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Human Development and Health

Effect of omega-3 fatty acids in non-alcoholic fatty liver disease

Volume 1 of 1

by Eleonora Scorletti (MD)

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To Andrea, *“per aspera ad astra”*.

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Human development and health

Thesis for the degree of Doctor of Philosophy

Title: EFFECT OF OMEGA-3 FATTY ACIDS IN NON-ALCOHOLIC FATTY LIVER DISEASE

Eleonora Scorletti

The first chapter (Introduction) of the thesis summarises the pathogenesis of NAFLD and its associated risk factors such as type 2 diabetes and cardiovascular disease. Moreover, it describes: a) the potential beneficial effects of long chain omega-3 fatty acid treatment [docosahexaenoic acid (DHA) plus eicosapentaenoic acid (EPA)] in NAFLD; b) the effect of genotypes patatin-like phospholipase domain-containing protein-3 (PNPLA3 I148M) and the transmembrane 6 superfamily member 2 protein (TM6SF2 E167K), on the level of DHA and EPA enrichment and end of study liver fat percentage after DHA+EPA treatment; and c) the effect of fatty acid desaturase (FADS) and Elongase (ELOVL) polymorphisms influencing omega-3 fatty acid metabolism. The second chapter describes the overall aim of this thesis. The aim of my research was to investigate in patients with NAFLD: a) the effect of long-chain omega-3 fatty acid treatment on liver fat percentage and liver fibrosis biomarkers; b) the effect of genotypes influencing NAFLD severity on treatment with DHA+EPA; and c) the effect of genotypes influencing omega-3 fatty acid metabolism in NAFLD. The third chapter describes in details the design and methods used in my research. Chapter four highlights my novel results from the WELCOME study. This chapter describes the baseline and end of study characteristics of the WELCOME study participants and shows the results of the DHA+EPA treatment on liver fat percentage and liver fibrosis biomarkers. This chapter also describes the association between DHA erythrocyte enrichment and decrease in liver fat percentage after DHA+EPA treatment. Chapter five illustrates the association between PNPLA3 I148M and DHA erythrocyte enrichment percentage and end of study liver fat percentage after DHA+EPA treatment. The chapter shows that PNPLA3 I148M was associated with higher end of study liver fat percentage and lower DHA tissue enrichment. Chapter six shows the negative association between FADS polymorphisms and omega-3 fatty acid metabolism in NAFLD. The chapter also shows that there was a gene-DHA+EPA interaction between the minor allele of the FADS1 rs174556 and Δ-5 desaturase activity after treatment with DHA+EPA. Finally, chapter seven, summarises my results in the context of current evidence and knowledge about the subject matter.

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Papers

1. Afolabi PR, **Scorletti E**, Smith DE, Almehmadi AA, Calder PC, Byrne CD; The characterization of hepatic mitochondria dysfunction in patients with non-alcoholic fatty liver disease using the 13C-Ketoisocaproate breath test. Journal of Breath Research. Accepted with minor revisions March 2018.
2. Welsh JA, **Scorletti E**, Clough GF, Englyst NA, Byrne CD; Leukocyte extracellular vesicle concentration is inversely associated with liver fibrosis severity in NAFLD. Journal of Leukocyte Biology. Accepted March 2018.
3. **Scorletti E**, Byrne CD; Omega-3 fatty acids and non-alcoholic fatty liver disease: evidence of efficacy and mechanism of action. Invited review, accepted March 2018, Molecular Aspects of Medicine.
4. **Scorletti E**, Afolabi PR, Miles EA, Smith DE, Almehmadi A, Alshathry A, Moyses HE, Clough GF, Wright M, Patel J, Bindels L, Delzenne NM, Calder PC, Byrne CD; Design and rationale of the INSYTE study: A randomized, placebo-controlled study to test the efficacy of a synbiotic on liver fat, disease biomarkers and intestinal microbiota in non-alcoholic fatty liver disease. Submitted Contemp Clin Trials January 2018.
5. Mann J, Valenti L, **Scorletti E,** Byrne C, Nobili V; Nonalcoholic Fatty Liver Disease in children. Seminars in Liver Disease. Accepted December 2017.
6. Nobili V, Mosca A, De Vito R, Raponi M, **Scorletti E**, Byrne CD; Liver zonation in children with NAFLD: associations with dietary fructose and uric acid concentrations. Liver Int. 2017 Dec 9.
7. Nobili V, Mosca A, **Scorletti E**, Byrne CD. Reply to: "Fructose, uric acid and zonal differences in NASH". J Hepatol. 2017 Nov;67(5):1118-1119.
8. Nobili V, Mosca A, **Scorletti E**, Byrne CD. Reply to: "Energy drinks and adolescents - A hepatic health hazard?" J Hepatol. 2017 Nov 23.
9. Byrne CD, Patel J, **Scorletti E**, Targher G; Tests for the diagnosing and monitoring non-alcoholic fatty liver disease in adults. Submitted to the BMJ, September 2017.
10. Nobili V, Alisi A, Mosca A, Della Corte C, Veraldi S, De Vito R, De Stefanis C, D'Oria V, Jahnel J, Zohrer E, **Scorletti E**, Byrne CD. Hepatic farnesoid X receptor protein level and circulating fibroblast growth factor 19 concentration in children with NAFLD. Liver Int. 2017 Jul 26.
11. Hodson L, Bhatia L, **Scorletti E**, Smith DE, Jackson NC, Shojaee-Moradie F, Umpleby M, Calder PC, Byrne CD. Docosahexaenoic acid enrichment in NAFLD is associated with improvements in hepatic metabolism and hepatic insulin sensitivity: a pilot study. Eur J Clin Nutr. 2017 Mar 15
12. Wainwright P, **Scorletti E**, Byrne CD. Type 2 Diabetes and Hepatocellular Carcinoma: Risk Factors and Pathogenesis. Curr Diab Rep. 2017 Apr;17(4):20.

13. Mosca A, Nobili V, De Vito R, Crudele A, **Scorletti E**, Villani A, Alisi A, Byrne CD. Serum uric acid concentrations and fructose consumption are independently associated with NASH in children and adolescents. J Hepatol. 2017 May;66(5):1031-1036.
14. **Scorletti E**, Byrne CD. Extrahepatic Diseases and NAFLD: The Triangular Relationship between NAFLD, Type 2-Diabetes and Dysbiosis. Dig Dis. 2016; 34 Suppl 1:11-8.
15. Clough GF, McCormick KG, **Scorletti E**, Bhatia L, Calder PC, Griffin MJ, Byrne CD. Higher body fat percentage is associated with enhanced temperature perception in NAFLD: results from the randomised Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with OMacor thErapy trial (WELCOME) trial. Diabetologia. 2016 Jul;59(7):1422-9.
16. Bhatia L, **Scorletti E**, Curzen N, Clough GF, Calder PC, Byrne CD. Improvement in non-alcoholic fatty liver disease severity is associated with a reduction in carotid intima-media thickness progression. Atherosclerosis. 2016 Mar;246:13-20.
17. Nobili V, Alisi A, Musso G, **Scorletti E**, Calder PC, Byrne CD. Omega-3 fatty acids: Mechanisms of benefit and therapeutic effects in pediatric and adult NAFLD. Crit Rev Clin Lab Sci. 2016;53(2):106-20.
18. **Scorletti E**, West AL, Bhatia L, Hoile SP, McCormick KG, Burdge GC, Lillycrop KA, Clough GF, Calder PC, Byrne CD. Treating liver fat and serum triglyceride levels in NAFLD, effects of PNPLA3 and TM6SF2 genotypes: Results from the WELCOME trial. J Hepatol. 2015 Dec;63(6):1476-83.
19. McCormick KG, **Scorletti E**, Bhatia L, Calder PC, Griffin MJ, Clough GF, Byrne CD. Impact of high dose n-3 polyunsaturated fatty acid treatment on measures of microvascular function and vibration perception in non-alcoholic fatty liver disease: results from the randomised WELCOME trial. Diabetologia. 2015 Aug;58(8):1916-25.
20. **Scorletti E**, Bhatia L, McCormick KG, Clough GF, Nash K, Hodson L, Moyses HE, Calder PC, Byrne CD; WELCOME Study. Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology. 2014 Oct; 60(4):1211-21.
21. **Scorletti E**, Bhatia L, McCormick KG, Clough GF, Nash K, Calder PC, Byrne CD; WELCOME Trial Investigators. Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chainomega-3 fatty acid treatment in non-alcoholic fatty liver disease [corrected]. Contemp Clin Trials. 2014 Mar;37(2):301-11. Erratum in: Contemp Clin Trials. 2014 May; 38(1):156.
22. **Scorletti E**, Byrne CD. Omega-3 fatty acids, hepatic lipid metabolism, and nonalcoholic fatty liver disease. Annu Rev Nutr. 2013 ; 33:231-48.
23. Targher G, **Scorletti E**, Mantovani A, Byrne CD. Nonalcoholic fatty liver disease and reduced serum vitamin D(3) levels. Metab Syndr Relat Disord. 2013 Aug;11(4):217-28.
24. **Scorletti E**, Calder PC, Byrne CD. Non-alcoholic fatty liver disease and cardiovascular risk: metabolic aspects and novel treatments. Endocrine. 2011 Dec;40(3):332-43

Academic Thesis: Declaration Of Authorship

I, Eleonora Scorletti

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

“Effect of omega-3 fatty acids in non-alcoholic fatty liver disease”

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:
    a. Scorletti E, West AL, Bhatia L, et al. Treating liver fat and serum triglyceride levels in NAFLD, effects of PNPLA3 and TM6SF2 genotypes: Results from the WELCOME trial. Journal of hepatology 2015;63:1476-83
    b. Scorletti E, Bhatia L, McCormick KG, et al. Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chainomega-3 fatty acid treatment in non-alcoholic fatty liver disease [corrected]. Contemporary clinical trials 2014;37:301-11
    c. Scorletti E, Bhatia L, McCormick KG, et al. Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology (Baltimore, Md) 2014;60:1211-21.

Signed:

Date: 18.06.2018

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"E quindi uscimmo a riveder le stelle"
(And thence we came forth to see again the stars)
(Dante Alighieri, Inferno XXXIV, 139)

List of abbreviations

AA: Arachidonic acid

ALA: Alpha-linolenic acid

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

BMI: Body mass index

ChREBP: Carbohydrate response element binding protein

CVD: Cardiovascular disease

CYP7A1: Cholesterol 7α-hydroxylase

DEXA: Dual-energy X-ray absorptiometry

DGLA: Di-homo-gamma-linoleic acid

DHA: Docosahexaenoic acid

ELOVL: Elongation-of-very-long-chain-fatty acid

EPA: Eicosapentaenoic acid

ETA: Eicosatetraenoic acid

FADS: Fatty acid desaturase

FXR: Farnesoid X receptor

HA: Hyaluronic acid

HDL: High density lipoprotein

IL: Interleukin

LA: Linoleic acid

LDL: Low-density lipoprotein

MRI: Magnetic resonance imaging

MRS: Magnetic resonance spectroscopy

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NEFA: Non-esterified fatty acid

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PIIINP: Procollagen-III N-terminal Propeptide

PNPLA3: Patatin-like phospholipase domain-containing protein-3

PPAR: Peroxisome proliferator-activated receptor

SNP: Single nucleotide polymorphism

SREBP-1c: Sterol regulatory element binding protein-1-c

TG: Triglycerides

TIMP-1: Tissue inhibitor of matrix metalloproteinase-1

TNF: Tumour necrosis factor

TM6SF2: Transmembrane 6 superfamily member- 2 protein

VLDL: Very-low-density lipoproteins

1. Introduction

1.1 Background and epidemiology of non-alcoholic fatty liver disease

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]. Across the world, the prevalence of NAFLD varies as it is influenced by demographics and diagnostic methodologies [9,12,13]. In research studies that have used liver enzymes as a diagnostic tool, the prevalence of NAFLD ranges 3 – 12% in the general population [14] and increases up to 33% when the diagnosis is made using a more precise method such as magnetic resonance spectroscopy. 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 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 [15]**.** Importantly, the progression of liver disease to NASH is often followed with the development of fibrosis (in 25%-33% of people with NAFLD); liver cirrhosis (5%–15% of people with NAFLD); liver failure (38% of people after 7-10 years of the diagnosis of NAFLD); and potentially hepatocellular carcinoma (2%-5%) [16]. (http://www.worldgastroenterology.org/UserFiles/file/guidelines/nafld-nash-english-012.pdf)

Historically, NAFLD was defined as "the liver manifestation of the metabolic syndrome"[17]. However, more recent data showed that NAFLD is a strong predictor for type 2 diabetes and metabolic syndrome [18]. Besides, there is growing evidence that describes NAFLD as a multisystem disease, affecting several extra-hepatic organs and regulatory pathways [5,18]. NAFLD is strongly related to metabolic disorders such as insulin resistance [19], obesity, dyslipidaemia, cardiovascular disease and microvasculature inflammation [20-24]. When NAFLD occurs in people with type 2 diabetes, there is an alteration of insulin sensitivity associated with a risk to develop insulin resistance [15]. Moreover, the risk of cardiovascular disease (CVD) increases with severity of NAFLD and is independent of traditional CVD risk factors [20,25,26], thus increasing risk for myocardial infarction by an additional 50% even after adjustment for all known cardiovascular risk factors. Diabetes is a significant risk factor for the development and progression of NAFLD, and up to 70% of type 2 diabetics have NAFLD. It is difficult to be certain of the true prevalence of steatohepatitis in people with type 2 diabetes due to lack of liver histology data, but studies have suggested it occurs in up to 80% of diabetics with NAFLD and abnormal levels of liver enzymes [15,18,27].

NAFLD has a multifactorial pathophysiology involving genetic polymorphisms [28] and metabolic factors[13], such as sedentary lifestyle, increased intake of energy-rich foods [29], malnutrition due to an imbalanced intake of nutrients (e.g. high fat, high carbohydrates and high protein diet [30], low fiber intake [31], high fructose intake[32]), altered gut microbiota composition (dysbiosis) [33-35], obesity [21,29].

Several research studies have attempted to test treatment in NAFLD: however, these studies have produced controversial results with limited success and serious safety concerns about long-term therapy [36-40]. For example, the results of trials testing treatment with metformin [41-44] thiazolidinediones [36-38,45] and anti-oxidants such as vitamin E [38], that have focussed primarily on modifying pathways affecting insulin resistance [46], oxidative stress [38] and lipid metabolism [47] have produced variable results. In a pilot study, Loomba et al. tested the effect of 1000mg of metformin twice daily for 48 weeks. The primary outcome was improvement in liver histology (NASH activity). At the end of the intervention, there was a three-point improvement on the NASH activity index; however, there was a positive significant correlation between decrease in NASH activity index score and decrease in body weight (Kg) ($r = 0.78$: $P < 0.0001$). During the study the average weight loos was 6Kg, and the major improvement in the NASH activity index was mainly in people with weight loss [43]. Sanyal et al. tested the effect of pioglitazone or vitamin E for the treatment of NAFLD and found that both agents were associated with reductions in hepatic steatosis and lobular inflammation compared with placebo. Vitamin E treatment resulted in an improvement in steatosis, inflammation and fibrosis in 43% of the patients treated (43% vs. 19% in the placebo group, $P=0.001$). For the pioglitazone group, there was an improvement in

steatosis and inflammation in ~60% of patients, whereas improvement in ballooning and fibrosis was only on ~44% of patients treated (34% patients with overall improvement in pioglitazone group and 19% patients with overall improvement in the placebo group; P=0.04) [38].
Currently, the EASL-EASD-EASO Clinical Practice Guidelines and the American Association for the Study of Liver Diseases recommend pioglitazone or high dose vitamin E for the treatment of NASH [1,48]. However, there are limitations in the use of pioglitazone and vitamin E. Pioglitazone is associated with weight gain, leg swelling, and a small risk of exacerbating heart failure and high dose of vitamin E might be associated with increased risk of prostate cancer[49] and haemorrhagic stroke [50].
Other studies tested the effect of statins and orlistat demonstrated no clinically important benefit on the progression of NAFLD when compared to placebo [51,52].

Currently, lifestyle changes may ameliorate steatosis [48 1,53]. Several research trials showed that weight loss is the most effective way to reduce liver fat; a 5% weight loss improves steatosis (25% reduction in MRI liver fat) [54,55] and there is evidence that ≥10% weight loss could be associated with a reduction of fibrosis [56-59]. Overall, any type of caloric restriction diet is considered effective, to reduce liver fat in patients with NAFLD [60]. A very low calories diet (43% carbohydrate, 34% protein, and 19.5% fat *plus* non-starchy vegetables for a total energy intake of 624–700 kcal/day) for 6 moths proved to be effective in reducing liver fat and in normalising hepatic insulin sensitivity [61,62]. However, Kirk et al. evaluated the longitudinal metabolic effects of short-term (48 h; 2% weight loss) and longer-term (11 wks; 7% weight loss) calorie restriction (1000 kcal/d energy deficit) with either a high- or low- carbohydrate diet. When compared a low-carbohydrates high-fat diet with a high-carbohydrates low-fat diet, the low-carbohydrate diet caused approximately a 3 times greater reduction in hepatic triglycerides content than the high carbohydrates in short and long period of calorie restriction [63]. In a small study, after obtaining equal weight loss with two dietary interventions that differed in macronutrient composition (low-carbohydrate and low-calorie diet), the low-carbohydrate diet resulted in significantly greater intrahepatic triglyceride reduction than the low-calorie diet [64]. Therefore, a hypocaloric low-carbohydrate diet is more effective in reducing liver fat than a low-calorie diet. Composition of fat in the diet also important to improve liver fat reduction. In a randomised controlled trial testing the effect of a isocaloric diet high in saturated fatty acids or polyunsaturated fatty acids, the reduction in liver fat was higher in the polyunsaturated fatty acids diet compared with the saturated fatty acids diet [65].

Typically, within a Westernized diet, omega-6 fatty acid consumption is markedly greater than omega-3 fatty acid consumption. 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 [66]. Several studies have shown that a diet with an inadequate intake of "omega-3 essential fatty acids" is associated with metabolic syndrome [67], cardiovascular disease [68], dyslipidaemia and fatty liver disease [69,70].

The EASL-EASD-EASO Clinical Practice Guidelines recommends the Mediterranean diet as the diet of choice for the treatment of NAFLD. Mediterranean diet is rich in vegetables, fruits, whole grains, seeds, nuts, legumes, and fish[48]. This diet is rich in monounsaturated fatty acids and polyunsaturated fatty acids as well as fibres and antioxidants and has been shown to reduce hepatic fat and improve hepatic insulin sensitivity even without weight loss [71].

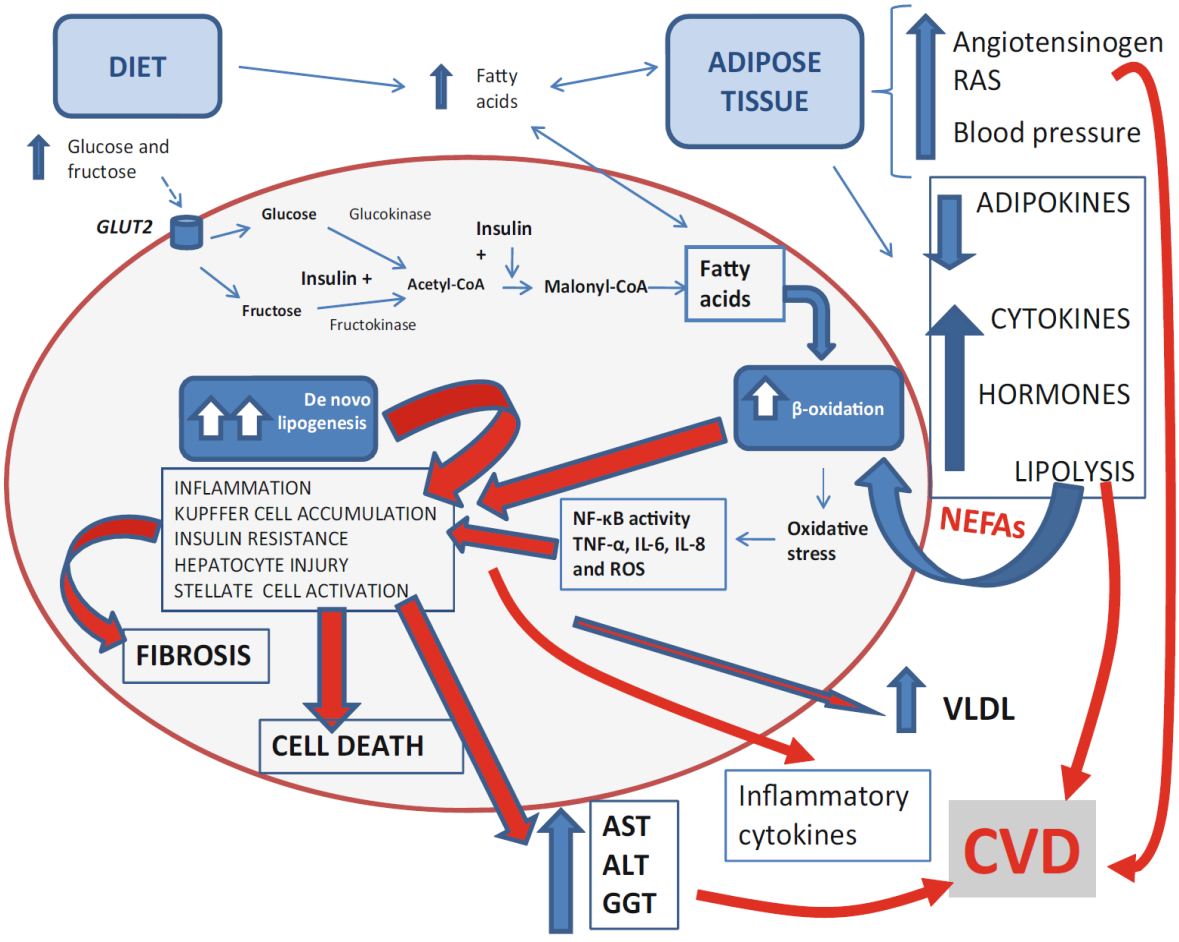
Physical activity is also beneficial for reducing liver fat [72,73] and may be promoted in patients who find difficult to modify their diet and follow dietary recommendation [74,75]. In a recent study, Houghton et al. showed that 12 weeks of exercise significantly produced a 16% reduction in liver fat compared with an 8% increase in the control group[76].

