Non-Alcoholic Fatty Liver Disease: Bases for Therapeutic
Roles of Vitamin E and n-3 PUFA

Abstract
Non-alcoholic fatty liver disease (NAFLD) is the first cause of liver disease worldwide, leading to non-alcoholic steatohepatitis (NASH), cirrhosis and finally death. The available evidence suggests that oxidative stress plays a key role in the pathophysiology of NAFLD, participating in mitochondrial increased lipid peroxidation involved in mitochondrial dysfunction. Polyunsaturated fatty acids (PUFAs) are present in the cell and mitochondrial membrane, and can act through several molecular pathways such as the peroxisome proliferator-activated receptor-α (PPAR-α) activation and sterol regulatory element-binding protein 1c (SREBP-1c) regulation to finally promote an antioxidant status within cell physiology. Vitamin E has antioxidant and anti-inflammatory properties that have been proved to reduce liver steatosis and inflammation, acting through free radical trapping and preventing docosahexaenoic acid (DHA) molecules damage through a membrane stabilization action. These two molecules could thereby be a potential target for promising therapies within the multiple pathways of this disease.

Keywords: NAFLD; Oxidative stress; n-3 PUFA; Vitamin E; Diabetes mellitus

Introduction
In the last years the amount of people affected with non-alcoholic fatty liver disease (NAFLD) has increased: reaching today the first cause of liver disease worldwide. The prevalence of NAFLD in the United States is between 10% and 30% with similar rates reported from Europe and Asia [1]. It should be considered that the development of cirrhosis in non alcoholic patients has been recognized as an important and frequent cause of aminotransferases elevation [1-3].

NAFLD etiopathogenesis has not been completely elucidated and should be understood as a multifactorial process that includes changes in metabolic homeostasis: inflammation: insulin resistance: fibrosis and oxidative stress [4]. Moreover it is known that patients who have metabolic syndrome, obesity, insulin resistance, diabetes mellitus (DM) or hyperlipidemia have an increased risk of developing non-alcoholic fatty liver (NAFL); probably because those diseases have similar multifactorial causes. In particular oxidative stress has an important role in the progression from NAFL to non-alcoholic steatohepatitis (NASH) and finally to cirrhosis and hepatocellular carcinoma (HCC) [5].

Oxidative stress is the result of an imbalance that favours the increase of oxidative species: which are normally managed by antioxidant mechanisms produced inside the hepatocyte. Specifically: the loss of this balance allows to the formation and increase of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both species target essential biomolecules within the hepatocytes: producing injury in cellular structure and compromising many biological functions: being this a critical event that could finally lead to hepatotoxicity [1,6,7].

Changes in lifestyle and daily diet are the main treatment in NAFLD [8]. Latest evidence has shown possible beneficial effects of vitamin E and n-3 polyunsaturated fatty acids (n-3 PUFAs) in NAFLD patients. Vitamin E and n-3 PUFAs are essential nutrients that have essential functions in human physiology and both are distinguished for their antioxidant properties.

The purpose of this review is to present a summary about the pathogenesis of NAFLD and to analyse the vitamin E and n-3 PUFAs role in the current treatment of this disease.

NAFLD and Cardiovascular Risk
One of the most important causes of death in patients with NAFLD: in particular: patients with non-alcoholic steatohepatitis: are cardiovascular diseases [9]. Cohort’s studies have shown ischemic heart disease represent a 25% of the total of death in patients with NAFLD: being this the second cause of mortality [10].
Recent studies show a relationship between NAFLD and atherosclerosis. Cardiovascular mortality in NAFLD is associated with the severity of liver disease; representing the most part of NAFLD mortality [11]. NAFLD may contribute to cardiovascular diseases development: by an increase of insulin resistance and atherogenic dyslipidemia, which both are proven cardiovascular risk factors [12,13]. An increased release of chemical messengers from the visceral adipose tissue: such as inflammatory cytokines: leads to inflammation of adipose tissue. This process intensifies insulin resistance: and consequently participates in the development of atherosclerosis and cardiovascular diseases [13,14]. Despite, NAFLD may be an independent factor for adipose tissue inflammation and posterior cardiovascular risk increasing as adipose tissue inflammation has been observed in individuals with NAFLD independent of the obesity grade [15].

