

## Nonalcoholic Fatty Liver Disease (NAFLD), a Manifestation of the Metabolic Syndrome: New Perspectives on the Nutritional Therapy

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### Abstract

Nonalcoholic fatty liver disease (NAFLD) is a multifactorial hepatic disease that develops through complex mechanisms that may be strongly influenced by dietary composition. NAFLD treatment is based on multidisciplinary intervention, which includes nutritional aspects. The objective of this review was to elucidate the influence and role of dietary composition, including fatty acid types, antioxidant nutrients, pre and probiotics and vitamin D in the nutritional treatment and prevention of NAFLD. Increased intake of Monounsaturated fatty acids (MUFAs) and omega-3 polyunsaturated fatty acid (PUFA), particularly as replacements for saturated fat and in a higher proportion than carbohydrates, is beneficial to NAFLD patients, improving insulin resistance; increasing plasma levels of adiponectin and its synthesis by the adipose tissue; and restoring the expression of hepatic peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ), which in turn reduces cholesterol levels and triacylglycerol accumulation. n-3 PUFAs can reduce lipotoxicity caused by excessive saturated and trans fatty acid ingestion and exert a protective role in inflammatory pathways, promoting resolvins and protectins. Several mechanisms linking gut flora to NAFLD have been proposed, such as inflammation and energy extraction. Studies are often designed to explore the beneficial effects of probiotics, prebiotics and vitamin D in these pathways. The results of this review reveal that the strong positive influence bioactive compounds have on these inflammatory processes must be considered when developing treatment and prevention plans for NAFLD patients.

**Keywords:** NAFLD; Nutrition; Fatty acids; Intestinal microbiota; Vitamin D

### Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as the abnormal accumulation of lipids, primarily in the form of triglycerides in individuals who do not consume significant amounts of alcohol ( $\leq 20$  g ethanol/d) [1]. NAFLD is characterized by a spectrum of disease varying from simple steatosis through to steatohepatitis with fibrosis and scarring, which can lead to cirrhosis [2].

The prevalence of individuals diagnosed with NAFLD varies from 10-39% of the world population. The disease can be associated with other co-morbidities and affects 50% of diabetics, 57 to 74% of obese people, and 90% of morbidly obese people. In the pediatric population, NAFLD affects 2.6% of eutrophic children and up to 60% of obese children and adolescents [3-5]. NAFLD is currently recognized as a clinically emergent problem among obese patients [6,7].

NAFLD is a multifactorial disease and is the hepatic manifestation of metabolic syndrome. The pathogenesis of NAFLD involves complex mechanisms and has been extensively discussed in the literature. Insulin resistance (IR), inflammatory states, nutrients, genetic factors and lifestyle all play key roles in its development [8]. The central mechanism responsible for NAFLD is insulin resistance, which causes an influx of free fatty acids into hepatocytes, elevates de novo hepatic lipogenesis that can exceed the rate of  $\beta$ -oxidation of fatty acids and causes very low density lipoprotein (VLDL) exportation, ultimately resulting in hepatic fat accumulation [7]. This disequilibrium, caused by hepatic lipid influx, can induce reactive oxygen species (ROS) production, which increases oxidative stress and activates stellar hepatic cells. Molecular lipotoxicity can occur once the influx of free fatty acids activates complex intracellular pathways, including the c-jun N-terminal Kinase enzyme and the Toll-like 4 receptor (TLR-4). Moreover, increased lipid peroxidation leads to the generation of ROS, which are toxic mediators that ultimately promote mitochondrial dysfunction in hepatocytes [9,10].

Visceral adipose tissue accumulation, which contributes to inflammatory pathways and the development of peripheral insulin resistance, has also been explored as a potential mechanism for

developing NAFLD [11]. Inflammatory cells, such as macrophages, infiltrate visceral adipose tissue, which increases inflammatory adipokine secretion, leptin, interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- $\alpha$ ) and angiotensinogen as well as reduces adiponectin production [12]. When the tissue becomes insulin-resistant, it induces major lipolytic activity and increases the levels of free fatty acid influx into the portal vein, potentially causing hepatotoxicity. Small intestinal bacterial overgrowth (SIBO) may also promote the progression of NAFLD into nonalcoholic steatohepatitis by enhancing intestinal permeability and favoring absorption of endotoxins with pro-inflammatory and pro-fibrogenetic effects on the liver [13]. It is worthwhile to consider that the microbiota and nutrients involved in intestinal health can inform strategies for NAFLD treatment.

Several studies have reported the important role that dietary composition plays in the development and progression of NAFLD [14-16]. A high-fat diet converts the pathology from bland NASH with fibrosis, which leads to cirrhosis in humans [17]. It has been proposed that the levels and types of fatty acid composition, especially saturated and trans fatty acids, promote the accumulation of free fatty acids (FFA) in hepatocytes, which causes apoptosis by diverse inflammatory pathways. These may include microbiota modification, insulin resistance, and ROS-induced stress that affect the mitochondrial membranes, endoplasmic reticulum and lysosomes (see review Estadella et al., [10]).

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Lipid peroxidation increases the levels of reactive oxygen species, which may be partially responsible for hepatocyte dysfunction. Antioxidant nutrients reportedly exert an important role in reducing ROS production [18]. Moreover, mono and polyunsaturated fatty acids can play a protective role in NAFLD development, mainly because n-3 PUFAs (polyunsaturated fatty acids) have many beneficial effects on most metabolic syndrome features and have anti-inflammatory properties [19].

The objective of this review was to elucidate the influence and role of dietary composition, including the type of consumed fatty acids, antioxidant nutrients, pre and probiotics and vitamin D in the nutritional treatment and prevention of NAFLD.

## NAFLD and Lipids

Fatty acids are an essential constituent of the cell membrane, as they modulate the activity of membrane-bound transporters and enzymes, modify membrane fluidity, serve as intracellular messengers and alter intracellular functions. One animal study reported that dietary lipids can change both the chemical composition and lateral organization of rat hepatocyte plasma membranes [20].

In humans, dietary fat intake may play a critical role in the development of NAFLD. It is now clear that both the levels of lipids introduced and the types of fatty acids ingested affect the development of the disease [21].