Therefore, the combination of calories restriction as well as dietary pattern associated with physical activity needs to be considered in the treatment of NAFLD.

1.2 Pathogenesis of non-alcoholic fatty liver disease

Hepatic fat accumulation is a consequence of an imbalance between the accumulation and catabolism of TG in the liver. **Figure 1** illustrates the metabolic and pathophysiological processes contributing to NAFLD and potential links between NAFLD and CVD [20]. There are three sources of metabolites for the hepatic synthesis of TG: a) dietary supply of fatty acids and glucose; b) *de novo* synthesis of fatty acids; and c) adipose tissue supply of fatty acids from lipolysis [21].

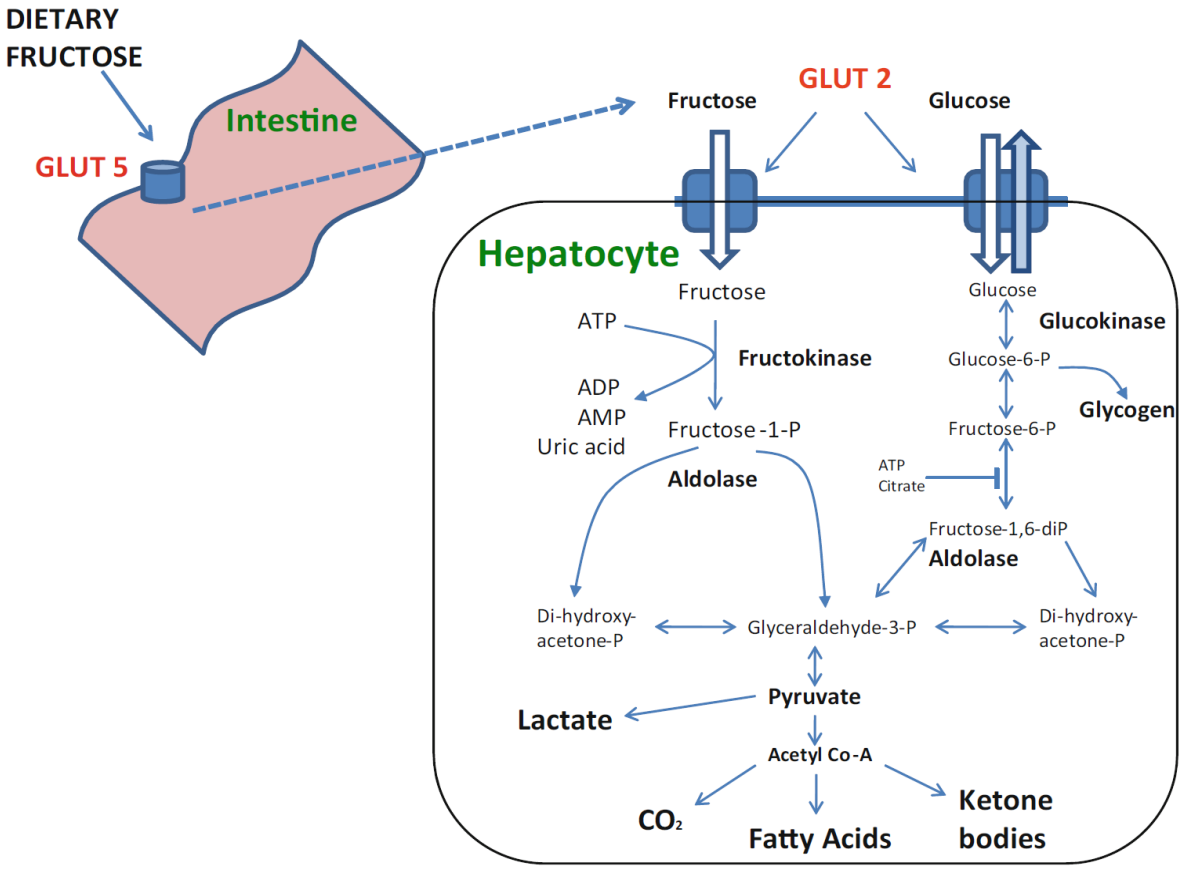
Figure 1. Mechanisms linking NAFLD to cardiovascular disease (CVD). Schematic figure showing potential pathogenetic mechanisms linking dietary fat and carbohydrate intake with NAFLD, increased adiposity, blood pressure and cardiovascular disease [21].



1.2.1 Role of carbohydrates and fructose in the pathogenesis of NAFLD

High-carbohydrate diets increase hepatic de novo lipogenesis [77]. This is a multi-stage process in which i) dietary glucose is transported into the liver by Glucose Transporter-2 (GLUT-2) [78]; ii) dietary glucose is phosphorylated by the enzyme Glucokinase and converted by liver-specific pyruvate kinase into acetyl-CoA; iii) acetyl-CoA is converted into malonyl-CoA in the presence of insulin (which activates acetyl CoA carboxylase); and iv) malonyl-CoA is the committed substrate on the fatty acid synthesis pathway. The activity of the glycolytic and lipogenic enzymes is controlled at the transcriptional level by SREBP-1c (sterol regulatory element binding protein-1-c) and ChREBP (carbohydrate response element binding protein). SREBP-1c and ChREBP are up-regulated in conditions of hyperinsulinaemia and hyperglycaemia respectively [78-80]. Interestingly, fructose metabolism differs markedly from that of glucose as it is insulin independent and is not regulated by the levels of ATP and citrate. In the liver, fructose is taken up by hepatocytes though the glucose transporter (GLUT-2) and subsequently phosphorylated to fructose-1-phosphate by the specific enzyme Fructokinase. Fructose-1-phosphate is metabolized to glyceraldehyde-3-phosphate by aldolase B; thereafter, glyceraldehyde-3-phosphate is converted into pyruvate and subsequently into acetyl-CoA. Fructose also promotes the expression of the transcriptional factors SREBP-1 and ChREBP, involved in the regulation of *de novo* lipogenesis [81,82] (**Figure 2**). The phosphorylation of fructose also stimulates adenosine monophosphate (AMP) deaminase to convert adenosine monophosphate in inosine monophosphate (IMP) and then inosine monophosphate is converted to uric acid [83,84]. Abdelmalek et al. showed that a daily fructose consumption was associated with fibrosis; cumulative odds ratios [95% CI] of ‘‘daily consumption’’ versus ‘‘no fructose consumption’’ were 2.6 [1.4, 5.0] (P =0.004). This was confirmed by a recent study conducted by Nobili et al. in children and adolescents with NAFLD. They showed that serum uric concentration and dietary fructose consumption were independently and positively associated with histologically diagnosed NASH (OR for uric acid = 2.488, 95% CI: 1.87–2.83, p = 0.004; OR for fructose = 1.612, 95% CI: 1.25–1.86, p = 0.001) [85]. In this study fructose consumption was significantly higher in the NASH group compared with the non-NASH group (70.4 g/day vs. 52.6 g/day; p = 0.002)[86]. However, clinical studies to investigate the potential benefit of lowering uric acid should also be performed.

Figure 2. Mechanism linking dietary fructose intake and hepatic fatty acid metabolism. Fructose is transported into enterocyte by the specific fructose transporter Glucose Transporter-5. In the liver, fructose metabolism is independent of insulin action and is also not regulated by the levels of ATP and citrate. Dietary fructose intake increases intrahepatic fructose-1-P. Fructose-1-P can be converted into glyceraldehyde-3-phosphate and thereby into acetyl Co-A (the precursor for fatty acid synthesis). Thus, dietary fructose intake may stimulate hepatic *de novo* lipogenesis and thus potentially be involved in the pathogenesis of NAFLD.



Moreover, recent studies have also investigated the effect of fructose on intestinal bacteria. In the intestine, fructose is absorbed by a specific transporter located at the apical side of the enterocytes: Glucose Transporte-5. Chronic high intake of fructose may cause overgrowth of intestinal bacteria and increase intestinal permeability, leading to a translocation of bacterial endotoxin into the bloodstream, which in turn activates Kupffer cells, ultimately causing inflammation and accumulation of fat in the liver [21,87,88]. Currently there are animal studies

showing the effect of fructose on intestinal barrier leading to NAFLD. After 16 weeks of feeding mice with 30% fructose solution, liver fat was significantly higher not only compared with liver of mice fed chow control diet but also compared with mice fed high-fat diet. Moreover, after chronic fructose consumption there was high bacterial endotoxin levels in the portal plasma and low level of tight junction protein occluding and zonula occludens 1 in the duodenum[89].

1.2.2 Lipids metabolism and the pathogenesis of NAFLD

A prolonged period of imbalanced intake of nutrients leads to a state of allosteric overload that may cause obesity, insulin resistance, metabolic syndrome features, alteration of gut microbiota and diabetes [29,88]. With a twenty-first century lifestyle that includes physical inactivity and excess calorie intake, there is a surplus of fat accumulated in ectopic visceral sites in insulin resistant individuals. For example, with insulin resistance, fat accumulates in visceral organs and tissues, such as skeletal muscle myocytes, hepatocytes and β-cells, and accumulation of fat in insulin-sensitive tissues (such as liver and muscle) tends to impair insulin signalling in these tissues. Dietary fat is absorbed in the intestine, assembled into chylomicrons and released into the systemic circulation. About 80% of chylomicrons are hydrolyzed by lipoprotein lipase liberating the constituent fatty acids, and the residual remnant is delivered to the liver [90]. *De novo* lipogenesis contributes to very-low-density lipoproteins (VLDL) assembly and may contribute between 2 and 5% of VLDL-TG production in healthy people and 20 to 30% in pathophysiological states [91-93]. Adipose tissue is the major source of non-esterified fatty acids (NEFAs) which accrue in the liver and are responsible for approximately 60% of TG accumulation [93]. The high flow of NEFAs to the liver causes a decrease of mitochondrial oxidation and a reduction of TG secretion into the systemic circulation as VLDL. This mechanism contributes to hepatic TG storage resulting in liver steatosis and liver inflammation [5].

In the presence of high energy intake and low energy expenditure, adipose tissue is overloaded by an excess of energy stored as lipid (triacylglycerol). The ability of expansion of the peripheral adipose tissue provides a temporary buffering capacity that protects the liver from an excessive flux of free fatty acid which would otherwise promote hepatic lipid accumulation. If this protective mechanism fails, excessive free fatty acid causes hepatic/peripheral insulin resistance, hepatic inflammation and in turn increases the risk of progressive liver disease with fibrosis, cirrhosis and hepatocellular carcinoma [94]. Insulin-sensitive individuals tend to easily expand

adipose tissue in subcutaneous adipose tissue depots in the presence of a twenty-first century lifestyle, thereby protecting their visceral organs from ectopic fat accumulation. In the presence of insulin resistance, adipose tissue hormone-sensitive lipase fails to be regulated, causing uncontrolled lipolysis and thus an increased flow of non-esterified fatty acids to the liver. In addition, in this scenario, there is increased hepatic gluconeogenesis in insulin-resistant states with diversion of glucose to the hepatic lipogenesis pathway which further increases the accumulation of fat in the liver. Excessive storage of fatty acids in hepatocytes and insulin resistance may increase mitochondrial β-oxidation free-radical production and the production of reactive oxygen species may induce NF-κB activity, leading to a production of proinflammatory cytokines, such as TNF-α, IL-6 and IL-8. The increase in oxidative stress may cause mitochondrial damage and dysfunction, leading to a decrease of oxidative capacity, which in turn may produce an imbalance between fat oxidation and lipogenesis pathways, ultimately resulting in liver-fat accumulation and NAFLD [6,19,94]. Lifestyle modifications, such as increased physical activity, positively affect nutritional behaviour, leading to a gradual weight loss, which in turn improves metabolic syndrome, reduces CV [95] risk and decreases NAFLD[96-98]. However, the effects of lifestyle change are not instantaneous, and weight loss should be a gradual process. Moreover, weight loss and increases in physical activity are often difficult to achieve and consequently there is a need for novel pharmacological treatments that are capable of controlling risk factors that contribute to NAFLD and CVD.

1.2.3 Hepatic and Adipose Tissue Function, Inflammation, and NAFLD

The liver has a central anatomic location in the gastrointestinal tract and is the major metabolic organ for anabolic and catabolic processes. The liver is linked to the intestine (a) through a unique vasculature that converges in the portal vein and (b) through the enterohepatic biliary circulation. The liver has a wide range of functions, inter alia detoxification, hormone production, and plasma protein synthesis. A major function of the liver is to affect lipid metabolism (e.g., cholesterol synthesis, de novo lipogenesis, and synthesis of apolipoprotein B100). Among other functions, the liver stores glycogen, lipid soluble vitamins, iron, and copper. The liver is not designed to store lipid, and lipid accumulation in hepatocytes is toxic. The quantity and the composition of ingested food are relevant factors with regard to fat accumulation in the hepatocytes. Over the past three decades, the food habits of the general population have changed toward a diet characterized by an increased consumption of fat and carbohydrate. NAFLD is a spectrum of fat-related liver conditions, and hepatic lipid accumulation plays a pivotal role in the pathogenesis and

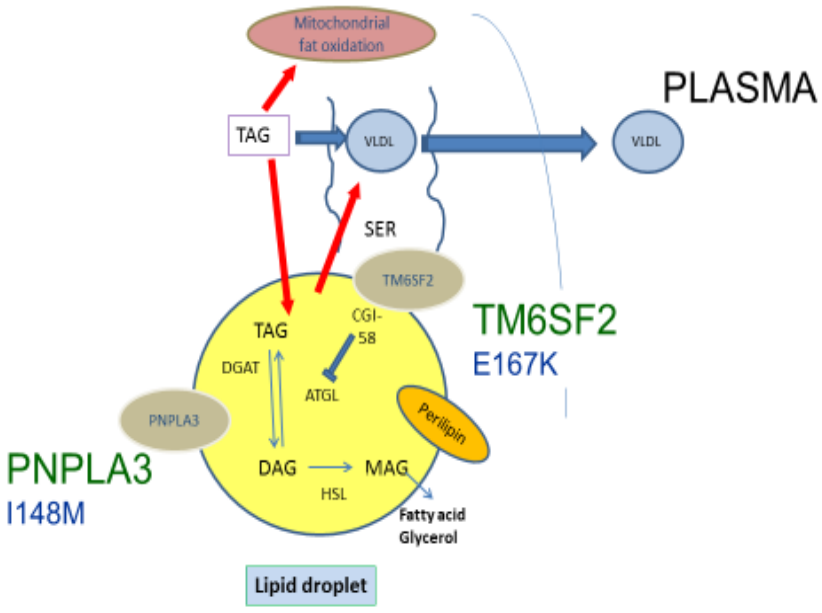
progression of NAFLD. In the jejunum, dietary TG are hydrolysed by pancreatic lipase, resulting in the release of fatty acids and monoacylglycerol that in turn are absorbed in the small intestine and utilized to synthesize TG. Subsequently, TG can be synthesized and stored in the adipose tissue or metabolized in the liver [99]. Dietary TG are packaged into chylomicrons, and hepatic TG are packaged into VLDL in order to transport TG to peripheral tissues. Chylomicrons are assembled in the enterocytes during the absorptive phase (exogenous pathway), whereas VLDL particles are secreted from the liver, particularly during the fasting state (endogenous pathway). In the exogenous pathway, chylomicrons are formed from the coalescence of dietary TG together with apolipoproteins apoB48, apoAIV, and apoAV. Subsequently, chylomicrons are released into the lymphatic system, and later they acquire apoAI, apoAII, apoC (CI, CII, CIII), and apoE. ApoCIII is an inhibitor of lipoprotein lipase, and hypertriglyceridemia can be further exacerbated by low activity of lipoprotein lipase or by high level of apoCIII. In the peripheral tissues, TGs from the chylomicrons are hydrolysed by lipoprotein lipase into free fatty acids, and Petersen et al.[100] have shown that a genetic variation of apoCIII is associated with NAFLD, which suggests that variation in lipoprotein lipase activity may be important in the pathogenesis of NAFLD. In the endogenous pathway, TGs derived from de novo lipogenesis and circulating NEFA contribute to form nascent VLDL particles that are secreted from the liver. Nascent VLDL contains only apoB100; subsequently, it acquires apoA (AI, AII, AIV), apoC (CI, CII and CIII), and apoE. TGs from VLDL particles are hydrolysed by lipoprotein lipase to form VLDL remnants, and then they are internalized in the liver, binding to the apoE receptor. In the liver, VLDL remnants are hydrolyzed by hepatic triglyceride lipase to form low-density lipoprotein (LDL) particles. This overflow from the transport pathway is clinically crucial, as an excessive transport of VLDL remnants to the liver increases LDL production, perhaps contributing to NAFLD. Ceramide and sphingolipids can be transported by VLDL, and in the liver, intestine, and heart, de novo ceramide could be secreted as part of apolipoprotein B–containing lipoproteins [101]; this alteration of lipoprotein structure may affect hepatic degradation of lipoproteins, with consequent intracellular accumulation of lipid [102,103], thereby potentially predisposing to NAFLD.

1.2.4 Influence of genotype polymorphism on liver lipid metabolism

There is evidence showing that genetic variation in both patatin-like phospholipase domain-containing protein-3 (PNPLA3) (I148M) and the transmembrane 6 superfamily member 2 protein (TM6SF2) (E167K) influences severity of liver disease, and serum TG concentrations in NAFLD **(Figure 3)**. Exome wide association study identified the rs738409 C>G single nucleotide

polymorphism (SNP) in the PNPLA3 gene, encoding for the isoleucine to methionine substitution at position 148 (I148M). In humans PNPLA3, also called adiponutrin, encodes a 481 amino acid membrane protein localised in the endoplasmic reticulum and at the surface of lipid droplets [104]. In human, this protein has the highest expression in hepatic stellate cells, retina and hepatocytes. PNPLA3 has a TG and retinyl-palmitate esterase activity. The functional PNPLA3 148I variant allows optimal TG hydrolysis whereas the isoleucine to methionine substitution leads to a loss of lipolytic activity leading an impairment of lipid catabolism, lipid droplets remodelling, and impairment of VLDL secretions. Interestingly, PNPLA3 I148M gene variant is linked to increased risk of developing cirrhosis and hepatocellular carcinoma independently from NALFD [105]. The variant protein was found to have reduced enzymatic activity for the hydrolysis of emulsified TG in hepatocytes. As a result, the secretion of VLDL is impaired. Free fatty acids and TG are stored in the hepatocytes and hepatic insulin resistance increases. PNPLA3 retinyl-palmitate activity in hepatic stellate cells may influence hepatic regeneration and differentiation by altering availability of retinol, a potent regulator of these phenomena. Recently, it has been identified the rs58542926 C>T genetic variant of the transmembrane 6 superfamily member 2 gene (TM6SF2), which encodes the loss-of-function lysine (E) to glutamic acid (K) at position 167 substitution (E167K), as a determinant of hepatic TG content, serum aminotransferases and lower serum lipoproteins.

Figure 3. Adiponutrin/patatin-like phospholipase domain-containing 3 gene (PNPLA3) and Transmembrane 6 superfamily 2 human gene (TM6SF2) modulation of the liver lipid droplet.



1.2.5 Enterohepatic Circulation and NAFLD

Imbalances in gut microbiota can increase fat absorption and energy harvest, causing liver fat accumulation [106]. Additionally, recent studies have shown the potential role of the gut microbiota in the pathogenesis and progression of NAFLD. The liver, biliary tract, intestine, portal venous circulation, colon, systemic circulation, and kidney are all involved in the enterohepatic circulation of bile acids [107]. Bile acid, water, electrolytes, phosphatidylcholine, cholesterol, and bilirubin are all components of bile, an iso-osmotic micellar solution produced by the liver. Bile acid synthesis is important for lipid digestion and absorption, cholesterol catabolism, fat-soluble vitamin absorption, and glucose and energy homeostasis. Bile acids are produced by cholesterol via two pathways: (*a*) a "classic" or natural pathway in which cholesterol is converted to 7-α hydroxycholesterol by a rate-limiting enzyme, cholesterol 7α-hydroxylase (CYP7A1); and (*b*) an "alternate" acidic pathway, in which cholesterol is converted to 27-hydroxy-cholesterol by 27-hydroxylase [108]. These two pathways form the primary bile acids, i.e., chenodeoxycholic acid and cholic acid. In the intestine, gut microbiota deconjugate and dehydroxylate primary bile acids to form secondary bile acids, i.e., urodeoxycholic acids, deoxycholic acid, and lithocholic acid. Bile acids are natural ligands for farnesoid X receptor (FXR), a nuclear receptor expressed in the liver, intestine, kidney, and adipose tissue. Chenodeoxycholic acid is the most effective endogenous ligand for FXR [109,110]. CYP7A1 is a rate-limiting enzyme that has a pivotal role in the regulation of bile acid synthesis. CYP7A1 transcription is inhibited by bile acids, steroid hormones, inflammatory cytokines, insulin, and growth factors. In physiological conditions, insulin stimulates CYP7A1 expression. In contrast, in the presence of insulin resistance that is characteristic of patients with NAFLD, high concentrations of insulin activate SREBP-1c, which inhibits CYP7A1 expression. CYP7A1 expression is also inhibited by increased bile acid synthesis and by an increased bile acid pool size, returning cholesterol to the liver via the enterohepatic circulation [110]. Inhibition of CYP7A1 decreases chenodeoxycholic acid production and FXR activation, causing hypercholesterolemia. Moreover, reduced activity of FXR decreases biliary cholesterol content and decreases the expression of hepatic but not intestinal expression of cholesterol transporters (ABCG5/G8) [111]. Therefore, inhibition of CYP7A1 leads to accumulation of cholesterol and alteration of enterohepatic circulation, with consequent hepatic lipotoxicity that may have a deleterious impact on the liver in NAFLD.

