between the liver and vascular wall might specifically account for the strong associations between liver steatosis and accelerated atherosclerosis. It has been shown that severe hepatic steatosis was linked to increased atherogenesis in diabetic patients by increased plasma TG, LDL, and VLDL concentrations and decreased HDL level and insulin resistance [6].
Table 1: Composition of the experimental diets.

<table>
<thead>
<tr>
<th>Ingredients* [g/kg]</th>
<th>C</th>
<th>C + c9t11</th>
<th>C + t10c12</th>
<th>F</th>
<th>F + c9t11</th>
<th>F + t10c12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>632.486</td>
<td>632.486</td>
<td>632.486</td>
</tr>
<tr>
<td>Corn starch</td>
<td>532.486</td>
<td>532.486</td>
<td>532.486</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>56.67</td>
<td>58.89</td>
<td>70</td>
<td>56.67</td>
<td>58.89</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>c9t11-CLA</td>
<td>—</td>
<td>13.33</td>
<td>—</td>
<td>11.11</td>
<td>—</td>
<td>11.11</td>
</tr>
<tr>
<td>t10c12-CLA</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*The experimental diets ingredients were obtained from Sigma-Aldrich, St. Louis, MO, USA, with the exception of the following: fructose obtained from Biofan, Poland; corn starch obtained from Agrotrade, Poland; and Casein obtained from Kazeina Polska Sp. z o.o., Poland.

CLA comprises a group of positional and geometric isomers of linoleic acid (C18:2, n-6). In contrast to the chain structure of the linoleic acid, in CLA the double bonds are separated by only one single bond and occur most frequently at carbons 9 and 11 as well as 10 and 12 forming c9t11 and t10c12-CLA isomers [7]. Studies have shown that CLA isomers actively inhibit carcinogenesis [8] and prevent atherosclerosis [9], diabetes [10–12], obesity [13, 14], and osteoporosis [15–17]. Recently, it has been shown that CLA can prevent NAFLD induced by a high-fat diet [18] and influence endothelium-mediated vascular homeostasis, by reducing the release of proinflammatory mediators, such as prostaglandin E\(_2\) and the vasoconstrictive agent thromboxane A\(_2\), within vascular endothelial cells [19, 20]. However, the effects of individual CLA isomers, c9t11 and t10c12-CLA, on fructose-induced NAFLD and endothelial dysfunction have not been investigated.

2. Materials and Methods

2.1. Animals and Experimental Design. Male Wistar rats at an initial body weight of approximately 100 g were divided into 6 experimental groups and fed for 4 weeks with either the control AIN-93G diet (C), a high-fructose diet (F, 63% fructose), or C and F diets supplemented with 1% c9t11 and t10c12-CLA isomers (Larodan Fine Chemicals, Malmo, Sweden). The addition of individual CLA isomers was calculated considering the purity of c9t11 (75%) and t10c12 (90%) and balanced at the expense of quantity consisted of soybean oil (Table 1). The control and experimental diets were prepared freshly according to Reeves et al. [21] and stored in darkness at 4°C to avoid lipid peroxidation.

Rats were housed in collective cages (3 rats per cage) in a room with a 12 h light-dark cycle and were given ad libitum access to diet and water. Rats were weighed weekly, and leftover food was measured daily for the calculation of food intake.

All procedures involving animals were conducted according to the Guidelines for Animal Care and Treatment of the European Union and were approved by the Local Animal Ethics Commission.

2.2. Blood Biochemistry. Blood samples were collected from the heart and centrifuged (1000 x g for 10 min.) to obtain serum. Serum samples were analysed using commercially available kits for total cholesterol (CHOL), HDL, and TG (Liquick Cor no. 2-211, 2-217, 2-262, resp., Cormay, Lublin, Poland). LDL + VLDL concentration was calculated as the difference between CHOL and HDL concentrations. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by commercially available kits (numbers A6624-050 and A6661-050, Alpha Diagnostics, Warsaw, Poland) according to the manufacturer’s instructions. Blood glucose concentrations were measured using an Accu-Chek Active glucose meter (Roche Diagnostics GmbH, Mannheim, Germany). Uric acid (UA) was analysed using commercially available kit (no. K6681-050, Alpha Diagnostics, Warsaw, Poland).

2.3. Histological Evaluation. Fragments of livers were fixed in 4% buffered formalin and prepared according to the standard paraffin method. Paraffin-embedded 5 μm sections were stained with hematoxylin and eosin (HE) for general histology and the Periodic Acid-Schiff (PAS) method was used for glycogen visualization. Sections were photographed under the 200x magnification with a Nikon Eclipse E400 light microscope equipped with a Nikon digital sight DS-Fi1 camera and NIS-Elements software (Nikon GmbH, Düsseldorf, Germany).

2.4. Liver Fat Content. Liver fragments were lyophilized and ground. The determination of fat content was performed with a Foss Tecator Extraction system Soxtec Avanti 2050 (Tecator Foss, Hilleröd, Sweden) by continuous extraction of
the fat from the material and then evaporating the solvent and drying and weighing the residue.

2.5. Liver Fatty Acid Composition. The liver lipid extraction was obtained according to the previously described method [22]. Briefly, the extracted fat was saponified (10 min. at 75°C) with a 0.5 M solution of KOH/MethOH and subjected to methylation (10 min. at 75°C) in 14% (v/v) BF3/MethOH (Sigma-Aldrich, St. Louis, MO, USA). Then, the methyl esters of fatty acids were extracted with hexane (Avantor Performance Materials Poland S.A., Gliwice, Poland) and analysed using a gas chromatograph coupled with a mass spectrometer (Shimadzu GCMS QP 5050, Shimadzu, Kyoto, Japan). Separation was achieved using SP-2560 capillary column with a length of 100 m, inner diameter of 0.25 mm, and film thickness of 0.25 μm (Supelco, St. Louis, MO, USA). Helium was the carrier gas.

Identification of methyl esters of long-chain fatty acids was based on the reference standards (FAME Mixture, Lardodan Fine Chemicals, Malmo, Sweden) and a library of mass spectra (NIST 1.7). Percentage of methyl esters of fatty acids was calculated from the analytical signal with the formula \( \frac{\sum_i A_i}{\sum A} \cdot 100 \) where \( A_i \) is the \( i \)th signal of the ester and \( \sum A \) is the sum of all identified analytical signal esters [23].