The lipid metabolic perturbations in NAFLD are complex and may be more fully explored. The evaluation of the amounts and quality of different lipid classes, comparison of fatty acid distribution and comprehension of the functions fatty acids have in different NAFLD mechanisms can be an important tool for NAFLD treatment [21]. Recently, lipotoxicity and plasma lipidome have emerged as novel targets for potential therapeutic strategies [21,22]. The possible mechanisms by which lipids affect NAFLD treatment will be further explored in the present review.

## NAFLD and mono and polyunsaturated fatty acids

Diet composition may directly and indirectly influence the NAFLD pathways. Dyslipidemia is considered a risk factor for NAFLD development [23]. Several studies have demonstrated that a high intake of monounsaturated fat improves postprandial glucose, decreases oxidized low density lipoprotein (LDL), LDL cholesterol, and triacylglycerol concentrations, particularly for a diet in which monounsaturated fats replace both saturated fat and high levels of carbohydrates [8,24-26].

A recent study has demonstrated that in only 6 weeks, an olive-oil rich diet, the Mediterranean diet, which contains high levels of monounsaturated fatty acids, promotes a decrease in hepatic steatosis accompanied by an improvement in peripheral insulin sensitivity and a reduction in circulating insulin concentrations [27]. These authors also suggested that an increased intake of monounsaturated fatty acids (MUFAs) and omega-3 PUFAs, particularly as a replacement for saturated fat and as a higher proportion of the diet than carbohydrate intake, is beneficial for NAFLD patients. Some studies suggest that insulin resistance may be accompanied by a change in the composition of fatty acids in the blood and tissues, with deficiency of omega 3polyunsaturated fatty acids. Moreover, there have been promising results in animal models and humans with the use of omega 3 in NAFLD [28,29].

The beneficial effects of supplementing with omega 3 can be partially explained by the increase in the plasma levels of adiponectin as

well as the increased synthesis of adiponectin in the adipose tissue and the restored expression of hepatic peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ), promoting a reduction in the cholesterol levels and accumulation of triacylglycerol [30].

Several authors have shown that n-3 PUFA may have beneficial effects in preventing the complications of lipotoxicity that are primarily caused by excess saturated and trans fatty acids. PUFA dietary intake has beneficial effects on intra-hepatic fat accumulation in patients with NAFLD [19,21,31]. In a study on a pediatric NAFLD population, n3-docosahexaenoic acid treatment for 6 months improved the fatty liver and insulin sensitivity [29].

Omega 3 (n-3) PUFAs, especially eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA), exert protective roles in the inflammatory pathways involved in NAFLD development and progression [19].

The omega-3 (n-3) fatty acids are essential, polyunsaturated fatty acids (PUFAs) that cannot be synthesized *in vivo*. Instead, they are consumed in the diet, especially from fish oil, flaxseed and some nuts. These fatty acids, which are derived from  $\alpha$ -linolenic acid and mainly occur as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are anti-inflammatory. The other key group of PUFAs, n-6 fatty acids, is predominantly found in grain and derived from linolenic acid; arachidonic acid is proinflammatory and prothrombotic [28].

Omega-6 (n-6) and omega-3 (n-3) fatty acids (PUFAs) are competitively metabolized by the same pathway. In this way, the ratio of n-6 to n-3 should be approximately 3:1; however, there is no consensus on the adequate ratio for achieving beneficial effects in NAFLD. Although this ratio has not been established, it is strong confirmation that a low total level of n-3 is found in NAFLD and is associated with steatosis, increased oxidative stress and progression of the disease [28].

Briefly, the n-6 and n-3 fatty acids compete for enzymes involved in chain desaturation and elongation reactions. Although these enzymes have higher affinity for the n-3 family, the conversion of  $\alpha$ -linolenic acid in PUFA-LC is strongly influenced by the levels of linoleic acid in the diet. Thus, the ratio between the daily intake of food sources of fatty acids n-6 and n-3 is important in human nutrition [32-34].

Studies have revealed a significant reduction in the mortality rate of patients with cardiovascular disease, inflammation, and symptoms resulting from asthma when the ratio of linoleic/ $\alpha$ -linolenic in the diet is 4:1; the symptoms were intensified for a ratio of 10:1 [32-34].

Moreover, there was a study in which the exposure of human hepatocytes to different mixtures of linoleic and  $\alpha$ -linolenic affected the transcription levels of a selection of genes encoding regulatory proteins that are involved in several stages of fatty acid metabolism. This effect strongly depends on the ratio of n6/n3 fatty acids, revealing the importance of ingesting not only appropriate levels of fatty acids but also an appropriate ratio of n6 and n3 fatty acids [35].

Arachidonic acid, di-homo- $\gamma$ -linolenic acid (20:3 n-6), and eicosapentaenoic acid (20:5 n-3) are precursors of series 1, 2 and 3 prostanoids and series 4, 5 and 6 leukotrienes, respectively. Cyclooxygenase (COX), which converts these free fatty acids in cyclic endoperoxides, produces prostaglandins and thromboxane, the prostanoids. Lipoxygenase (LOX), which is also linked with the production of lipoxins 1,9, contributes to the production of leukotrienes [36].

Increased levels of fish oil decrease the levels of arachidonic acid in the inflammatory cell membranes, resulting in less substrate availability

for the synthesis of eicosanoids that are derived from arachidonic acid. Additionally, EPA inhibits arachidonic acid release from phospholipids, which is mediated by phospholipase A2, and competitively inhibits the COX-mediated oxygenation of arachidonic acid. Thus, n-3 fatty acids have important anti-inflammatory properties, including decreasing the production of series 2 prostaglandins and thromboxanes [37].

The excessive production of series 2 prostanoids may be related to immune disorders, cardiovascular disease, and inflammation. Thus, people are encouraged to increase their intake of n-3 fatty acids to increase the production of series 3 prostanoids [36].

Alterations in the liver long chain polyunsaturated fatty acids (LCPUFA) status in NAFLD are characterized by significant depletion of the n-3 LCPUFA content and an increased in the n-6/n-3 LCPUFA ratio, which can favor the development of NAFLD [38].

