Omega-3 fatty acids and non-alcoholic fatty liver disease: evidence of efficacy and mechanism of action.

[*]Eleonora Scorletti [1,2], Christopher D. Byrne [1,2,]

Affiliations: 1. *Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton, UK.*

2. National Institute for Health Research, Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, Southampton, UK.

***Corresponding author: Dr Eleonora Scorletti**. Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton General Hospital, Southampton, UK. E-mail: e.scorletti@soton.ac.uk

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1 Abstract

For many years it has been known that high doses of long chain omega-3 fatty acids are beneficial in the treatment of hypertriglyceridaemia. Over the last three decades, there has also been a wealth of in vitro and in vivo data that has accumulated to suggest that long chain omega-3 fatty acid treatment might be beneficial to decrease liver triacylglycerol. Several biological mechanisms have been identified that support this hypothesis; notably, it has been shown that long chain omega-3 fatty acids have a beneficial effect: a) on bioactive metabolites involved in inflammatory pathways, and b) on alteration of nuclear transcription factor activities such as peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP), involved in inflammatory pathways and liver lipid metabolism. Since the pathogenesis of non alcoholic fatty liver disease (NAFLD) begins with the accumulation of liver lipid and progresses with inflammation and then several years later with development of fibrosis; it has been thought in patients with NAFLD omega-3 fatty acid treatment would be beneficial in treating liver lipid and possibly also in ameliorating inflammation. Meta-analyses (of predominantly dietary studies and small trials) have tended to support the assertion that omega-3 fatty acids are beneficial in decreasing liver lipid, but recent randomised controlled trials have produced conflicting data. These trials have suggested that omega-3 fatty acid might be beneficial in decreasing liver triglyceride (docosahexanoic acid also possibly being more effective than eicosapentanoic acid) but not in decreasing other features of steatohepatitis (or liver fibrosis). The purpose of this review is to discuss recent evidence regarding biological mechanisms by which long chain omega-3 fatty acids might act to ameliorate liver disease in NAFLD; to consider the recent evidence from randomised trials in both adults and children with NAFLD; and finally to discuss key ‘known

unknowns' that need to be considered, before planning future studies that are focussed on testing the effects of omega-3 fatty acid treatment in patients with NAFLD.

2 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a pathologic condition defined by the presence of triglycerides (TG) deposition in the liver greater than 5% of the total liver weight (1-4). The term NAFLD encompasses a spectrum of liver diseases where the first stage is characterized by simple steatosis with liver fat accumulation in the hepatocytes (4-7). The second stage is non-alcoholic steatohepatitis (NASH) characterized by hepatocyte injury due to inflammation, ballooning and possible collagen deposition. NASH is a progressive form of fatty liver that can worsen over time and may lead to cirrhosis and liver failure (2). NAFLD has become one of the most common causes of chronic liver disease and liver related mortality worldwide, and is now becoming a major reason for liver transplantation (8, 9). According to the World Gastroenterology Organization's global guidelines (http://www.worldgastroenterology.org/UserFiles/file/guidelines/nafld-nash-english-2012.pdf) approximately 10-20% of people with NAFLD progress to NASH.

Although NAFLD also occurs in normal weight people, the burgeoning epidemic of overweight, obesity and type 2 diabetes is also contributing to a marked increase in the burden of chronic disease caused by NAFLD. Whilst national mortality data show that the majority of liver disease deaths were previously attributed to alcoholic cirrhosis, there is emerging evidence of the importance of non-alcoholic fatty liver as a risk factor for severe chronic liver disease (10, 11). The prevalence of disease progression from NAFLD to NASH is approximately 10-20% in the general population; however, the prevalence increases up to 37% in the presence of obesity (2). Furthermore, it is now clear that NAFLD is also a risk factor for type 2 diabetes and cardiovascular disease (12) and therefore NAFLD has an important adverse impact not only on hepatology and gastroenterology services but also

diabetes, cardiology and cardiac surgery services within the National Health Service. The prognosis of NAFLD is hard to establish due to the heterogeneity of the condition and to the fact that most studies are small with relatively short follow-up (13). The pathophysiology of NAFLD is complex and involves several metabolic and genetic aspects. The multifactorial mechanisms responsible for the development and progression of NAFLD include genetic polymorphisms (14) and metabolic factors (15), such as sedentary lifestyle, increased intake of energy-rich foods (16), malnutrition due to an imbalanced intake of nutrients (e.g. high fat, high carbohydrates and high protein diet (17), low fibre intake (18), high fructose intake (19)), altered gut microbiota composition (dysbiosis) (20-22), obesity (16, 23). Several research studies have attempted to test various treatments in NAFLD: however, these studies have produced controversial results with limited success and serious safety concerns about long-term therapy (24-28). Currently, lifestyle changes may ameliorate steatosis, but sometimes weight loss and its maintenance are difficult to achieve (29, 30). Typically, within a Westernized diet, omega-6 fatty acid consumption is markedly greater than omega-3 fatty acid. The potential consequences of an increased ratio of omega-6 to omega-3 fatty acid consumption are increased production of pro-inflammatory arachidonic acid-derived eicosanoids and impaired regulation of hepatic and adipose function, predisposing to NAFLD (31). Several studies have shown that a diet with an inadequate intake of "omega-3 essential fatty acids" is associated with metabolic syndrome (32), cardiovascular disease (33), dyslipidaemia and fatty liver disease (34, 35).

3 Omega-3 fatty acids

Omega-3 polyunsaturated fatty acids are long-chain fatty acids characterized by the presence of a double bond (C=C) at the third carbon atom of the hydrocarboxylic chain counting form the methyl end (36). α-linolenic acid is the simplest fatty acid with an 18-carbon hydrocarboxylic chain and three double bonds. α-linolenic acid is one of the two "essential fatty acids" (the other is linoleic acid from the omega-6 series), namely they cannot be synthesised by animals, including humans; therefore, these fatty acids need to be obtained through diet. Both α-linolenic acid and linoleic acid are synthesized in plants and consequently are found in numerous seeds, nuts, and seed oils such as linseeds (flaxseeds), and their oil, soybean oil, rapeseed oil, walnuts and sunflower oil (37). Typically, linseeds contain 45–55% of fatty acids as α-linolenic acid whereas sunflower oil is highly rich in linoleic acid. Eicosapentaenoic acid [EPA; 20:5(ω-3)], docosapentaenoic acid [DPA; 22:5(ω-3)], and docosahexaenoic acid [DHA; 22:6(ω-3)] are functionally the most important very long chain highly unsaturated omega-3 fatty acids **(Figure 1)**. Although these very long chain fatty acids are found in a variety of foods, fish (especially oily fish) and other seafood are the richest sources of EPA, DPA and DHA. α -linolenic acid is the precursor of the long chain omega-3 fatty acid series and the recommended daily intake is 1.6 g/day for men and 1.1 g/day for women (≥0.5% total fat) (36). With regard to EPA and DHA, an adequate intake ranges between 0.25 and 2 g/day (corresponding to two fish potions per week, with at least one oily fishmeal consumed) (38). With a healthy diet, the physiological ratio between omega-6 and omega-3 fatty acids should be 4:1. Whereas, in the Western diet, the consumption of linoleic acid for the omega-6 series is 5 to 20-fold higher than the consumption of α-linolenic acid. In the presence of a physiological equilibrium, desaturases and elongases enzymes exhibit greater affinity to metabolize omega-3 fatty acids (38). Interestingly, several studies described the associations between SNPs of the fatty acid