Primary bile acids such as chenodeoxycholic acid and cholic acid are influenced in three ways by gut microbiota to produce secondary bile acids such as urodeoxycholic acid, deoxycholic acid and lithocholic acid : i) the key process is deconjugation of primary bile acids to form unconjugated bile acids, that are passively or actively absorbed and returned directly to the liver for

reconjugation; ii) some chenodeoxycholic acid is modified through epimerization to produce urodeoxycholic acid; and iii) bacterial 7α-dehydroxylase converts cholic acid to deoxycholic acid and chenodeoxycholic acid to lithocholic acid in the colon.

Secondary bile acids are highly hydrophobic and toxic, and increased concentrations in the liver have been linked to inflammation, cholestasis and carcinogenesis [112]. Secondary bile acids have the following toxic effects that have the potential to influence liver disease development and progression in NAFLD: i) Increased intestinal permeability with decreased expression of tight junctions. This allows transfer of endotoxin products to the liver. ii) The hydrophobicity of secondary bile acids allows their interaction with the phospholipids in cell membranes of hepatocytes inducing perturbations of mitochondrial membranes.

Recent evidence provided by the Farnesoid X Receptor (FXR) Ligand Obeticholic Acid in NASH Treatment (FLINT) Trial with a FXR agonist (obeticholic acid) created by adding an ethyl group to chenodeoxycholic acid [113]. Treatment with obeticholic acid produced an improvement in liver disease (in ~45% of patients with NASH). However, obeticholic acid is not well tolerated and produces side effects such as cholestasis with itching, and a substantial increase in low density lipoprotein cholesterol.

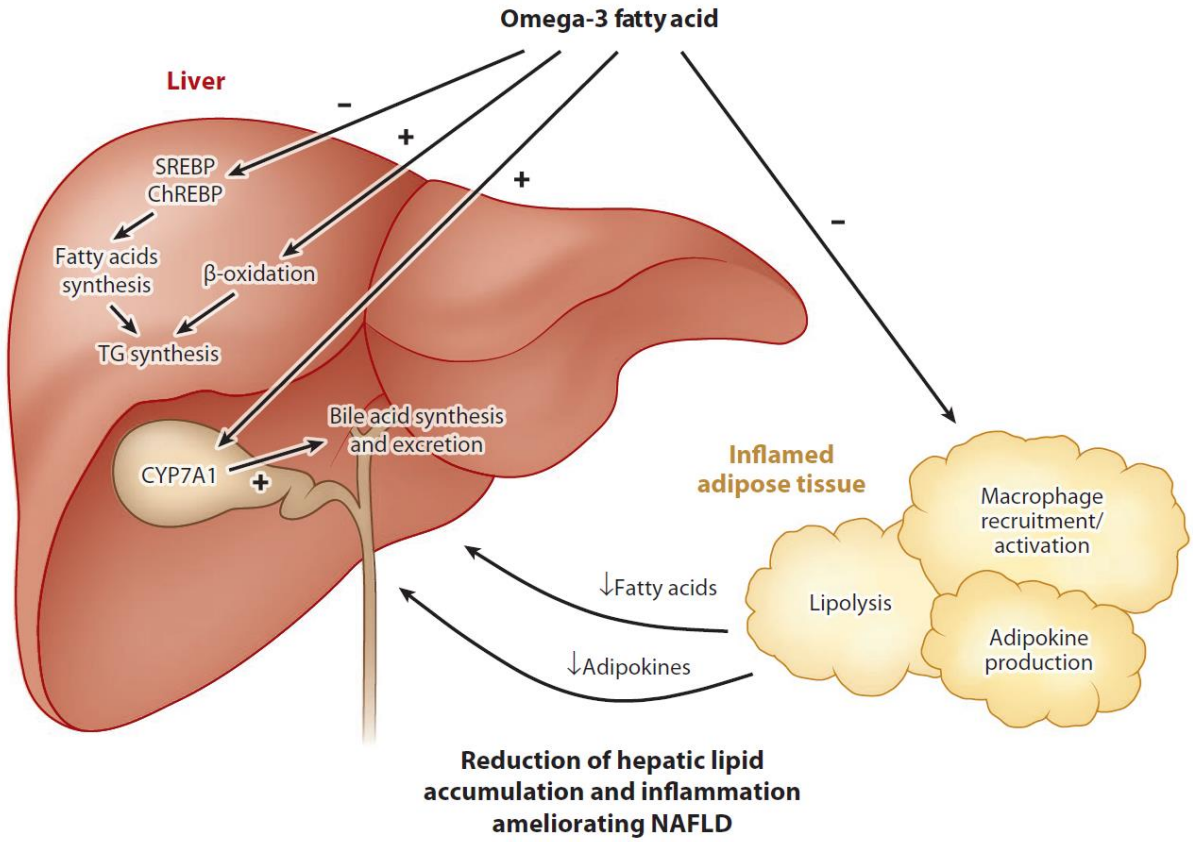
There is evidence that omega-3 fatty acids can affect lipid metabolism not only in the liver but also in the intestine. Omega-3 fatty acids increase mRNA expression of the intestinal cholesterol gene transporter (Abcg5/8) and bile acid transporters that promote cholesterol excretion in faeces [114]. Liver X receptor (LXR) and FXR are two nuclear receptors involved in cholesterol and bile acid metabolism. The activation of liver X receptor by oxysterols (oxygenated derivatives of cholesterol) promotes the conversion of cholesterol into bile acids that in turn increases hepatic TG synthesis and storage by promoting the expression of SREBP-1c. FXR is activated by bile acids to prevent bile acid accumulation. Moreover, FXR activation decreases hepatic and plasma TG through suppression of the expression of SREBP-1c. Bile acid–activated FXR also induces the expression of apoCII that stimulates lipoprotein lipase activity, and it suppresses the expression of apoCIII (an LPL inhibitor). FXR, via induction of small heterodimer partner, represses the expression of SREBP-1c and ChREBP, which are responsible for the hepatic *de novo* lipogenesis[115]. Consequently, several potential pathways and mechanisms can be altered to cause a disturbance in liver lipid homeostasis and may be important in the pathogenesis of NAFLD. There is evidence that omega-3 fatty acids act as FXR ligands and regulate FXR to affect lipid metabolism [116]. In transgenic mice, capable of converting omega-6 fatty acids into omega-3 fatty acids, Kim et al. [117] have demonstrated that endogenously synthesized omega-3 fatty acids have beneficial effects on high-fat-diet-induced NAFLD. In this study, endogenously synthesized omega-3 fatty acids are

shown to upregulate genes involved in cholesterol uptake (Ldl-r), bile acid synthesis (Cyp7a1), and excretion (Abcg5 and Abcg8) [117]. Thus, these collective data suggest that omega-3 fatty acids increase primary bile acid synthesis and bile acid excretion from the liver and that increased bile acid synthesis activates FXR, induces the expression of apoCII, and suppresses the expression of apoCIII, thereby suggesting a mechanism of potential benefit by which omega-3 fatty acids may ameliorate NAFLD.

1.3 Omega-3 polyunsaturated fatty acids

Recently, several studies have shown that a diet with an inadequate intake of "omega-3 essential fatty acids" is associated with metabolic syndrome, cardiovascular disease, dyslipidaemia and fatty liver disease [66]. Long-chain omega-3 fatty acids belong to a family of polyunsaturated fatty acids that are known to have important beneficial effects on metabolism and inflammation. Omega-3 and omega-6 fatty acids were found to be of primary importance for normal growth thus they were termed "essential fatty acids". Several studies have analysed the relevance of essential fatty acids deficiency in the development of metabolic syndrome, cardiovascular disease, dyslipidemia, fatty liver accumulation [e.g., NAFLD], and hepatic steatosis [e.g., NASH] [66]. Typically, with a Westernized diet, long-chain omega-6 fatty acid consumption is markedly greater than omega-3 fatty acid consumption. The potential consequences of an alteration in the ratio of omega-6 to omega-3 fatty acid consumption are: increased production of proinflammatory arachidonic acid–derived eicosanoids and impaired regulation of hepatic and adipose function, predisposing to NAFLD [66]. If the adipose tissue is inflamed with widespread macrophage infiltration, the production of adipokines may act to exacerbate liver inflammation and NASH. Omega-3 fatty acid treatment may have beneficial effects in regulating hepatic lipid metabolism, adipose tissue function, and inflammation. Recent studies testing the effects of omega-3 fatty acids in NAFLD are showing promise and suggesting that these fatty acids may be useful in the treatment of NAFLD[66]. To date, further research is needed in NAFLD to (a) establish the dose of long-chain omega-3 fatty acids as a treatment, (b) determine the duration of therapy, and (c) test whether there is benefit on the different component features of NAFLD (hepatic fat, inflammation, and fibrosis) (**Figure 4**).

Figure 4. Potential beneficial effects of omega-3 fatty acids in liver and adipose tissue to ameliorate NAFLD. In liver, long-chain omega-3 fatty acids regulate hepatic lipid metabolism by increasing hepatic fatty acid oxidation and inhibition of SREBP-1c and ChREBP activity (nuclear transcription factors that stimulate hepatic de novo lipogenesis). In adipose tissue, omega-3 fatty acids have a potential anti-inflammatory effect by inhibiting macrophage recruitment and activation; decreasing fatty acid release; decreasing adipokine and cytokine secretion, and favourably affecting the enterohepatic circulation. Furthermore, omega-3 fatty acids upregulate CYP7A1 expression, increasing bile acid synthesis and excretion. The potential beneficial consequence of these effects is to ameliorate NAFLD. Abbreviations: ChREBP, carbohydrate regulatory element–binding protein; CYP7A1, cholesterol 7α-hydroxylase; NAFLD, non-alcoholic fatty liver disease; SREBP, sterol regulatory element–binding protein; TG, triglyceride [66].



1.3.1 Chemical structure and nomenclature

Omega-3 fatty acids together with omega-6 fatty acids belong to the family of polyunsaturated fatty acids; these are long-chain fatty acids characterized by the presence of more than two double bonds in the molecule [66]. Polyunsaturated fatty acids contain a carboxyl group at one end and a methyl group at the other end of the carbon chain; the first double bond is counted from the methyl end (or omega or n end) of the carbon chain. These fatty acids are classified on the basis of distinct systems of nomenclature. Common names (e.g., α-linolenic acid, the simplest omega-3) are vernacular names that often do not follow any classification. Systematic names (e.g., all-cis-9,12,15-octadecatrienoic acid) describe the molecule counting the double bond beginning from the carboxylic group; the double bond is labelled as cis or trans depending on the orientation of the double bond and the shape of the molecule. Shorthand nomenclature is based on the number of carbon atoms in the molecule and the number and position of double bonds. There are three different shorthand nomenclatures: (a) delta-x (Δ^x), in which the double bond is counted from the carboxylic acid and the prefix cis or trans indicates the stereochemical conformation of the molecule (e.g., cis-Δ^9, cis-Δ^{12} octadecadienoic acid); (b) n minus x or omega-x (ω-x), in which "x" is the number of the position of the double bond counting from the terminal "n or ω" methyl group (CH3) (e.g., α-linolenic acid is an n-3 or ω-3 or omega-3 fatty acid); and (c) lipid number, whereby the molecule is described according to the number of carbon atoms forming the fatty acid chain and the number of double bonds present in the fatty acid chain (e.g., α-linolenic acid is described as 18:3).

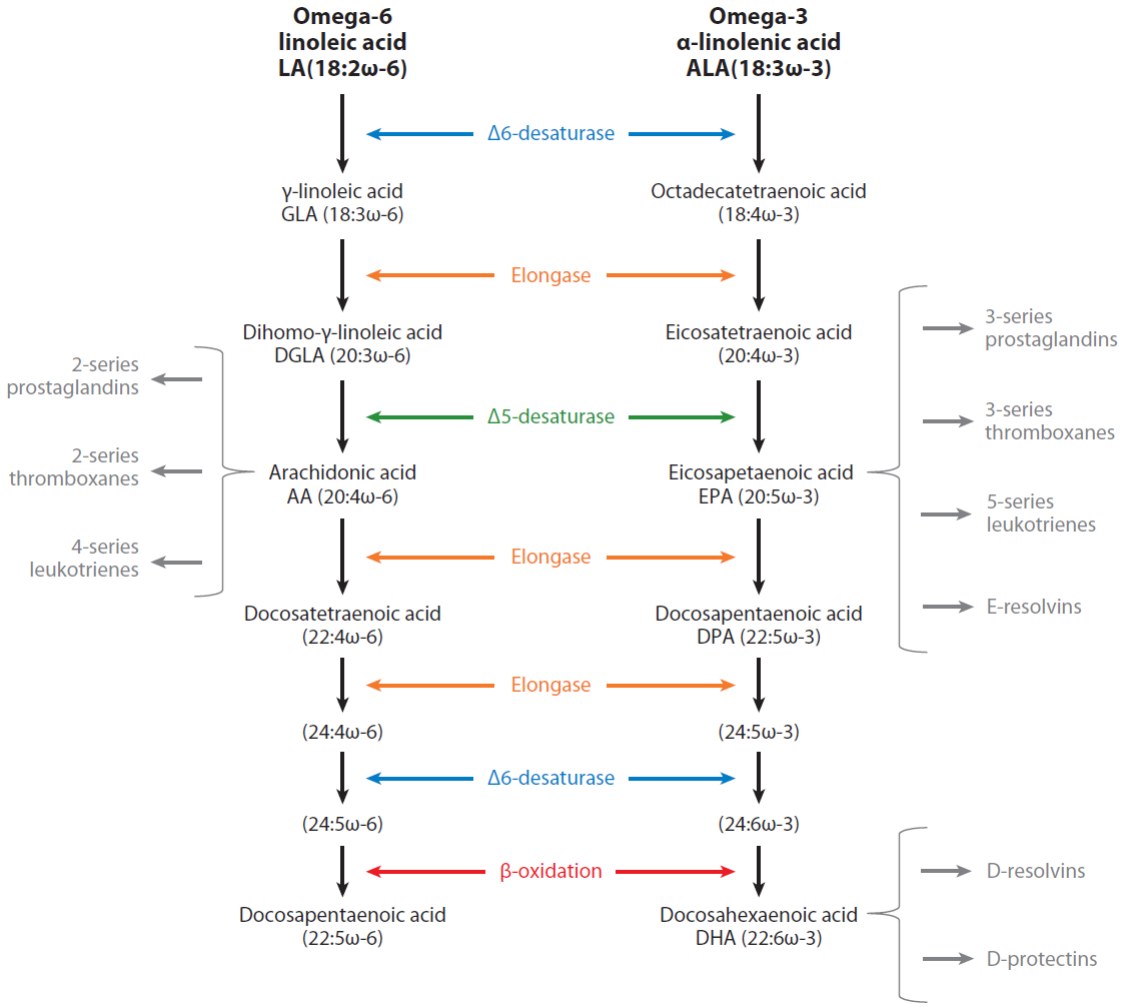
1.3.2 Synthesis and metabolism

Omega-3 fatty acid tissue levels are regulated by both dietary intake and endogenous synthesis via consecutive elongation and desaturation of omega-3 fatty acid precursors **(Figure 5)**. Δ-5 and Δ-6 desaturase are key enzymes in the omega-3 fatty acids metabolism as they are responsible for the double bond formation between two carbons leading to more unsaturated fatty acids [118,119]. In the liver, dietary α-linolenic acid is first metabolized to stearidonic acid [SDA; 18:4(ω-3)] by Δ6-desaturase. This first reaction is a rate-limiting step and competes with the conversion of linoleic acid to arachidonic acid [AA; 20:4(ω-6)] in omega-6 fatty acid metabolism. SDA can be elongated to form eicosatetraenoic acid that can be desaturated by Δ5-desaturase to form eicosapentaenoic acid [EPA; 20:5(ω-3)]. At this stage EPA can be further elongated to form docosapentaenoic acid [DPA; 22:5(ω-3)], and DPA can be converted to docosahexaenoic acid [DHA; 22:6(ω-3)] by Δ6-desaturase with the involvement of limited peroxisomal β-oxidation. In omega-3 fatty acid metabolism, Δ6-desaturase participates twice, once in the first step in which α-linolenic acid is

desaturated to SDA and subsequently in the conversion of DPA to DHA. In contrast, Δ5-desaturase activity is utilized for omega-6 fatty acid metabolism only once [120,121]. Several factors can influence the equilibrium of Δ5-desaturase and Δ6-desaturases, including diet, oxidative stress, SNPs, and liver disease [122]. The optimal ratio between omega-6 and omega-3 fatty acids should be 1–4:1. In a Western diet, however, omega-6 fatty acid consumption is significantly higher than omega-3 fatty acid consumption; as a result, the aforementioned ratio can increase to 10:1 or even 20:1 [123]. In the presence of an optimal ratio of omega-6 and omega-3 fatty acids, Δ5-desaturase and Δ6-desaturase compete to metabolize these two polyunsaturated fatty acids, although both enzymes have a greater affinity for omega-3 fatty acids. A diet rich in omega-6 fatty acids causes an accumulation of AA in the cell membrane, influencing cell transport and favouring the production of AA–derived eicosanoids. In physiological and pathological conditions, cyclooxygenases and lipoxygenases can convert AA and EPA into eicosanoids. Eicosanoids are lipid molecules with signalling functions that have an important role in regulating inflammation. EPA-derived eicosanoids are 3-series prostaglandins and thromboxanes (with three double bonds in the carbon chain) and 5-series leukotrienes (with five double bonds in the carbon chain). These EPA-derived eicosanoids have an anti-inflammatory effect compared with arachidonic acid–derived eicosanoids [66,124]. These types of eicosanoids are associated with an increased release of proinflammatory cytokines, neutrophil activation, increased production of reactive oxygen species, and increased vascular permeability.

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 [125-129]. 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. 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 [130,131]. Omaga-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 [129].

Figure 5. Synthesis and metabolites of omega-6 and omega-3 fatty acids. In the liver, dietary α-linolenic acid is metabolized to stearidonic acid by Δ6-desaturase; this first reaction is a rate-limiting step and competes with the conversion of linoleic acid to arachidonic acid in omega-6 fatty acid metabolism. In omega-3 fatty acid metabolism, Δ6-desaturase participates twice, once in the first step in which α-linolenic acid is desaturated to stearidonic acid and subsequently in the conversion of docosapentaenoic acid to docosahexaenoic acid; in contrast, Δ6-desaturase activity is utilized for omega-6 fatty acid metabolism only once. Δ5-Desaturase and Δ6-desaturase compete to metabolize these two polyunsaturated fatty acids. Omega-6 fatty acids produce 2- and 4-series prostaglandins, thromboxanes, and leukotrienes, whereas omega-3 fatty acids produce 3- and 5-series prostaglandins and leukotrienes as well as resolvins and protectins. Moreover, in physiological and pathological conditions, cyclooxygenases and lipoxygenases can convert arachidonic acid and EPA into eicosanoids. Eicosanoids are lipid molecules with signalling functions that have an important role in regulating inflammation [66]. Figure modified from Reference [132].