Studies investigating whether dietary n-3 LCPUFA supplementation triggers an antioxidant response that prevents liver steatosis have demonstrated that supplementation results in a reduction in the hepatic lipid content; increased insulin sensitivity; and decreased levels of serum transaminase, TNF- $\alpha$ , soluble TNF receptor 1 and 2, and oxidative stress markers, with improvement of hepatic steatosis. These outcomes are mediated by either n-3 LCPUFA derived resolvins and protectins and/or direct PPAR- $\alpha$ -dependent factor nuclear kappa B (NF- $\kappa$ B) and inhibition of the activator protein 1 (AP-1) [39-41].

Omega-3 fatty acids are key regulators of the transcription of hepatic genes, such as PPAR $\alpha$  and sterol regulatory element binding protein-1 (SREBP-1). PPAR $\alpha$  reduces plasma lipids and increases mitochondrial beta oxidation, and omega-3 fatty acids activate this transcription factor, improving the expression of genes associated with fatty acid and lipid metabolism [42]. The SREBP-1 influences the genes involved in fatty acid and cholesterol synthesis, reducing endogenous lipid production and improving peripheral insulin sensitivity [28]. Moreover, the levels of omega 3 fatty acids in the diet can substantially influence the stimulation of adiponectin, an anti-inflammatory adipokine, gene expression [43].

EPA and DHA promote decreases in the levels of anti-inflammatory and inflammation resolving resolvins and protectins [37]. The resolvins and protectins are new families of locally acting mediators that are derived from essential fatty acids. The synthesis of resolvins and protectins involves the COX and LOX pathways, and their biological effects of have been widely evaluated in cell culture and animal models as anti-inflammatory and inflammation resolving; for example, they inhibit the transendothelial migration of neutrophils, preventing the infiltration of neutrophils into sites of inflammation and inhibiting IL-1 $\beta$  production. Furthermore, protectin D1 specifically inhibits the synthesis of TNF- $\alpha$  and IL-1  $\beta$  [37].

Several studies have confirmed that n-3 fatty acids are involved in the regulation of hepatic gene expression, cell membrane composition, insulin sensitivity, anti-inflammatory effects, and the reduction of TNF- $\alpha$  levels, decreasing liver steatosis [19,21,28,31,44,45].

### **NAFLD, intestinal health and inflammatory pathways**

Multi-targeted therapy could optimally treat NAFLD patients. Recently, interesting nutritional strategies like the use of dietary supplements, such as probiotics and long chain omega-3 polyunsaturated fatty acids, have been adopted in NAFLD treatment [28,46,47].

Among the pathogenic factors leading to NAFLD, crosstalk between the gut and liver is critical [48]. Currently, specific nutrients that are capable of increasing the intestinal permeability to bacterial

endotoxins promote the inflammatory response of liver cells, leading to a profibrogenic state [49]. A recent study utilizing animal models [50] demonstrated that restoring the gut microflora is critical to protecting the liver from fat accumulation, which reinforces that nutrients that act on inflammatory pathways to improve gut health and promote adequate microbiota colonization, such as pro and prebiotics and vitamin D, should be considered in NAFLD treatment.

## **NAFLD: Probiotics and Prebiotics**

### **Microbiota**

The human gut contains substantial microorganisms, including bacteria, archaea, viruses, and some unicellular eukaryotes, called microbiota, which may provide energy, nutrients, and immunological protection that benefit the host [51].

The intestinal flora is important to normal gut function and maintenance of health, and the dietary composition can influence the intestinal colonization. In healthy adults, 80% of the identified fecal microbiota can be classified into the following three dominant phyla: Bacteroidetes, Firmicutes and Actinobacteria. The Firmicutes to Bacteroidetes ratio is important in the human gut microbiota composition [52]. Studies have demonstrated that human obesity is associated with low levels of intestinal Bacteroidetes and high levels of Firmicutes, favoring the development of metabolic syndrome and NAFLD [53,54].

Several mechanisms have been proposed that link the gut flora to obesity; for example, the gut microbiota increase energy extraction from indigestible dietary polysaccharides and elevate plasma lipopolysaccharide levels, resulting in chronic low-grade inflammation. Additionally, the gut microbiota in regulate the host genes that control metabolic processes [55,56].

## **Gut Microbiota: Inflammation and Energy Extraction**

### **Inflammatory process**

Germ-free mice are protected from obesity and metabolic syndrome. There may be complex mechanisms for the potential increased energy harvest from the diet through dietary-induced or genetically induced obese microbiota [57]. However, experimental protocols that colonize germ-free mice with selective human flora are essential for investigating the influence of diets and other confounding factors on the microbiota and their consequent implications for the host metabolism [58].

The quality of the diet influences the gut microbiota composition. Diet explains 57% of the bacterial variation in the gut while genetic background only accounts for 12% of the variation in animals, suggesting the importance of diet in determining the composition of the gut microbiota [59].

Cani et al. [55] found that mice treated with a high-fat diet have a significant change in the composition of their dominant bacterial populations within the gut microflora, including a decrease in the number of Bifidobacteria, Eubacterium rectal-Clostridium coccoides group and Bacteroides, favoring an increase in the gram-negative to gram-positive ratio. This change in the gut microflora composition was associated with a significant increase in plasma lipopolysaccharide (LPS) levels accompanied by an increase in the intestinal permeability and a reduction in the expression of genes coding for tight junction proteins [60,61]. These studies demonstrate the possible pathways that are responsible for the gut microbiota in metabolic endotoxemia, inflammation, obesity, liver hepatic triacylglycerol accumulation, insulin resistance and type 2 diabetes [62,63].



Increasing evidence suggests the gut microbiota in humans controls obesity and visceral fat storage [64]. Small intestinal bacterial overgrowth (SIBO), a common condition in obese individuals, is mainly stimulated by slowing of the oro-coecal transit time, may promote NAFLD progression to nonalcoholic steatohepatitis by enhancing intestinal permeability and favoring absorption of endotoxins with pro-inflammatory and pro-fibrogenetic effects on the liver [65].

In previous clinical studies performed by our research group, we found a positive correlation between the calories derived from SFA intake and visceral fat in NAFLD patients [66,67]. Moreover, we recently demonstrated a positive correlation between the plasma endotoxin concentration and pro-inflammatory cytokines, especially IL-6, and insulin resistance in obese adolescents. After long-term (one year) interdisciplinary therapy, endotoxemia, pro-inflammatory status and insulin resistance were decreased [68]. These results demonstrate the efficiency of lifestyle changes (i.e., nutritional modification) in reducing the pro-inflammatory state of obese individuals [69].