desaturases (FADS1 and FADS2) gene cluster and members of the elongation-of-very-long-chain-fatty-acids (ELOVL) gene family with plasma levels of AA, EPA, DPA and DHA (39-43). Hepatic Elovl5, *Fads1* & *Fads3* expression are suppressed by increases in dietary omega-3 fatty acids (44). Since these enzymes are involved in both omega-3 and omega-6 fatty acid synthesis, dietary omega-3 fatty acids will increase tissue omega-3 and lower tissue omega-6 fatty acid downstream products. FADS1 and FADS2 are genes encoding for key enzymes in the omega-3 and omega-6 fatty acid series, the Δ-5 and Δ-6 desaturase respectively. Elongases are enzymes encoded by genes within the ELOVL family and are responsible for catalysing the elongation of the aliphatic chain of carbons leading to the formation of long-chain omega-3 polyunsaturated fatty acids (43). Interestingly, FADS and ELOVL polymorphisms are associated with reduced Δ-5 and Δ-6 desaturase activity and accumulation of desaturase substrates and a reduction of desaturase products (45, 46). Omega-3 fatty acid supplementation can improve Δ-5 and Δ-6 desaturase activity through a gene-treatment interaction. Cormier et al. showed that 6 weeks supplementation with 2 g of EPA plus 1 g of DHA daily in 210 healthy people increased Δ-5 desaturase activity and decreased Δ-6 desaturase activity increasing omega-3 and omega-6 fatty acid plasma levels (43).

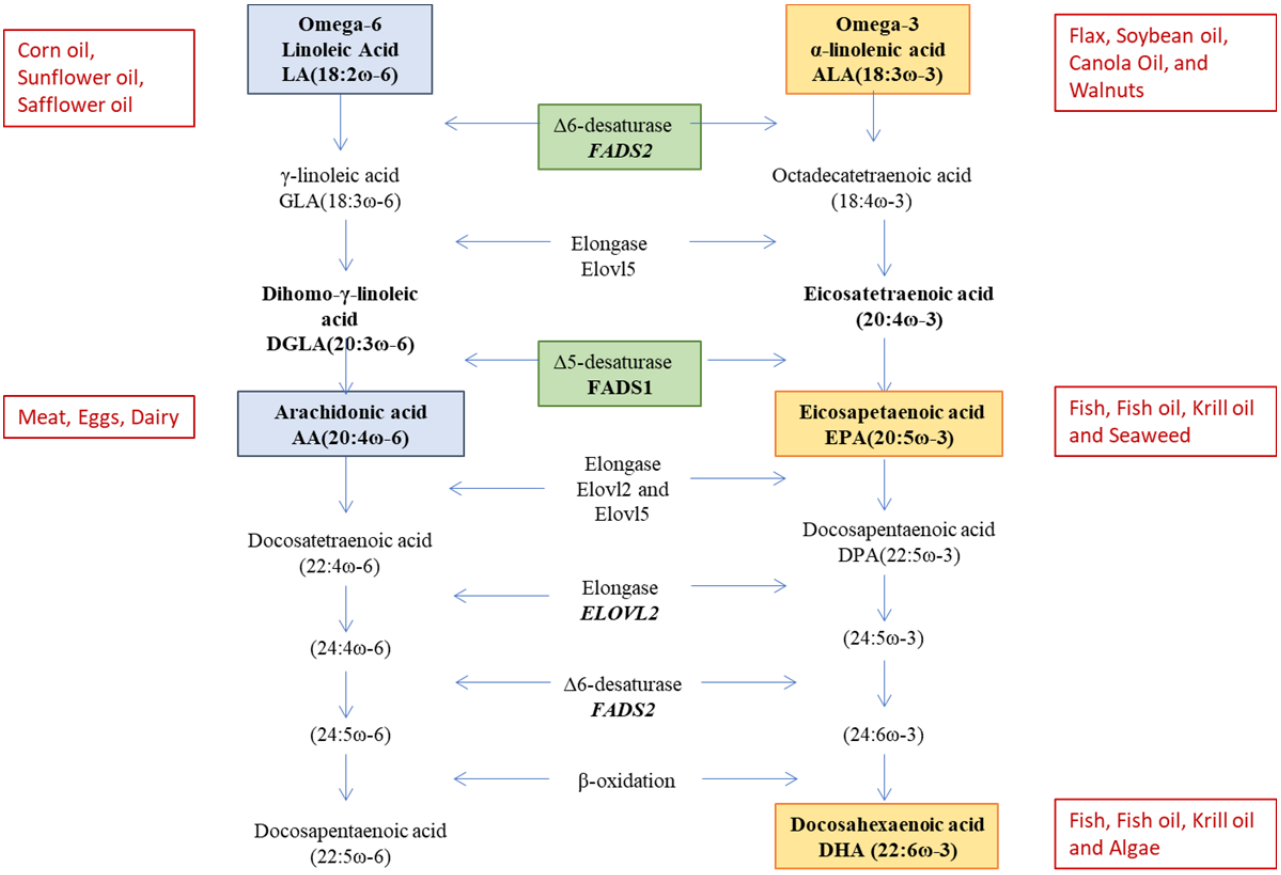

Figure 1. Metabolism of essential fatty acids.

Figure 1. Linoleic acid and α-linoleic acid are converted to polyunsaturated fatty acids by enzymatic elongase and desaturase enzymes. Essential fatty acids are found in seeds and vegetable oils; whereas polyunsaturated fatty acids are found in fish, fish oil, algae, meat, and eggs.

4 Pathogenesis of NAFLD and treatment with omega-3 fatty acids

4.1 Obesity, insulin resistance and adipose tissue dysfunction

Overweight and obesity are major risk factors for several chronic diseases such as type 2 diabetes, metabolic syndrome and NAFLD. In particular, excessive intake of fat and carbohydrates can affect lipid metabolism. Dietary lipids are essential macronutrient in the human diet because provide energy for the body. However, there are different types of dietary fat, some of them are potentially harmful (such as saturated fat and trans-fat) some other types of fat are beneficial for health (such as monounsaturated fatty acids, polyunsaturated fatty acids and omega-3 fatty acids (31, 47)). For example, a low intake of omega-3 fatty acids as well as a high omega-6/omega-3 fatty acid ratio is associated with a risk to develop NAFLD (47, 48). In the presence of obesity there is an excessive accumulation of fat, in form of triglycerides, in the adipose tissue, causing expansion of the visceral and peripheral adipose depots. After a meal, in a physiological condition, the production of insulin by pancreatic β-cells suppresses hepatic glucose output via inhibition of gluconeogenesis and glycogenolysis; and increases hepatic glycogen synthesis. In the adipose tissue, insulin inhibits lipolysis through suppression of hormone-sensitive lipase and upregulates lipogenesis (49). The adipose tissue is an endocrine organ secreting adipokines (such as leptin, adiponectin, resistin, apelin, and visfatin; chemokines such as monocyte chemotactic protein (MCP)-1 and IL-8; other proinflammatory cytokines such as IL-6, IL-1, angiotensin-II, and TNF-α; and antiinflammatory cytokines such as IL-10) involved in the regulation of energy balance, glucose homeostasis, inflammation and immune function (50). The overload of triglycerides in the adipose tissue results in an increase in adipocyte size accompanied by