2.6. Real-Time qRT-PCR. Total RNA was isolated from liver samples with a “Total RNA” kit (A&A Biotechnologie, Gdynia, Poland) according to the manufacturer’s instruction and quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, Delaware, USA). Subsequently, samples were purified with an RNeasyMinElute Cleanup kit (Qiagen, Hilden, Germany) and analysed with a BioAnalyzer (Agilent, Santa Clara, California, USA) to measure final RNA quality and integrity.

Expression of the FAS and SCD-1 genes was checked by qRT-PCR with the following primers: \( 5' \)-TGACGCTGC-TGACGTCTATG-3' (forward), \( 5' \)-ATTGTCTCTGGATGATTGA-3' (reverse) and \( 5' \)-GTGCTAAAGACCTTATGCAACAG-3' (forward), \( 5' \)-GGATGTTCTCCGGAGATTGA-3' (reverse). FAS and SCD-1 mRNA sample concentrations were analysed using a LightCycler (Roche Diagnostics, Basel, Switzerland) with a SYBR-green fluorochrome (Qiagen, Valencia, CA, USA). Results are presented as the ratio of the expression of each gene to \( \beta \)-actin expression (\( \beta \)-actin primers: \( 5' \)-ACATCCGTAAGACCTTATGCAACAG-3' (forward), \( 5' \)-GTGCTAGAGCCAGGCATCTT-3' (reverse)).

2.7. Assessment of Endothelial Function. Thoracic aortas were carefully dissected out of the surrounding tissues. The assessment of NO-dependent endothelial function in the isolated rings of rat aortas was determined by dose-dependent relaxation to acetylcholine (Ach, 0.01–10 μM, Sigma-Aldrich, St. Louis, MO, USA) both without and in the presence of the inhibitor of nitric oxide synthase N\textsubscript{N} -Nitro-L-Arginine Methyl Ester (L-NNAME, 300 μM, Sigma-Aldrich, St. Louis, MO, USA) as previously described [24]. The endothelium-independent response was tested by a response evoked by sodium nitroprusside (SNP, 0.001–1 μM, Sigma-Aldrich, St. Louis, MO, USA).

2.8. Statistical Analysis. Data are expressed as means ± SEM. The Shapiro-Wilk test was applied to test the assumption of normality. Based on those results, either a nonparametric Kruskal-Wallis test or two-way analysis of variance (ANOVA) and Duncan post-hoc test were used to assess the statistical significance at a significance level of \( P \leq 0.05 \). The results were analysed using STATISTICA 10.0 software.

3. Results

3.1. Body Weight and Food Intake. The final body weight gain of the high-fructose-fed rats (group F) was significantly less than that of rats in group C. The CLA isomers had no effect on body weight in rats fed with diet C, while rats fed with diets F + c9t11 and F + t10c12-CLA gained significantly more weight than rats from group F, reaching weights similar to those of C group (Figure 1(a)). The changes in body weight did not depend on the food intake, which did not differ between experimental groups (Figure 1(b)).

3.2. Plasma Biochemistry. No changes in serum lipid profile were observed after four weeks of feeding rats with a high-fructose diet (group F) compared with group C. The CLA isomers had no effect on body weight in rats fed with diet C, while rats fed with diets F + c9t11 and F + t10c12-CLA gained significantly more weight than rats from group F, reaching weights similar to those of C group (Figure 1(a)). The changes in body weight did not depend on the food intake, which did not differ between experimental groups (Figure 1(b)).

Although no differences were observed in serum ALT and AST between groups C and F, addition of c9t11-CLA to a high-fructose diet (F + c9t11 group) significantly decreased ALT concentration in rats compared with rats in the C + t10c12 group. In turn, c9t11-CLA decreased LDL + VLDL by 50% only in the group receiving the C + c9t11 diet. No changes were observed in TG concentration between groups C and F; however, TG concentration decreased in the F + t10c12 group compared with the F + c9t11 group and increased in F + c9t11 versus C + c9t11.

Although no differences were observed in serum ALT and AST between groups C and F, addition of c9t11-CLA to a high-fructose diet (F + c9t11 group) significantly decreased ALT concentration in rats compared with rats in the C + c9t11 group. The glucose and UA concentration did not differ between experimental groups (Table 2).

3.3. Liver Weight and Fatty Acid Composition. The high-fructose diet increased liver weight in rats (20% versus control group, \( P < 0.05 \)) in comparison with control group. The CLA isomers (c9t11 and t10c12) had no effect on rats liver weight. Interestingly, the liver lipids content did not differ between groups C and F, while the addition of the t10c12-CLA isomer to diets C and F led to a significant reduction in the liver lipids. The c9t11-CLA isomer had no effects on lipids storage in the rats’ livers (Figure 2).

Analysis of liver fatty acid composition revealed that CLA isomers (c9t11 and t10c12) were incorporated into liver lipids, whereas they were not found in the livers of rats fed with control or high-fructose diets (Table 3). Moreover, in C + t10c12 and F + t10c12 groups a small amount of c9t11-CLA isomers was observed.