An increase in serum LPS has been associated with the proinflammatory state, development of insulin resistance and type 2 diabetes. LPS stimulates Toll-like receptors in the cell membranes, which activate specific kinases and lead to insulin resistance [68].

These pathways also activate NF- $\kappa$ B, which results in the expression of inflammatory genes. Similar to LPS, saturated fatty acids are also recognized by membrane receptors that trigger proinflammatory signaling pathways [56,62,63,68].

### Energy Extraction: Metabolic Processes

The bacterial metabolites, TMAO (trimethylamine-N-oxide) and SCFA (short chain fatty acids) are markers of risk for disease and have varied effects on metabolism [51].

TMAO is a phospholipid that is integral to cell membranes and is present in foods with higher fat contents that are associated with the atheroprogession process, which is influenced by the microbiota composition [51]. This phospholipid is a new biomarker for developing CVD as well as a novel biomarker for food choice behavior. In fact, NAFLD has recently been associated with the pro atherogenic state [70].

The SCFAs produced by microbiota fermentation are acetate, propionate and butyrate, which are used as an energy source for colonocytes, and the overproduction of SCFAs could increase the liver lipogenesis because they are a lipogenic substrate [71]. Indeed, SCFAs are affected by the gut microbiota composition and influence the regulation of blood lipids as well as affect pathways related to food intake and lipid metabolism. The intestinal microbiota break down indigestible polysaccharides (i.e., fiber) to short-chain fatty acids (SCFAs) providing 80 to 200 kcal per day or approximately 4–10% of the daily energy intake in normal adults [72].

The mechanisms supporting that the absence of adequate gut microbiota is associated with the development of obesity and NAFLD are related, in part, to the effect of healthy microbiota on the reduction of hepatic de novo lipogenesis as well as to the inhibition of triacylglycerol storage in the white adipose tissue. The latter effect is thought to be caused by an excessive production of FIAF (fasting-induced adipocyte factor) or angiopoietin-like protein 4 (ANGPTL4) in the intestines of the germ-free mice [73].

FIAF is a circulating lipoprotein lipase inhibitor produced by the intestines, liver and adipose tissue. FIAF inhibits lipoprotein lipase (LPL), blocking the disassociation of fatty acids from triacylglycerols

for uptake into tissues as well as upregulating fatty acid oxidation and uncoupling proteins, potentially reducing the fat storage in germ-free mice [74]. FIAF also plays a role in the metabolic adaptation to fasting via PPAR activation [75].

Several systems in people can be affected by bacterial components and metabolites of the gut microbiota detecting, especially in the case of the development of metabolic diseases [51]. The gut microbiota can directly and indirectly affect the host's health through bacterial components and metabolites.

### Probiotic Effects

The probiotics promote gastrointestinal health and beneficial consequences for the liver.

In colonic epithelia, probiotics stimulate mucin production and improve the self-protecting properties of the intestinal epithelium by stimulating tight junctions, which competitively exclude microbial pathogens. Indeed, the tight junction proteins remain intact and, thereby, prevent both the uptake of macromolecules and translocation of viable organisms to mesenteric lymph nodes and the liver [76].

Moreover, the integrity of the intestinal barrier decreases the LPS exposure and proinflammatory signaling, improving the anti-inflammatory cytokines, such as interleukin-10, which results in less vulnerability to hepatotoxins and limits intestinal bacteria overgrowth (IBO) and LPS production [76]. Altogether, probiotic supplementation is an important tool for treating NAFLD patients.

Corroborating these findings, studies have demonstrated that gut microbiota manipulation with probiotics in rodents with fatty livers reduces intestinal inflammation and improves the function of the epithelial barrier [60,61]. Hence, probiotics could be used to treat NAFLD human patients [13].

Loguerio and colleagues [77] showed that probiotics may reduce NAFLD liver injury and improve liver function tests. Another study found that treatment with 500 million *Lactobacillus bulgaricus* and *Streptococcus thermophiles*/day in adults with biopsyproven NAFLD promotes a significant reduction of the levels of liver transaminases [78].

These data suggest that the diet composition has an important role on the development and treatment of NAFLD [67]. Additionally, it is essential to consider the use of probiotics to treat and prevent NAFLD [62].

To further elucidate the role of the gut microbiota and gut-liver-axis both in causing or worsening obesity itself and/or related complications including NAFLD and NASH, robust, well-designed studies should be performed. These studies should focus on the mechanisms involved in the possible imbalances of the numerous metabolic, toxic, and immunological actors participating in the gut-liver-axis. The probiotic-mediated gut microbiota modulation is a promising tool for treating NAFLD, NASH, and obesity due to the safety, tolerability and efficacy of this treatment method [78,79].

Using probiotics as an intervention for intestinal microbiota can modulate the expression of nuclear receptors, improving insulin resistance in the liver and adipose tissue as well as protecting against the development of NAFLD. Randomized placebo controlled trials on the use of probiotics in NAFLD are ongoing in humans [46-48].

### NAFLD and Prebiotics

Prebiotics are beneficial, non-digestible food ingredients that

affect the host by selectively stimulating growth and/or modifying the metabolic activity of select intestinal bacteria [80]. Various fermented ingredients are classified as prebiotics, including inulin-type fructans and galactans [81].

The main health effects of prebiotics are due to three principal mechanisms that include selective modulation of the gut microbiota to reduce inflammation; improvement of glucoregulation and modification of lipid metabolism including reduced de novo fatty acid synthesis and SCFA production; and reduction of body fat [81,82].

Modulation of the gut microbiota is linked to improvement in the glucose, energy intake, insulin, satiety hormones, hepatic cholesterol, and triacylglycerol accumulation. Prebiotics have been reported as a potential therapeutic approach for reducing the risk of obesity and altering the composition of the gut microbiota (Figure 1) [78].

Prebiotic fibers have a bifidogenic effect and are associated with reduced LPS levels. As stated before, LPS, also known as lipoglycans, are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; LPS are found in the outer membrane of gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals [83].