adipocytes hypertrophy, activation of the death receptor and mitochondrial pathways of adipocyte apoptosis, macrophage recruitment and activation, and dysregulation of adipokine secretory patterns (51, 52). The imbalance between secretions of pro- compared to anti-inflammatory adipokines causes an inflammatory state of subcutaneous and visceral adipose tissue. This condition of adipose tissue overload and inflammation leads to ectopic deposition of fat in the liver (NAFLD), metabolic inflammation and insulin resistance. Inflamed adipocytes fail to suppress intracellular production of reactive oxygen species, with consequent upregulation of nuclear factor-κB (NF-κB) causing adipocyte secretion of adipokines, furthering inflammation. Omega-3 fatty acids have anti-inflammatory effects by regulating NF-κB subunit abundance (53). In a state of insulin resistance, there is an impairment in insulin-mediated suppression of hepatic glucose production and inhibition of lipolysis. This leads to hyperglycaemia and increases plasma levels of non-esterified fatty acids (NEFAs) (54). The rate of NEFA entering the circulation is a reflection of the balance between lipolysis of triglycerides in the adipose tissue and uptake of post prandial NEFA in the adipocyte for re-esterification into triglycerides and storage. In the presence of insulin resistance, adipose tissue lipolysis is inhibited as insulin fail to suppress hormone-sensitive lipase. In addition, fatty acid esterification is diminished as this mechanism is dependent on the supply of glycerol-3-phosphate derived from insulin-mediated glucose uptake and glycolysis in the adipocyte. The anatomic connection between adipose tissue and liver through the vasculature, facilitate the venous effluent of visceral fat into the portal vein resulting in NEFA flux to the liver (49). Omega-3 fatty acids have beneficial effects in regulating hepatic lipid metabolism, adipose tissue function, and inflammation reducing hepatic TG accumulation (31, 55, 56) **(Figure 2)**.

4.2 De novo lipogenesis

The excess intake of dietary carbohydrates together with insulin resistance promotes hepatic *de novo* (*de novo* lipogenesis: DNL) synthesis of free fatty acids form acetyl-coenzyme A (CoA). In the liver, DNL can be increased by activation of transcription factors such as sterol regulatory element-binding protein-1 (SREBP-1), carbohydrate response element-binding protein (ChREBP). There are three isoforms of SREBP-1: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a is expressed only at low levels in the liver, SREBP-1c is the predominant isoform in adult liver. Both SREBP-1a, SREBP-1c activate genes required for fatty acid synthesis and is involved in the regulation of enzymes that catalyse lipogenesis, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and TG synthesis. SREBP-2 is involved in cellular cholesterol homeostasis activating the LDL receptor gene and various genes required for cholesterol synthesis (57). ChREBP is a transcriptional regulator expressed in the liver that activates glycolytic and lipogenic gene expressions for DNL in response to high glucose. Omega-3 fatty acids regulate ChREBP activity by controlling the cellular abundance of max-like factor X (MLX), the ChREBP heterodimer partner that is required for ChREBP/MLX to bind DNA and activate glycolytic and lipogenic gene expressions (58).

A chronic stimulation of DNL (and reduced fatty acid oxidation) in the liver can in turn enhance intracellular availability of triglyceride, promoting fatty liver (59). Although in a physiological condition, the contribution of DNL to VLDL-TG synthesis is only around 5%, this percentage increase to 20–30% in the presence of high carbohydrate diet (60). Omega-3 fatty acids can suppress DNL in two ways: by reducing the activities of the lipogenic enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) and by suppressing the nuclear abundance of SREBP1c. Omega-3 fatty acids accelerate SREBP1c proteasome-

mediated degradation with little effect on SREBP1c precursors (61). ~~and suppressing SREBP1c activity by inhibiting proteolytic activation and decreasing mRNA stability~~ (31, 62) **(Figure 2)**.

Figure 2. Mechanisms potentially responsible for a beneficial effect of omega-3 fatty acid treatment in NAFLD.

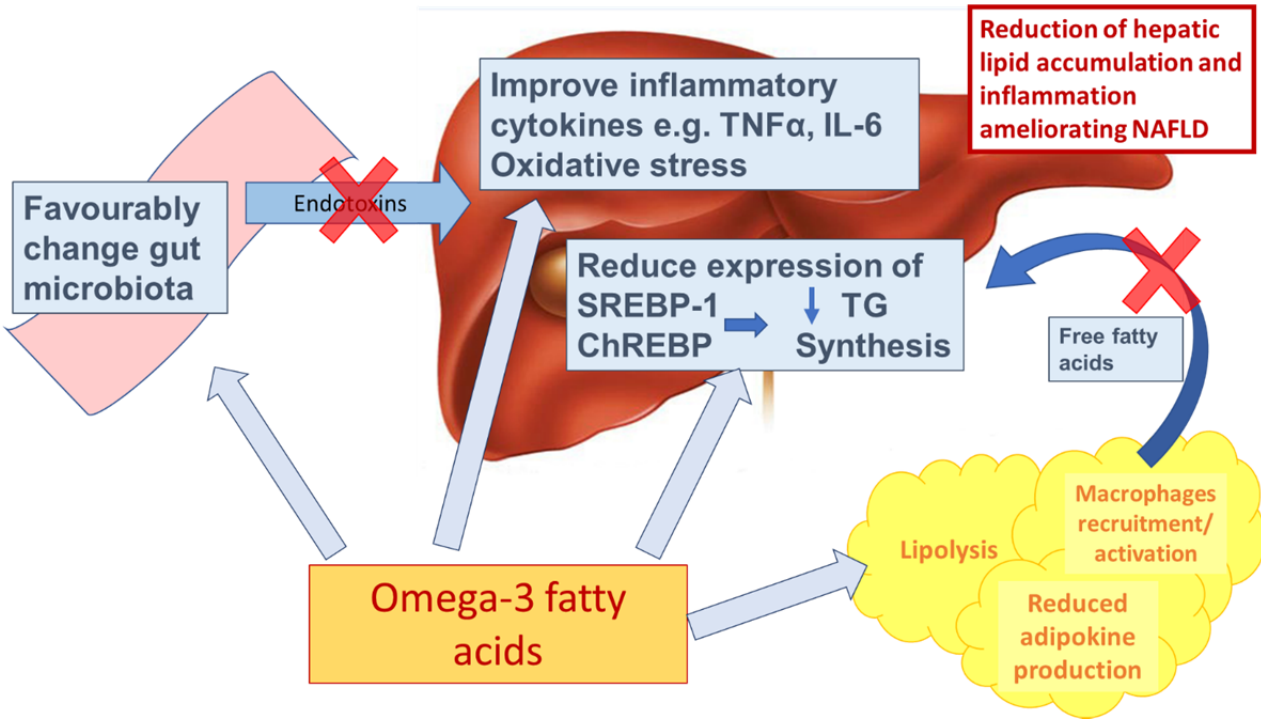

Figure 2. Mechanisms potentially responsible for a beneficial effect of omega-3 fatty acid treatment in NAFLD.

Omega-3 fatty acids increase hepatic fatty acid oxidation and reduce triglycerides (TG) synthesis by inhibiting the expression of SREBP-1c and ChREBP activity (nuclear transcription factors that stimulate hepatic de novo lipogenesis). In adipose tissue, omega-3 fatty acids decrease fatty acid and adipokine release and have a potential anti-inflammatory effect by inhibiting macrophage recruitment and activation. Omega-3 PUFA treatment also increases abundance of butyrate-producing bacterial species in the intestine reducing lipopolysaccharide production.