1.3.3 Effect of omega-3 fatty acids in NAFLD

The effect of omega-3 fatty acids on TGs primarily involves the suppression of hepatic VLDL apoB production and apoB pool size. Several tracer studies have demonstrated the effects of omega-3 fatty acids on VLDL metabolism. Chan et al. [133] showed a reduction of TG plasma concentration in obese people after six weeks of treatment with high doses (4 g) of fish oil capsules comprising 45% EPA and 39% DHA. This reduction was mainly due to the effects of omega-3 fatty acids on VLDL apoB pool size; the effect of omega-3 fatty acids on VLDL particles was to favour the conversion of VLDL to LDL [133]. This effect involves a decrease in TG synthesis by 35% and an increase in fatty acid mitochondrial oxidation. In particular, omega-3 fatty acids induce the aggregation of apoB after its secretion from the endoplasmic reticulum. In the Golgi, this aggregate material is oxidized and remains in the cell, where it is susceptible to the autophagic process [134]. In a study using cultured hepatocytes, the effects of incubation with palmitic acid, oleic acid, and DHA on endoplasmic reticulum stress and apoB100 secretion were compared [135]. The investigators found that a long period of incubation with oleic acid provoked endoplasmic reticulum stress. In contrast, a short incubation period with palmitic acid was sufficient to cause the same effect on endoplasmic reticulum stress. In addition, palmitic acid favoured ceramide production. DHA did not induce endoplasmic reticulum stress at any time. All three fatty acids inhibited the secretion of apoB100, but only DHA induced autophagic degradation. Furthermore, it has been shown that DHA may interfere with apoCIII gene transcription, thereby potentially decreasing the negative effect of apoCIII on Lipoprotein lipase activity [136]. Fatty acids may have different chain lengths (short, medium, long, and very long) and may be saturated or unsaturated. Fatty acids have different metabolic fates, depending on their chain length and degree of saturation. Short-chain (2–5 carbon atoms) and medium-chain (6–12 carbon atoms) fatty acids are directly absorbed from the intestine into the blood through intestinal branching capillaries. Medium chain fatty acids can form medium-chain TG that are not incorporated into chylomicrons. Medium-chain fatty acids can be rapidly oxidized to form acetyl-CoA in the liver and thus are not stored in adipose tissue [137]. Long-chain (13–21 carbons) and very-long chain (22 or more carbons) fatty acids are constituents of glycerolipids (mono-, di-, and triglycerides) and sphingolipids with a backbone comprising ceramide. Increased intake of these fatty acids promotes the accrual of long chain fatty acyl CoAs, diacylglycerol, sphingolipids, and ceramide in tissue lipid deposits. Increased plasma levels of sphingolipids have been implicated in the pathogenesis of obesity, insulin resistance, and NAFLD. Ceramide can be produced by three pathways: (a) *de novo* ceramide synthesis from palmitate and serine, (b) sphingomyelin hydrolysis by a neutral sphingomyelinase and acidic sphingomyelinase, and (c) ceramide salvage by the catabolism of other complex

sphingolipids. A diet rich in glycerolipids and omega-6 polyunsaturated fatty acids supplies substrate for *de novo* ceramide synthesis [138]. Sphingomyelinase activity has been found to be increased in the adipose tissue of obese mice fed a high-fat diet, and these mice had increased plasma ceramide levels [139]. Ceramide may be implicated in the pathogenesis of NASH [140]. Recently, Moles et al. [141] demonstrated that the liver expression of acidic sphingomyelinase is increased in subjects with NASH. These authors found that acidic sphingomyelinase contributes to the pathogenesis of liver fibrosis through activation of hepatic stellate cells [141]. Haus et al. [142] have shown that ceramide also induces liver inflammation through activation of nuclear factor-κB (NF-κB), tumour necrosis factor-α (TNF-α), and proinflammatory cytokines that in turn induce ceramide synthesis through activation of plasma membrane enzyme sphingomyelinase. The accumulation of ceramides in the liver increases the production of reactive oxygen species that together with proinflammatory cytokines may contribute to the progression of liver steatosis. Furthermore, ceramide may inhibit insulin action by decreasing phosphorylation and activation of Akt, a protein kinase involved in the regulation of glucose and lipid metabolism, contributing to insulin resistance [142]. All of these mechanisms are implicated in the pathogenesis of NAFLD. Ceramide can be produced in caveolae in response to inflammation. Caveolae are dynamic plasma membrane–located assemblies of cholesterol, sphingolipids (sphingomyelin, ceramide), glycerophospholipids, TNF-α, and interleukin (IL)-1β receptors. In the presence of inflammation, the action of TNF-α and IL- 1β can stimulate apoptosis and sphingomyelinase activity, with consequent production of ceramide [143]. Ma and colleagues [144] noted that feeding mice omega-3 fatty acids, but not omega-6 fatty acids, altered the caveolae microenvironment and modified membrane lipid composition by increasing phospholipid omega-3 fatty acyl content. This modification positively influences cellular signalling and apoptosis and reduces cholesterol content in caveolae by 46% compared with omega-6 fatty acids [144]. Furthermore, the incorporation of DHA into the phospholipid in caveolae inhibits the activity of sphingomyelinase, with a consequent reduction of ceramide production that in turn decreases activity of proinflammatory cytokines TNF-α and IL-1β [145]. High concentrations of glucose and insulin activate glycolytic/lipogenic enzymes such as glucokinase and liver-pyruvate kinase. Glucose activates liver-pyruvate kinase, which in turn catalyses the formation of acetyl-CoA from pyruvate, providing citrate for fatty acid synthesis. The metabolism of fatty acids is regulated by several nuclear receptors and transcription factors; the former includes the PPAR family, retinoid X receptor α (RXRα), liver X receptor α (LXRα), and hepatic nuclear factor α and γ (HNF4 α and γ); among the latter are sterol regulatory element–binding protein-1 (SREBP-1), carbohydrate response element–binding protein (ChREBP), and max-like factor X (MLX). Notably, these transcription factors have a pivotal role in controlling hepatic carbohydrate and lipid synthesis and

oxidation relevant to the pathogenesis of NAFLD [146]. Peroxisome proliferator-activated receptors (PPARs) are transcriptional factors that regulate the expression of genes involved in lipids, carbohydrates, and protein metabolism. There are three types of PPAR: PPARα, PPARβ/δ, and PPARγ. PPARα is mainly expressed in the liver and is involved in fatty acid uptake, transport, and oxidation. Omega-3 fatty acids are potent ligands for PPARs. The activation of PPARα by omega-3 fatty acids promotes mitochondrial and peroxisomal fatty acid oxidation, decreasing their intracellular accumulation. PPARγ is expressed in liver, muscle, and adipose tissue, and its activation decreases fatty acid synthesis and TG formation. PPARβ/δ is expressed in the liver, adipose tissue, and intestine, and the activation of PPARβ/δ enhances fatty acid oxidation [147,148]. In the presence of high insulin levels and oxysterols (oxidized derivatives of cholesterol) there is an increased activation of LXRα that stimulates the fatty acid biosynthetic pathway through upregulation of SREBP-1c [149-151]. SREBP-1c is one of the three helix-loop-helix leucine zipper transcription factors (SREBP-1a, SREBP-1c, and SREBP-2) involved in the regulation of enzymes that catalyze lipogenesis, such as acetyl-CoA carboxylase, fatty acid synthase, and TG synthesis (e.g., glycerol-phosphate acyltransferase). SREBP-1c is expressed in the liver, adipose tissue, and skeletal muscle and is very important in the regulation of lipid metabolism. High-carbohydrate diets increase hepatic de novo lipogenesis through a multistage process in which (a) dietary glucose is transported into the liver by glucose transporter-2, (b) dietary glucose is phosphorylated by the enzyme glucokinase and converted by liver-specific pyruvate kinase into acetyl-CoA, (c) acetyl-CoA is converted into malonyl-CoA in the presence of insulin that activates acetyl CoA carboxylase, and (d) malonyl-CoA is the committed substrate on the fatty acid synthesis pathway. The activity of the glycolytic and lipogenic enzymes is controlled at the transcriptional level by SREBP-1c and ChREBP. Hepatic de novo lipogenesis accounts for 5% of the total lipid production in healthy subjects but can increase to 26% in subjects with NASH [93]. Omega-3 fatty acid metabolism is important for the biosynthesis of EPA and DHA. In hepatocytes, omega-3 fatty acids downregulate expression of several genes involved in lipogenesis by inhibiting SREBP-1c [152] and upregulate lipid oxidation by activation of PPARα [153], which facilitates fatty acid transfer into the mitochondria. In hepatocytes, both omega-3 and omega-6 fatty acids downregulate the activity of SREBP-1c, depressing its lipogenic function [154,155]. In particular, DHA appears to have a specific effect on SREBP-1c gene expression. Jump et al. [156] have shown that DHA has a unique role in the suppression of the mature nuclear form of SREBP through the inhibition of 26S proteasome by accelerating 26S proteasome–dependent degradation of nuclear SREBP-1 [156,157]. Increased concentrations of blood glucose stimulate hepatic lipogenesis, activating ChREBP, which is a transcriptional regulator expressed in the liver and modulated by glucose [158]. Under basal conditions, ChREBP is phosphorylated and localized in the cytosol of hepatocytes.

High glucose concentrations stimulate the glycolytic pathway, resulting in increased production of glyceraldehyde 3-phosphate and fructose-6-P, which results in the formation of xylulose 5-phosphate. Xylulose 5-phosphate activates protein phosphatase that dephosphorylates ChREBP, and the protein phosphatase thereby stimulates translocation of ChREBP from the cytosol to the nucleus [159]. Dephosphorylated ChREBP moves into the hepatocyte nuclei in the form of a heterodimer ChREBP/max-like factor X. In the nucleus, the heterodimer binds to its response element (ChoRE), thereby activating glycolytic and lipogenic gene expressions that include liver-pyruvate kinase, acetyl-CoA carboxylase, and fatty acid synthase [160]. Hepatocyte nuclear factor 4 alpha binds the liver-pyruvate kinase element adjacent to the carbohydrate receptor for full glucose activation of liver-pyruvate kinase gene transcription. SREBP-1c and ChREBP are upregulated in conditions of hyperinsulinemia and hyperglycaemia, respectively. Thus, in situations of increased insulin and increased glucose concentrations, such as in type 2 diabetes, liver lipid accumulation is promoted, potentially affecting NAFLD development. Interestingly, it has been shown that DHA reduces ChREBP activity by reducing ChREBP and max-like factor X nuclear abundance and by interacting with HNF4α and liver-pyruvate kinase promoter activity [161], therefore suggesting a novel mechanism by which DHA may act to decrease lipogenesis in NAFLD. Patients with NAFLD often develop insulin resistance and type 2 diabetes mellitus [162]. Moreover, the presence of NAFLD and type 2 diabetes increases the risk of NASH. With normal insulin sensitivity, insulin binds to the insulin receptor substrate protein, resulting in the phosphorylation of tyrosine residues. Tyrosine phosphorylation of insulin receptor substrate-1 and -2 initiates a cascade of events that lead to translocation of glucose transporter-4 into the cell membrane for glucose transport into the cytoplasm [163]. With liver fat accumulation, an excess of diacylglycerol in hepatocytes causes insulin resistance by inhibiting the tyrosine kinase activity [164]. Omega-3 fatty acids can favourably affect lipid metabolism and insulin sensitivity by inducing phosphorylation of adenosine monophosphate-activated protein kinase in the liver and in adipose tissue. DHA activates adenosine monophosphate-activated protein kinase and stimulates lipid oxidation in hepatocytes, adipocytes, and skeletal muscle, modulating glucose and lipid metabolism. Similarly, EPA-activated adenosine monophosphate-activated protein kinase stimulates muscle glucose uptake, improving insulin sensitivity [165]. Adipose tissue has an important role in regulating energy homeostasis and produces several proinflammatory adipokines, such as TNF-α, IL-8, IL-6, IL-1β, and monocyte chemoattractant protein-1, as well as hormones such as leptin, adiponectin, and resistin. Excessive calorie intake may induce defective adipose tissue expansion, leading to adipocyte injury, inflammation, and death; this typically occurs in centrally obese people. In adipocytes overloaded with lipids, insulin fails to suppress hormone-sensitive lipase-mediated lipolysis, increasing release of NEFAs into the circulation. When this occurs, NEFAs are redirected

to the liver, where they are oxidized or re-esterified into TG and secreted via VLDL. The aforementioned rise of NEFAs in the circulation combined with insulin resistance caused by excessive calorie intake produces an increase in CD36 fatty acid transporter expression [166]. CD36 is a lipid chaperone for oxidized LDL and NEFAs. In monocytes and macrophages, its expression is regulated by PPARα/γ. CD36 interacts with oxidized LDL and jun-N-terminal kinase, triggering adipocyte inflammation. Additionally, the Toll-like receptor (TLR)/CD36 complex stimulates NF-κB, causing adipocyte secretion of adipokines, furthering inflammation [167]. Proinflammatory adipokines may cause liver inflammation, and DHA (and to a lesser extent EPA) suppresses PPARα/γ-mediated upregulation of CD36 expression [168]. Adiponectin is a hormone produced by adipose tissue that is decreased in obesity and NAFLD. Adiponectin has a beneficial effect on the liver by decreasing lipid accumulation and by protecting the liver from inflammation and fibrosis. Inflammation of adipose tissue causes decreased production of adiponectin, resulting in a failure to suppress intracellular production of reactive oxygen species, with consequent upregulation of NF-κB. Omega-3 fatty acids can increase levels of adiponectin secondary to PPARγ stimulation [169], thereby potentially decreasing the risk of progression from hepatic steatosis to NASH. The production of proinflammatory adipokines causes the recruitment of macrophages in adipose tissue, stimulating the production of TNF-α, IL-6, and reactive oxygen species and increasing adipocyte lipolysis. The combined effect increases the flow of fatty acids and cytokines to the liver, causing endoplasmic reticulum stress and activating Kupffer cells. Subsequently, endoplasmic reticulum stress and Kupffer cell activation trigger liver inflammation, potentially promoting development of NASH [170].

2. Aims and Hypotheses

2.1 Aims

This thesis aims are:

1) to assess whether 15-18 months treatment with 4 g of highly purified DHA+EPA daily has a beneficial effect on:
    a. liver fat percentage measured by magnetic resonance spectroscopy (MRS) scan in three discrete liver zones and
    b. two algorithmically derived, histologically validated liver fibrosis scores [7,171].
2) to test whether either PNPLA3 (I148M) or TM6SF2 (E167K) genotypes affect:
    a. the level of DHA and EPA enrichment;
    b. end of study liver fat percentage; and
    c. end of study fasting TG concentration, in patients with NAFLD treated for 15–18 months with 4 g of DHA+EPA daily.
3) to test whether:
    a. SNPs of the FADS gene cluster and ELOVL gene family affect:
        i. Δ-5 and Δ-6 desaturase activities as determined by product-to-precursor ratios (see **Figure 11**),
        ii. DHA, EPA and AA percentage in erythrocyte membranes, and
        iii. liver fat percentage measured by MRS;
    b. to test whether there is a gene-DHA+EPA interaction affecting
        i. both Δ-5 and Δ-6 desaturase activities,
        ii. DHA, EPA and AA percentage in erythrocyte membranes.

2.2 Hypotheses

First hypothesis:

Treatment with high dose (4 g/day) of highly purified DHA+EPA for 15-18 months will have a beneficial effect on:

a. liver fat percentage measure by magnetic resonance spectroscopy (MRS) scan in three discrete liver zones and
b. two algorithmically derived, histologically validated liver fibrosis scores [7,171].

Second hypothesis:

In patients with NAFLD, either PNPLA3 (I148M) or TM6SF2 (E167K) genotypes:

a) reduce the level of DHA and EPA enrichment;

b) increase end of study liver fat percentage; and

c) reduce end of study fasting TG concentration, after 15–18 months treatment with 4 g of DHA+EPA daily.

Third hypothesis

a. In patients with NAFLD, SNPs of the FADS gene cluster and ELOVL gene family affect
    i. Δ-5 and Δ-6 desaturase activities as determined by product-to-precursor ratios (see **Figure 11**),
    ii. DHA, EPA and AA percentage in erythrocyte membranes, and
    iii. liver fat percentage measured by MRS;
b. to test whether there is a gene-DHA+EPA interaction affecting
    i. both Δ-5 and Δ-6 desaturase activities,
    ii. DHA, EPA and AA percentage in erythrocyte membranes.

3. Methods

3.1 Study design

The WELCOME study [172] (Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with OMacor thErapy; www.clinicalTrials.gov registration number NCT00760513) was a randomized double-blind placebo controlled trial testing the effects of high dose omega-3 fatty acid ethyl esters in participants with NAFLD recruited from six hospitals in the South of England. The WELCOME study was approved by the local ethics committee (REC: 08/H0502/165).

3.2 Patient selection

Participants with fatty liver disease were identified from secondary care clinics, at University Hospital Southampton diagnosed on either radiological or biopsy criteria for NAFLD.

3.3 Recruitment

At University Hospital Southampton NHS Foundation Trust, potential participants with a diagnosis of NAFLD established as part of their attendance at Hospital Clinic were contacted by means of personal contact from the research team. Contact occurred at their Hospital Clinic attendance or by letter of invitation from the research team. Outside Southampton, at Poole Hospital NHS Trust, Portsmouth Hospitals NHS Trust, Royal Bournemouth and Christchurch Hospitals NHS Trust, Basingstoke and North Hampshire NHS Trust, Winchester and Eastleigh Healthcare NHS Trust, and the Isle of Wight NHS Trust, collaborators (medical doctors responsible for the care of people with NAFLD) acted as 'post boxes' and informed potential participants about the study, providing them with a patient information sheet and asking them if interested to get in touch with the research team members in Southampton listed on the patient information sheet.

3.4 Randomisation

All participants gave written, informed consent. After completion of baseline study tests, 103 participants who met the inclusion criteria were randomised **(See figure 8 for the consort diagram)** in groups of four to either omega-3 fatty acids 4 g/day or placebo (olive oil) 4 g/day for 15–18 months treatment. Patients were randomised according to standardized procedures (computerized block randomisation) by a research pharmacist at University Hospital Southampton NHS Foundation Trust. Simple randomisation in blocks of four, either to trial medication or placebo was used. During the study period, there was no specific intervention to advocate change in lifestyle that might influence NAFLD. As part of usual patient care in the region, all patients with NAFLD attending clinic were routinely given general healthy lifestyle advice.

3.5 Intervention

Omacor and placebo capsules were of similar appearance and taste; capsules were stored at the hospital pharmacy and dispensed at baseline visit and at 6 and 12 months follow-up. Compliance to the treatment was evaluated by capsule count at every visit, and serious adverse events were recorded. Omacor and placebo were provided by Pronova Biopharma through Abbott Laboratories.

3.5.1 Active group

51 participants were randomised to receive 4 g per day of omega-3 fatty acid ethyl esters: Omacor 1 g contains eicosapentaenoic acid (EPA) 460 mg and docosahexaenoic acid (DHA) 380 mg as ethyl esters (Omacor/Lovaza, Pronova, Sandefjord, Norway; approved by the Food and Drug Administration and the European Medicines Agency for the treatment of hypertriglyceridemia).

3.5.2 Placebo group

52 participants were randomised to receive 4 g per day of olive oil. Olive oil that contained ~67% oleic acid, ~15% linoleic acid, ~15% palmitic acid, ~2% stearic acid and ~1% alpha linolenic acid was chosen as the placebo. Olive oil (4 g/day) has been chosen because oleic acid is common within the diet, and this dose of olive oil was isocaloric with the intervention. This dose and choice of placebo is the same as has been used by others, testing the effects of 8 weeks treatment with 4 g/day omega-3 oil (4 × 1000-mg capsules of 56% docosahexaenoic acid and 27% eicosapentaenoic acid) on NAFLD in women with polycystic ovarian syndrome [173].

Participants were advised to take 2 g b.d.; but if participants preferred to take 4 g o.d., that was considered acceptable.

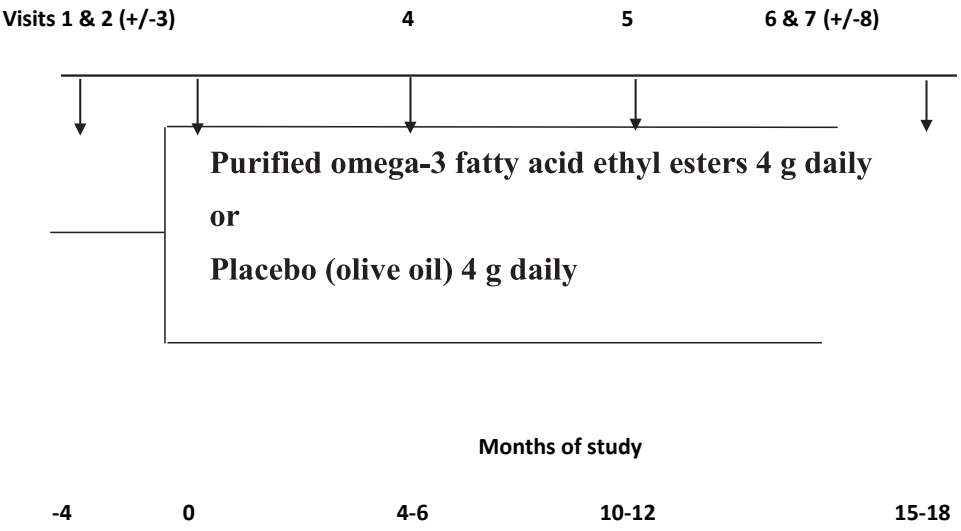
3.6 Inclusion and exclusion criteria

The inclusion criteria for participation in the study were age >18 years and: 1) a recent (<3 years) histological diagnosis of non-alcoholic steatosis or steatohepatitis in keeping with NAFLD [174]; or 2) steatosis diagnosed by ultrasound, CT or magnetic resonance imaging in a patient who also had either diabetes and/or features of the metabolic syndrome. All participants underwent an assessment of liver fat percentage by magnetic resonance spectroscopy (MRS) examination at recruitment, to establish the baseline liver fat percentage at entry into the trial. Exclusion criteria included known other causes of liver disease (e.g. hepatitis A, B or C, primary biliary cirrhosis, Wilson's disease, autoimmune hepatitis and haemochromatosis). These conditions were excluded with blood tests. Subjects were also excluded if alcohol consumption was >35 units per week for women and >50 units per week for men. These thresholds of alcohol consumption were chosen because at the time of the study design alcohol intake above these thresholds was considered harmful to the liver [175]. Nonetheless, at recruitment, only one man was consuming >21 units of alcohol per week and one woman was consuming >14 units per week. Additional exclusion criteria were: decompensated acute or chronic liver disease; cirrhosis; pregnancy or breast feeding; and hypersensitivity to Omacor, soya or any of the excipients.

3.7 Study visit overview and follow-up

During the trial, participants attended the clinical research facility (Wellcome Trust Clinical Research Facility at University Hospital Southampton NHS Foundation Trust) at 6 months and at 12 months for simple haematological and biochemical blood tests that were undertaken as a safety check **(Figure 6)**.

Figure 6. Schedule, visits and timescale of the randomized controlled trial. Schematic figure of study design. The period of time from −4 to 0 months of study was dedicated to recruitment of potential participants, screening and baseline visits (visit 1 and visit 2). At time point 0 we block-randomised participants to purified omega-3 fatty acid ethyl esters 4 g daily or placebo olive oil 4 g daily. During the trial, participants attended the clinical research facility at 6 months and at 12 months for simple blood tests undertaken as a safety checklist (visit 4 and visit 5). The end of study visits were performed at 15–18 months (visit 6 and visit 7). Visit 3 and visit 8 were extra visits to allow participants with restricted time to complete at their convenience the clinical tests.



At each visit, participants were specifically asked about any adverse events. Medication records were also reviewed during the study visits. After completing 15–18 months randomisation to either Omacor or placebo, subjects returned for end of study investigations that included a repeat measurement of liver fat by MRS examination. 95/103 randomised participants completed the study. Details of reasons for participant withdrawal from the study are presented in **Table 1.**

The progress of the trial was divided into 6 steps:

Months 0–3 Staff recruitment, Ethics application

Months 4–15 Recruitment of retrospective cohort & baseline phenotyping

Months 4–15 Randomisation to 15–18 months placebo controlled trial

Months 4–15 Recruitment of prospective new referrals

Months 4–41 Placebo controlled trial and analyses of baseline data

Months 41–48 Follow-up phenotyping and analyses of results.