Jumpertz et al. [84] reported that the stool energy in proportion to the ingested calories is positively correlated with the abundance of the

phylum Bacteroidetes and negatively correlated with the abundance of the phylum Firmicutes in the feces of people.

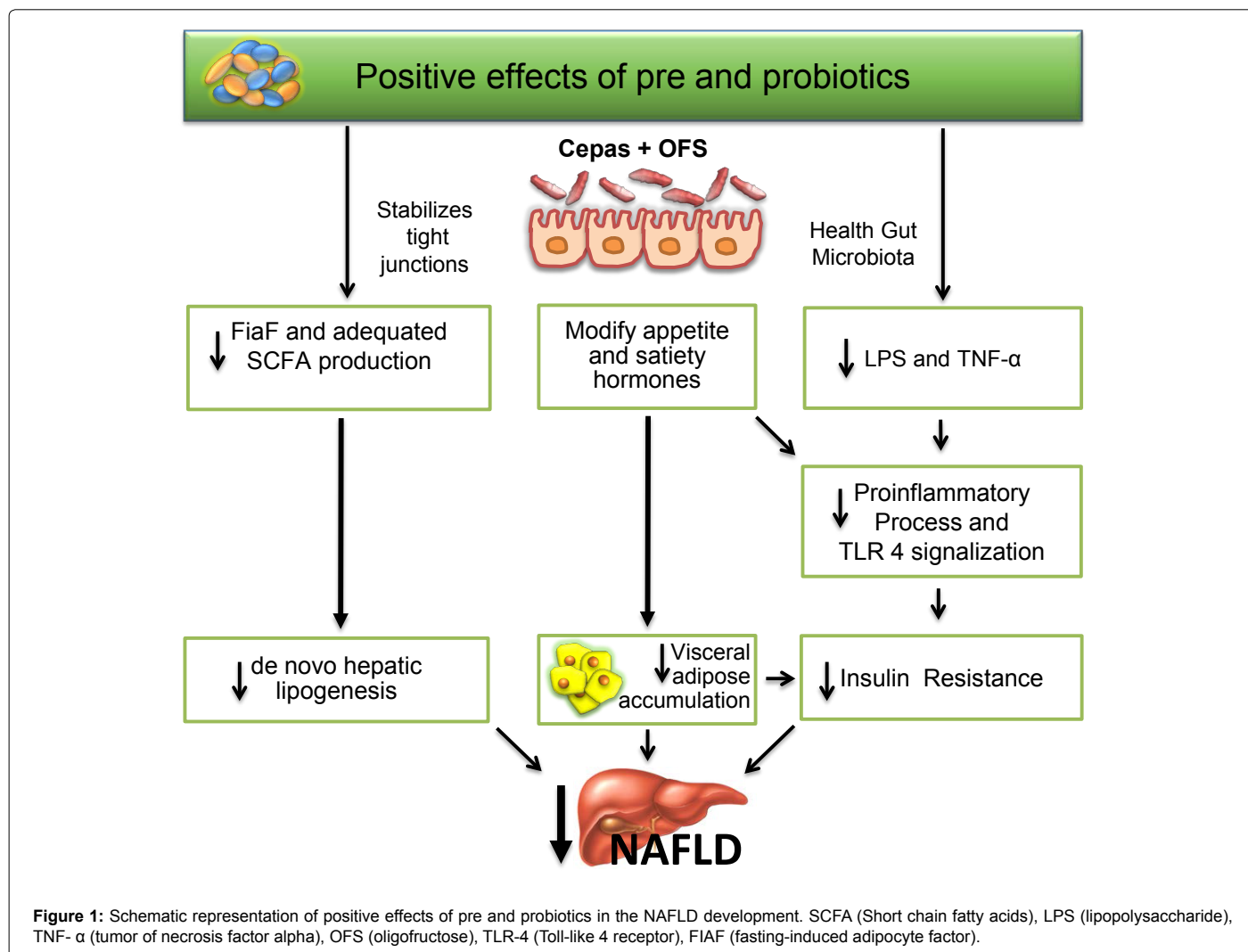
Dysbiosis in the gut microbiota refers to a condition with microbial imbalances in the bowel that may activate hepatic de novo lipogenesis by increasing the expression of lipogenic enzymes, acetyl co-A carboxylase (ACC), and fatty acid synthase (FAS) [64].

A recent study in obese rats showed that prebiotic fibers improve or normalize gut microbiota dysbiosis by decreasing Firmicutes and increasing Bacteroidetes phylae [82].

Finally, prebiotic induced changes in the gut microbiota also influence the production of the gut trophic hormone, glucagon-like peptide-2, which could potentially decrease the lipid and LPS concentrations due its effects on the intestinal permeability and epithelial tight junctions [85].

Prebiotic-rich diets may ameliorate NAFLD by attenuating de novo fatty acid synthesis as has been shown in animal models [86-89]. Fiordaliso et al. [89] verified that animals treated with oligofructose (OFS) have a modified liver capacity that can synthesize triacylglycerols from free fatty acids in isolated hepatocytes.

However, in another animal model of germ-free mice, there was a doubling of the hepatic triacylglycerol content and a concomitant



increase in the hepatic mRNA levels of sterol-responsive element-binding protein (SREBP-1) and carbohydrate-responsive element-binding protein (ChREBP), both of which are positive regulators of the aforementioned lipogenic enzymes [90,91].

Moreover, prebiotics associated with probiotics promote the production of short chain fatty acids (SCFA), leading to the growth of indigenous bifidobacteria and/or lactobacilli and lowering the luminal pH, which in turn prevents the growth of pathogens [92]. Acetate, propionate and butyrate are the main SCFAs produced in the large intestine. As described previously, although the greater part of butyrate is metabolized by colonocytes, propionate and acetate are delivered to the liver via the portal vein [93].

Butyrate is a major metabolite in the colonic lumen arising from bacterial fermentation of dietary fiber and is a critical mediator of the colonic inflammatory response. This SCFA is associated with the inhibition of HMG-CoA reductase, an enzyme involved in the metabolic pathway that produces cholesterol. Studies have shown that a diet enriched with prebiotic fibers promotes an increase in the ratio of propionate to acetate, decreasing lipogenesis as propionate inhibits lipogenesis, whereas acetate promotes the process [88,93].