4.3 Genetic polymorphisms

i. Influence of PNPLA3 genotype on NAFLD pathogenesis and progression

There is evidence showing that the genetic variation in patatin-like phospholipase domain-containing protein-3 (*PNPLA3*-I148M) influences severity of liver disease, and serum TG concentrations in NAFLD. *PNPLA3* encodes a 481-amino acid membrane protein, also called adiponutrin, localised in the endoplasmic reticulum and at the surface of lipid droplets. Adiponutrin promotes either triacylglycerol hydrolase or acylglycerol transacetylase activity in the liver. Exome wide association study identified a single nucleotide polymorphism (SNP) rs738409 in exon3 of the *PNPLA3* gene, encoding for the isoleucine to methionine substitution at position 148 (I148M). The loss-of-function 148M variant is characterised by dysfunctional PNPLA3 (adiponutrin) protein that accumulates on the surface of lipid droplets (63). This variant is associated to a loss of lipolytic activity and impairment of liver lipid catabolism, with consequent lipid droplets remodelling, and impairment of VLDL secretions. This would favour hepatocellular accumulation of triglycerides with consequent impairment of mobilization of fatty acids from the hepatocytes. Romeo et al. first showed that this *PNPLA3*-I148M variation was strongly associated with NAFLD (64).

4.4 Fads

Fatty acid desaturase 1 and fatty acid desaturase 2 (FADS1 and FADS2) genes encode for Δ-5 and Δ-6 desaturases enzymes. This are key enzymes mainly expressed in the liver and are responsible for catalysing the formation of double bonds at the Δ-5 and Δ-6 positions in long chain polyunsaturated fatty acids, respectively. Both genes, FADS1 and FADS2, are oriented head-to-head and localized in a cluster on chromosome 11 (11q12-13.1). Elongases are enzymes responsible for catalysing the elongation of the aliphatic chain of carbons adding two carbon units to the carboxylic end of a fatty acid chain, leading to the formation of long-chain omega-3 and omega-6 polyunsaturated fatty acids (65). Elongases are encoded by members of the elongation-of-very-long-chain-fatty-acids (ELOVL) gene family located on chromosome 6 (66). Several genome-wide association (GWA) studies have shown that single nucleotide polymorphisms (SNPs) in both FADS1-FADS2 gene clusters and ELOVL gene family were strongly associated with: higher EPA and lower DHA proportions (39, 67), NAFLD (68), metabolic syndrome, (69) and dyslipidaemia (39-43, 67). These studies have also shown that low Δ5-desaturase enzyme activity was associated with accumulation of desaturase substrates and low desaturase products (45, 46). Other investigations have found that the concurrence of low Δ-5 desaturase activity and high Δ6-desaturase activity was associated with dyslipidaemia, suggesting that heritable differences in omega-3 and omega-6 fatty acids metabolism also influenced plasma lipid profiles (70-73). Currently, it is unclear whether treatment with omega-3 fatty acids can affect Δ-5 and Δ-6 desaturase enzyme activities in people with NAFLD through a gene-DHA+EPA interaction. Interestingly, FADS and ELOVL polymorphisms are associated with reduced Δ-5 and Δ-6 desaturase activity and

accumulation of desaturase substrates and a reduction of desaturase products (45, 46). Omega-3 fatty acid supplementation can improve Δ-5 and Δ-6 desaturase activity through a gene-treatment interaction. Cormier et al. showed that 6 weeks supplementation with 2 g of EPA plus 1 g of DHA daily in 210 healthy people increased Δ-5 desaturase activity and decreased Δ-6 desaturase activity increasing omega-3 and decreasing omega-6 fatty acid plasma levels (43).

4.5 Gut microbiota dysbiosis

The development and consequences of NAFLD involve not only altered liver function, but also dysfunction of key extra-hepatic tissues, such as intestine, with the production of endotoxin and bacterial products derived from the gut microbiota. Imbalances in the gut microbiota (dysbiosis) can lead to metabolic endotoxemia, obesity, insulin resistance and inflammation, all factors implicated in NAFLD. In the presence of dysbiosis, there is an increased production of endotoxins from the Gram-negative bacteria that can damage the intestinal barrier. These endotoxins are then released in the blood stream causing a subclinical elevation in circulating levels of lipopolysaccharide (74). Although this is a very new area of research, there is evidence that omega-3 PUFA supplementation increases abundance of butyrate-producing bacteria which decrease production of endotoxins (lipopolysaccharide) and contribute to gut health (75, 76). Moreover, Rajkumar at al. showed that the combination of probiotic (*Bifidobacteria, Lactobacilli,* and *Streptococcus thermophilus*) and omega-3 fatty acids supplement (180 mg EPA and 120 mg of DHA) was more effective in improving lipid profile, insulin sensitivity and inflammatory biomarkers in overweight healthy adults, than probiotic alone **(Figure 2)**.

5 Omega-3 fatty acid treatment for NAFLD

Several studies have attempted to test the effects of long chain omega-3 fatty acid treatment in NAFLD. However, these studies have produced controversial results with limited success and serious safety concerns about long-term therapy (24-28, 77). Lifestyle changes (exercise and diet) (78, 79) may ameliorate steatosis, but sometimes weight loss and its maintenance are difficult to achieve (29, 30). Studies that have attempted to treat NAFLD by targeting specific pathways in the pathogenesis of NAFLD have to date met with limited success. The use of medications for glucose control and insulin resistance (metformin, glitazones (24, 25) or GLP1 agonist) (80), lipid metabolism (PPAR and FXR agonists (81, 82), and oxidative stress (vitamin E) (26) produced variable results. These relatively small trials have also generated controversy, not least because any positive effects of treatment are limited by side-effects and concerns about long term safety of glitazones (83) and the high dose of vitamin E required (84). Initial clinical trials testing the effects of omega-3 fatty acid treatment in NAFLD differed markedly in four cardinal areas: 1) duration of the treatment, (2) composition of the omega-3 fatty acid treatment, (3) dosage of omega-3 fatty acids, and (4) testing for both adherence to the omega-3 intervention and for contamination with omega-3 fatty acids obtained from other readily available sources **(Table 1)**.

Table 1. Studies investigating the effect of omega-3 fatty acids in patients with NAFLD