Table 1. Reasons for withdrawal

○ Pre-existing difficulties with venesection/priority of other on-going clinical treatment
○ Death from bronchopneumonia diagnosed following randomisation
○ Allergy to salmon discovered following recruitment
○ Insufficient time to continue with study
○ Reliant on friends and family to travel long distance for visits and felt that health was also not up to the commitment. Offers of travel assistance and reduced visit schedule declined. Also could not tolerate magnetic resonance imaging (MRI) and MRS examination
○ Serious health concerns that, although successfully treated, meant the volunteer felt unable to continue frequent hospital visits and tests
○ Withdrawal for personal/family reasons that volunteer was unwilling to discuss with study team
○ No longer able to fulfil time commitment due to family problems
○ Relocation of work and no longer able to fulfil time commitment
○ Unwilling to continue. Research team unable to contact participant

N.B. Some participants had more than one reason for withdrawal.

3.7.1 Detail of follow-up of non-compliant subjects & withdrawal of subjects.

- Subjects were withdrawn if they developed a serious chronic illness that affected their liver condition.
- Subjects were withdrawn if they required treatment with a known hepatotoxic drug.
- Subjects were withdrawn if they developed a medical condition that would affect their consumption of trial medication.
- In all instances, a member of the research team discussed withdrawing from the trial with the volunteer.
- Data were collected up to the point of withdrawal.
- Withdrawn subjects were offered a follow up in the liver clinic at Southampton University Hospitals Trust.
- Data storage and handling and record keeping was taken place on password protected Personal Computers operated by members of the research team. Members of the research team were responsible for data collection, recording and quality. Data were collected and analysed using standard software packages such as Excel and SPSS statistics. Double entry and cross validation techniques were used to ensure the quality of the data.
- Source data were stored in a secure research office at the Wellcome Trust Clinical Research Facility at Southampton University Hospitals Trust during the Trial. After cessation of the study, records were stored in a secure room at The Institute of Developmental Sciences (IDS) Building, University of Southampton, MP 887 Southampton General Hospital, Tremona Rd, Southampton SO16 6YD, in accordance with adherence to Data Protection Act 1998.
- The Investigator(s)/Institution will permit monitoring, audits, REC and MHRA review (as applicable) and provide direct access to source data and documents.

3.8 Baseline and end of study measurements

3.8.1 Biochemical and anthropometric measurements

Glucose, insulin, total cholesterol, HDL-cholesterol, TG, platelets, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase were measured in fasting serum using commercially available kits according to the manufacturers' instructions. Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and hyaluronic acid (HA) were analysed using an ELISA kit Dynex DS2 platform. The Procollagen-III N-terminal Propeptide (PIIINP) assay was performed with a UniQ radioimmunoassay kit supplied by Orion Diagnostica (Product no.68570). We generated two different histologically-validated liver fibrosis scores [7,171]. The first of these scores comprised measurement of HA, PIIINP and TIMP-1 [171] and the second validated algorithmically-derived score (NAFLD fibrosis score) used age (years), body mass index (BMI), impaired fasting glucose/diabetes (yes/no), ALT/AST ratio, platelet count and albumin concentration [7]. Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Little Chalfont, Bucks, UK) on the non-dominant arm after subjects had become acclimatised and had rested for at least 60 min; the mean of three measurements was calculated. Waist circumference was measured over bare skin midway between the costal margin and the iliac crest. Hip circumference was measured at the widest part between the greater trochanter and lower buttock level. BMI was calculated as height (m) squared divided by weight (kg). Bioelectrical impedance (Bodystat 1500; Bodystat, Isle of Man, UK) was used to determine body composition. Metabolic syndrome was defined using the International Diabetes Federation criteria [176].

3.8.2 Assessment of compliance and measurement of erythrocyte DHA and EPA enrichment

DHA and EPA concentrations were measured in erythrocytes at the beginning and at the end of the study to evaluate erythrocyte enrichment during the study in all participants. Enrichment was defined as the difference between end of study and baseline measurements. Measurement of omega-3 fatty acids in erythrocytes is a validated proxy for liver tissue concentrations of omega-3 fatty acids [177,178]. Measurement of omega-3 fatty acids in erythrocytes at baseline and end of study was also used as a measure to assess compliance with study allocation to Omacor in that arm of the trial. To quantify the magnitude of tissue enrichment with omega-3 fatty acids due to the effects of Omacor treatment, erythrocyte fatty acids were analysed by gas chromatography at both baseline and upon completion of the trial period of intervention. Thawed packed red cells (1 ml) were mixed vigorously with 5 ml chloroform:methanol (2:1 vol/vol); butylated hydroxytoluene

(50 mg/L) was included in the chloroform:methanol as an antioxidant. After centrifugation the organic phase that includes the extracted total lipid was collected. This was dried down under nitrogen at 40 °C and redissolved in 0.5 ml toluene. Fatty acid methyl esters (FAMEs) were formed by incubation of the entire lipid extract with 1 ml methanol containing 2% (vol/vol) H2SO4 at 50 °C for 2 h. After cooling, samples were neutralized by addition of 1 ml of a solution of 0.25 M KHCO3 and 0.5 M K2CO3. Then FAMEs were extracted into 1 ml hexane, dried down, redissolved in a small volume (150 µl) of hexane, and separated by gas chromatography. Gas chromatography was performed on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column (30 m × 0.22 mm × 0.25 µm). Inlet temperature was 300 °C. Oven temperature was initially 115 °C and this was maintained for 2 min post-injection. Then the oven temperature was programmed to increase to 200 °C at the rate of 10 °C/min, to hold at 200 °C for 16 min, and then to increase to 240 °C at the rate of 60 °C/min and then to hold at 240 °C for 2 min. The total run time was 37 min. Helium was used as the carrier gas. FAMEs were detected by a flame ionization detector held at a temperature of 300 °C. The instrument was controlled by, and data were collected using, HPChemStation (Hewlett Packard). FAMEs were identified by comparison of retention times with those of authentic standards run previously. Intra-assay coefficients of variance (CVs) for EPA, DPA and DHA were 3.0%, 1.0% and 2.0% respectively. Inter-assay CVs for EPA, DPA and DHA were 5.0%, 6.1% and 2.2% respectively.

3.8.3 Body fat (total body fat, regional body fat and visceral fat)

Radiological assessments of body fat i.e. dual-energy X-ray absorptiometry (DEXA), and magnetic resonance imaging (MRI) and liver fat magnetic resonance spectroscopy (MRS) were undertaken at both baseline and end of study. DEXA and horizontal five-slice cross-sectional MRI were used to evaluate in detail absolute amount and relative percentages of body fat, truncal fat and visceral fat. DEXA scanning was undertaken with a Delphi W instrument (Hologic, Bedford, MA, USA) using a standard visual method to divide images into trunk, limb and head. MRI images were acquired from five non-contiguous slices of the abdomen, extending from 5 cm below to 15 cm above L4–L5, to obtain a more accurate estimation of visceral fat than from a single slice. Axial scans were acquired with participants in the supine position. Participants were scanned on a 1.5 T MR scanner (Siemens Avanto, Syngo software release B17; Siemens AG, Munich, Germany) using a 32-channel body coil. A gradient echo 2D FLASH (fast low angle shot) sequence (TR = 111 ms, TE = 4.18 ms, flip angle = 70°, slice width = 10 mm, slice spacing = 50 mm) was used to obtain T1-weighted images. In order to accommodate the circumference of the individual being scanned within the image, the field of view was varied. The MR images were analysed using a proprietary software package (Mimics 14.0; Materialise NV, Leuven, Belgium) to identify regions of

subcutaneous and visceral fat within the cross-sectional abdominal MR images. This package enabled identification of subcutaneous and visceral fat. By examining the histogram of pixel values present in the image, threshold levels could be set. Since fat pixels were the highest value pixels in the image, fat tissue could be identified from other tissue in the images. Some manual intervention was required when using this technique, as there was some variation in signal intensity across the image, which is often the case in large field-of-view MR images. Three different masks were created; one comprising the whole cross-section of the body, one containing the visceral fat region and one containing the subcutaneous fat region. It was possible to determine the number of pixels contained within each of these masks, and hence calculate the areas of subcutaneous fat and visceral fat, and compare them with the total cross-sectional area. Adipose tissue volume was converted to mass in kg using a density of 0.92 kg/l for adipose tissue.

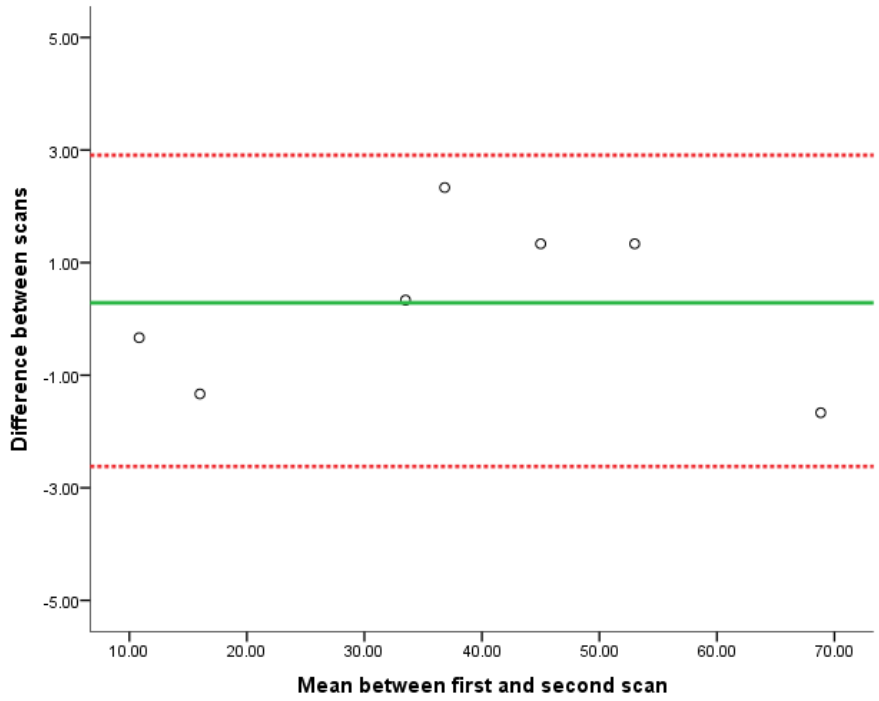
3.8.4 Mean liver fat percentage

Participants underwent MR spectroscopy (MRS) of the liver to measure the quantity of liver fat accumulated in three discrete liver zones, at baseline and follow-up. Three 20 × 20 × 20 mm^3 spectroscopic volumes of interest (VOI) were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margin of the liver. For the second visit scan, these VOI positions were copied from the first scan, to ensure consistency. A PRESS (point resolved spectroscopy) spin echo pulse sequence was used to acquire the spectroscopic data. The pulse sequence used a TR = 1500 ms, TE = 3 ms, flip angle = 90°, bandwidth = 1000 Hz, 8 averages and acquisition duration of 1024 data points, with no water suppression. The acquisition was obtained in a breath hold examination of 18 s. Spectra were post-processed using Siemens scanner software. This was a fully automated process and involved several steps, starting with filtering the data using a Hanning filter, zero-filling the data, baseline correction, phase correction and finally curve fitting was performed (with 4 iterations) to identify the water and lipid peaks. Values for the lipid and water peak integrals were produced for each VOI and recorded for each subject.

With regard to precision for the liver MRS, I analysed the data of repeated scan measurements of liver fat percentage (7 patients were scanned twice by the same radiographer). The mean and standard deviation (SD) of MRS liver fat percentage for the first scan was 37.86% (20.34) and for the repeated scan was 37.57% (20.24). I also performed a paired sample t-test that showed no significant difference between the two means (p=0.98). A Bland-Altman plot is shown as **Figure 7**. As all pairs of observations lie within 1.96-SD of the mean it is reasonable to assume that the

method is reasonably precise. Moreover, the result of the regression analysis confirmed that there are no proportional bias (Mean between first and second scan: β=0.005; p=0.88).

Figure 7. Bland-Altman plot shows that the MRS scans provide precise data.



With regard to accuracy for the liver MRS, there is recent evidence showing that MRS results correlate strongly with histopathology results [179-181]. In one recent study Nasr et al. showed a strong correlation between MRS with liver histopathology results (r=0.87; P < .001). With a cut-off value of 3% the sensitivity and specificity were 79% and 100% respectively [AUROC (95% CI) = 0.969 (0.933-1.000)] [181]. Although I have liver biopsy data for approximately 50 patients, I was not able to measure accuracy for the liver MRS estimation of liver fat percentage for the following reasons:

Although histological assessment of liver fat is the current 'gold standard' for assessing liver fat accumulation, in the WELCOME Study some patients had their liver biopsy two or three years before the baseline MRS measurement. As it is well accepted that liver fat changes with lifestyle alterations such as weight loss, change in diet or increases in physical activity, comparisons

between liver histology and MRS estimation of liver fat would be unreliable in the WELCOME study because of this separation in time. I have added this as a limitation to my final Discussion.

Additionally, although liver biopsy is the reference procedure for grading liver fat accumulation, this procedure has limitations [182]. First, the liver is not necessarily uniformly affected by fat accumulation and since the volume of a needle biopsy sample represents a very minor fraction of the whole organ (0.05 cm^3, 25-mm length), sampling variation is a relevant issue to consider since a too small biopsy (sampling error) can result in substantial misdiagnosis and staging inaccuracy [183,184]. Secondly, since these grading estimations are subjective, there is considerable inter- and intra-individual variation in steatosis grading at histopathology [185-187].

3.8.5 Diet

Change in diet during the study period was assessed by completion of a food frequency questionnaire (FFQ) at baseline and at the end of the study. This questionnaire comprises more than twenty groups of food and the frequency options where based on weekly consumption. From these reported weekly frequencies of foods consumption, a 'prudent diet score' using principal component analyses was generated [188]. These components are independent linear combinations of dietary variables that account for maximum variance and each component identifies a pattern of consumption of certain foods. A "prudent diet" was identified as being a healthy diet specifically rich in fruit and vegetables and that contained fewer dietary calories from simple carbohydrates and saturated or trans-fatty acids (e.g. prudent diet: wholemeal bread, yoghurt, and fish). The prudent diet score is a continuous variable and changes in this measurement during the trial will be analysed by comparing baseline measurement with end of study measurement, in order to assess whether participation in the trial results in a change in the participant's diet.

3.8.6 Cardiorespiratory fitness

Cardiorespiratory fitness measured in terms of maximal oxygen uptake (peak VO_2) was determined from breath-by-breath analysis of oxygen consumption and CO_2 production using a Cortex metalyser 3B instrument (Cortex Biophysik, Germany) during maximal treadmill exercise (Woodway P55 treadmill) utilising a modified Bruce protocol, with 12-lead ECG monitoring throughout the test. Volunteers were advised to avoid strenuous exercise and alcohol for 24 h prior to testing. Volunteers were encouraged to continue until the respiratory exchange ratio was N1.1 and they reached at least 90% of their predicted maximum heart rate (as determined by 220 − age), unless

they experienced chest pain or felt unwell. Cardiorespiratory fitness was measured by peak VO_2 which was corrected for total body weight.

3.8.7 Physical activity energy expenditure

Physical activity levels were assessed at baseline and at the end of the study by measuring total energy expenditure using a SenseWear Pro3 armband (Bodymedia, Pittsburgh, USA) [189] for approximately 4 days on each occasion. The SenseWear armband is a compact and lightweight ~82 g device worn around the upper arm that is well tolerated and contains sensors for 2 plane accelerometry, near body temperature, skin temperature and the galvanic skin response. The SenseWear Pro3 armband allows reliable measurement of physical activity energy expenditure levels and calculation of total energy expenditure recordings. Assessment of any change in physical activity at the end of the trial was undertaken by comparing baseline and end of study data to assess whether participation in the trial resulted in any change in this behaviour.

3.8.8 DNA analyses

Blood was collected from participants at baseline for determination of polymorphisms in the gene encoding PNPLA3. DNA was extracted from 200 µl whole blood using QIAamp blood DNA blood mini kit (Qiagen, 51106), as per manufacturer's instructions. All samples were eluted in 200 µl DNAse free water. Quality and quantity of DNA was confirmed by spectrophotometry using a Nanodrop 2000 (Thermo Scientific), where all samples had a 260/280 ratio ≥1.8. PCR primers were designed using PyroMark Assay Design 2.0 software (Biotage) to investigate the SNP rs738409 in the human PNPLA3-I148M gene variant. PCR was carried out on 25 ng DNA using 25 µl KAPA2G Robust Hot Start Taq (Anachem, KK5702) and 0.2µM of forward (5' AGCAGAGAAAGCCGACTTACCAC 3') and reverse (5' GGGTGCTCTCGCCTATAACTTC 3') primers in a 50 µl reaction. PCR products were immobilised on streptavidin–sepharose beads (GE Healthcare UK Ltd., 17-5113-01), washed, denatured and released into annealing buffer containing the sequencing primer (5' ATGTTCCTGCTTCAT 3'). PNPLA3 SNP genotype was analysed using Pyromark ND 1.0 Software (Biotage). TM6SF2 genotype was determined using TaqMan C_89463510_10 Genotyping assay (Life technologies 4351379) and TaqMan Genotyping Master Mix (Life Technologies, 4371353) and analysed using LightCycler 480 SW 1.5.1 software (Roche). The rs58542926 C>T (E167K, TM6SF2) single-nucleotide polymorphisms were assessed in duplicate. Analytical pass rate was 100%.

3.9 Sample size and power calculation

Based on previous published literature at the time of the design of the protocol in 2007/2008, a 15% decrease in liver fat was estimated after omega-3 fatty acid treatment. Subsequently, in 2012 a systematic review and meta-analysis of omega-3 fatty acid supplementation in the treatment of NAFLD produced a similar estimate of the effect size of treatment to decrease liver fat [190]. This meta-analysis suggested a Hedge's g pooled effect size of omega-3 fatty acid treatment to decrease liver fat of -0.97 (95%CIs -1.35, -0.58, $p<0.0001$). With a sigma of 0.3, a pooled effect size of -0.97 represents a ~30% decrease in liver fat percentage, with omega-3 fatty acid treatment. Assuming a sigma of 0.3, and an alpha of 0.05; and allowing for a 15% drop out rate, as specified originally in the protocol, the estimated sample size of 50 people in each group provides 99% power to detect a 30% change in liver fat (two tailed test). There would be 86% power to detect the more conservative estimate of a 20% difference in liver fat with omega-3 treatment. With an estimated value for sigma of 0.3, and an alpha of 0.05, a sample size of 50 participants in each arm would give 94% power to detect this difference in liver fat.

4. Results

4.1 The effect of highly purified high dose Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) treatment in NAFLD.

4.1.1 Introduction

Since the prevalence of major risk factors for NAFLD (obesity, insulin resistance and type 2 diabetes) has increased worldwide, NAFLD is rapidly becoming an important problem for patients and health care professionals. Progression of liver disease is often silent and NAFLD may be overlooked as it presents with other serious conditions such as type 2 diabetes and cardiovascular disease (CVD) [21,191]. NAFLD is often associated with obesity and other features of the metabolic syndrome and strategies that produce weight loss can be very effective in lowering liver fat content, although whether such strategies are effective in improving other components of NAFLD is uncertain [6,94]. Although treatments are available for individual components of the metabolic syndrome (e.g. glucose intolerance, increased blood pressure, dyslipidaemia, and obesity) [192], whether these treatments are effective in NAFLD is unproven. Studies that have attempted to treat NAFLD by targeting specific pathways in the pathogenesis of NAFLD have to date met with limited success. For example, the results of trials testing treatment with thiazolidinediones [36,37] and anti-oxidants such as vitamin E [38], that have focussed primarily on modifying pathways affecting insulin resistance [46], oxidative stress [38] and lipid metabolism [47] have 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 [193]and the high dose of vitamin E required [194]. Typically, within a Westernized diet, omega-6 fatty acid consumption is markedly greater than omega-3 fatty acid consumption [69]. 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. Recently, several studies have shown that a diet with an inadequate intake of “omega-3 essential fatty acids” is associated with metabolic syndrome [67], cardiovascular disease [68], dyslipidaemia and fatty liver disease [70].

The aim of this chapter is to assess whether 15-18 months treatment with 4 gr of highly purified DHA+EPA daily has a beneficial effect on:

a. liver fat percentage measure by magnetic resonance spectroscopy (MRS) scan in three discrete liver zones and
b. two algorithmically derived, histologically validated liver fibrosis scores [7,171].

4.1.2 Hypothesis

Treatment with high dose (4 g/day) of highly purified DHA+EPA for 15-18 months will have a beneficial effect on:

a. liver fat percentage measure by magnetic resonance spectroscopy (MRS) scan in three discrete liver zones and
b. two algorithmically derived, histologically validated liver fibrosis scores [7,171]

4.1.3 Methods

4.1.3.1 Patients

One hundred and three participants were randomized **(Figure 8)** to either Omacor (DHA+EPA) 4 g per day [(n=51; 1 g of Omacor contains 460 mg of EPA and 380 mg of DHA as ethyl esters] or 4 g per day of placebo [(olive oil; n=52; 1 g of olive oil contains 600 mg of oleic acid plus lesser amounts of linoleic, palmitic, stearic, and α-linolenic acids]. DHA+EPA and placebo capsules were gelatine coated and of similar appearance and taste. Erythrocyte EPA and DHA enrichment (between baseline and end of study) were measured to test adherence to the intervention in the DHA+EPA group and monitor contamination with DHA and EPA in the placebo group. Compliance with the allocated medication was also monitored by recording returned unused capsules.

4.1.3.2 Inclusion and Exclusion Criteria.

Inclusion and exclusion criteria have been described previously (See chapter 3.6). Briefly, subjects were eligible (1) with histological confirmation of NAFLD or (2) imaging evidence of liver fat (ultrasound, MRI, or computed tomography scan), features of metabolic syndrome [195], and exclusion of other liver conditions causing liver fat accumulation or cirrhosis. Subjects were also excluded if alcohol consumption was >35 units (1 unit is 7.9 g of alcohol) per week for women and >50 units per week for men, which was the threshold for harmful alcohol consumption at the beginning of WELCOME study recruitment [175]. Additional exclusion criteria were pregnancy, breastfeeding, and hypersensitivity to DHA+EPA, soya, or the excipients.