**NAFLD Influences on macrophage polarization**

Macrophages are part of the innate immunity and play a key role in inflammation and host defense [16]. Depending on the environment stimuli these cells may undergo to different phenotypes [17]. Classical M1 activation leads to the release of pro-inflammatory cytokines: whereas M2 activation promoted the tissue remodelling and causes a regulation on immune-regulatory functions [18,19]. Macrophages plasticity allowed the switch from M1 to M2 and vice versa [20]. For example: adipose tissues in obesity promoted a polarization shift toward M1 [21].

**Oxidative Stress and NAFLD**

Oxidative stress is described as a mechanism of injury in different disease processes. The term refers to a condition in which there is an imbalance between the production of ROS and RNS and the antioxidant defense system within a particular biological system [22,23]. Mitochondrion is the principal source of ROS production. Mitochondria consume 90% of cell’s oxygen to produce ATP through oxidative phosphorylation. However about 2% of the oxygen used in cell metabolism is converted to ROS mainly through superoxide anion (O2-. ) production [24,25]. ROS can also be produced outside of the mitochondria in lesser significative amounts. These processes involve non enzymatic and enzymatic reactions. In the case of NASH extra-mitochondrial ROS are generated principally through NADPH oxidase xanthine oxidase D-amino oxidase p-450 cytochromes proline and lysine hydroxylase and uncoupled nitric oxygen synthase. It has been described a direct association between NASH and cytochrome P450 2E1 (CYP2E1) isoform pro-oxidant activity [26]. This microsomal enzyme promotes free fatty acids B-oxidation a potential mechanism of ROS production [27]. In addition ROS may be generated through NADPH oxidase in Kupffer cells [28].

Mitochondrial ROS production contributes to diverse liver diseases through an accumulation of mitochondrial DNA (mtDNA) mutations leading to dysfunction caused by several reactions involved in oxidative phosphorylation processes and final lethal cell injury [29]. There is some evidence that mtDNA mutations may affect NAFLD development as suggested by studies reporting reduced levels of mtDNA in patients with NASH [30]. Reports demonstrate that mtDNA increased levels are found in patients with fatty liver but with nor-inflammation/fibrosis [31]. Paradies has hypothesized that the accumulation of mtDNA mutations may lead to an impairment of oxidative phosphorylation reactions and mitochondrial respiratory chain dysfunction resulting in increased ROS production prior to accumulation of mtDNA mutations finally triggering a vicious cycle of oxidative damage in which ROS production promotes further mitochondrial dysfunction and oxidative damage [32]. This suggests that a link may exist between mitochondrial mtDNA mutations and NAFLD etiology and pathophysiological mechanisms. Moreover damage induced to mtDNA can be transferred through mitochondria and cell division [33].

PUFAs are an important component of mitochondrial phospholipids. In the literature lipid peroxidation has been related as a consequence of oxidative stress. Mitochondria present a high concentration of PUFAs being substracts for oxidizing reactions and generating lipid peroxidation products such as hydroperoxides and endoperoxides. Subsequently these products may undergo fragmentation resulting in the formation of aldehyde by-products such as 4-hydroxy-2-nonenal and malondialdehyde [34-36]. The importance of these molecules formed only by peroxidation of PUFAs [35] lies in the potential to migrate to distant intracellular and extracellular targets thus amplifying oxidative stress effects [35-37] (Figure 1).

![Figure 1: Relationship between oxidative stress and NAFLD.](image)