Authors, year	Study design	Intervention	Population	Outcome measurements	Results	Comments
Hatzitolios et al., (2004) (85)	Interventional	Fish oil (15ml daily; DHA=1.58 g/d and EPA 2.25 g/d for 24 weeks); atorvastatin (20 mg/daily); orlistat (120 mg thrice daily) **for 6 months**	64 Patients with non-alcoholic fatty liver disease associated with hyperlipidaemia Fish oil: Group A (n=23) NAFLD with predominant hypertriglyceridemia; Atorvastatin: Group B (n=28): NAFLD with predominant hypercholesterolemia; Orlistat: Group C (n=21): overweight patients with NAFLD	Liver function, lipid levels and liver ultrasonography	Ultrasonography showed resolution of fatty liver in 35% of patients in Group A, 61% in Group B, and in 86% in Group C ($p< 0.001$, Group C vs. A).	No control group. A significant decrease (13%) in BMI was found only in the Orlistat group.
Capanni et al., (2006) (86)	Open-label	Oral administration of omega-3 PUFA, 1-g capsule/day (EPA 375 mg/d and DHA 625 mg/d) **for 12 months**	56 patients with NAFLD (42 subjects receiving therapy; 14 controls)	Liver function, omega-6/omega-3 ratio, liver US and liver perfusion by DPI	Improvement in AST ($P=0.003$), ALT ($P=0.002$), GGT ($P =0.03$), and TG ($P = 0.02$)	Absence of blinding and randomization
Spadaro et al., (2008) (87)	Randomized; open-label	AHA diet + 2 g/day n-3 PUFA (fish oil) vs AHA diet **for 6 months**	36 patients with NAFLD (18 subjects receiving therapy +AHA diet; 18 controls AHA diet alone)	Liver fat assessed by abdominal US, ALT, AST, TNF-α serum levels, and HOMA	Reduction in ALT ($P<0.01$), TG ($P<0.01$), serum TNF-α ($P<0.05$) and HOMA (IR) ($P<0.05$)	Lack of placebo, and the non blinding of participants and investigators; the amount of EPA and DHA in fish oil was not reported.
Zhu et al., (2008) (88)	Randomized Placebo-control	Seal oils 2 g/three times a day plus caloric restriction to 25-30 kcal/d) vs placebo **for 6 months**	134 patients with NAFLD determined by ultrasound (66 subjects receiving Therapy; 68 receiving placebo)	Liver function test fatty liver assessed by US	Decrease in ALT, TG, LDL ($P < 0.05$); complete fatty liver regression ($P=0.004$)	The amount of EPA and DHA in fish oil and the composition of placebo was not reported.
Vega et al., (2008) (89)	Open label with washout period between placebo and treatment	Fish oil (9 g/d, EPA 4.63 g/d and DHA 2.15 g/d) vs Placebo capsules consisted of 6.5 g/d of vaccenic acid, 0.9 g/d of linoleic acid and 1.1 g/d of palmitic acid **for 2 months**	17 patients with previous elevated liver fat on MRS (17patientis: 4 weeks on placebo oil followed by 8 weeks on fish oil treatment)	Liver fat content assessed by MRS; liver enzymes, TG and adiponectin levels	Improvement of plasma triglyceride level by ($P<0.03$), VLDL + IDL ($P < 0.03$), ApoB ($P< 0.03$) and liver fat content.	Causes of liver disease other than NAFLD were not excluded and alcohol intake was not reported; short duration
Tanaka et al., (2008) (90)	Interventional	Purified EPA ethyl ester (2.7 g/d) **for 12 months**	23 patients with biopsy proven NASH	Improvement in steatosis, fibrosis and ballooning; improvement of liver enzymes.	Improvement of steatosis, fibrosis and ballooning in 6 out of 7 subjects who underwent second liver biopsy.	No control group; only 7 patients consented to undergo post-treatment liver biopsy.
Cussons et al., (2009) (91)	Double blind, crossover study with 4 weeks wash out	Fish oil (4 g/d: 1.08 g/d EPA and 2.24 g/d DHA); Placebo olive oil (4 g/d) **for 2 months** (8 weeks of fish oil or olive oil; wash out period 4 weeks)	25 patients with polycystic ovary syndrome and NAFLD determined by MRS	Liver fat measured by MRS	Improvement of liver fat percentage on MRS after fish oil treatment (14.8 fish oil vs 18.2% placebo).	In the fish oil supplement, DHA concentration was higher than EPA. This may have contributed to the improvement of liver fat percentage.

Nobili et al., (2011) (92)	Randomized	DHA (250 and 500 mg/day); Placebo: germ oil **for 6 months**	60 children with biopsy proven NAFLD. DHA 250 mg/d (n=20), DHA 500 mg/d (n=20) or Placebo (n = 20)	Primary: change in liver fat content as detected by US; secondary: changes in ALT, TG and BMI	Improvement of liver fat detected by US - DHA 250 mg vs placebo ($p<0.001$) and DHA 500 mg vs placebo ($p=0.01$)	
Sanyal et al., (2014) (93)	Double-blind randomised Placebo-controlled	Purified EPA ethyl ester (1.8 mg/day, and 2.7 g/day); Placebo (composition not reported) **for 12 months**	243 patients with biopsy proven NASH. EPA 1.8 g/d (n=82) EPA 2.7 g/d (n=86) Placebo (n=75)	Improvement of the NAS score by 2 points or more with contribution form more than 1 parameter and no worsening of fibrosis	No significant effect in hepatic fat and enzymes, insulin resistance and inflammatory markers	
Scorletti et al. (2014) (94)	Double-blind randomised Placebo-controlled	Purified EPA-EE (1.84 g/day) and DHA-EE (1.52 g/day) **For 15-18 months**	Patients with NAFLD confirmed by biopsy, MRS, computed tomography or ultrasound DHA+EPA (n=51) Placebo (n=52)	Reduction of liver fat measured by magnetic resonance spectroscopy (MRI) and improvement of two fibrosis scores	Erythrocyte DHA but not DHA+EPA enrichment was associated with a reduction in liver fat.	These results supported the evidence that DHA might be more effective than EPA in decreasing liver fat.
Pacifico et al. (2015) (95)	Double-blinded, parallel-group, randomized, placebo controlled	250 mg/day algal oil (39% DHA ≈97.5 mg/day). Low-caloric diet (25-30 kcal/kg/day) and daily exercise (60 min/day, 5 times/week) **For 6 months**	Children with NAFLD diagnosed by MRI DHA+diet (n=25) Placebo+diet (n=26)	Change in hepatic fat fraction as estimated by MRI	DHA supplementation reduced hepatic fat by 53.4% and the hepatic fat fraction from 14% to 6.5% assessed by MRI.	
Dasarathy et al., (2015) (96)	Prospective, randomized, double blind placebo-controlled study	EPA (2.16 g/d) and DHA (1.44 g/d); Placebo: corn oil **For 12 months**	37 patients with well controlled diabetes and biopsy proven NASH within 6 months prior to the start of study. EPA/DHA (n=18) Placebo (n=19)	Improvement of ≥ 2 points in the NAS determined by liver biopsy	EPA/DHA supplement did not provide beneficial effect over placebo in NASH patients with diabetes	No information regarding erythrocytes enrichment after treatment. No information regarding compliance with supplements, diet or life style.
Argo et al. (2015) (97)	Double-blind, randomized placebo-controlled	EPA (1.05 g/day) and DHA (0.75 g/day), aerobic exercise at least 150 min/week and decrease energy intake by 500-1000 calories/day **for 12 months**	34 patients with NASH diagnosed by biopsy EPA+DHA+diet+exercise (n=17) Placebo+diet+exercise (n=17)	Decrease of at least two points in the NAS score	Significantly decreased liver fat ($p=0.0009$) and markers of liver injury but not NAS score.	
Boyraz et al., (2015) (98)	Double-blinded, randomized, placebo controlled	Omega-3 fatty acids 1 g/d (containing 720 mg omega-3; 380 mg EPA and 200 mg DHA) plus a calorie restriction diet with 25-30 kcal/kg/day and Physical activity (one hour, three times per week). Placebo (no composition available) **For 12 months**	Obese adolescents with NAFLD Omega-3 fatty acids (n=56) Placebo (n=52)	Improvement in liver functions, liver brightness and insulin resistance	The combination of omega-3 fatty acids and lifestyle change showed a greater improvement in hepatic fat compared with placebo.	No mention was made about the placebo composition. The composition of omega-3 fatty acid supplement was available on supplement's manufacture website, no information was mentioned in the article
Janczyk et al., (2015) (99)	Randomized, double-blind, placebo controlled	Omega-3 fatty acids (DHA and EPA 3:2 proportion); Treatment was adjusted for weight:	64 Overweight/obese children with NAFLD. Omega-3 fatty acids (n=30) Placebo (N=34)	Decreased ALT activity by ≥0.3 times the upper limit of normal	Omega-3 fatty acids did not decrease serum ALT	Liver steatosis was a secondary outcome, omega-3 fatty acids did not affect liver steatosis on ultrasound