The high-fructose diet significantly increased total SFA and monounsaturated fatty acids (MUFA) and decreased...
Table 2: Lipid profile, UA, glucose concentration, and enzyme activities (AST, ALT) in rats fed with experimental diets.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>C + c9t11</th>
<th>C + t10c12</th>
<th>F</th>
<th>F + c9t11</th>
<th>F + t10c12</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG [mmol/L]</td>
<td>2.98 ± 0.42&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.28 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.08 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.86 ± 0.43&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.29 ± 0.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.75 ± 0.35&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>TCH [mmol/L]</td>
<td>2.09 ± 0.24&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>1.78 ± 0.29&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>1.98 ± 0.15&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>1.84 ± 0.06&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>1.85 ± 0.21&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>1.98 ± 0.06&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL + VLDL [mmol/L]</td>
<td>0.95 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.49 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.49 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86 ± 0.08&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.64 ± 0.22&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.36 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL [mmol/L]</td>
<td>1.14 ± 0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.29 ± 0.26&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.48 ± 0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.98 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.21 ± 0.16&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.62 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>UA [mg/dL]</td>
<td>3.41 ± 0.82&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>3.56 ± 0.99&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>3.48 ± 1.01&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>3.33 ± 0.35&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>3.46 ± 0.92&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>3.57 ± 0.75&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose [mg/dL]</td>
<td>103.00 ± 2.4&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>106.25 ± 4.2&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>104.33 ± 3.2&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>103.00 ± 5.2&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>110.25 ± 3.6&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>100.80 ± 2.3&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALT [UI]</td>
<td>23.22 ± 4.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>29.40 ± 8.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.86 ± 2.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.93 ± 0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.16 ± 3.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.51 ± 2.71&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AST [UI]</td>
<td>49.76 ± 2.62&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>44.81 ± 5.26&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>61.71 ± 8.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.44 ± 4.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.45 ± 7.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.05 ± 4.45&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values are means ± SEM (n = 6). Values in the same row with different superscript letters were significantly different (P ≤ 0.05).
The high-fructose diet resulted in a significant decrease in 16:1 to 16:0 and total polyunsaturated fatty acids (PUFA) in the liver compared with the control group. Among rats fed with a high-fructose diet, addition of the t10c12-CLA isomer increased total SFA and decreased total MUFA (\( P < 0.05 \)), while c9t11-CLA had no effect on SFA, MUFA, nor PUFA level. Moreover, a high-fructose diet significantly increased the 16:1 to 16:0 ratio in the liver, while addition of t10c12-CLA isomer resulted in a significant decrease in 16:1 to 16:0 and 18:1 to 18:0 ratios (Table 3).

### 3.4. Liver Histopathology

The high-fructose diet resulted in excessive glycogen storage into hepatocytes without the appearance of steatosis or inflammation, compared with the control group. The CLA isomers, c9t11 and t10c12-CLA, significantly decreased glycogen content in the liver in rats fed with the high-fructose diet (Figure 3).

### 3.5. Gene Expression Analyses (Real-Time PCR)

The high-fructose diet increased mRNA expression of FAS and SCD-1 in the liver compared with control animals. The addition of individual isomers to the control diet had no effect on gene expression levels in the liver. Among rats fed with the high-fructose diet, both c9t11 and t10c12-CLA isomers significantly decreased FAS expression, while SCD-1 expression was decreased only in the t10c12-CLA isomer group (Figure 4).

### 3.6. Assessment of Endothelial Function

The high-fructose diet had no effect on the magnitude of NO-dependent vasodilatation in aorta induced by Ach. The addition of individual CLA isomers (c9t11 and t10c12) to the high-fructose diet resulted in a slightly, but not significantly improved endothelial function observed as an increased NO-dependent vasodilatation at low concentration of Ach (0.03 \( \mu \)M), while no statistically significant difference in Ach-dependent response was at high concentration of Ach (1 \( \mu \)M) (Figure 5). Furthermore, in the presence of L-NAME (300 \( \mu \)M) the Ach-induced vasodilatation in aorta rings was substantially inhibited in all experimental groups. The endothelium-independent response to SNP was not affected by experimental treatment.

### 4. Discussion

In the present study, we found that a high-fructose diet decreased body weight gain without an effect on food intake and induced liver enlargement associated with excessive accumulation of glycogen in hepatocytes. Simultaneously, upregulation of mRNA expression of lipogenic genes, FAS and SCD-1, and changes in liver fatty acids composition were observed. The addition of CLA isomers (c9t11 and t10c12) to the high-fructose diet exhibited hepatoprotective effects manifested as reduced glycogen content in the liver. Moreover, t10c12-CLA, but not c9t11-CLA, decreased the concentration of serum TG (although not significantly) and LDL + VLDL cholesterol and increased HDL level. Additionally, t10c12-CLA reduced liver lipid content and affected fatty acid composition by downregulating liver SCD-1 and FAS expression. Similarly, c9t11-CLA decreased liver mRNA expression of FAS, though it had no significant effect on SCD-1. The slightly improved NO-dependent function in aortas from rats fed with the high-fructose diet and either c9t11 or t10c12 may suggest a vasoprotective effect of CLA isomers, but further studies would be necessary to confirm these findings.

Several studies have shown that high-fructose intake may lead to adverse metabolic alteration, in particular increase in plasma TG, hepatic insulin resistance, and liver steatosis [3, 25]. ALT and AST are commonly used in the assessment of liver function. An increase in ALT activity is often associated