The imbalance produced in the antioxidant/oxidant ratio within the hepatocyte leads to oxidative stress. ROS and RNS are principally enhanced by mitochondrial processes but, besides, a considerable amount is produced by other extra mitochondrial systems such as XO, NOS, NOX, and CYP450. Inside the mitochondria, the increased quantity of ROS and RNS produces an accumulation of mutated mtDNA, which leads to mitochondrial dysfunction and cell damage. This last event produces more ROS and RNS, which finally leads to a vicious cycle with more mutated mtDNA production, and so on. MDA and HNE are products from the peroxidation of PUFAs, and can amplify oxidative stress both intracellular and extracellular. XO: Xanthine Oxidase; NOS: Nitric Oxide Synthase; NOX: NADPH Oxidase; CYP450: Cytochrome P450; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; PUFAs: Polyunsaturated Fatty Acids; mtDNA: mitochondrial DNA; MDA: Malondialdehyde; HNE: 4-Hydroxy-2-Nonenal.
Therapeutic Approaches into NAFLD n-3 PUFAs and Vitamin E

n-3 polyunsaturated fatty acids

- **n-3 PUFAs generalities:** Polyunsaturated fatty acids (PUFAs) are safe and efficacious compounds [38] participating in numerous processes having a wide range of effects on biochemical and physiologic functions. PUFAs are key compounds of triglyceride and phospholipid membranes of cells and subcellular organelle membranes [39]. Two main groups of PUFAs can be identified n-3 PUFAs or omega 3 fatty acids and n-6 PUFAs also known as omega 6 polyunsaturated fatty acids (n-6 PUFAs). Both classes of PUFAs are essential nutrients since human body cannot synthesize them [40] n-6 PUFAs are usually obtained from corn products and soybean oil while n-3 PUFAs are part of fish oil canola walnuts among others [41].

The two fatty acids experience similar metabolic process including elongation and desaturation processes made by enzymes localized in the endoplasmic reticulum and the mitochondria [42,43]. Metabolized n-6 PUFAs lead to arachidonic acid while metabolized α-Linolenic acid the most abundant n-3 PUFAs in the western diet lead to eicosapentaenoic acid (EPA) which are further metabolized to DHA [40]. Evidence suggests a pro-inflammatory role of n-6 PUFAs and an anti-inflammatory profile of n-3 PUFAs through their metabolized species [44].

- **PUFAs and cardiovascular risk:** Multiple evidence has reported a supportive effect of n-3 PUFA in cardiovascular disease principally decreasing the risk of cardiac mortality [45]. Beneficial effects of n-3 PUFA ingest to reduce cardiovascular risk are decrease in triglycerides levels reduce heart rate and blood pressure [46,47] improve in endothelial and autonomic function [48-50] and anti-inflammatory effects [45]. In addition cross-sectional studies have been related n-3 PUFAs consumption with lower levels of atherosclerosis [51,52]. Recently pre-specified sub-study of the WELCOME study [53] has reported a beneficial effect in carotid intima media thickness (CIMT) progression when NAFLD severity was decreased. CIMT is a validated tool for the prediction of stroke or myocardial infarction [54]. Previously a meta-analysis described an important association between CIMT and patients with NAFLD this association might be responsible of an increase 13% of CIMT [55]. However in the pre-specified sub-study of the WELCOME trial [53] n-3 PUFA supplementation did not improve CIMT progression. Despite of this negative result it is necessary to perform more studies using n-3 PUFA as main strategy for NAFLD treatment with the objective to deepen the analysis of cardiovascular outcomes.

- **Effects on macrophage polarization:** PPAR-γ modulate the immune inflammatory response due to its anti-inflammatory properties [56] generating a negative interference of multiples genes including nuclear factor kappa B (NF-κ B) [57]. PPAR-γ is a regulator of macrophage M2 polarization [20]. Moreover it participated in acquisition and maintenance of M2 phenotype in adipose tissue [58] and its disruption in mice impaired M2 macrophage activation leading to a susceptible to obesity and insulin resistance [59]. Kupffer cells are the resident macrophages of the liver. Evidence has showed that Kupffer cell contributes to the pathogenesis of NAFLD. Studies have investigated the effect of DHA on Kupffer cells/macrophages polarization in vitro a NAFLD model. High fat (HF) diet-induced hepatic steatosis and local pro-inflammatory response was closely associated with M1-predominant polarization of Kupffer cells [20]. On the other hand PPAR-γ results to have the potential to balance lipid-induced M1/M2 macrophages/Kupffer cells polarization preventing the development of NAFLD in these HF diet mice. n-3 PUFAs administration favours a switch of Kupffer cells/macrophages to an M2 phenotype [20].

n-3 PUFAs antioxidant properties: Through its metabolism to structurally related prostaglandin and leukotrienes n-3 PUFAs derived species act as ligands that stimulate transcription genes. For example it joins nuclear receptor proteins such as peroxisome proliferator-activated receptors (PPARs) to defend against ROS production and PPARs excess [60-62]. Thus n-3 PUFAs produce an up regulation in both fat oxidation genes and antioxidant genes generating and imbalance that benefits antioxidants molecules [60].