		<40 kg: DHA=0.27 g/d and EPA=0.18 g/d; 40-60 kg: DHA= 0.53 g/d and EPA= 0.36 g/d; >60 kg: DHA= 0.8 g/d and EPA= 0.53 g/d; Placebo: sunflower oil **For 6 months**				
Li et al., (2015) (100)	Prospective, randomized, controlled unblinded	50 ml omega-3 fatty acids (1:1 ratio EPA:DHA) added into daily diet.	78 patients with biopsy proven NASH. Omega-3 fatty acid (n=39) Control (normal saline) (n=39)	Improvement of NASH	Improvements in liver histology and metabolic profile compared to control group.	No placebo group. BMI reduction in the treatment group
Nogueira et al. (2016) (101)	Double-blind, randomized and placebo-controlled	0.945 g n-3 per day (605 mg ALA, 143mg EPA and 177mg DHA) **For 6 months**	50 patients with NAFLD diagnosed by liver biopsy Treatment (n=27) Placebo (n=23)	Effect of treatment in patients with biopsy proven NASH	No changes in the treatment or placebo group were observed	There was an increase in plasma omega-3 fatty acids in both placebo and treatment group

These differences in the design of the clinical trial has added to the confusion about the efficacy of omega-3 fatty acid treatment in NAFLD (77, 102, 103). Hatzitolios et al, studied the effect of different lipid lowering treatments in patients with NAFLD and hyperlipidaemia for 6 months (85). The authors compared the effects of fish oil with those of atorvastatin and orlistat on liver fat. At the end of the study, there was a general improvement in liver fat assessed by ultrasound with 35% improvement in patients receiving fish oil, 61% improvement in patients receiving atorvastatin and 86% improvement in patients receiving orlistat. However, there was a significant decrease (13%) in BMI in the orlistat group resulting in a significant reduction in liver fat in the orlistat group compared with the fish oil group ($p< 0.001$ orlistat vs. fish oil) (85). Capanni, Sofi and Spadaro suggested a beneficial effect of omega-3 fatty acid treatment on liver enzymes after 6-12 months intervention (86, 87, 104, 105). The composition and dosage of omega-3 fatty acids used in a clinical trial is also potentially very important, as EPA and DHA are not absorbed and metabolised in the same way. For example, omega-3 fatty acids used in clinical trials have been fish oil, seal oil, or purified EPA, DHA or EPA+DHA. EPA and DHA are metabolised differently and they may not have equivalent effects on the liver in NAFLD. EPA supplements increases blood levels of EPA and DPA, but not DHA; whereas, DHA supplements increase blood levels of DHA, DPA and EPA. EPA and DHA may not have equivalent effects on oxidative stress (106), inflammation (107) or fibrosis (108). The range of effects of omega-3 fatty acids on NAFLD and/or NASH may depend on the composition and purity of omega-3 fatty acids used in the study and on the severity of liver disease. Previous studies used mixtures of EPA and DHA with different degree of purification. Two studies tested the effect of purified EPA, one in 2008 (90) and one in 2014 (93). The first study investigated the effect of 2.7 g/d of EPA for 12 months on markers of NAFLD and NASH in 23 patients with liver biopsy proven NASH. However, the end of study biopsy was performed in only 7 subjects (90). At the end

of the study, the ultrasound showed an improvement in liver steatosis in 12 patients and the liver biopsy showed a decrease in steatosis (29%), fibrosis (59%), lobular inflammation (48%), ballooning (44%) and NAS (39%) (90). After this small study, Sanyal et al. showed no improvement of NAS score after supplementation with purified EPA in patients with NASH (93). On the contrary, Pacifico and Nobili showed that DHA was effective in reducing liver fat and markers of liver fibrosis (95, 109). Cusson et al. showed an improvement in MRS liver fat percentage after fish oil treatment (14.8 fish oil vs 18.2% placebo). Interestingly, the fish oil supplement contained higher concentration of DHA (2.24 g/d) than EPA (1.08 g/d) and this may have contributed to the improvement of liver fat percentage (91). These opposing results may be explained not only by the composition of omega-3 fatty acid used in the trial, but also by the severity of liver disease in patients recruited to the trials. Omega-3 fatty acids maybe effective in the early stages of NAFLD, and further studies are needed to test the specific effects of DHA on NASH/fibrosis. To date, only one clinical trial has tested the effect of purified EPA in NASH. The primary outcomes were either a NAS≤3 without worsening of fibrosis on a drop in NAS by two or more points with no worsening of fibrosis (93). The authors reported no beneficial effect on the histologic features of NASH or on serum triglyceride levels. The very modest effect of EPA on triglyceride levels supports the possibility that the dosage of EPA may have been too low, as we might have expected a triglyceride-lowering effective even though patients did not have hypertriglyceridaemia. In addition, supplementation with EPA may suppresses the conversion of EPA to DHA (110). There is also evidence that EPA and DHA metabolism may be different in men and women. For example, in men, the conversion of EPA to DHA is <1%, whereas in women is up to 9% (111-118). Trials testing omega-3 fatty acid interventions have predominantly focussed on testing the effects of DHA. The effects of high dose purified DHA only has been tested in children by Nobili et al. (119). These authors studied the effect of 18 months treatment with

DHA in children with biopsy proven NAFLD. At the end of the study there was an improvement in hepatic steatosis ballooning, inflammation but there was no beneficial effect on fibrosis. However, it was not considered ethical to subject the children in the placebo-arm of the trial to an end of study liver biopsy. The combination of EPA+DHA has been tested in several clinical trials. Dasarathy et al. tested the effect of EPA+DHA on patients with diabetes and biopsy proven NASH for 12 months (96). At the end of the study, there was no beneficial effect over placebo in NASH patients with diabetes. The results of the biopsy showed a greater improvement in the placebo group compared to the EPA+DHA group. Interestingly, there was no information regarding compliance with supplements, diet or life style (96). In another study, Li et al. evaluated the effect of omega-3 fatty acid on NASH diagnosed by liver biopsy (100). Patients were randomised to receive 50 ml of omega-3 fatty acids (1:1 ratio EPA:DHA) added into daily diet or to receive normal saline for 6 months. Liver biopsies were undertaken at the beginning and at the end of the study. After 6 months of treatment, the EPA+DHA group showed improvement in steatosis grade, necro-inflammatory grade, fibrosis stage and ballooning score compared to control group. However, both groups showed an increase in physical activity and a decrease in BMI at the end of the study (100). In overweight and obese children with NAFLD, Janczyk et al. tested the effect of a weight-adjusted dosage of EPA+DHA (99). The primary outcome was an improvement of ALT activity by ≥0.3 times the upper limit of normal, whereas improvement in liver steatosis was a secondary outcome. After 6 months there was a decrease in liver function tests and liver steatosis in both placebo and EPA+DHA groups. There was no significant reduction of ALT or liver steatosis measured by ultrasound in the EPA+DHA group compared to placebo (99). Boyraz et al studied obese adolescents with NAFLD in a double-blinded, randomized, placebo-controlled trial (98) and the intervention was: 1 g/d of omega-3 fatty acids (380 mg of EPA and 200 mg of DHA) (as per the supplement's manufacturer website) or placebo

(composition not available in the published paper). All adolescents received lifestyle advice with a calorie restricted diet (25-30 kcal/kg/d) for weight loss and physical activity (one hour, three times per week). After 12 months of treatment, there was a decrease in weight and hepatic steatosis measured by ultrasound in both groups. The improvements were more pronounced in the PUFA group, showing an additional effect of omega-3 fatty acid supplementation in adolescents that underwent a lifestyle change (98).

Only one clinical trial the WELCOME study (Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with OMacor thErapy, undertaken by the authors) has assessed erythrocyte EPA and DHA enrichment to assess compliance during the study (14, 94, 120). Using erythrocyte DHA percentage enrichment or erythrocyte EPA percentage enrichment, we were able to test the specific contribution of each omega-3 fatty acid. Thus, it was possible to test associations between percentage DHA enrichment (or percentage EPA enrichment), and changes in liver fat percentage measured by magnetic resonance spectroscopy (94). In this study, we showed an independent association between a decrease in liver fat percentage and erythrocyte DHA enrichment (but not with erythrocyte EPA enrichment).