**Table 3: Liver fatty acid composition of Wistar rats after four weeks of experimental diets.**

<table>
<thead>
<tr>
<th>Fatty acid [%]</th>
<th>C</th>
<th>C + c9t11</th>
<th>C + t10c12</th>
<th>F</th>
<th>F + c9t11</th>
<th>F + t10c12</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.68 ± 0.13(^a)</td>
<td>1.35 ± 0.26(^ab)</td>
<td>1.44 ± 0.23(^ab)</td>
<td>1.29 ± 0.14(^ab)</td>
<td>1.14 ± 0.31(^ab)</td>
<td>0.88 ± 0.17(^ab)</td>
</tr>
<tr>
<td>C16:0</td>
<td>16.85 ± 0.67(^a)</td>
<td>24.95 ± 1.14(^b)</td>
<td>28.12 ± 2.12(^b)</td>
<td>23.16 ± 1.32(^b)</td>
<td>25.52 ± 2.92(^b)</td>
<td>26.03 ± 0.43(^b)</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.70 ± 0.54(^a)</td>
<td>5.13 ± 0.47(^ab)</td>
<td>2.77 ± 0.46(^a)</td>
<td>6.11 ± 0.72(^b)</td>
<td>5.91 ± 1.72(^b)</td>
<td>2.84 ± 0.11(^a)</td>
</tr>
<tr>
<td>C18:1</td>
<td>7.69 ± 0.65(^ab)</td>
<td>6.30 ± 0.81(^a)</td>
<td>14.89 ± 1.00(^b)</td>
<td>8.79 ± 1.13(^b)</td>
<td>10.41 ± 0.70(^bc)</td>
<td>13.34 ± 1.72(^cd)</td>
</tr>
<tr>
<td>C18:2</td>
<td>21.41 ± 0.53(^a)</td>
<td>19.06 ± 1.19(^a)</td>
<td>9.15 ± 2.92(^b)</td>
<td>25.22 ± 1.82(^a)</td>
<td>19.87 ± 3.88(^a)</td>
<td>17.37 ± 1.88(^a)</td>
</tr>
<tr>
<td>C18:3</td>
<td>34.57 ± 2.08(^d)</td>
<td>26.90 ± 2.11(^i)</td>
<td>25.24 ± 1.19(^bc)</td>
<td>18.76 ± 0.30(^a)</td>
<td>20.83 ± 1.48(^ab)</td>
<td>20.77 ± 1.21(^ab)</td>
</tr>
<tr>
<td>C20:4</td>
<td>7.72 ± 1.15(^a)</td>
<td>2.21 ± 0.29(^b)</td>
<td>1.70 ± 0.16(^b)</td>
<td>0.99 ± 0.11(^b)</td>
<td>1.24 ± 0.10(^b)</td>
<td>1.12 ± 0.07(^b)</td>
</tr>
<tr>
<td>c9t11-CLA</td>
<td>0.00 ± 0.00(^a)</td>
<td>2.46 ± 0.44(^a)</td>
<td>0.57 ± 0.22(^a)</td>
<td>0.00 ± 0.00(^a)</td>
<td>1.42 ± 0.36(^b)</td>
<td>0.23 ± 0.06(^a)</td>
</tr>
<tr>
<td>t10c12-CLA</td>
<td>0.00 ± 0.00(^a)</td>
<td>0.00 ± 0.00(^a)</td>
<td>1.38 ± 0.71(^b)</td>
<td>0.00 ± 0.00(^a)</td>
<td>0.08 ± 0.03(^a)</td>
<td>1.07 ± 0.40(^ab)</td>
</tr>
<tr>
<td>C22:4</td>
<td>7.22 ± 1.53(^a)</td>
<td>9.25 ± 0.80(^ab)</td>
<td>11.50 ± 1.10(^bc)</td>
<td>9.07 ± 0.86(^ab)</td>
<td>10.49 ± 0.82(^bc)</td>
<td>13.16 ± 1.48(^a)</td>
</tr>
<tr>
<td>C22:6</td>
<td>0.99 ± 0.66(^a)</td>
<td>0.17 ± 0.03(^a)</td>
<td>0.42 ± 0.06(^a)</td>
<td>1.13 ± 0.58(^a)</td>
<td>0.47 ± 0.11(^a)</td>
<td>0.30 ± 0.01(^a)</td>
</tr>
<tr>
<td>I6:1/I6:0</td>
<td>0.16 ± 0.03(^ab)</td>
<td>0.20 ± 0.01(^a)</td>
<td>0.10 ± 0.01(^a)</td>
<td>0.28 ± 0.02(^a)</td>
<td>0.22 ± 0.04(^ab)</td>
<td>0.11 ± 0.00(^a)</td>
</tr>
<tr>
<td>I18:1/I8:0</td>
<td>2.85 ± 0.26(^a)</td>
<td>3.28 ± 0.66(^a)</td>
<td>0.60 ± 0.18(^b)</td>
<td>3.03 ± 0.46(^a)</td>
<td>1.97 ± 0.45(^a)</td>
<td>1.38 ± 0.27(^bc)</td>
</tr>
<tr>
<td>SFA [%]</td>
<td>25.22 ± 1.19(^a)</td>
<td>32.61 ± 0.85(^a)</td>
<td>44.46 ± 1.79(^a)</td>
<td>33.24 ± 2.17(^a)</td>
<td>37.07 ± 3.55(^ab)</td>
<td>40.25 ± 2.13(^bc)</td>
</tr>
<tr>
<td>MUFA [%]</td>
<td>24.11 ± 0.82(^a)</td>
<td>24.19 ± 1.58(^a)</td>
<td>11.92 ± 2.48(^a)</td>
<td>31.82 ± 2.32(^b)</td>
<td>25.79 ± 2.28(^ab)</td>
<td>20.21 ± 1.84(^a)</td>
</tr>
<tr>
<td>PUFA [%]</td>
<td>50.68 ± 1.93(^a)</td>
<td>43.16 ± 1.89(^a)</td>
<td>43.63 ± 0.88(^a)</td>
<td>34.94 ± 3.13(^b)</td>
<td>37.14 ± 1.34(^ab)</td>
<td>39.54 ± 1.87(^ab)</td>
</tr>
</tbody>
</table>

*Values are means ± SEM (\( n=6 \)). Values in the same row with different superscript letters were significantly different (\( P \leq 0.05 \)).
with the development of fatty liver, although some authors do not confirm these observations and show that the absence of elevated liver enzymes does not exclude NAFLD diagnosis [26, 27]. In the current work, in which the effect of short-term high-fructose diet was studied, liver enzyme concentrations showed no differences among experimental groups; however, increased liver weight compared with control animals was observed. Some investigators have hypothesized that these effects may be due to increased lipid content in the liver [28–30]. To test this hypothesis, we assessed liver total lipid content and performed histological analysis of the liver samples. The results showed increased glycogen storage in rats fed with the high-fructose diet without a difference in liver fat content between the control and high-fructose groups, indicating the predominance of gluconeogenesis over de novo fatty acid synthesis in the early phase response to a high-fructose diet. Interestingly, further analysis of mRNA expression of two lipogenic genes, FAS and SCD-1, exhibited upregulation of both after consuming the high-fructose diet. These observations clearly show that the adverse effect of fructose on glucose metabolism in the liver is closely linked to the alterations in lipid metabolism. Although we did not observe any differences in liver lipid content after the high-fructose diet, we did observe significant alterations in liver fatty acid composition in rats. We found that the high-fructose diet increased liver SFA and MUFA with a simultaneous reduction in PUFA content. Previously, it was shown that the balance between SFA and UFA is a prognostic marker in NAFLD/NASH [31, 32], as well as in cardiovascular and total mortality in humans [33]. As lipids play a crucial role not only in energy storage but also as structural components of cellular membranes and actively participate in cellular signalling, observed in the current study, altered balance between SFA and UFA is of great importance. Furthermore, it has been previously shown that SFA and UFA had distinct impact on cell viability and apoptosis [34]. SFA (palmitic acid (C16:0) and oleic acid (C18:0)) were shown to increase apoptosis of endothelial cells, while UFA (palmitoleic acid (C16:1), oleic acid (C18:1), and linoleic acid (C18:2)) did not promote apoptosis but prevented stearate-induced apoptosis in endothelial cells [34]. Moreover, myotubes’ exposure to SFA (C16:0 and C18:0), but not to UFA (C16:1, C18:1, and C18:2), resulted in activation of an inflammatory response due to induction of COX-2 expression and subsequent prostaglandin E2 production [35]. In the current study, the fructose-induced alterations in SFA, MUFA, and UFA content were largely due to significant increases in C16:0 and decreases in C18:2 and linolenic acid (C18:3). An increased C16:0 level has been shown to induce accumulation of ceramides leading to a significant decrease in NO generation and insulin resistance [36, 37]. A depletion in NO bioavailability and insulin resistance is
associated with liver steatosis [38, 39], suggesting a prominent pathogenetic role of fructose in liver injury. Interestingly, in the current study, the high-fructose diet also increased C16:1 concentration; however, since C16:1 was showed to be, apart from C18:1, the most abundant monounsaturated fatty acid in various kinds of lipids, including phospholipids, triglycerides, cholesterol esters, wax esters, and alkylacylglycerols [40], its increased concentration in a state of upregulation of lipogenic genes is not surprising.