Within the properties of n-3 PUFAs evidence suggests a modulation of redox signaling pathways [63]. For example oxidized omega 3 react with keep 1 thereby inducing NF-F2-related factor 2 (Nrf2) participating in the expression of genes that encode proteins responsible to regulate detoxification of ROS such as heme-oxygenase-1 [61].

Relationship between n-3 PUFAs and NAFLD: Some patients with NAFLD have concomitantly diminished levels of n-3 PUFA. It has been observed that NAFLD condition associated with a decline in hepatic n-3 PUFAs is more severe than with decline in n-6 PUFAs [64,65]. High blood and hepatic ω6/ω3 PUFAs ratio is associated with inflammation and NAFLD progression [38]. DHA would be more effective than EPA at attenuating western diet-induced hepatic fat inflammation and fibrosis and controlling multiple liver lipid metabolism signaling pathways which take control over liver lipid metabolism inflammation and fibrosis [38,65].

It was reported that dietary supplementation with fatty acids can improve NAFLD associated with hyperlipidemia by modifying the function of platelets and leukocytes [66,67]. Also n-3 PUFAs especially DHA have demonstrated to reduce triglycerides (TG) accumulation and improve hepatic steatosis being important regulators of hepatic gene transcription [68]. In fact PUFAs might prevent NAFLD by the activation of PPARs and the inhibition of sterol regulatory element-binding protein-1c gene (SREBP-1c) by down regulating over expressed glycolytic and lipogenic genes [69].

As mentioned above n-3 PUFA ingestion leads to PPARα regulation and activation. Mice lacking PPARα have elevated free fatty acid levels and fatty livers consequences of their inability to combat fatty acids [62].

Humans studies support a role of SREBP-1c in the pathogenesis of steatosis [70,71]. Increased SREBP-1c leads to mixed insulin resistance and hepatic steatosis, finally leading to NAFLD [70].


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resistance and sensitivity in livers of lipodystrophic and ob/ob mice [72]. Shimomura et al. [73] described that combination of insulin resistance and insulin sensitivity establishes a vicious cycle that aggravates hyperinsulinemia and insulin resistance in lipodystrophic and ob/ob mice [73].

Clinical trials in NAFLD with n-3 PUFAs supplementation: Evidence has shown different results in the use of n-3 PUFAs in patients with NAFLD.

A. What has not worked: A double blind randomized placebo controlled multicenter clinical trial was performed by Dasarathy et al. [74] in NASH patients with DM. Participants were randomized into two study groups and received either an oral dose of 2160 mg of EPA and 1440 mg of DHA in divided two pills or placebo the study has a duration of 48 weeks. Primary endpoint was histology changes. Research showed that on adults there were no differences between n-3 PUFAs group and placebo in the mean serum transaminases and histological evaluations [74,75].

B. What has worked: Different reviews have summarized clinical trials performed in NAFLD patients such as one performed by jump et al. [38].

a) Analyses assess the clinical trials impacts of n-3 PUFAs supplementation on children with NAFLD. Disparate doses of DHA or DHA+EPA were used with treatment duration ranging from 3 to 24 months. Nobili et al. [76] performed studies that showed a decrease in plasma alanine transaminase (ALT) on ultrasound (US) hepatosteatosis [76-78] NAFLD activity score (NAS) [79] and aspartate aminotransferase (AST) HOMA-IR fasting insulin and plasma TG [78]. Janczyk et al. conducted a randomized control trial on 64 subjects treatment group received DHA+EPA in a 32 proportion weight adjusted dose treatment with n-3 PUFAs improved AST and gammaglutamyl transpeptidase levels in children with NAFLD compared with placebo no difference was observed at US [80]. Some studies such as one performed by Pacifico et al. [81] also informed a diminished-on body weight and waist circumference [81].

b) On adults results are similar of those seen in children. Moreover it has been demonstrated a decrease in hepatic steatosis joined to a diminished on hepatic fibrosis in studies performed in the past five years [82-84]. Hepatic enzymes such as ALT and AST decrease in the treated group compared to placebo one [84-86]. A diminish on plasma TG was also observed [82,84-86].