6 Areas of uncertainty and unanswered questions for future research

We consider there are several poorly answered questions in considering whether omega-3 fatty acids have efficacy in ameliorating liver disease in NAFLD. We have listed some of the important areas of uncertainty that we consider still need to be resolved in the planning of future studies testing the effects of omega-3 fatty acids on the different components of liver disease in NAFLD.

6.1 Duration of treatment with omega-3 fatty acids

Omega-3 fatty acid interventions of variable durations have been tested in different studies (102). The duration of the WELCOME study intervention (minimum 15 months, maximum 18 months) was informed by a meta-analysis by He et al. showing the effectiveness of ≥12 months treatment with omega-3 fatty acids on AST, TG and liver fat and studies from Capanni, Sofi and Spadaro showing a beneficial effect of omega-3 fatty acids on liver fat and liver enzymes after 6-12 months treatment (86, 87, 104, 105). Whether it is necessary to intervene for >12 months with omega-3 fatty acids to decrease liver triglyceride seems unlikely as other intervention that decrease liver lipid (such as weight loss) are known to decrease liver triglyceride concentrations over much shorter periods of time. However, it seems likely that a longer period of intervention would be needed to have an impact on NASH. Most of the biopsy-based end point trials that have tested the effects of other agents (or drugs) on histological end points (focussed on improving NAS by ≥2 points), have intervened for ~ 2 years. Such a timescale has been based on an assumption that the longer duration of intervention is needed to improve features of steatohepatitis such as inflammation and ballooning of hepatocytes (and to have a potential impact on fibrosis). Therefore, most of the studies to date that have tested the effects of omega-3 fatty acids on liver disease in NAFLD have been of too short a duration to be certain of whether there might be any benefit or not on NASH.

6.2 Appropriate dosage of omega-3 fatty acid

The dosage of 4 g of DHA+EPA daily was selected for the WELCOME study for two reasons.

First, in light of the proven effectiveness of this dose to decrease serum triglyceride levels in patients with hypertriglyceridemia (121). Secondly, it was hypothesised that this dosage would raise the erythrocyte level of EPA and DHA within phospholipids above the pre-specified level of 0.7% and 2% respectively in the phospholipid fraction of red blood cell membrane preparations. This level had been previously deemed to be the minimum increase for improving the DHA+EPA sum after treatment, to a value thought to result in decreased risk of CVD (122).

In the WELCOME study, we were concerned that there might be variable tissue enrichment with omega-3 fatty acids, despite good compliance from the participants, and consequently we tested enrichment of red blood cell membrane phospholipid (as a validated proxy for liver enrichment with DHA and EPA). To our surprise we found very variable tissue enrichment despite us using the highest licensed dose of DHA+EPA (as Omacor or Lovaza). Participants in the trial were also questioned about their compliance and virtually all assured us that they had been compliant with taking their allocated medication. We also counted returned unused capsules from participants at 6 monthly intervals during the trial to monitor compliance. Since an explanation for variable tissue enrichment with DHA and EPA is uncertain, it is possible that liver enrichment is sub-optimal in some individuals leading to little response to therapy.

6.3 Composition and chemical purity of omega-3 fatty acid treatment

All clinical trials used different composition of omega-3 fatty acid treatment, some studies used fish oil, others used DHA or EPA or a combination of DHA and EPA. One study used the highest licence dose of omega-3 fatty acids, 380 mg DHA and 460 mg EPA per g of

oil(94). Notably, contrary to fish oil preparations used in other studies no lipid-soluble vitamins A and D were present (102). This combination was chosen because of the different characteristics of DHA and EPA and specifically because they produce different lipid mediators: EPA-derived eicosanoids and DHA-derived resolvins and protectins. EPA-derived eicosanoids have an anti-inflammatory effect.; DHA-derived resolvins and protectins have a role in the resolution of inflammation and have thus been described as ‘‘specialized pro-resolving lipid mediators’’(123). The key function of these mediators is to reduce liver macrophage infiltration, and induce a specific hepatic miRNA signature, in order to reduce inflammatory adipokine expression (56, 124). Moreover, DHA also inhibits the secretion of apoB-100 by promoting its autophagic degradation causing a reduction in VLDL synthesis in the liver (31). With regard to the chemical purity of the treatment, only one study selected the most highly purified DHA and EPA available on the market(120). This decision was made to avoid contamination with polluting particles and reduce toxicity due to other compounds that might be present within the fish oil preparation. This was again a substantial departure from previous studies. It is noteworthy that many of the trials testing the effects of different omega-3 fatty acid/fish oil preparations have used various different preparations containing long chain omega-3 fatty acids. It is plausible that not only the dose of specific fatty acids, and their variable tissue enrichment, but also the purity of the preparations and their contaminants, may also influence whether these agents affect liver disease in NAFLD.

6.4 Methods used to assess severity of liver disease in NAFLD and heterogeneity of participants recruited within the spectrum of NAFLD

Different methods can be used to assess the severity of liver disease. Four studies measured liver fat with B-mode ultrasound imaging and liver echotexture was scored on a four-grade

scale by comparing it with the right kidney cortical echogenicity (86-88, 92). Although the ultrasound scan is a cheap procedure, it is operator dependant and therefore sometime could be difficult to identify different grade of echogenicity to quantify liver fat. Two studies used magnetic resonance spectroscopy (MRS) to quantify liver fat percentage (89, 95) and one study (94) used a synergistic combination of MRS and non-invasive markers of liver function, and NAFLD disease severity. The combination of MRS and markers of liver function was a unique feature and a substantial departure at the time from the traditional approach of carrying out assessment of liver disease solely through liver biopsy. Multiple reasons are in support of the choice to use MRS over liver biopsy. First, liver biopsy is invasive, expensive, and subject to sampling variability (125-129), and many investigators currently consider it a high-risk procedure that is unacceptable as a research test for monitoring NAFLD. Also, liver biopsy evaluates only a tiny portion (0.05 cm^3) of the liver (800-1,000 cm^3), and NAFLD is often a patchy disease. Secondly, MRS is currently considered the non-invasive gold-standard technique for assessing liver fat percentage and has excellent reproducibility and sensitivity (130, 131) with a coefficient of variance of only 8%, and liver fat signals of only 0.2% are clearly evident above the noise level (131). It is also plausible that omega-3 fatty acid treatment only affects liver fat without affecting inflammation or fibrosis in NAFLD. Interestingly, it has also been shown recently by Argo et al. (97), that omega-3 fatty acid treatment caused a significant reduction in liver fat on the paired analysis of MRI image-assisted lipid morphometry, regardless of weight loss or gain. In this study, Argo et al tested the effects of 3 g of fish oil/day for 1 year in 41 participants with non-cirrhotic NASH and in this study each 1000 mg capsule contained 70% total N-3 as triglyceride: 35% EPA, 25% DHA, 10% other omega-3's, and a scant amount of lemon oil.