In the present work we did not find any effects of high-fructose diet on endothelial function assessed as NO-dependent vasodilation in aortic rings [41]. This is probably due to the relatively short time of high-fructose feeding. Bartuš et al. [42] observed endothelial dysfunction after eight weeks of feeding rats with a high-fructose diet. Quite surprisingly, we found that both individual isomers tend to improve endothelium-dependent responses induced by a low concentration of Ach. As proper endothelial function inhibits leukocyte recruitment, smooth muscle cell proliferation, and development of atherosclerotic plaques [43] and both t10c12 and c9t11-CLA slightly improved endothelial function, these findings may have therapeutic implications. It is notable that improvement was comparable with control and high-fructose groups, as high-fructose diet showed no effect on endothelial function in the current study. Interestingly, the c9t11-CLA isomer reduced ICAM-1 and VCAM-1 expression in endothelium and reduced the adhesion of macrophages to endothelium [44], while the t10c12-CLA isomer activated vascular eNOS in an adiponectin-dependent manner [45]. Obviously, CLA isomers may display vasoprotective activity that warrants further studies.

In the present work, we have shown that both isomers exhibited hepatoprotective properties, although the effects of t10c12-CLA were more pronounced than those of c9t11-CLA. The action of t10c12-CLA, unlike c9t11-CLA, involved improvements not only in liver glycogen storage but also in plasma lipid profile and liver lipid content (some of them in treated rats compared with rats in the high-fructose and control groups). These observations may be partially explained by the increased β-oxidation or/and higher hepatic TG secretion induced by t10c12-CLA [46–48]. The involved mechanism may also be linked with inhibition of lipogenesis. In the current study, we showed that t10c12-CLA decreased mRNA expression of liver two lipogenic genes, FAS and SCD-1, in rats. Moreover, as a result, changes in the composition of liver fatty acids were observed. The modulation of liver fatty acid composition by mixture of CLA isomers has been confirmed previously [49]. Intake of t10c12-CLA has also been shown to cause an increase of C18:0 and a commensurate decrease of C18:1 in rat livers [50] and mouse adipocyte cultures [51]. In the current study, we also observed a decrease
in 18:1 to 18:0 and 16:1 to 16:0 ratios in liver lipids, indicating diminished SCD-1 activity. Thus, downregulation of hepatic FAS and SCD-1 expression may contribute to the mechanism of hepatic lipid content reduction and improvement in lipoprotein profile by the intake of t10c12-CLA. Although the c9t11-CLA isomer induced reduction of FAS expression, its effect was not visible in other measurements, suggesting that its effect on lipid metabolism is modest and probably differs from t10c12-CLA.

Reports on the biological effects of CLA isomers differ among species used in the studies. Results from rats and mice are especially ambiguous. While t10c12-CLA increased TG concentration and induced liver steatosis in mice [52, 53], data from studies on rats, similar to results obtained in the current study, showed protective effects of t10c12-CLA against hyperlipidemia and increased liver weight and lipid content [54]. There are many data in the literature that show important differences in metabolic regulation of lipids among different hamster [55], mice [56], and rat strains [57].

In conclusion, in the current study we showed that a short-term high-fructose diet leads to liver enlargement associated with excessive glycogen storage in hepatocytes. This is accompanied by a simultaneous alteration in liver fatty acid composition and upregulation of genes involved in de novo lipogenesis, indicating a predominance of gluconeogenesis over de novo fatty acids synthesis in the early phase of liver response to fructose feeding. Although both CLA isomers (c9t11 and t10c12) showed hepatoprotective effects, the hypolipemic action of the t10c12-CLA isomer was more pronounced than that of c9t11-CLA due to its favourable alteration of the plasma lipid profile, decreased liver lipid content, and beneficial changes in liver fatty acid composition. These results are intriguing, and possible effects of CLA on endothelial function are not conclusive and warrant further studies.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgments**

This study was supported by the State Committee for Scientific Research in Poland (NCN), Grant no. N N312 110838, and partially by the European Union from the resources of the European Regional Development Fund under the Innovative Economy Programme (Grant coordinated by JCET-UJ, no. POIG.01.01.02-00-069/09).

**References**


[19] P. M. Coen, P. M. Cummins, Y. A. Birney, R. Devery, and P. A. Cahill, “Modulation of nitric oxide and 6-keto-prostaglandin F-


[24] M. Bartuš, M. Lomnicka, R. B. Kostogrys et al., “1-Methylnic-otaminide (MNA) prevents endothelial dysfunction in hyper-


[27] H. S. Usłusoy, S. G. Nak, M. Gültener, and Z. Biyikli, “Non-


Nonalcoholic Fatty Liver Disease Relationship with Metabolic Syndrome in Class III Obesity Individuals

A. Cordeiro, S. E. Pereira, C. J. Saboya, and A. Ramalho

Introduction

Obesity is represented mainly by abdominal obesity and insulin resistance (IR), both present in most individuals diagnosed with metabolic syndrome (MS). IR is the key risk factor in the pathogenesis of nonalcoholic fatty liver disease (NAFLD).

Objective

To relate NAFLD to MS in class III obese individuals.

Methodology

A descriptive cross-sectional study with class III obese individuals, aged ≥20–60 years. Blood pressure measurement, weight, height, body mass index (BMI), waist circumference (WC) and blood glucose, insulin, high-density lipoprotein cholesterol (HDL-c), and triglycerides data were obtained. HOMA-IR (homeostatic model assessment insulin resistance) calculation was carried out with a cutoff value of 2.71 for IR evaluation. The diagnosis of NAFLD was performed by liver biopsy and the diagnosis of MS was performed in accordance with the National Cholesterol Education Program/Adult Treatment Panel III (NCEPATP III).