4.1.3.3 Biochemical Measurements, Body Composition and Energy Expenditure.

All measurements (including MRS liver fat percentage) were undertaken at baseline and end of study. Fibrosis markers, including hyaluronic acid (HA), procollagen-III N-terminal propeptide (PIIINP), and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), were measured along with cytokeratin 18 (CK18), which has been used to assess non-alcoholic steatohepatitis (NASH) [196]. Energy expenditure was assessed by measurement of metabolic equivalent of task (MET); SenseWear Pro 3 Armband monitor; BodyMedia, Inc., Pittsburgh, PA). Any change in diet during the study was assessed by food frequency questionnaire. A “prudent diet score” as healthy diet index was also generated using principal component analyses [188].

4.1.3.4 Measurement of erythrocyte DHA and EPA enrichment

DHA and EPA concentrations in erythrocytes membrane were measured in all participants at the beginning and at the end of the study to evaluate erythrocyte enrichment. Enrichment was defined as the difference between end of study and baseline measurements. Measurement of omega-3 fatty acids in erythrocytes is considered a validated proxy for liver tissue concentrations of omega-3 fatty acids [177,178]. Measurement of omega-3 fatty acids in erythrocytes at baseline and end of study was also used as a measure to assess compliance with study allocation to DHA+EPA in that arm of the trial. To quantify the magnitude of tissue enrichment with omega-3 fatty acids after DHA+EPA treatment, erythrocyte fatty acids were analysed by gas chromatography at both baseline and upon completion of the trial period of intervention.

4.1.3.5 Mean liver fat percentage

Participants underwent MR spectroscopy (MRS) of the liver to measure the quantity of liver fat accumulated in three discrete liver zones, at baseline and follow-up. Three 20 × 20 × 20 mm^3 spectroscopic volumes of interest (VOI) were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margin of the liver. For the second visit scan, these VOI positions were copied from the first scan, to ensure consistency.

4.1.3.6 Statistical analysis

Statistical analyses were performed using SPSS for Windows. The normal distribution of the data was tested by the Shapiro–Wilk and Kolmogorov–Smirnov tests. Data were adjusted for potential confounders (e.g. age, sex, alcohol consumption, and weight loss, increased physical activity and change in diet during the study). Data are reported as mean and standard deviation for normally distributed variables, or as median and interquartile range for non-normally distributed variables. Comparison of mean values of continuous variables from the two groups was undertaken using paired t-test and when variables were not normally distributed, the Wilcoxon Signed rank test was undertaken. The Pearson and Spearman rank correlation coefficients were used to investigate factors associated with liver fat percentage at baseline. Multivariable linear regression was undertaken with liver fat percentage and each of the algorithmically-derived liver fibrosis scores, as the respective outcomes to test the effects of tissue omega-3 fatty acid enrichment due to the intervention. Explanatory variables: 1) Percentage enrichment of EPA or DHA, measured in erythrocytes as the difference between baseline and end of study percentages; and separately 2)

allocation to DHA+EPA or to placebo, as a binary indicator variable were included in the regression model to test the effects of omega 3 fatty acid enrichment due to DHA+EPA treatment. Where variables were not normally distributed, logarithmic transformation was undertaken to normalise the distribution. In order to adjust the regression models for potential confounding factors such as change in weight, change in diet, or change in physical activity during the period of the intervention, the mathematical difference between baseline and end of study data for weight, prudent diet score [188] and metabolic equivalent of tasks (METs) (as a measure of energy expenditure) were calculated [197]. Analyses were also adjusted for NAFLD severity (as indicated by the measurement of liver fat percentage, NAFLD fibrosis biomarker scores, and Cytokeratin-18) and BMI. Both intention-to-treat (ITT) analysis and per protocol analysis were undertaken. For ITT analyses, complete case analysis was undertaken with exclusion of cases with missing data. Analytic comparisons of baseline data in participants with missing end of study data and participants with complete data was undertaken. Per protocol analysis included all participants who consumed ≥50% of their supplement in the time period from randomization to final visit and had a baseline average liver fat percentage ≥5% for the mean of the three liver zones. A p-value of <0.05 was considered statistically significant for all analyses. No interim analyses during the trial were planned.

4.1.4 Results

4.1.4.1 Baseline and end of study characteristics according to randomisation group

At baseline, 103 people were randomised to either DHA+EPA or placebo (**Figure 8**). **Tables 2** shows baseline characteristics in placebo and DHA+EPA groups at randomisation. At baseline, there were clear differences in some of the parameters between DHA+EPA and placebo group. For example, by chance, participants in the treatment group were approximately six years younger than participants in the placebo group. Moreover, again by chance, the ratio of men to women in each group showed that there was a higher proportion of men in the placebo group; whereas, there was an equal proportion of men and women in the DHA+EPA group. In addition, some anthropometric data such as BMI, waist circumference, total fat (g) measured with DEXA and visceral fat measured with MRI scan were higher by chance in the DHA+EPA group compared to placebo. With regard to lipid and liver profile, plasma TG were higher in the DHA+EPA group, whereas serum hyaluronic acid was lower in the DHA+EPA group compared to placebo.

Therefore, these differences between placebo and treatment group were taken in account during the data analysis, with adjustment for age, sex, BMI, waist circumference, DEXA total fat, MRI visceral fat, TG and HA) as potential confounders, depending on the outcome examined. Ninety-

five participants completed the trial (55 men and 40 women) and inadequate MRS data were obtained for 4 of these 95 participants. Therefore, the end-of-study liver fat percentage was available on 91 participants for the primary and secondary analyses.

Figure 8. Consort diagram showing recruitment to the study.

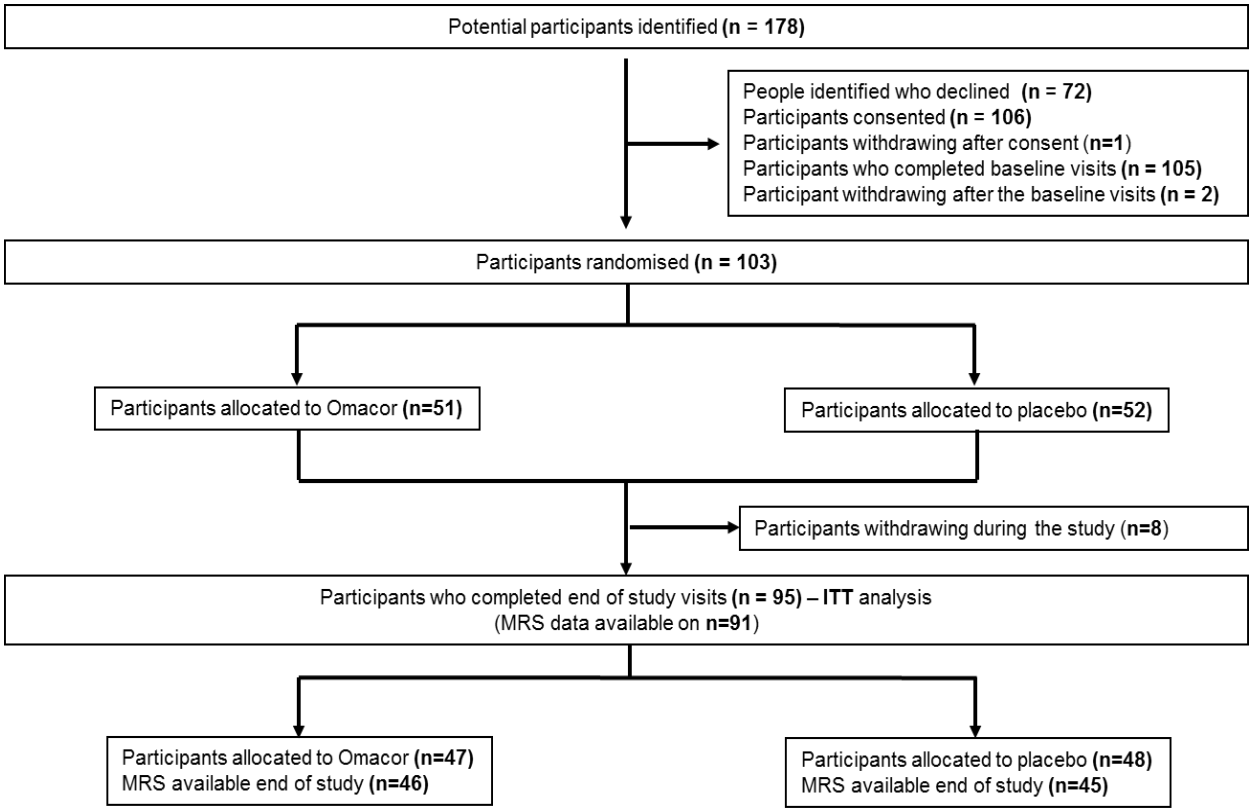


Table 2. Baseline variables in placebo and DHA+EPA groups at randomisation

Variables	Placebo	DHA+EPA	p value
Age (yr)	54.0 (9.6)	48.6 (11·1)	0.09
Sex (M/F)	35/17	25/26	0.08
Diabetes (%)	9.0	9.0	0.9
Weight (kg)	93 (14.4)	97 (17)	0.2
BMI (kg/m2)	32.0 (4.3)	34.3 (5.8)	0.02
Waist circumference (cm)	108.1 (11.9)	112.8 (12.0)	0.06
Diastolic blood pressure (mmHg)	85.3 (8.1)	84.7 (11.8)	0.8
Systolic blood pressure (mmHg)	137.7 (15·9)	138.2 (16.7)	0.9
*Fasting plasma glucose (mmol/L)	6.2 (2.0)	6.2 (2.8)	0.3
*Fasting plasma insulin (µUnit/ml)	11.3 (12.2)	13.6 (11.9)	0.8
*HbA1c (% total)	6.1 (1.6)	5.9 (1.2)	0.2
*Plasma triglycerides (mmol/L)	1.4 (0.9)	1.8 (1.2)	0.04
Plasma cholesterol (mmol/L)	4.8 (1.3)	4.9 (1.1)	0.4
LDL-cholesterol (mmol/L)	2.7 (0.8)	3.0 (0.9)	0.3
HDL-cholesterol (mmol/L)	1.1 (0.3)	1.0 (0.2)	0.1
*ALT (IU/L)	56.0 (34)	54.0 (43)	0.6
*AST (IU/L)	41.5 (19)	38.0 (24)	0.2
*DEXA total fat mass (g)	32390.1 (8140)	37500.7 (15109)	0.08
DEXA total lean mass (g)	58536.4 (10933.0)	58033.9 (12370.1)	0.8
DEXA andro/gynoid (ratio)	1.2 (0.2)	1.2 (0.1)	0.1
MRI subcutaneous fat (%)	30.2 (9.5)	34.2 (9.8)	0.05
MRI visceral fat (%)	16.7 (4.5)	15.6 (5.1)	0.3
*Hyaluronic acid (µg/L)	22.5 (27.0)	18.0 (21.0)	0.04
*PIIINP (µg/L)	5.1 (1.9)	5.4 (3)	0.6
*MRS liver fat %	21.7 (19.3)	23.0 (36.2)	0.75
NAFLD Fibrosis score	-1.7 (1.3)	-1.5 (1.4)	0.6
** Liver fibrosis score	9.0 (0.8)	8.8 (0.8)	0.34
Erythrocyte DHA (%)	4.2 (1.4)	3.9 (1.2)	0.32
Erythrocyte EPA (%)	0.9 (0.4)	0.8 (0.3)	0.43

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed (*) are expressed as median (inter-quartile range (IQR)).

** Fibrosis score calculated from PIIINP, HA and TIMP-1[171]

Table 3. Comparison between baseline and end of study data in all participants randomised to placebo or DHA+EPA.

	Placebo			DHA+EPA		
Variables	**Baseline**	**End of study**	***p*-value**	**Baseline**	**End of study**	***p*-value**
Age (yr)	54.0 (9.6)	55.4 (9.6)	n.a	48.6 (11.1)	50.1 (11.1)	n.a
Sex (M/F)	35/17	32/16	n.a	25/26	23/24	n.a
Diabetes (%)	9.0	7.5	n.a	9.0	7.6	n.a
Weight (kg)	94.5 (15.8)	90.4 (16.3)	0.76	96.5 (17)	94.1 (13)	0.38
BMI (kg/m^2)	32.4 (4.5)	30.8 (4.5)	0.74	34.0 (5.8)	33.4 (4.9)	0.30
Diet (FFQ-PCA)	0.09 (0.97)	-0.07(0.9)	0.46	-0.1 (1.0)	0.03 (1.0)	0.55
Waist circumference (cm)	108.1 (11.5)	107.7 (10.3)	0.65	114.4 (13.4)	112.3 (10.4)	0.96
Diastolic blood pressure (mmHg)	86.3 (7.4)	84.9 (6.5)	0.75	85.4 (12.3)	81.7 (8.2)	**0.006**
Systolic blood pressure (mmHg)	137.7 (15.1)	13 5.9 (11.3)	0.2	138.2 (17.4)	133.3 (13.7)	**0.004**
*Fasting plasma glucose (mmol/L)	5.4 (1.9)	5.5 (2.8)	0.4	5.4 (1.3)	5.4 (2.0)	0.3
*Fasting plasma insulin (µUnit/ml)	11.7 (11.2)	10.2 (9.3)	0.68	12.3 (7.1)	13.9 (6.4)	0.18
*HbA1c (% total)	6.1 (1.6)	6.2 (1.6)	0.2	5.9 (1.2)	5.9 (1.4)	0.3
*Plasma triglycerides (mmol/L)	1.5 (0.5)	1.8 (0.6)	**0.05**	1.8 (0.7)	1.5 (1.2)	**0.02**
Plasma cholesterol (mmol/L)	4.5 (0.8)	4.8 (1)	0.28	4.9 (1.1)	4.7 (1.1)	0.17
LDL-cholesterol (mmol/L)	2.7 (0.7)	2.8 (0.8)	0.38	3.0 (0.9)	2.8 (0.9)	0.12
HDL-cholesterol (mmol/L)	1.1 (0.3)	1.1 (0.2)	0.91	1.0 (0.2)	1.1 (0.3)	**< 0.0001**
*ALT (iU/L)	56.5 (43)	46.5 (32)	0.4	55.0 (43)	44.0 (37)	0.6
*AST (iU/L)	42.5 (31)	35.0 (21)	0.1	39.0 (24)	34.0 (28)	0.7
Platelets (x10^9/L)	215.4 (52.7)	210.0 (49.5)	0.21	213.0 (70.0)	207.3 (71.8)	0.34
VO_{2max} (ml/min/kg)	27.8 (5.1)	25.4 (5.3)	**0.0001**	25.9 (7.6)	23.9 (9.2)	**0.001**
METs	1.5 (1.4)	1.2 (0.2)	0.22	1.1 (0.2)	1.1 (0.2)	0.32
*DEXA total fat mass (g)	33251.9 (8734)	30821.5 (8136)	0.34	38127.7 (10565)	35694.1 (8061.6)	0.09
DEXA total lean mass (g)	59017.2 (11343.8)	56395.5 (11237)	0.14	56352.8 (11563.7)	56218.2 (10799.3)	0.61
DEXA andro/gynoid (ratio)	1.2 (0.2)	1.2 (0.1)	0.13	1.2 (0.1)	1.1 (0.1)	0.72
MRI subcutaneous fat (%)	30.4 (9.7)	28.8 (9)	0.43	35.4 (10.5)	32 (9.6)	0.47
MRI visceral fat (%)	16.7 (4.7)	16.5 (5.4)	0.36	15.2 (5.1)	15.9 (4.7)	0.67
*Hyaluronic acid (µg/L)	22.5 (28.0)	34.0 (38.0)	**0.003**	18.0 (21.0)	29.0 (31.0)	**< 0.0001**
*PIIINP (µg/L)	5.1 (1.9)	4.8 (2.5)	0.18	5.4 (2.6)	4.9 (2.2)	0.49
TIMP-1 (ng/ml)	309.1 (90.2)	358.9 (88.4)	**0.002**	303.4 (126.5)	351.3 (72.6)	**0.01**
CK-18 M65 (IU/L)	688.6 (424.6)	389.5 (416)	**0.007**	515.1 (326.4)	701.1 (623.4)	0.31
*MRS liver fat %	21.7 (13.7, 32.3)	19.7 (11.3, 28.0)	**0.03**	23.0 (12.0, 47.5)	16.3 (9.0, 30.7)	**0.04**
NAFLD Fibrosis score	-1.7 (1.3)	-0.82 (1.2)	< 0.0001	-1.5 (1.4)	-0.7 (1.5)	< 0.0001
** Liver fibrosis score	9.0 (0.8)	9.2 (0.8)	0.01	8.8 (0.8)	9.1 (0.9)	0.002
Erythrocyte DHA (%)	4.1(1.6)	5.0 (1)	0.002	3.8 (1.2)	7.1 (1.3)	< 0.0001
Erythrocyte EPA (%)	0.9 (0.4)	1.0 (0.2)	0.17	0.8 (0.3)	2.4 (1.8)	< 0.0001

Variables that are normally distributed are expressed as mean ± standard deviation (SD). *Variables that are non-normally distributed are expressed as median ± inter-quartile range (IQR).
** Fibrosis score calculated from PIIINP, HA and TIMP-1 [171]

4.1.4.2 End of study results

Ninety-five participants completed the study (55 men and 40 women). **Table 4** shows the changes between baseline and end-of-trial measurements, stratified by randomisation group, for the main anthropometric and biochemical variables. From capsule counts at 6 and 12 months and at the end of study, we estimated that all participants consumed >50% of their study medication and 78% consumed >75%. No serious adverse events occurred that were attributed to medication. Alcohol consumption was not associated with baseline liver fat percentage (p=0.93).

Table 4. Main anthropometric and biochemical variables at baseline and end of study according to randomization group.

	Placebo			DHA+EPA		
Variables	**Baseline**	**End of study**	***p*-value**	**Baseline**	**End of study**	***p*-value**
BMI (kg/m^2)	32.0 (4.5)	30.8 (4.5)	0.31	34.4 (5.8)	33.4 (4.9)	0.3
Waist circumference (cm)	108.1 (11.9)	107.7 (10.3)	0.45	112.8 (12.0)	112.3 (10.4)	0.87
*Fasting plasma glucose (mmol/L)	6.2 (2.0)	6.7 (3.0)	0.12	6.2 (2.8)	6.1 (2.0)	0.81
MRI subcutaneous fat (%)	30.2 (9.5)	28.8 (9)	0.46	34.2 (9.8)	32 (9.6)	0.47
MRI visceral fat (%)	16.7 (4.5)	16.5 (5.4)	0.35	15.6 (5.1)	15.9 (4.7)	0.67
*HbA1c (% total)	6.1 (1.6)	6.0 (2.0)	0.14	5.9 (1.2)	5.7 (2.0)	0.53
*Plasma triglycerides (mmol/L)	1.4 (0.5)	1.8 (0.6)	0.08	1.8 (1.2)	1.5 (1.2)	0.018
Plasma cholesterol (mmol/L)	4.8 (1.3)	4.8 (1)	0.25	4.9 (1.1)	4.7 (1.1)	0.18
LDL-cholesterol (mmol/L)	2.7 (0.8)	2.8 (0.8)	0.44	3.0 (0.9)	2.8 (0.9)	0.14
HDL-cholesterol (mmol/L)	1.1 (0.3)	1.1 (0.2)	0.87	1.0 (0.2)	1.1 (0.3)	< 0.0001
*ALT (iU/L)	56.0 (34)	48.5 (25)	0.06	54.0 (43)	44.0 (34)	0.70
*AST (iU/L)	41.5 (19)	35.0 (17)	0.04	38.0 (24)	30.0 (27)	0.83

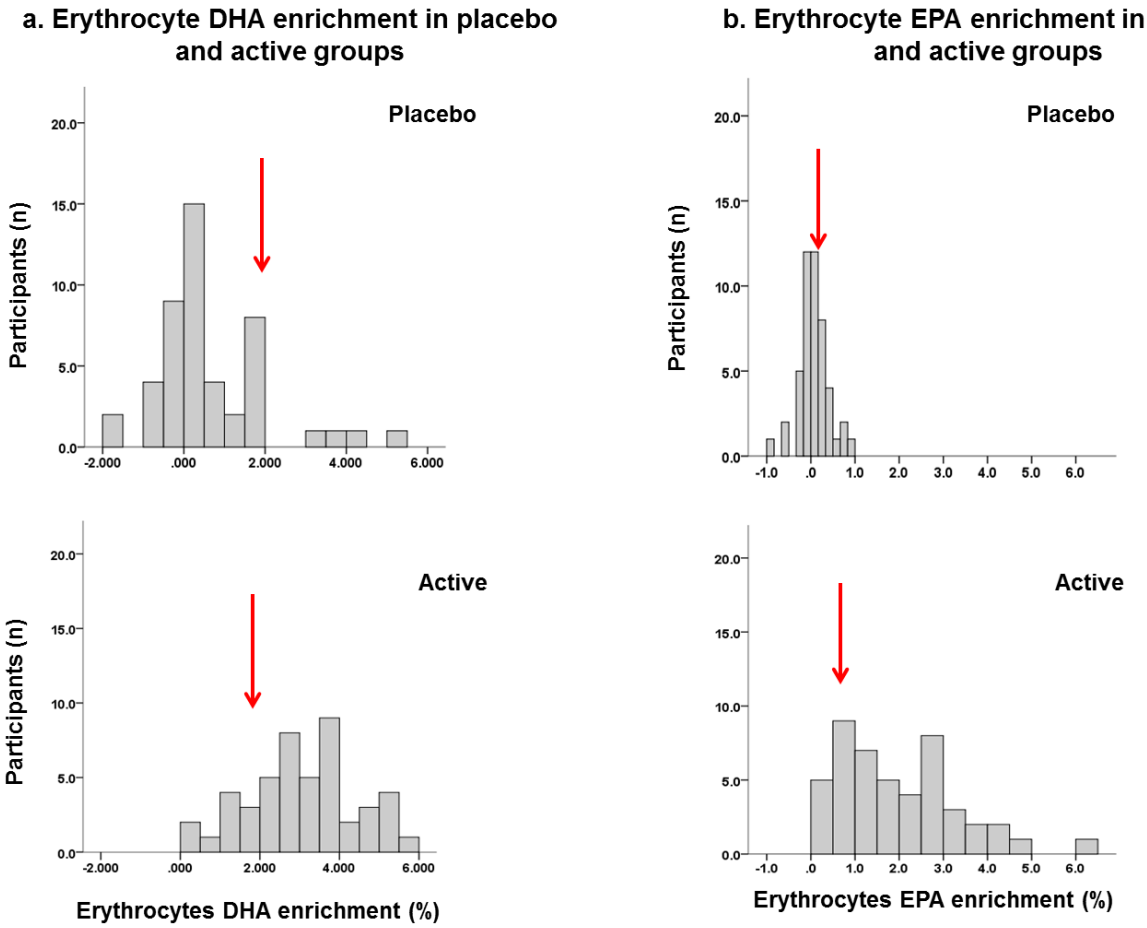
Variables that are normally distributed are expressed as mean (SD). Variables that are non-normally distributed (*) are expressed as median (IQR).