C. What is new: Based on the hypothesis that supplementation with DHA and vitamin D would benefit the whole spectrum of NAFLD. Della Corte et al. [87] evaluated in a randomized double-blind placebo-controlled trial the effect of daily DHA (500mg) plus vitamin D (800 IU) in 41 obese children with biopsy-proven NAFLD and vitamin D deficiency after 12 months of use. Results demonstrated that DHA plus vitamin D treatment improved insulin-resistance lipid profile ALT and NAFLD Activity Score.

Hodson et al. [88] conducted a randomized control trial double-blind and placebo-controlled study. This was a pre-specified sub-study of the WELCOME trial (Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with Omacor therapy). They measured whether the treatment with 3 PUFA in patients was associated with changes in hepatic fatty acids (FA) synthesis post prandial FA partitioning and hepatic and peripheral insulin sensitivity. Twenty-four subjects were allocated to two groups group A 12 received EPA+DHA 4 g/day for 15-18 months and group B received placebo during the same time. Results showed that patients who have an increase in erythrocyte DHA enrichment of >2% (a surrogate marker of changes in liver enrichment) have significantly lower concentrations of plasma TG and very-low density lipoproteins pre- and post-prandial (P<0.001) a reduction on de-novo lipogenesis and favourable changes in both hepatic insulin sensitivity and hepatic FA metabolism. No changes were observed in whole-body insulin sensitivity or peripheral glucose disposal.

Vitamin E

Vitamin E sources: Vitamin E is an essential micronutrient for humans and achieving an optimal status is assumed to produce beneficial health outcomes [89]. There are eight lipophilic forms of vitamin E naturally occurring which include four tocopherols (αT βT γT δT) and four tocotrienols (αTET βTET γTET δTET) being the γ-tocopherol the most abundant of them [90,91]. These natural forms of vitamin E are made by plants and can be found in many food oils such as corn soybean and peanut oil [92-94].

Effects on Cardiovascular Risk: Vitamin E cardiovascular benefits have been suggested as it inhibits the oxidation of low-density lipoprotein cholesterol in plasma [95]. Clinical trials have not provided convincing evidence of a vitamin E protective effect for cardiovascular risk in general population [96]. However a meta-analysis showed vitamin E treatment significantly reduced the risk of myocardial infarction and death by 40% and 50% respectively in a diabetic homozygous for haptoglobin allele population [97].

Diabetes mellitus has been associated with a low concentration of antioxidants in particular vitamin E concentration [98]. A recent study suggested a relationship between both vitamin E deficiency and oxidative status with prediabetes in a sample of apparently healthy individuals [99]. These outcomes suggest vitamin E may be effective to reduce cardiovascular risk in patients exposed to oxidative stress related damage such as the case of diabetes and NAFLD.

Vitamin E antioxidant properties: All tocopherols and tocotrienols are potent antioxidants assuming its role by scavenging lipid peroxyl which can produce severe damage on the cell membranes via lipid peroxidation [89]. Some anti-inflammatory properties have been seen with the vitamin E γT natural form such as inhibiting pro-inflammatory eicosanoids and being able to trap electrophiles including reactive nitrogen species which could diminish the pro-oxidant environment enhanced during inflammation [89,100,101].

Relationship between vitamin E and NAFLD: As seen before oxidative stress plays a key role in NAFLD pathogenesis and in
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fact patients with this disease have an enhanced oxidative stress and an antioxidant deficiency which may lead to increased lipid peroxidation and cell death due to mitochondrial impairment [91]. Even though there are no standard protocols for the treatment of NAFLD a higher intake of vitamin E is thought to counteract the increased oxidative stress found in this kind of patients and it is commonly prescribed as a supplement in the clinical practise [91,102] being nowadays considered to be a first line pharmacological treatment in the management of NASH especially when diet and other lifestyle changes are insufficient [103].