Results

Of the 50 individuals evaluated, 86% were women and BMI means were 45.4 ± 3.6 Kg/m². The overall individuals had NAFLD, 70% steatosis, and 30% steatohepatitis. The diagnosis of MS occurred in 56% but showed no significant association with NAFLD (P = 0.254). Triglycerides (178 ± 65.5 mg/dL) and insulin (28.2 ± 22.6 mcU/mL) mean values were significantly higher in steatohepatitis (P = 0.002 and P = 0.042, resp.) compared to individuals with steatosis. IR was confirmed in 76% and showed a relationship with NAFLD severity.

Conclusion

NAFLD was not related to MS; however, MS components, evaluated in isolation, as well as IR, were related to the presence and severity of NAFLD.
described a strong correlation between NAFLD presence and metabolic changes in obese patients. The odds ratio for NAFLD development in this group was 10.77 regarding eutrophic individuals. Even if it is not part of the diagnostic criteria for metabolic syndrome (MS), NAFLD is often associated with this metabolic disorder [6].

MS is a condition intensely related to the global epidemic of obesity, since in its physiopathological basis the excess of visceral fat is a precursor of metabolic changes. It can be defined as a group of interrelated risk factors of metabolic origin which directly contributes to the development of cardiovascular disease (CVD) and/or diabetes mellitus type 2 (DM2). Still it has not been established whether MS development is due to a single cause or to multiple causes, but it has been acknowledged that abdominal obesity and insulin resistance (IR) seem to play a fundamental role in this syndrome genesis [7].

Considering that NAFLD relationship with MS and with its constituent components has been increasingly acknowledged, such findings have stimulated the interest in studies focusing the role played by liver disease on the increase of cardiometabolic risk, aspects that constitute the objective of the present investigation.

2. Methods

Class III obesity individuals, of both sexes, aged ≥ 20 to 60 years, with indication for bariatric surgery, were included in this study. These patients were from a clinic specialized in controlling obesity in Rio de Janeiro municipality, being the study conducted from August 2012 to October 2013. Pregnant women, nursing mothers, and patients with malabsorption bowel syndrome, acute and chronic infections, alcohol consumption exceeding 20 g/day in women and 40 g/day in men, and any other liver disease, except NAFLD, were excluded from the study.

2.1. Anthropometric Evaluation. Class III obesity classification was based on the World Health Organization (WHO) criteria [8]. BMI calculation was conducted using the weight (kg) and height (m²) [9] anthropometric measurements.

Waist circumference (WC) measurement was performed with the patient in a standing position with abdomen relaxed, arms at the sides, and feet together, using a nonextensible tape measure. The tape involved the individual in the largest abdominal diameter. The measurement was carried out at the completion of the patient’s normal expiration [10].

2.2. Biochemical Evaluation. For biochemical evaluation, blood sample was obtained by venipuncture, after a 12 h fast period. Laboratory tests were conducted to evaluate metabolism and liver function and damage: albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), gamma-glutamyl transpeptidase (GGT), lipid profile [total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c)], glucose, and insulin. Determinations of triglycerides, total cholesterol, HDL-c, and glucose were carried out by enzymatic colorimetric method.

### Table 1: NCEP/ATP III criteria.

<table>
<thead>
<tr>
<th>Components</th>
<th>Presence of ≥3 components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>≥100 mg/dL</td>
</tr>
<tr>
<td>HDL-c</td>
<td>&lt;40 mg/dL for men, &lt;50 mg/dL for women</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>≥150 mg/dL</td>
</tr>
<tr>
<td>WC</td>
<td>≥102 cm for men, ≥88 cm for women</td>
</tr>
<tr>
<td>Systemic arterial hypertension</td>
<td>≥130 × 85 mmHg</td>
</tr>
</tbody>
</table>

HDL-c: high-density lipoprotein cholesterol; WC: waist circumference.

Reagents for these biochemical evaluations were purchased from Labtest Diagnóstica S.A., Minas Gerais, Brazil. The LDL-c fraction was determined in accordance with Friedewald’s formula. Basal insulin was quantified by reversed-phase high performance liquid chromatography (RP-HPLC) (Labtest Diagnóstica S.A., Minas Gerais, Brazil). The cutoff points for total cholesterol and fractions, triglycerides, and glucose were those established by the NCEP-ATP III criteria for diagnosing MS, a disease confirmed when there is presence of three or more risk factors [11], as shown in Table 1.

IR was identified by the HOMA-IR index [12] obtained from the following calculation: HOMA-IR = fasting insulinemia (mU/L) × fasting blood glucose (mmol/L)/22.5. The reference values used were found in the literature for adult individuals [13], and the value above 2.71 was the cutoff point.

2.3. Systemic Blood Pressure Measurement. The blood pressure measurement by indirect method was conducted and the OMRON HEM 705 CP portable tensiometers (OMRON Healthcare Europe B.V., Hoofddorp, Netherlands) were used with appropriate clamps to measure the brachial circumference of the patient with a range of 0 to 300 mmHg and an accuracy of ±3 mmHg. The overall technical requirements for obtaining the appropriate systemic blood pressure, as well as the definition of the cutoff point equal to or above 130/85 mmHg that is already considered prehypertensive, obeyed the VI Brazilian Guidelines of Arterial Hypertension specifications (Diretrizes Brasileiras de Hipertensão Arterial VI-2010) [14].

2.4. The Diagnosis of NAFLD: Liver Biopsy. Histological evaluation was performed by liver biopsy through the withdrawal of 4 mm of the liver left lobe thickness, obtained via puncture with a 16 G × 15 cm Menghini needle (Euromed, Minas Gerais, Brazil). Biopsies were conducted by the medical surgeon along the bariatric surgery. All histological evaluations were performed by the same pathologist, who had no knowledge of the patients’ clinical and biochemical data, through hematoxylin-eosin, Masson and Perl's trichrome stainings (Interlab, São Paulo, Brazil). Hematoxylin-eosin allows a general view of acinar architecture, inflammatory infiltrates, and alterations in hepatocytes. Masson verifies the presence of fibrosis, be it portal, perisinusoidal, or around centrlobular veins and Perl verifies the presence of iron deposits [15].
NAFLD gradation and the staging of liver fibrosis were determined in accordance with the proposal of Brunt and coworkers [15]. Gradation was performed considering the presence of macrovesicular steatosis (simple steatosis), necroinflammatory activity (presence of steatohepatitis), and presence of fibrosis and cirrhosis.