Animals receiving a high fat diet and prebiotic treatment have decreased steatosis, fat storage in the white adipose tissue, systemic inflammation, and insulin resistance [93,94]. Prebiotic treatment in people results in a reduction in BMI, waist circumference, fat mass, and insulin resistance [81,95].

Prebiotic fiber supplementation is associated with reduced body weight or attenuated weight gain in lean, high-fructose fed high-fat, high-sucrose fed and genetically obese rodent models [96].

Although the majority of animal studies use a 10% inulin dose by weight with a minimum 4 week supplementation period, Sugatani et al. [97] reported reductions in the liver TAG and cholesterol in cafeteria diet-fed rats supplemented with 5% synthetic inulin for 3 weeks. In comparison, improvements in serum and liver lipids with prebiotic doses are reported to be as high as 20% [87]. The minimum inulin dose and necessary duration has not been fully defined and requires further study.

Human studies assessing the effects of prebiotic fiber supplementation on NAFLD patients are lacking; however, we can verify clinical protocols to identify the effects of prebiotic fiber supplementation on serum lipids. Parnell and Reimer [98] reported significant weight loss following 3 months of oligofructose supplementation in overweight and obese adults. Other possible mechanisms for the action of prebiotics in humans include improvements in glycemia and modifications to plasma glucagon-like peptide-1, peptide YY and ghrelin [82].

In summary, the prebiotic-induced, gut-mediated changes in luminal and peripheral metabolism include a reduction in bacteria-derived hepatotoxins, improved gut epithelial barrier, reduced inflammation, decreased de novo lipogenesis, modified appetite and satiety, and improved glycemic control [82] (Figure 1).

## NAFLD and Vitamin D

Recent studies suggest that vitamin D is associated with obesity, diabetes, cardiovascular diseases, metabolic syndrome and NAFLD [99-101]. In a multiple logistic regression analysis study, performed on Korean men, the tertiles with lower 25(OH)D(3) levels presented with a significantly increased risk for NAFLD compared with the highest tertile, even after adjusting for the body mass index and metabolic syndrome. Accordingly, individuals with higher serum 25(OH)D3 presented with a significantly reduced risk for NAFLD independent of

obesity and metabolic syndrome [99]. Additionally, inadequate 25(OH) D status progressively increased the odds of NAFLD when classified categorically as sufficient (25(OH)D (>30 ng/mL), insufficient (15-30 ng/mL) or deficient (<15 ng/mL) [102].

In a recent meta-analysis, the vitamin D role in the pathogenesis of NAFLD was discussed. Deficiency of vitamin D, considering a cut-off serum level ranging from 12 to 30 ng/mL, was associated with a higher NAFLD risk. The normal range of vitamin D remains controversial; however, the most recent Institute of Medicine (IOM) Committee report endorses the use of 20 ng/mL [103].

The lower 25(OH) D concentrations in overweight and obese individuals needs to be confirmed and the possible reasons for differences in these concentrations must be studied in more detail to better manage the vitamin D status [104]. To prevent and treat vitamin D deficiency by maintaining serum 25(OH)D levels above 30 ng/mL, the Endocrine Society Guidelines recommend 400-1000 UI for children and 1000-2000 UI for adults [105].

The vitamin D is a hormone implicated in several aspects of metabolism and the human immune system [106]. This vitamin can be obtained from exogenous sources and can be synthesized in the skin by conversion of 7-two hydroxylation in the liver and kidney, generating active vitamin D (vitamin D, 1,25(OH)2D3) [107]. The hepatocytes are exclusively responsible for 25-hydroxylation, which is mediated by CYP27A1 and CYP2R1, two cytochromes expressed in the liver [108]. Barchetta et al. [109] demonstrated that the expression of these cytochromes in NASH patients is lower than in the control group, but this difference was not significant.

Moreover, liver vitamin D receptor (VDR) expression has a strong association with the NASH diagnosis independent of BMI, insulin resistance or adiponectin, suggesting a loss in the hydroxylation of hepatocytes and the hepatoprotective role of vitamin D [109]. The VDR is expressed in many tissues, such as the liver, pancreas, and several immune cells, and its expression is most abundant on the epithelial cells of the gastrointestinal tract. The VDR modulates the expression of several other genes that are involved in converting dehydrocholesterol to previtamin D3 by ultraviolet radiation [107]. The VDR is expressed by macrophages; 1,25(OH)2D3 upregulates the inhibitor of nuclear factor (NF)- $\kappa$ B (I $\kappa$ B- $\alpha$ ) by increasing mRNA stability and decreasing I $\kappa$ B- $\alpha$  phosphorylation, suggesting that 1,25(OH)2D3 has an anti-inflammatory effect on macrophages [110].

Vitamin D increases the VDR expression in the rat ileum and liver as well as in the ileum of humans [111]. The VDR stabilizes tight junctions in the intestinal epithelial cells [112], suggesting a possible reduction of LPS action and the proinflammatory process.

There is a negative correlation between VDR expressions on hepatocytes with the NAFLD activity score in humans [109]. In an animal study performed in rats fed on a high-fat/high-fructose corn syrup diet alone or with vitamin D depletion, the vitamin deficient group presented with significantly greater hepatic steatosis, lobular inflammation and higher hepatic messenger mRNA levels for TLR2, TLR4 and TLR9 and other proinflammatory and oxidative stress markers compared to the control group.

Vitamin D deficiency can exacerbate NAFLD through TLR activation accompanied by increased inflammation and oxidative stress [113]. In a recent investigation developed by our research group, vitamin D3 supplementation for a high fatty diet exerts an anti-inflammatory effect once it decreases the IL-6 production in epididymal adipose tissue in mice as well as in 3T3-L1 adipocytes stimulated with LPS [114].