4.1.4.3 DHA and EPA erythrocytes enrichment after 15-18 months treatment with DHA+EPA.

DHA and EPA erythrocytes enrichment was measured after 15-18 months of treatment with 4 g of DHA+EPA daily. Based on previous literature a minimum 2% increase in erythrocyte DHA and a minimum 0.7% increase in erythrocyte EPA was hypothesized after DHA+EPA intervention [198,199]. The omega-3 fatty acid index is the sum of DHA and EPA in erythrocyte membranes and is expressed as a percentage of total erythrocyte fatty acids. At baseline, the omega-3 fatty acid index was between 4 and 5%, which is associated with an intermediate level of cardiovascular risk. To improve the omega-3 fatty acid index to a value proposed to result in significantly decreased CV risk [200], a 0.7% increase in EPA and a 2% increase in DHA would be sufficient. **Figure 9** shows the percentage enrichment in erythrocyte DHA and EPA between baseline and end of study in participants randomized to placebo or DHA+EPA.

Enrichment was highly variable in the DHA+EPA group and 5 and 6 participants in the DHA+EPA group did not reach the pre-specified threshold for EPA and DHA enrichment, respectively. In the placebo group, no enrichment was expected between baseline and end of study in all participants in this group, but 3 and 4 participants reached the thresholds set for the DHA+EPA group, for EPA and DHA, respectively. One participant in the placebo group admitted to taking cod liver oil during the study and another markedly increased consumption of fish.

Figure 9. Percentage change in erythrocyte DHA and EPA concentration between baseline and end of study in placebo and DHA+EPA groups.



4.1.4.4 ITT Analyses and secondary analyses

Table 5 shows the results of the regression models for the ITT analyses and secondary analyses at baseline and end-of-study. Data for change in plasma TG concentration are shown for comparison (DHA+EPA is licensed for lowering plasma TG concentrations). In the ITT analyses, in the fully adjusted model, there was a 3.64% decrease in liver fat % (β=-3.64; 95% CI: -8.0, 0.8; p=0.1) with DHA+EPA treatment. Secondary analyses **(Table 5)** were undertaken to test the association between DHA or EPA enrichment and each of the primary outcomes (adjustments as per the ITT analysis). Erythrocyte DHA enrichment was independently associated with a decrease in liver fat percentage (-1.7% for each 1% DHA enrichment; β=-1.7; 95% CI: -2.9, -0.5; p=0.007 in the fully adjusted model). In this cohort, there were only 9 patients with high NAFLD fibrosis score and 14 with high liver fibrosis score. There was no improvement in either liver fibrosis score with DHA+EPA or with DHA or EPA enrichment. At the end of the study, there was no significant difference in changing MRS liver fat between placebo and treatment group. The t-test comparison between placebo and treatment group showed no significant benefit of DHA+EPA treatment in reducing MRS liver fat (MRS liver fat difference between placebo and treatment group p=0.3). In a regression model using MRS liver fat percentage difference as outcome and randomisation group as explanatory variable, the unstandardized β coefficient for the randomisation group was -1.6 (95% CI -6.3, 2.9) and the p value was not significant (p=0.4). Therefore, the decrease in liver fat at the end of the study was similar in both placebo and treatment group.

Because there was clear evidence of contamination with DHA and EPA enrichment in some participants in the placebo group, and there was also poor enrichment with DHA and EPA in some participants in the treatment group **(Figure 9)**, the relationships between liver fat percentage at recruitment and the change in liver fat percentage between baseline and end of study was assessed in all participants, stratifying by the pre-specified threshold for erythrocyte DHA enrichment (<2% and ≥2%) and regardless of randomization group **(Figure 10)**. For those individuals achieving a ≥2% absolute increase in DHA between baseline and end of study, there was a strong inverse association between liver fat percentage at recruitment and the change in liver fat percentage between baseline and end of study.

Table 5. Baseline and end of study results and regression models testing the effects of the intervention on liver fat percentage and two biomarker scores for liver fibrosis (and triglyceride), adjusted for baseline measurement of outcome only* and fully adjusted**

	Placebo		Treatment		Difference in change from baseline to end of study* (95% CI) DHA+EPA treatment Primary analysis	Adjusted difference in change from baseline to end of study** (95% CI) DHA+EPA treatment Primary analysis	Adjusted difference in change from baseline to end of study** (95% CI) % Erythrocyte EPA enrichment Secondary analysis	Adjusted difference in change from baseline to end of study** (95% CI) %Erythrocyte DHA enrichment Secondary analysis
Primary outcomes	**Baseline**	**End of study**	**Baseline**	**End of study**				
Liver fat %	21.7 (19.3)	19.7 (18.0)	23.0 (36.2)	16.3 (22.0)	-1.7 (-6.3, 3.0) p=0.48	-3.64 (-8.0, 0.8) p=0.1	-1.0 (-2.7, -0.6) P=0.20	-1.7 (-2.9, -0.5) P=0.007
#Liver fibrosis score	9.0 (0.8)	9.2 (0.8)	8.8 (0.8)	9.1 (0.9)	-0.001 (-0.3, 0.3) p=1.0	0.14 (-0.26, 0.33) P=0.8	0.01 (-0.1, 0.1) P= 0.86	0.03 (-0.1, 0.1) P= 0.50
NAFLD fibrosis score	-1.7 (1.3)	-0.8 (1.2)	-1.5 (1.4)	-0.7 (1.5)	-0.03 (-0.4, 0.3) p=0.9	0.01 (-0.3, 0.3) p=0.9	0.00 (-0.1, 0.1) p=0.9	0.01 (-0.1, 0.1) p=0.6
Plasma triglyceride (mmol/L)	1.4 (0.5)	1.8 (0.6)	1.8 (1.2)	1.5 (1.2)	-0.2 (-0.6, 0.1) P=0.2	-0.4 (-0.8, -0.1) P=0.02	-0.2 (-0.3, -0.1) P=0.002	-0.1 (-0.2, -0.0) P=0.007

Baseline and end of study results for primary outcomes and for serum triglyceride concentrations are reported either as means and SDs or median and IQRs (liver fat % and plasma triglyceride concentration). Results of regression models testing the effects of DHA+EPA randomisation (ITT analyses) and EPA and DHA percentage enrichment (secondary analyses) between baseline and end of study are shown.

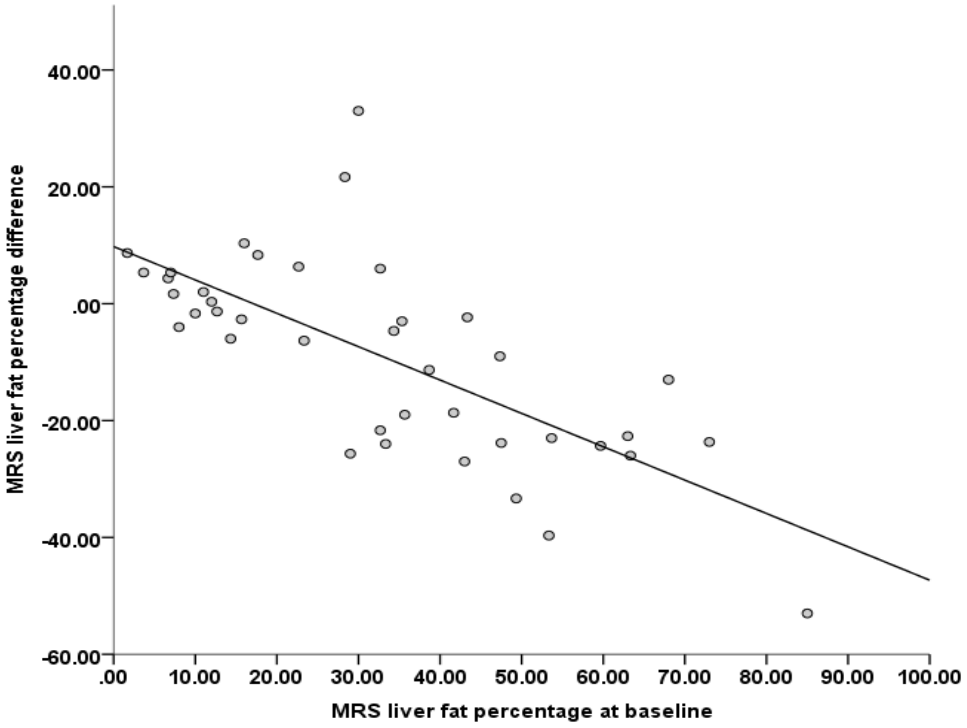
For each primary outcome (liver fat %, # Liver fibrosis score and NAFLD fibrosis score); the difference in outcome, represents the change in the outcome between baseline and end of study (* adjusted for baseline measurement of the outcome variable only, and ** adjusted for baseline measurement of the outcome plus age, sex, change in weight (Kg) between baseline and end of study and change in CK18 concentration between baseline and end of study and change in DHA or EPA).

#Liver fibrosis score (score derived from TIMP-1, HA and PIIINP concentrations, see Methods).

Triglyceride data are presented for comparison as this dose of Omacor treatment is licensed for the treatment of high triglyceride concentrations.

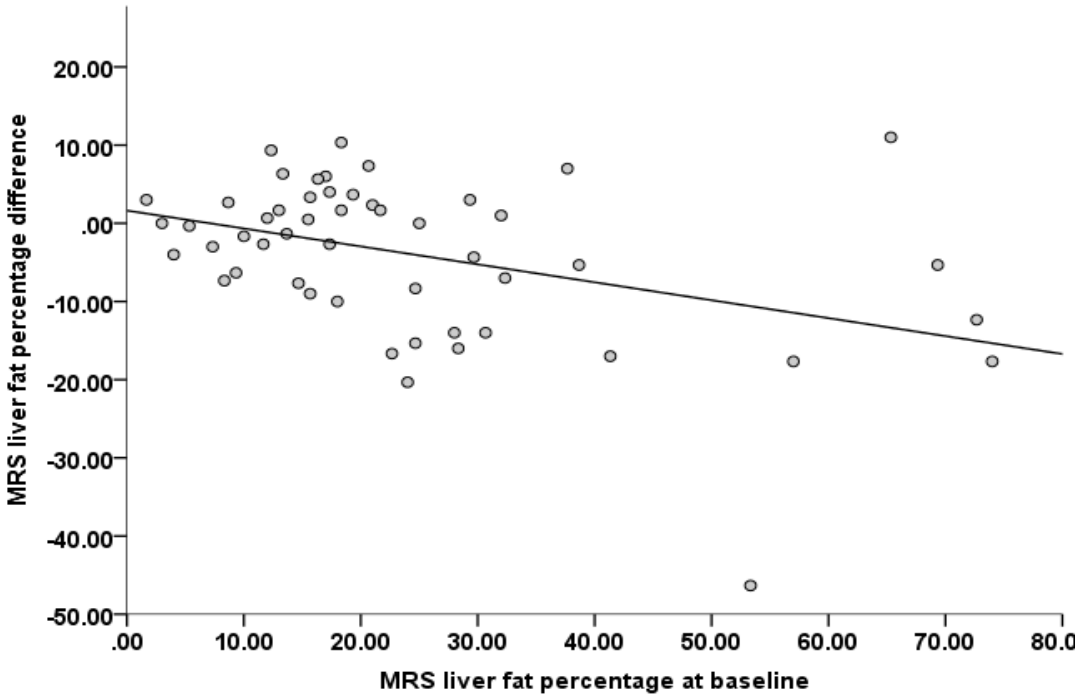
Figure 10. Scatter plots showing the relationships between baseline liver fat % at recruitment and the change in liver fat % between baseline and end of study for all participants stratified by erythrocyte DHA enrichment (A) ≥2% DHA enrichment (between baseline and end of study) or (B) <2% erythrocyte DHA enrichment.

A. Participants achieving erythrocyte DHA enrichment ≥2%.



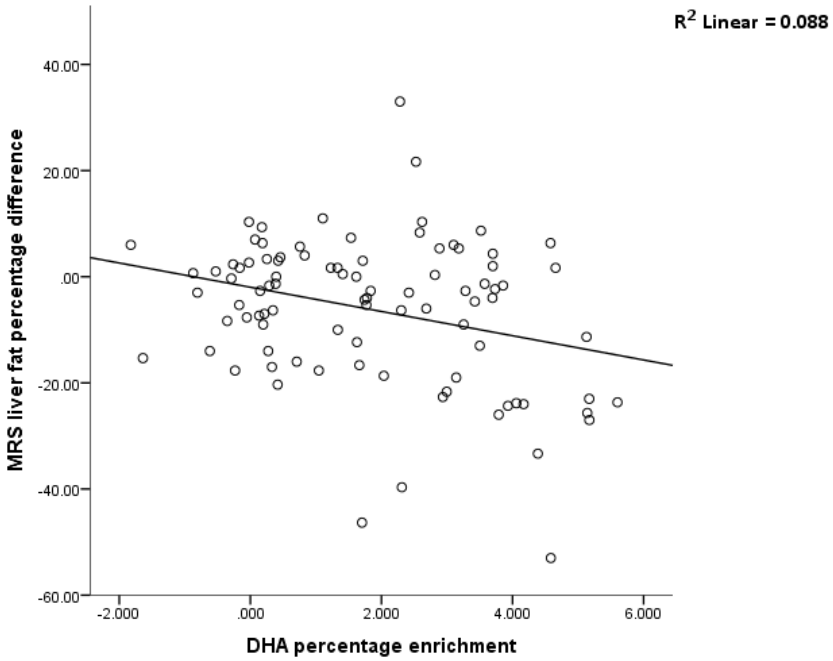
[R^2 =0.51, p < 0.0001]

B. Participants with erythrocyte DHA enrichment <2%.

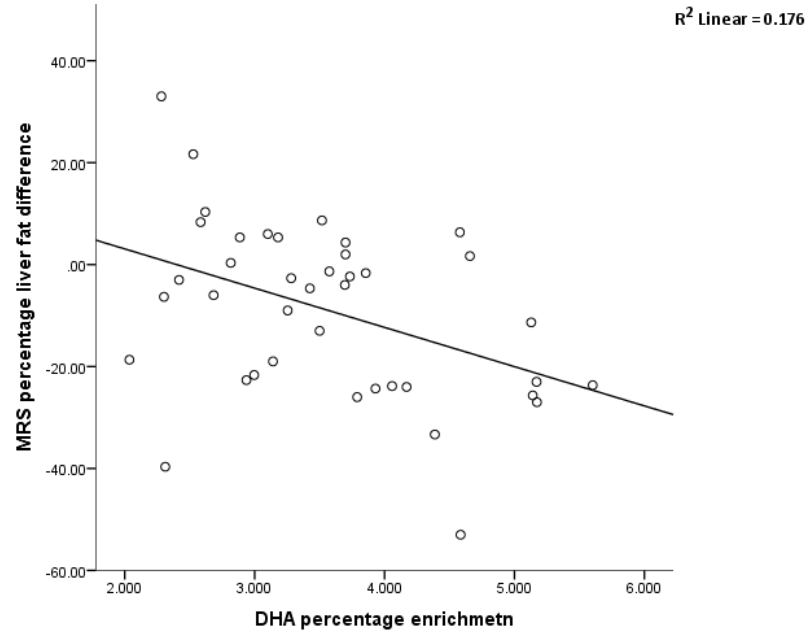


[R^2 =0.16, p =0.004]

C. Scatterplot showing the relationship between DHA enrichment and MRS liver fat percentage difference in all participants recruited in the study.



D Scatterplot showing the relationship between DHA enrichment and MRS liver fat percentage difference considering only participants who reached the ≥2% DHA enrichment threshold.



4.1.4.5 Magnitude of the Effect to Decrease Percentage of Liver Fat by Percentage of DHA Enrichment

The magnitude of the effect of each 1% DHA enrichment to decrease liver fat percentage adjusting for change in physical activity during the study (available in n=82 of 91 participants) was further investigated. In a regression model that included liver fat percentage difference as the outcome, and age, sex, difference in body weight (kg), difference in CK18 (as a marker of apoptosis and necrosis), difference in MET, between baseline and follow-up, and DHA percentage enrichment as the explanatory variables; each 1% DHA enrichment was associated with a 3.3% reduction in liver fat percentage (β=-3.3; 95% CI: -4.8, -1.8; $p<0.0001$).

4.1.5 Discussion

The results described in this chapter show that treatment with high dose of highly purified DHA and EPA for 15-18 months in people with NAFLD did not have a beneficial effect on liver fat percentage and on two biomarker scores for liver fibrosis. At the end of the study, there was no significant difference in changing MRS liver fat between placebo and treatment group.
When the ITT analysis was performed, the results show that treatment with high dose of highly purified DHA and EPA for 15-18 months in people with NAFLD did not have a beneficial effect on liver fat percentage and on two biomarker scores for liver fibrosis. Despite the negative result on the primary outcomes, the ITT analyses showed a trend toward a decrease in liver fat percentage with DHA+EPA treatment. However, the results of the ITT analysis were attenuated by the poor adherence to the DHA+EPA intervention (in the treatment group), and by the contamination with DHA and EPA (in the placebo group). Further regression analysis was undertaken after excluding the two participants who admitted to being protocol violators.
With these two participants excluded, there was an improvement in the effect of DHA+EPA treatment to decrease liver fat percentage (-4.2%; β=-4.2; 95% CI: -8.6, 0.1; p=0.057).
In the secondary analysis, this problem was circumvented by using erythrocyte DHA percentage enrichment or erythrocyte EPA percentage enrichment as the exposure variable instead of using randomisation group. Thus, it was possible to test the effect of percentage DHA enrichment (or percentage EPA enrichment), regardless of randomization group on each of the primary outcomes.
Interestingly, in the secondary analyses, there was a substantial decrease in liver fat percentage with high-percentage DHA erythrocytes enrichment. The model for the secondary analysis was adjusted for several confounders such as age, sex, difference in body weight (kg), MET difference and CK18 difference. In this adjusted model, the variable that was significantly correlated with MRS liver fat percentage difference was DHA enrichment. For each 1% DHA enrichment was associated with a 3.3% reduction in liver fat percentage (β =-3.3; 95% CI: -4.8, -1.8; p<0.0001). Consequently, for a 6% enrichment in DHA, there was a (6x3.3%)=~20% decrease in liver fat percentage. Recently, Nobili et al. tested the effect of 18 months of treatment with DHA in children with NAFLD. Liver biopsy was undertaken before and after treatment. In this study, the investigators found that DHA improved hepatic steatosis, ballooning, and inflammation[201].

With regard to liver fibrosis, there was no suggestion of benefit of DHA+EPA treatment on the two liver fibrosis scores that might have indicated an effect of treatment in NASH (See **Table 5**). Probably, the treatment with DHA+EPA needs to be tested for a longer period than 18 months if any improvement in liver fibrosis is secondary to improvements in liver fat or liver inflammation.

Importantly, we did not observe any deterioration in either fibrosis score during the period of the study.

In the exclusion criteria, the alcohol threshold was based on the UK Governmental Guidelines (21 and 14 units of alcohol per week for men and women, respectively); however, only one man and one woman had consumed alcohol above the limits preceding enrolment. Exclusion of these 2 subjects did not affect the results, and inclusion of baseline alcohol consumption as a continuous exposure variable in the regression model shown in **Table 5** did not affect the association between DHA and liver fat percentage. Furthermore, in this model, alcohol intake was not associated with liver fat percentage (β=0.005; 95% CI: -0.304, 0.314; p=0.98). However, the alcohol consumption was documented only at baseline and at end of study; therefore, there is the possibility that, during the study, alcohol consumption may have changed.

In conclusion, erythrocyte DHA enrichment with DHA+EPA treatment is linearly associated with decreased mean liver fat percentage, calculated from liver fat percentage in three discrete liver regions, in patients with NAFLD. These data suggest that substantial decreases in liver fat percentage can be achieved with high levels of erythrocyte DHA enrichment in patients with NAFLD.