Clinical trials in NAFLD with vitamin E supplementation: Many studies have been run to prove the impact of the use of vitamin E on NAFLD patients. For example there is a cohort study on patients with NAFLD and metabolic syndrome treated with vitamin E for six months showing a reduction on ALT levels compared with the control group [104]. Magosso et al. [105] examined the effects of a one-year treatment of mixed palm tocotrienols on the echogenic response of hyper cholesterolemic adult patients with NAFLD showing hepatoprotective effects. Another clinical trial showed that obese children with NAFLD treated with a six-month lifestyle change plus vitamin E therapy is associated with a significant reduction of oxidative stress represented with decreased levels of prostaglandin F2α and ALT and increased levels of endogenous secretory receptor for advanced glycation end products compared with the lifestyle only group leading to conclude that vitamin E supplementation have potential and positive results on the oxidative profile of this kind of patients [106].

PIVENS trial compared non-diabetic biopsy-proven NASH patients who received vitamin E to patients receiving pioglitazone and placebo showing that vitamin E was better than placebo in reducing ALT levels liver steatosis and inflammation [107]. Another trial called TONIC performed in children and adolescents recorded similar findings [108]. Finally a meta-analysis was published by Sawangjit et al. [109] in which they analysed 44 randomized controlled trials up to November 2015 comparing different interventions for NAFLD that involved a total of 3802 patients. Vitamin E therapies were supported by high quality evidence in resolution of NASH and improvement in NAS characterized by a decrease in steatosis ballooning and lobular inflammation.

Combined n-3 PUFAs and vitamin E therapy

A mixed vitamin E and n-3 PUFA could be an interesting therapeutic strategy in NAFLD. This hypothesis is based on antioxidant effect of these molecules that was described above. Both nutrients are related vitamin E has an antioxidant effect that consists in avoiding PUFA oxidation through free radical scavenging by hydrogen donor mechanisms [110]. Moreover a recent theory has been postulated to explain that vitamin E acts as a membrane stabilizator protecting DHA molecules from oxidative stress [111,112]. Human scale studies have demonstrated that vitamin E would play an important role in n-3 PUFA plasma concentration existing a positive association between high n-3 PUFA levels and vitamin E intake probably because vitamin E has a role in the support of plasma n-3 PUFAs [113]. This has to be taken into account when a combined antioxidant therapy with Vitamin E and n-3 PUFA is planned. However there are no studies performed on NAFLD animal models likewise these nutrients have not been mixed at the same time in humans. Considering the beneficial results that were obtained in clinical trials with vitamin E or n-3 PUFA further studies could investigate a potential synergistic effect from the association of vitamin E and n-3 PUFA in NAFLD models (Figure 2).

Conclusion

NAFLD remains as an unsolved problem in the clinical practice. Despite the pertinent pathophysiologic mechanisms of the disease have not been fully elucidated oxidative stress is a key factor within the development of the metabolic damage. Nevertheless evidence suggests that both vitamin E and PUFAs could be beneficial individually to reduce the pejorative molecular effects of the disease and improve outcomes in clinical practice through diverse oxidative stress associated pathways. The combination of both therapies seems to be an interesting novel therapeutic strategy to reinforce the antioxidant system and finally ameliorate the lethal cellular consequences of oxidative stress in NAFLD patients. However nowadays no studies have been carried with this proposal. We suggest that clinical trial realization is more than relevant to prove the effectiveness of this treatment and finally contribute to reduce and prevent the actual increase in prevalence and global impact of this disease.

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Conflict of Interest

The authors declare that there are no conflicts of interests.

Figure 2: General overview of n-3 PUFA and vitamin E effects on oxidative stress.

Vitamin E plays its role via lipid peroxide scavenging: avoiding the potential damage to the cellular and mitochondrial membranes. N-3 PUFA act inducing both antioxidant and oxidant genes producing an imbalance that favours the antioxidant mechanisms. Besides: n-3 PUFA can reduce inflammation: diminishing its signalling pathways.

ROS: reactive oxygen species; n-3 PUFA: n-3 polyunsaturated fatty acids.
References

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