2.5. Statistical Analysis. Statistical calculations were performed by the SPSS program version 17.0. The statistical analyses used were Student’s t-test (for mean of unpaired samples), Pearson’s Correlation, and Spearman’s Correlation (between nonparametric variables), and association was verified by the chi-square ($\chi^2$) test. A 5% significance level was considered.

This study was approved by the Research Ethics Committee of Hospital Universitário Clementino Fraga Filho of Universidade Federal do Rio de Janeiro (Research Protocol n° 011/06-CEP).

3. Results

The sample comprised 50 individuals, being 43 (86%) female and 7 (14%) male. Mean age was 43 ± 10.5 years, ranging from 26 to 60 years. Table 2 shows the sample general characteristics.

Regarding WC, in accordance with the cutoff set by WHO [8], 100% of the patients had a very high risk of metabolic complications associated with obesity.

The diagnosis of NAFLD was confirmed in 100% of the patients according to histological evaluation data after liver biopsy: 70% of the individuals had steatosis and 30% had steatohepatitis. Among those who had steatohepatitis, 13% presented fibrosis.

Means of the anthropometric variables were observed according to the staging of NAFLD (Table 3).

Table 4 shows the mean serum concentrations of biochemical indicators of liver function and damage according to the staging of NAFLD. GGT activity was higher in individuals with steatohepatitis (significantly for steatohepatitis with fibrosis versus steatosis); ALT and AST liver enzyme activities were within the normality standard as well as AP levels. Albumin dosages were significantly lower in individuals with steatohepatitis with fibrosis.

Among the obese patients studied, 56% had MS and 72% had systemic arterial hypertension (SAH), but there was no significant association between MS and NAFLD ($P = 0.254$) or between NAFLD and SAH ($P = 0.105$).

Table 5 shows the mean serum concentrations of MS and IR biochemical indicators according to the staging of NAFLD, and it reveals that the highest insulin and triglycerides means were observed in steatohepatitis (significantly for steatohepatitis with fibrosis versus steatosis).

Among the obese individuals with steatohepatitis those who had fibrosis showed HDL-c mean below the recommended cutoff point for MS corresponding to 38.9 ± 7.2 mg/dL ($P = 0.038$).

It was found that 76% of the individuals had IR according to the HOMA-IR calculation, 68% of these individuals had steatosis, 32% had steatohepatitis, and individuals who had fibrosis also had IR.

4. Discussion

In this study, we observed that individuals with the highest mean of WC showed a significantly more advanced degree of NAFLD confirming previous results, where excess of fatness, particularly in the abdominal region (central obesity), is strongly associated with NAFLD and IR. This predisposes to SAH, dyslipidemia, and inflammation, factors present in MS. The association between these clinical evidences and the excess of adipose tissue involves metabolic and inflammatory mechanisms [16]. Intra-abdominal fat in eutrophic individuals constitutes around 10% of body deposits. It is formed by fat surrounding the internal viscera, the omentum, and mesentery [17], and the omentum and mesentery venous drainage is given by the portal venous system. Thus, the liver is directly exposed to cytokines and free fatty acids (FFAs) released by the visceral adipose tissue, while the same metabolites released by the adipose tissue from other regions reach the liver diluted by the systemic circulation. In the NAFLD “portal theory”, there is an exacerbated release of FFAs, endotoxins, and proinflammatory cytokines of visceral fat that reach the liver, via portal system, promoting the development of liver resistance to insulin, hepatic steatosis, and inflammation in obese individuals [16, 18].

In general, albumin is a good marker of liver disease severity and classically evaluates liver function [19]. In our study, serum albumin levels were lower in the group with

Table 2: Sample general characteristics ($n = 50$).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean/SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43.0 ± 10.5</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>44.1 ± 3.8</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>121.4 ± 21.4</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>121.6 ± 12.7</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>25.1 ± 18.3</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>274 ± 15.9</td>
</tr>
<tr>
<td>AP (U/L)</td>
<td>76.1 ± 23.4</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>33.4 ± 21.8</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>132.0 ± 50.9</td>
</tr>
<tr>
<td>C (mg/dL)</td>
<td>197.7 ± 36.1</td>
</tr>
<tr>
<td>LDL-c (mg/dL)</td>
<td>121.8 ± 34.5</td>
</tr>
<tr>
<td>HDL-c (mg/dL)</td>
<td>48.3 ± 11.9</td>
</tr>
<tr>
<td>Insulin (mcU/mL)</td>
<td>19.0 ± 12.5</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>100.2 ± 19.0</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.5 ± 4.7</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>180 ± 125</td>
</tr>
</tbody>
</table>

AST: aspartate aminotransferase; ALT: alanine aminotransferase; AP: alkaline phosphatase; BMI: body mass index; C: total cholesterol; GGT: gamma-glutamyl transpeptidase; HDL-c: high-density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment insulin resistance; LDL-c: low-density lipoprotein cholesterol; SBP: systemic blood pressure; TG: triglycerides; WC: waist circumference.
Table 3: Mean of the anthropometric variables according to the staging of NAFLD.

<table>
<thead>
<tr>
<th>Variables</th>
<th>The staging of NAFLD by liver biopsy</th>
<th>Comparison of staging of NAFLD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steatosis (n = 31)</td>
<td>Steatohepatitis without fibrosis (n = 17)</td>
<td>Steatohepatitis with fibrosis (n = 2)</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>120.4 ± 20.3</td>
<td>119.9 ± 19.7</td>
<td>148.5 ± 47.4</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>119.5 ± 11.1</td>
<td>123.8 ± 11.9</td>
<td>144.0 ± 18.3</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>43.6 ± 3.4</td>
<td>40.5 ± 0.7</td>
<td>45.6 ± 2.2</td>
</tr>
</tbody>
</table>

Mean and standard deviation; BMI: body mass index; WC: waist circumference.

Table 4: Mean serum concentrations of biochemical indicators of liver function and damage according to the staging of NAFLD.