Another study developed in human adipocytes and in 3T3-L1 adipocytes reported decreases in the levels of inflammatory markers such as IL-6, MCP-1, and IL-1 $\beta$  (mRNA and protein levels) after 1,25-dihydroxyvitamin D3 (1,25-(OH)(2) D(3) treatment. In basal and TNF- $\alpha$ -stimulated conditions, this treatment decreased the expression of the proinflammatory marker in 3T3-L1 and human adipocytes. Finally, the 1,25-(OH)(2) D(3) treatment promoted improvement in glucose uptake and AKT phosphorylation. These data support the involvement of the vitamin D receptor gene and NF- $\kappa$ B in NAFLD, suggesting that low-grade inflammation could be linked to vitamin D deficiency [115].

The effects of 1,25(OH)2D3-VDR have not yet been fully defined, but it appears that vitamin D, likely cooperating with other regulators, exerts immunoregulation, antimicrobial defense, xenobiotic detoxification, anti-cancer actions, control of insulin secretion and, possibly, cardiovascular effects [110]. Indeed, the influence of vitamin D in the NAFLD mechanisms should be further explored [99].

The bioavailability of vitamin D is disturbed by obesity, wherein adiposity is associated with lower bioavailability of this vitamin, leading to impairment in insulin secretion and sensitivity [104]. Corroborating this finding, an investigation of two common VDR polymorphisms suggested that 2 major VDR gene polymorphisms may be linked to insulin secretion and resistance [107].

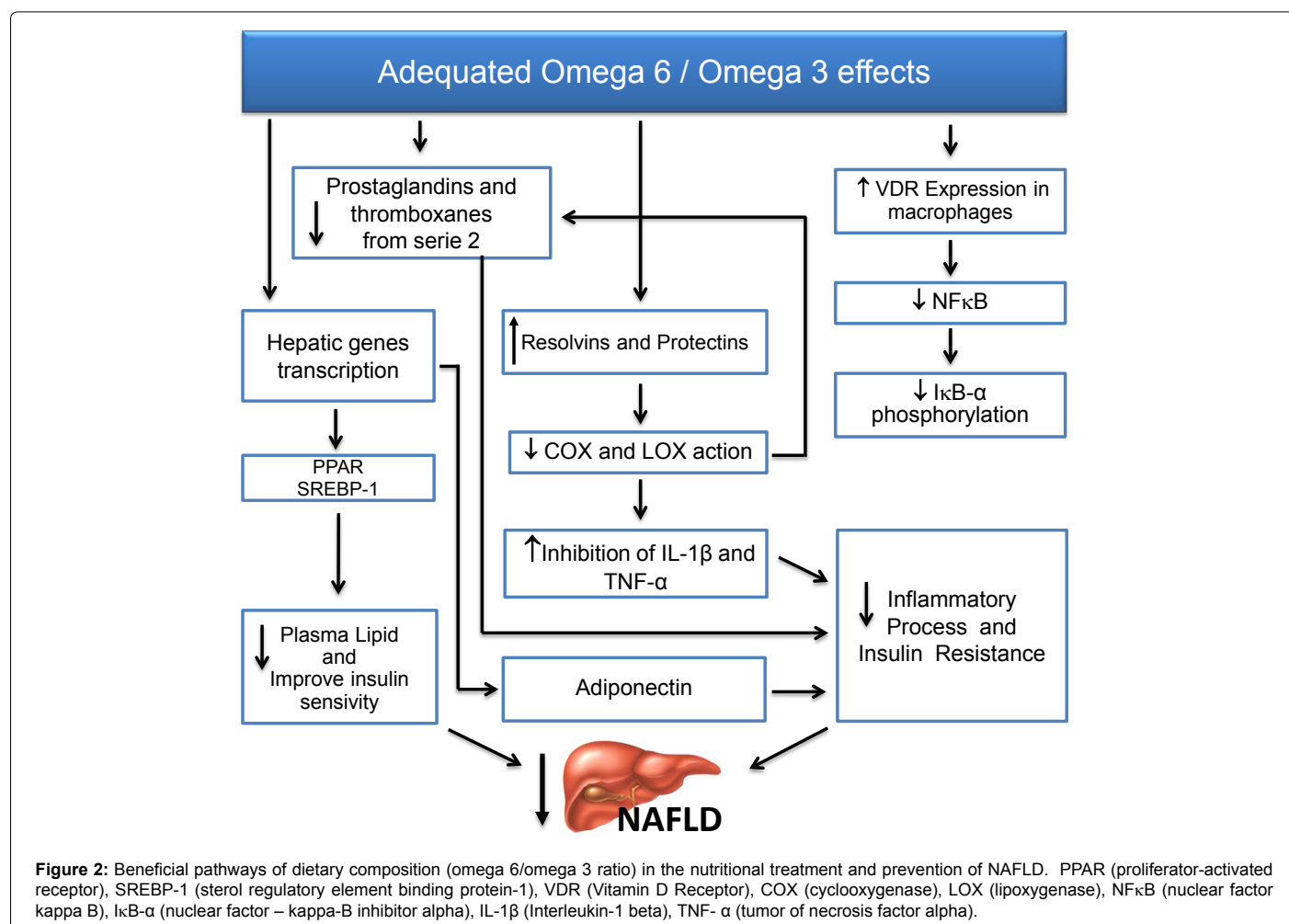
Pancreatic insulin secretion is inhibited by vitamin D deficiency,

suggesting a role for this vitamin in the regulation of endocrine pancreatic function, especially in the  $\beta$  cell [116]. It is already known that 1,25(OH)2D3 directly influences  $\beta$  cell insulin secretion through the induction of increases in the intracellular free calcium concentration through voltage-dependent Ca $^{2+}$  channels [117]. This mechanism is implicated in insulin cascade signaling, which exerts a fundamental role in preventing several diseases, such as NAFLD.