6.5 Effect of genotype to modulate the influence of omega-3 fatty acid treatment.

Scorletti et al. showed that there was a beneficial effect of achieving high levels of DHA tissue enrichment. Specifically, they showed that high levels of erythrocyte DHA enrichment (≥2%), was effective for reducing liver fat. Patients with high liver fat percentage obtained the most benefit from achieving good DHA enrichment (≥2%): a 6% enrichment in DHA resulted in a (6x3.3%)= ~20% decrease in liver fat percentage. These results were consistent with previous published literature (92, 95, 103, 109, 132-134). Nobili et al. conducted two clinical trials testing the effect of DHA supplementation in children with NAFLD. The authors showed specific beneficial effects of DHA on liver biopsy with improvement on hepatic steatosis, ballooning, and inflammation NAS, but DHA was ineffective on fibrosis (92, 109). Pacifico and colleagues, showed that after 6 months of DHA supplementation in children with NAFLD, MRS liver fat was reduced by 53.4% (95% CI, 33.4-73.4; p= 0.04) in the DHA group. Interestingly, there is evidence showing that PNPLA3-148MM genotype adversely affected response to DHA+EPA treatment in NAFLD (14). Although, the numbers of subjects studied in the trial with PNPLA3-148MM genotype was small (~13%), there was a suggestion that subjects with this genotype had lower levels of DHA tissue enrichment only (with no effect on EPA enrichment), and also no decrease in liver fat, with omega-3 fatty acid treatment.

PNPLA3 is a multifunctional enzyme with both triacylglycerol lipase and acylglycerol O-acyltransferase activity that participates in triacylglycerol hydrolysis. Whereas, the isoleucine to methionine substitution leads to a loss of lipolytic activity leading to an impairment of lipid catabolism, lipid droplets remodelling, and impairment of VLDL secretions, increasing liver fat accumulation and affecting DHA metabolism. PNPLA3-I148M variant is attached on

the surface of lipid droplets reducing TG breakdown leading to lipid retention in the hepatocyte lipid droplet slowing down the conversion of ethyl ester to TGs. Thus, it is plausible that ethyl esters of omega-3 fatty acid preparations maybe less effective in subjects with NAFLD, who have this particular genotype.

6.6 The present state of recommendations and possible future evolution of guidelines

At present, the NICE NAFLD guidelines (ng49) for England and Wales do not recommend omega-3 fatty acids to adults with NAFLD because there is insufficient evidence of their beneficial effect. To date, these guidelines, the joint European Association for the Study of Diabetes, European Association for the Study of Liver Disease and the European Association for the Study of Obesity (135) and the US Guidelines (1) recommend pioglitazone or vitamin E for adults with NASH, whether they have diabetes or not. In the NICE Guidelines these treatments are advocated for use only in secondary and tertiary care settings because of their side-effects and concerns about long term safety (83, 84).

However, there is increasing evidence that the paradigm on which these guidelines were originally based is shifting. The results of trials testing treatment with thiazolidinediones (24) and anti-oxidants such as vitamin E (26), have produced variable results with ~50% of patients with NASH, not responding to treatment and to date it is uncertain why liver disease in some patients responds to therapy and in other patients, there is no improvement in liver disease. With no easy way to identify non responders, there is a reluctance amongst many clinicians to use agents that have potential side effects and at the same time might not ameliorate the liver disease. There is some support for the hypothesis that omega-3 fatty acids treatment might have a beneficial effect on NAFLD, and at the same time produce minimal side effects (31). As discussed above several biological mechanisms have been identified that

support this hypothesis; notably, it has been shown that these omega-3 fatty acids have a beneficial effect on bioactive metabolites, alteration of transcription factor activity such as peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) (31). There is evidence showing that EPA has modest effects on fatty acid oxidation and triglyceride catabolism by binding to and activating PPAR (α, β, and g) (136-138). As far as we know, to date there are no clinical studies testing the effect of long term treatment with omega-3 fatty acids in humans. Nonetheless, there are divergent opinions regarding the beneficial effect of omega-3 fatty acid treatment on liver fat accumulation. In an animal model, supplemental feeding with ALA or EPA and DHA decreased 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity and increased biliary secretion causing more oxidative stress, and consequently more liver damage (139). It is possible that hydroxyl (OH) radical attack on the highly unsaturated omega-3 fatty acids creates lipid peroxyl radicals and then lipid peroxides which would be the obvious route to creating oxidative liver damage initiated by high tissue levels of omega-3 fatty acids. An *in vitro* study testing the effects of various concentrations and durations of incubation of saturated (palmitate), mono-unsaturated fatty acids (oleate) and omega-3 fatty acids (eicopentanoate) on triglyceride and apo-B metabolism in HepG2 cells showed over 25 years ago that in contrast to palmitate and oleate, 250 micromolar eicosapentanoate adversely affected cell viability after only 72 hours incubation. Whether such high concentrations of serum EPA could ever be reached with high dose EPA treatment is uncertain but this evidence suggests that very high supplementation levels over months or years of exposure could have a deleterious impact on a damaged liver (NAFLD) in vivo (140). In contrast, EPA and DHA supplementation has been shown to attenuate a Western diet-mediated induction of hepatic inflammation and oxidative stress; and in particular, DHA and not EPA attenuated hepatic fibrosis (47, 141). Thus, although it is

not proven, this body of evidence suggests it is important to err on the side of caution, as further human studies are needed to show that long term high dose supplementation with omega-3 fatty acids is safe.

A recent systematic review and meta-analysis has shown a benefit of omega-3 fatty acid treatment on liver fat in NAFLD (102), emphasising that omega-3 fatty acid treatment might confer a benefit early in the course of the liver disease in NAFLD. We suggest this body of evidence should not be diminished by the results of a recent trial in which treatment involving high doses of EPA failed to show an improvement in NAFLD histological score in patients with more advanced disease who had NASH at recruitment (93). As there is considerable evidence that EPA and DHA have different biological effects and also different metabolism in men and women (e.g. in men, the conversion of EPA to DHA is <1%, whereas in women is up to 9%) (113-118) it is possibly not surprising that Sanyal et al. did not find that EPA treatment had a beneficial effect in NASH.

7 Conclusions

There is increasing support for the hypothesis that omega-3 fatty acids treatment might have a beneficial effect on liver disease in NAFLD and cause minimal side effects (31). First, several biological mechanisms have been identified that support this hypothesis; notably, it has been shown that these omega-3 fatty acids have a beneficial effect on bioactive metabolites, alteration of transcription factor activity such as peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) (31). Additionally, as discussed

above there is some evidence of benefit from omega-3 fatty acid treatment from randomised trials in patients with NAFLD. However, as discussed above, we consider that certain factors ('known unknowns') need to be considered further, before planning future studies that are focussed on investigating the effects of omega-3 fatty acids in NAFLD. Moreover, the mechanistic basis for omega-3 fatty acid action is based on studies in rodents and cells. These studies have established that omega-3 fatty acids are pleiotropic regulators of multiple pathways, many of which are involved in the onset and progression of NAFLD. Whether these same mechanisms are operative in humans is less clear. Further human studies are needed to better understand the beneficial effect of omega-3 fatty acids, specifically the different effect of EPA and DHA, on NAFLD.

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Conflict of interest: CDB was Principal Investigator for the **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD (non alcoholic fatty liver disease) with **OM**acor th**E**rapy) WELCOME Study. The WELCOME study was a randomised placebo-controlled trial in patients with NAFLD that tested the effects of high dose purified n-3 long chain fatty acids (Omacor/Lovaza Abbott/Pronova) 4 grames o.d. on a range of liver and cardio-metabolic outcomes. The trial was funded independently of the drug manufacturers/suppliers whose only input was to provide the active compound and matched placebo at no cost. There was no pharmaceutical input into the study design, conduct of the

trial, analyses of the data, or writing and submission, of the papers resulting from this trial. The trial had core funding from NIHR and also attracted extra grant support from Diabetes UK, the NHS and the University of Pavia (Italy), and was registered with the Diabetes and Hepatology networks. The trial was adopted by the NIHR Portfolio. (www.clinicaltrials.gov registration number NCT00760513).

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