<table>
<thead>
<tr>
<th>Variables</th>
<th>The staging of NAFLD by liver biopsy</th>
<th>Comparison of staging of NAFLD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steatosis (n = 31)</td>
<td>Steatohepatitis without fibrosis (n = 17)</td>
<td>Steatohepatitis with fibrosis (n = 2)</td>
</tr>
<tr>
<td>AP (U/L)</td>
<td>75.1 ± 24.9</td>
<td>78.3 ± 20.2</td>
<td>73.1 ± 1.4</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>22.9 ± 12.8</td>
<td>29.9 ± 26.7</td>
<td>30.2 ± 7.6</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>27.1 ± 15.5</td>
<td>28.2 ± 17.5</td>
<td>28.9 ± 10.3</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>29.4 ± 17.8</td>
<td>42.6 ± 27.7</td>
<td>62.4 ± 14.6</td>
</tr>
<tr>
<td>Alb (g/dL)</td>
<td>4.5 ± 0.7</td>
<td>3.9 ± 0.3</td>
<td>3.3 ± 0.2</td>
</tr>
</tbody>
</table>

Mean and standard deviation; Alb: albumin; ALT: alanine aminotransferase; AP: alkaline phosphatase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transpeptidase.

steatohepatitis without fibrosis versus the steatosis group but still within the normality range. When individuals with steatohepatitis with fibrosis are considered, the albumin values found were even lower and just below the normality value recommended (3.4 g/dL). Such results are close to the values found by Sarma and coworkers [20] in patients with cirrhosis resulting from alcoholic etiology or cryptogenic origin (3.74 g/dL). Hypoalbuminemia is an important feature of liver disease worsening. Yennu and coworkers [21] observed that an albumin level less than 3.5 g/dL was an independent predictor of moderate to severe inflammation, as well as advanced fibrosis.
Among the hepatocellular damage markers mostly used in the literature are the transaminases, GGT, and AP [22]. In this study, the ALT, AST, and AP mean levels among individuals with hepatic steatosis and steatohepatitis showed no significant differences. Although many studies reported an increase in the liver function and damage markers in individuals with various degrees of NAFLD [23, 24], generally these enzymes are normal in over 78% of the individuals [25]. Furthermore, the increases described, most often, are moderate and are restricted to AST and ALT, suggesting that the diagnosis and severity of the disease cannot be defined solely by the evidence of liver damage. Papadia and coworkers [26] pointed out that the increase in AST and ALT means was regarded as a marker of the degree of fat infiltration in hepatocytes, with significant increase in individuals with triglycerides accumulation in the liver reaching over 70%, but not as a marker of inflammation and fibrosis. Lomonaco and coworkers [27] observed normal ALT levels in the overall NAFLD stages.

With regard to GGT values, the present study showed higher values in the individuals with steatohepatitis, significantly in the presence of fibrosis. These results are in line with the findings that demonstrate that liver disease worsening may be a possible explanation for GGT increase with the progression of liver fibrosis [28]. However, Silva and Duarte [29] found no association between GGT levels and hepatic steatosis, suggesting that the increase is due to GGT increased hepatic synthesis, like an adaptive answer to changes arising from the disease, associated with a greater GGT release into blood circulation.

Considering the MS biochemical components, hypertriglycerideremia and HDL-c low concentrations are the lipid profile impairments usually associated with the presence of NAFLD [30]. In the present study, HDL-c serum concentrations, as assessed in stages of liver disease, showed no significant changes. However, in individuals with steatohepatitis who also had fibrosis, HDL-c mean concentrations were significantly lower. Boza and coworkers [31] have observed significantly HDL-c lower means in class III obese individuals with NAFLD in comparison to the group without the disease, and this variable was the only lipid fraction associated with the diagnosis of NAFLD. Similarly, a study developed by Chaves and co-workers [24] reported that the only lipid fraction related to the presence of steatosis was HDL-c, showing significantly lower median in patients with NAFLD. In the study of Dias and coworkers [32], which assessed possible predictors of NAFLD in obese individuals, no correlation for lipid fractions was observed occurring in the most advanced stages of the liver disease. However, a weak negative correlation was observed between HDL-c levels and the degree of simple steatosis, classified as mild, moderate, or severe.

In the present study, we observed that triglycerides mean was higher in steatohepatitis (especially in fibrosis patients)
as found in the study of Júnior and Nonino-Borges [33], which suggests that such changes are steatohepatitis predictors since triglycerides synthesis increases in patients with NAFLD, mainly in those with obesity and DM2. Triglyceride accumulation in the liver tissue occurs as a consequence of adipose tissue lipolysis and hepatic de novo lipogenesis [34]. Triglycerides can be exported from the liver by very low-density lipoprotein (VLDL) particles formed by the incorporation of triglycerides to apolipoprotein B (apoB) [35]; thus, changes in the synthesis and secretion of apoB also contribute to hepatic triglyceride accumulation [36].

Insulin mean was high in obese individuals with steatohepatitis (especially in fibrosis patients), and IR was higher (although not significant) in the most advanced stage of liver disease. This metabolic change is considered a key risk factor in NAFLD pathogenesis being connected with the increase in oxidative stress and lipotoxicity [37–39]. It is believed that IR, oxidative stress, and inflammatory cascade play an essential role in the disease pathogenesis and progression. In addition to these facts, there are a number of interactions between hepatocytes, stellate cells, fat cells, Kupffer cells, inflammatory mediators, and reactive oxygen species resulting in inflammation or cirrhosis. In IR states, fat and muscle cells have a preference for oxidizing lipids and a high proportion of the higher levels of FFA released from fat cells are taken by the liver, leading to steatosis [40]. Macrovesicular steatosis arises from the increased hepatic synthesis of fatty acids, esterification of these fatty acids in triglycerides, and decreased triglyceride transport out of the liver [41]. Peripheral resistance to insulin also contributes to the increased entry of FFAs in the liver, which causes unbalance between oxidation and export of FFAs, resulting in fat accumulation in the liver parenchyma [42].

5. Conclusion

NAFLD was not related to the diagnosis of MS; however, MS components, evaluated in isolation, as well as IR, which is often associated with MS, were related to the presence and severity of liver disease. These results suggest the importance of monitoring these components in the screening for NAFLD.

Conflict of Interests

The authors declare no conflict of interests.

References