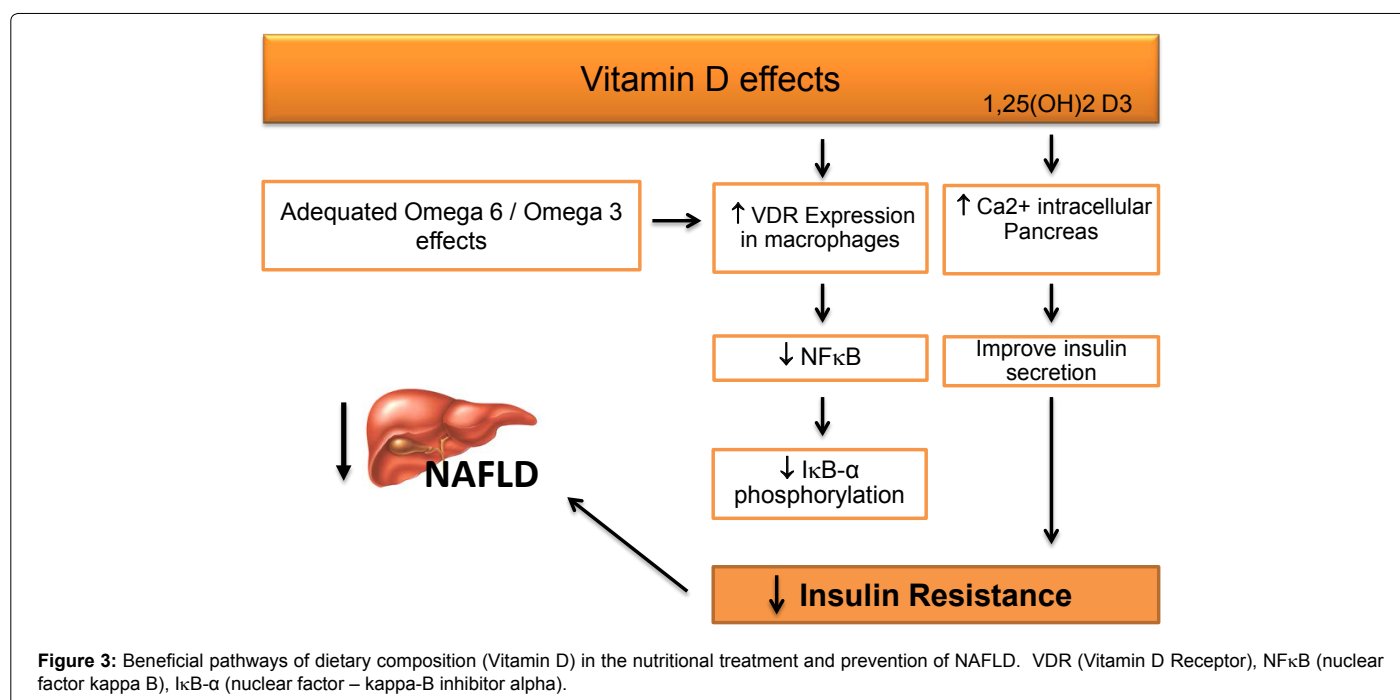
A study on young adults showed that the prevalence of metabolic syndrome components is significantly lower across quintiles of vitamin D intake, reinforcing the usefulness of vitamin D intake [100]. Vitamin D intake may modulate the risks of metabolic syndrome and, as cited previously, vitamin D is involved in the promotion of calcium influx, regulation of insulin secretion and glucose uptake [100].

Based on these findings, measuring vitamin D levels may be an important strategy in NAFLD treatment, and vitamin D supplementation may be associated with an adequate n3/n6 ratio and pre and probiotics, contributing to healthy gut microbiota [103] (Figures 2 and 3).

Finally, another possible mechanism linking vitamin D, the PUFAs and NAFLD has been studied. Researchers have identified several additional nutritional lipids as candidate, low-affinity VDR ligands that may function locally in high concentrations. The novel putative VDR ligands include w3- and w6-essential polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and arachidonic acid. The ligand binding of VDR triggers tight association between VDR and its







heterodimeric partner, retinoid X receptor (RXR), and only the VDR-RXR heterodimer can penetrate the deep groove of DNA and recognize vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D-regulated genes. These VDR-RXR controlled genes encode proteins that determine bone growth and remodeling, intestinal calcium absorption, phosphate homeostasis, the mammalian hair cycle, cell proliferation, and lipid detoxification [110]. The n-3 PUFAs, such as DHA and EPA, and n-6 PUFAs, such as linoleic acid and arachidonic acid, compete with titrated 1,25(OH)2D3 for binding to VDR with affinities for the receptor that are four orders of magnitude lower than that of the 1,25(OH)2D3 hormonal ligand. Nevertheless, the authors concluded that high local concentrations of PUFAs could be present in select cells or tissues and, if VDR is expressed, result in VDR-mediated anti-proliferation/ pro-differentiation effects, which may partially explain the chemoprotective nature of diets rich in PUFAs [110].

### New perspectives on the nutritional treatment and prevention of NAFLD

Knowledge of the inflammatory pathways involved in the development of NAFLD and new studies in nutrition, which reveal the strong positive influence of bioactive compounds in these inflammatory processes, are essential for guiding treatment and prevention recommendations. Based on these findings, we suggest the follow steps for NAFLD treatment:

Analyzing the dietary intake using different tools, such as the 24-hour recall, three-day food diary, food frequency and diet history, focusing on verifying the w3/w6 ratio, pre and probiotics and vitamin D ingestion;

The nutritional plan must be designed based on reducing the saturated and trans fatty acids and substituting monounsaturated and polyunsaturated fatty acids, corresponding to the following: saturated fat <7%, monounsaturated fat 10% and polyunsaturated fat 10% of the total energetic value. Supplementation with w3 (1 g/fish oil) is beneficial at a suggested ratio of n-6 to n-3 of approximately 3:1;

Considering that dietary composition can influence the health of

gut microbiota, the dietary plan should include daily alimentary sources of probiotics and a prebiotic-rich diet, such as with a 10% of inulin dose by weight with a minimum 4 week supplementation period. However, the optimal dose of pre and probiotics need to be further investigated.

Performing a serum analysis of vitamin D to verify possible deficiency and determine the necessary vitamin D supplementation;

Based on the cut-off serum level, 12 to 30 ng/mL of vitamin D was associated with an increased NAFLD risk, and the recommended vitamin D was 400-1000 UI for children and 1000-2000 UI for adults to correct the deficiency status and promote NAFLD improvement.

Altogether, these guidelines are important for nutritional therapy. Finally, the multidisciplinary team should always look for joint strategies and treat all aspects of the disease in question as well as delve deeper into promising areas such as nutrigenomics and translational investigations, which improve our understanding of the interaction between potential bioactive components and track the triggering of these comorbidities, all of which are new strategies for the treatment of NAFLD.

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