4.2 The Treating liver fat and serum triglyceride levels in NAFLD, effects of PNPLA3 and TM6SF2 genotypes

4.2.1 Introduction

Genetic variation in both patatin-like phospholipase domain-containing protein-3 (PNPLA3) (I148M) and the transmembrane 6 superfamily member 2 protein (TM6SF2) (E167K) influences severity of liver disease, and serum TG concentrations in non-alcoholic fatty liver disease (NAFLD), but whether either genotype influences the responses to treatments is uncertain. In NAFLD, patients with the homozygous I148M/M gene variant of the patatin-like phospholipase domain-containing protein-3 (PNPLA3) have higher levels of liver fat accumulation [202,203] and lower serum fasting TGs concentration, compared to those without the homozygous gene variant[204]. An exome wide association study identified the rs738409 C>G single nucleotide polymorphism (SNP) in the PNPLA3 gene, encoding for the isoleucine to methionine substitution at position 148 (I148M). In humans, PNPLA3, also called adiponutrin, encodes a 481 amino acid membrane protein localised in the endoplasmic reticulum and at the surface of lipid droplets [104]. In humans, this protein has the highest expression in hepatic stellate cells, retina and hepatocytes. PNPLA3 has a TG and retinyl-palmitate esterase activity. The functional PNPLA3 148I variant allows optimal TG hydrolysis whereas the isoleucine to methionine substitution leads to a loss of lipolytic activity leading an impairment of lipid catabolism, lipid droplets remodelling, and impairment of VLDL secretion[205]. Interestingly, PNPLA3 I148M gene variant is linked to increased risk of developing cirrhosis and hepatocellular carcinoma independently from NALFD [105]. The variant protein was found to have reduced enzymatic activity for the hydrolysis of emulsified TG in hepatocytes. As a result, the secretion of VLDL is impaired. PNPLA3 retinyl-palmitate activity in hepatic stellate cells may influence hepatic regeneration and differentiation by altering availability of retinol, potent regulators of these phenomena. Recently, it has been identified the rs58542926 C>T genetic variant of the transmembrane 6 superfamily member 2 gene (TM6SF2), which encodes the loss-of-function lysine (E) to glutamic acid (K) at position 167 substitution (E167K), as a determinant of hepatic TG content, serum aminotransferases and lower serum lipoproteins **(Figure 3)** [206-208]. Although genetic variation in both PNPLA3 and TM6SF2 is known to influence severity of liver disease, and fasting serum TG concentrations in NAFLD, it is presently uncertain whether either genotype influences the responses to treatments for NAFLD.

The aim of this chapter is to test whether either PNPLA3 (I148M) or TM6SF2 (E167K) genotypes affected:

a) the level of DHA and EPA enrichment in erythrocytes;

b) end of study liver fat percentage; and

c) end of study fasting TG concentration, in patients with NAFLD treated for 15–18 months with 4 g of DHA+EPA daily.

4.2.2 Hypothesis

In patients with NAFLD, either PNPLA3 (I148M) or TM6SF2 (E167K) genotypes affect:

a) the level of DHA and EPA enrichment in erythrocytes;

b) end of study liver fat percentage; and

c) end of study fasting TG concentration, after 15–18 months treatment with 4 g of DHA+EPA daily.

4.2.3 Methods

4.2.3.1 Study design

PNPLA3 and TM6SF2 genotypes were measured in 103 participants of the WELCOME study. Consort diagram and the baseline characteristics of both placebo and active groups have been reported in Chapter 3.8. All participants had NAFLD and features of metabolic syndrome. Metabolic syndrome was defined using the International Diabetes Federation criteria [195], the criteria for defining NAFLD were reported in Chapter 3.8. Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Little Chalfont, Bucks, UK) in the non-dominant arm. Waist circumference was measured over bare skin, midway between the costal margin and the iliac crest. Hip circumference was measured at the widest part between the greater trochanter and lower buttock level. Radiological assessments of body fat (dual-energy X-ray absorptiometry, DEXA) were undertaken at both baseline and end of study. DEXA scanning was undertaken with a Delphi W instrument (Hologic, Bedford, MA, USA) to assess percentage body fat, fat distribution and lean mass. Glucose, insulin, total cholesterol, HDL-cholesterol, TG, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and cytokeratin-18 (CK-18 M65 as a marker of liver apoptosis [196]) were measured in fasting serum. For measurement of serum TG concentration, the intra-assay coefficient of variation (CV) was 1% and the inter-assay CV was 1.7%, for TG concentrations <1 mmol/L. Magnetic resonance imaging (MRI) of abdominal visceral and subcutaneous fat and MR spectroscopy of hepatic lipid content were undertaken at the beginning and at the end of the study. Any change in diet during the study was assessed by food frequency questionnaire and we also generated a 'prudent diet score' as a healthy diet index, using principal component analyses [188]. Drug medication was recorded at each study visit. At 6 months, 12 months and end of study, participants returned all used study capsules. Returned capsules were counted to assess compliance to allocated DHA+EPA or placebo.

4.2.3.2 DNA analyses

Blood was collected from participants at baseline for determining polymorphisms in the gene encoding PNPLA3 and TM6SF2. DNA was extracted from 200 µl whole blood using QIAamp blood DNA blood mini kit (Qiagen, 51106), as per manufacturer's instructions. All samples were eluted in 200 µl DNAse free water. Quality and quantity of DNA was confirmed by spectrophotometry using a Nanodrop 2000 (Thermo Scientific), where all samples had a 260/280 ratio ≥1.8. PCR primers were designed using PyroMark Assay Design 2.0 software (Biotage) to investigate the SNP rs738409 in the human PNPLA3-I148M gene variant. PCR was carried out on 25 ng DNA using 25

µl KAPA2G Robust Hot Start Taq (Anachem, KK5702) and 0.2 µM of forward (5'AGCAGAGAAAGCCGACTTACCAC 3') and reverse (5'GGGTGCTCTCGCCTATAACTTC 3') primers in a 50 µl reaction. PCR products were immobilised on streptavidin–sepharose beads (GE Healthcare UK Ltd., 17-5113-01), washed, denatured and released into annealing buffer containing the sequencing primer (5'ATGTTCCTGCTTCAT 3'). PNPLA3 SNP genotype was analysed using Pyromark ND 1.0 Software (Biotage). TM6SF2 genotype was determined using TaqMan C_89463510_10 Genotyping assay (Life technologies 4351379) and TaqMan Genotyping Master Mix (Life Technologies, 4371353) and analysed using LightCycler 480 SW 1.5.1 software (Roche). The rs58542926 C>T (E167K, TM6SF2) single-nucleotide polymorphisms were assessed in duplicate. Analytical pass rate was 100%.

4.2.3.3 Statistical analyses for the secondary

All statistical analyses were performed using SPSS for Windows (version 21.0; SPSS). Baseline and end of study characteristics in DHA+EPA and placebo groups were examined. Data are reported as means and 95% CIs or SDs for normally distributed variables, or as median and interquartile range (IQR), or ranges with maxima and minima for non-normally distributed variables. Comparisons of means between groups were performed by using t-tests for normally distributed variables and Mann Whitney U for non-normally distributed unpaired variables. Comparisons of paired data were tested by paired t tests, Wilcoxon signed rank and McNemar's tests. Multivariable linear regression modelling with backwards elimination was undertaken to test independent associations with these outcome variables: a) end of study TG concentration, b) end of study liver fat % c) DHA or EPA percentage enrichment in erythrocytes (end of study % minus baseline %). In each of these separate regression models, we adjusted for the baseline measurement of the outcome measure in question. Covariates, potential confounders and change in potential confounders between baseline and end of study were included, to test the independence of associations between key exposures and the outcome in question. Explanatory variables that were included in the models were continuous or categorical. For PNPLA3 and TM6SF2 genotype, a binary indicator variable was created and coded as 148I/M+148I/I=0 and 148M/M=1, or TM6SF2 167E/E=0 and TM6SF2 167E/K+167K/K=1 respectively. Differences between variables in PNPLA3 genotype groups and in TG quartile groups were tested by ANOVA or Kruskal Wallis depending on whether variables were normally or non-normally distributed. A P value of <0.05 was considered to be statistically significant. ANCOVA analyses were also undertaken test the effects of allelic variation of the genotypes for PNPLA3 and TM6SF2 on the study outcomes and to estimate the adjusted mean differences, according to genotype, between baseline and end of study in the

study outcomes. Each ANCOVA model was adjusted for the same potential confounders as shown in the linear regression models.

4.2.4 Results

4.2.4.1 Subject characteristics

Table 6 summarises the baseline and end of study results for the main anthropometric and biochemical variables, according to randomisation group. Ninety-five participants completed the study (55 men and 40 women) and 32 of these had diabetes. At baseline, fasting TG concentration and BMI were higher in the DHA+EPA group compared with the placebo group (p=0.04 and p=0.02, respectively). At baseline, there were no differences between the groups in MRI visceral fat measurements, waist circumference and weight. At baseline, 7.4% (n=7) of participants were taking fibrates, 6.3% (n=6) were taking ezetimibe, 44.2% (n=42) were taking statins, 4.2% (n=4) were taking orlistat and 9.5% (n=9) were taking levothyroxine. At the end of the study, there were 9.5% (n = 9) of people taking fibrates, 5.3% (n = 6) people were taking ezetimibe, 49.5% (n = 47) people taking statins, 1.1% (n = 1) were taking orlistat **(Table 6)**, 10.5% (n=10) were taking levothyroxine. With regard to other metabolic and biochemical variables, there were no significant differences between randomisation groups **(Table 6).** Diet (assessed by prudent diet score) and alcohol intake did not change significantly during the study in either group. Capsule count at 6 months, 12 months and at end of study confirmed that compliance with treatment was at least 78% of all allocated capsules in all participants during the duration of the study. **Table 6** also shows fatty acid percentages in erythrocytes at baseline and at the end of study in the placebo and DHA+EPA groups. Despite very good compliance with the DHA+EPA intervention in the treatment arm, there was very variable DHA and EPA enrichment between individuals. Some individuals had only limited enrichment of either fatty acid while others had excellent enrichment, which did not relate to numbers of capsules consumed. For example, for DHA and EPA, the range of change in concentrations in erythrocyte membranes was from -1.8% to +5.6% (DHA) and from -0.9% to +6.2% (EPA). In the placebo group, there was no change in enrichment in EPA and there was a very small increase in DHA enrichment. 42 participants had the PNPLA3 148I/I genotype, 43 had the 148I/M genotype and 13 had the 148M/M genotype. **Tables 7** describe the baseline characteristics of the cohort stratified by PNPLA3 genotype. With regard to MRS liver fat percentage, there was a trend towards an increase in liver fat in the group of participants carrying the GG genotype (mean liver fat GG genotype=25.1%; SD=1.8) compared to the group of participants carrying the CC and CG genotype (mean liver fat CC genotype=19.4%; SD=2.5 and mean liver fat CG genotype=19.9%; SD=2.3) (Table 7). However, there were no significant differences between the three genotypes groups (p=0.3). Moreover, there was no difference in

liver fat percentage between GG and CC group (mean liver fat GG genotype=25.1%; SD=1.8 and mean liver fat CC genotype=19.4%; SD=2.5; p=0.1).

There were between group differences for DHA percentage (p = 0.03). With regard to TM6SF2 (E167K) genotype, 80 participants had the TM6SF2 167E/E genotype, 15 had the TM6SF2 167E/K genotype and three had the TM6SF2 167K/K genotype **(Table 8)**.

Table 6. Baseline and end of study biochemical variables, anthropometric characteristics and lipid lowering medication.

	Placebo group			DHA+EPA group		
Variables	**Baseline**	**End of study**	***p*-value**	**Baseline**	**End of study**	***p*-value**
Age (y)	54.0 (9.6)	55.4 (9.6)	n/a	48.6 (11.1)	50.1 (11.1)	n/a
Sex (M/F)	35/17	32/16	n/a	25/26	23/24	n/a
Weight (kg)	94.5 (15.8)	90.4 (16.3)	0.76	96.5 (17)	94.1 (13)	0.38
BMI (kg/m^2)	32.4 (4.5)	30.8 (4.5)	0.74	34.0 (5.8)	33.4 (4.9)	0.30
Prudent Diet Score*	0.09 (0.97)	-0.07(0.9)	0.46	-0.1 (1.0)	0.03 (1.0)	0.55
Alcohol intake (Units/week)	6.3	6.8	0.88	2.3	2.2	0.67
Waist circumference (cm)	108.1 (11.5)	107.7 (10.3)	0.65	114.4 (13.4)	112.3 (10.4)	0.96
Diastolic blood pressure (mmHg)	86.3 (7.4)	82.9 (6.5)	0.75	85.4 (12.3)	81.7 (8.2)	0.006
Systolic blood pressure (mmHg)	137.7 (15.1)	133.9 (11.3)	0.13	138.2 (17.4)	133.3 (13.7)	0.004
Fasting plasma glucose (mmol/L)	6.2 (2.0)	6.7 (3.0)	0.07	6.2 (1.2)	6.1 (2.0)	0.77
Fasting plasma insulin (µUnit/ml)	11.7 (11.2)	10.2 (9.3)	0.68	12.3 (7.1)	13.9 (6.4)	0.18
HbA1c (%)	6.7 (1.2)	6.0 (2.0)	0.18	5.9 (0.9)	5.7 (2.0)	0.64
Serum triglycerides (mmol/L)	1.5 (0.5)	1.8 (0.6)	0.05	1.8 (0.7)	1.5 (1.2)	0.018
Serum cholesterol (mmol/L)	4.5 (0.8)	4.8 (1)	0.28	4.9 (1.1)	4.7 (1.1)	0.17
LDL-cholesterol (mmol/L)	2.7 (0.7)	2.8 (0.8)	0.38	3.0 (0.9)	2.8 (0.9)	0.12
HDL-cholesterol (mmol/L)	1.1 (0.3)	1.1 (0.2)	0.91	1.0 (0.2)	1.1 (0.3)	< 0.0001
ALT (IU/L)	59.5 (45)	48.5 (25)	0.03	55.0 (51)	44.0 (34)	0.89
AST (IU/L)	50.0 (25)	35.0 (17)	0.02	39.0 (24)	30.0 (27)	0.97
DEXA total fat mass (g)	33252 (8734)	30822 (8136)	0.34	38128 (10565)	35694 (8061.6)	0.09
DEXA total lean mass (g)	59017 (11344)	56396 (11237)	0.14	56353 (11564)	56218 (10799)	0.61
DEXA andro/gynoid (ratio)	1.2 (0.2)	1.2 (0.1)	0.13	1.2 (0.1)	1.1 (0.1)	0.72
MRI subcutaneous fat (%)	30.4 (9.7)	28.8 (9)	0.43	35.4 (10.5)	32.0 (9.6)	0.47
MRI visceral fat (%)	16.7 (4.7)	16.5 (5.4)	0.36	15.2 (5.1)	15.9 (4.7)	0.67
MRS liver fat %	21.7 (13.7, 32.3)	19.7 (11.3, 28.0)	0.006	23.0 (12.0, 47.5)	16.3 (9.0, 30.7)	0.01

Erythrocyte DHA (%) enrichment	4.1 (1.6)	5.0 (1)	0.002	3.8 (1.2)	7.1 (1.3)	< 0.0001
Erythrocyte EPA (%) enrichment	0.9 (0.4)	1.0 (0.2)	0.17	0.8 (0.3)	2.4 (1.8)	< 0.0001
Fibrates n	4	4	1.0	3	5	0.5
Statins n	23	23	1.0	19	24	0.06
Ezetimibe n	5	3	0.5	1	2	1.0

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed are expressed as median (inter-quartile range (IQR)).
*Prudent Diet Score (continuous variable derived from food frequency questionnaire).

Table 7. Baseline characteristics according to PNPLA3 genotype (I148M)

Phenotypes	**Genotypes**			
	C/C N=42	C/G N=43	G/G N=13	*P value (difference between groups)*
Weight (kg)	96.8 (20.2)	97.9 (15.4)	89.3 (10.8)	0.5
BMI (kg/m^2)	34.6 (6.5)	33.2 (4.8)	31.7 (4.5)	0.2
Fasting plasma glucose (mmol/L)	5.3 (1.9)	5.5 (1.2)	5.4 (2.0)	0.3
HOMA-IR	2.5 (2.4)	3.1 (4.6)	2.6 (1.1)	0.3
Serum triglyceride (mmol/L)	1.6 (1.2)	1.7 (0.9)	1.4 (0.3)	0.4
Serum cholesterol (mmol/L)	4.9 (0.9)	4.6 (1.1)	4.6 (0.7)	0.3
HDL-cholesterol (mmol/L)	1.1 (0.4)	1.1 (0.3)	1.1 (0.3)	0.9
LDL-cholesterol (mmol/L)	3.0 (0.9)	2.7 (0.9)	2.9 (0.7)	0.5
ALT (IU/L)	53 (44)	55 (33)	70 (65)	0.4
AST (IU/L)	38 (21)	39 (26)	45 (54)	0.5
Erythrocyte EPA (%) baseline	0.8 (0.3)	0.9 (0.4)	0.7 (0.3)	0.1
Erythrocyte DHA (%) baseline	3.7 (1.2)	4.3 (1.4)	3.6 (1.4)	0.03
MRS liver fat (%)	19.4 (2.5)	19.9 (2.3)	25.1 (1.8)	0.3

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed are expressed as median (inter-quartile range (IQR)). (CC = 148II, C/G=148 IM, GG= 148MM).

Table 8. Baseline characteristics according to TM6SF2 genotype

Phenotypes	Genotypes		
	CC	**CT+TT**	***P value (difference between groups)***
Weight (kg)	94.9 (15.1)	98.5 (20.9)	0.5
BMI (kg/m^2)	32.6 (4.5)	35.4 (8.1)	0.05
Fasting plasma glucose (mmol/L)	6.3 (2.7)	6.1 (1.3)	0.6
HOMA-IR	2.7 (2.9)	4.0 (5.8)	0.7
Serum triglyceride (mmol/L)	1.6 (1.1)	1.5 (0.7)	0.02
Serum cholesterol (mmol/L)	4.7 (1.0)	4.5 (0.9)	0.1
HDL-cholesterol (mmol/L)	1.0 (0.4)	1.0 (0.3)	0.6
LDL-cholesterol (mmol/L)	2.8 (1.0)	2.8 (0.6)	0.3
ALT (IU/L)	55.0 (51)	55.0 (58)	0.8
AST (IU/L)	42.0 (24)	39.0 (40)	0.8
MRS liver fat %	28.5 (20.3)	29.0 (19.1)	0.9
Erythrocyte EPA (%) baseline	1.1 (1.4)	0.5 (0.7)	0.1
Erythrocyte DHA (%) baseline	4.0 (1.4)	4.1 (0.8)	0.6

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed are expressed as median (inter-quartile range (IQR)). (CC = TM6SF2 167E/E, C/T= TM6SF2 167E/K, TT= TM6SF2 167K/K).

4.2.4.2 The effects of PNPLA3 (I148M) and TM6SF2 (E167K) genotypes on DHA and EPA enrichment

The results of this chapter show that, PNPLA3 148M/M genotype, and not either the TM6SF2 (E167K), TM6SF2 167E/K+K/K or TM6SF2 167E/E genotypes, was associated with an increase in liver fat percentage (+1.2%) at the end of the study regardless of randomisation group. In the treatment group, there were two participants carrying the PNPLA3 148M/M genotype, therefore it was not possible to compare the treatment group with the placebo group. However, in the treatment group there was a trend towards an increase in MRS liver fat percentage at the end of the study. Whereas, in the placebo group there were eight people carrying the PNPLA3 148M/M genotype and at the end of the study there was no change in the MRS liver fat percentage. The independent association of PNPLA3 (I148M) 148M/M genotype with either erythrocyte DHA or EPA % enrichment between baseline and end of study was tested. For DHA enrichment, the multivariable linear regression model included % change in erythrocyte DHA enrichment (end of study – baseline % enrichment) as the outcome, and age, sex, baseline liver fat %, PNPLA3 148M/M genotype, TM6SF2 genotype TM6SF2 167E/K+167K/K, BMI, diabetes, % DHA enrichment at baseline, total fat mass, change in CK- 18 M65 (between end of study and baseline), TG concentration at baseline, use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline and capsule count as explanatory variables. In this model, PNPLA3 148M/M was independently associated with % DHA enrichment (unstandardized β coefficient enrichment -1.02 (95%CI -1.97, -0.07), p=0.036). In contrast, when this regression model was adjusted to test associations between PNPLA3 148M/M genotype and % EPA enrichment, there was no significant association (unstandardized β coefficient -0.31 (95%CI -1.38, 0.75), p=0.56). TM6SF2 genotype was not independently associated with either EPA or DHA enrichment.

4.2.4.3 Effect of PNPLA3 and TM6SF2 genotypes on end of study liver fat percentage and end of study triglyceride concentration

Between baseline and end of study the change in liver fat % was -7.0 (14) (median and IQR) in the PNPLA3 148I/I+148M/M genotype groups compared with +1.2 (9.0) (median and IQR) in the 148M/M group (p=0.027). The change in liver fat was -4% (20) (median and IQR) in the TM6SF2 167EE genotype and in the TM6SF2 167E/K+K/K genotype it was 0.75 (12.3) (median and IQR). In the DHA+EPA group, the mean of MRS liver fat difference in people carrying the CC genotype (n=21) was -6%, whereas in people carrying the GG genotype (n=2) was +8% (p=0.2). In the placebo group, the mean and standard deviation (SD) of MRS liver fat difference in people carrying the CC genotype (n=19) was -7% (SD 10), whereas in people carrying the GG genotype

(n=8) was -0.4% (SD 9) (p=0.1). Between baseline and end of study the change in serum TG concentration was (median and IQR) -0.1 (0.8) in the PNPLA3 148I/I+148M/M genotype groups compared with +0.3 (0.9) in the 148M/M group (p=0.22). In the presence of the TM6SF2 167EE genotype the changes in TG were -0.1 mmol/L (0.8) (median and IQR) and -0.1 mmol/L (0.6) (median and IQR) in the TM6SF2 167E/K+K/K genotype group respectively. The effect of PNPLA3 I148M/M genotype on end of study liver fat percentage and end of study TG concentration was then investigated adjusting for covariates and potential confounders. **Table 9** shows the factors independently associated with the end of study liver fat %. DHA enrichment and decrease in weight (kg) during the 15-18 months of the trial, were both independently associated with end of study % liver fat and baseline % liver fat was also independently associated with end of study % liver fat.

Table 9. Factors independently associated with end of study liver fat percentage with DHA+EPA treatment.

Variables	Unstandardized B coefficient	95%CI	*p*-value
Baseline liver fat (%)	-0.39	-0.60, -0.28	<0.0001
Total body fat mass (kgs)	0.58	0.09, 1.07	0.021
Change in body weight (kg)	0.79	0.25, 1.32	0.004
PNPLA3 (I148M) genotype GG	9.5	2.53, 16.39	0.008
% DHA enrichment	-1.50	-2.82, -0.19	0.025

Factors included in the model, age, sex, baseline liver fat %, PNPLA3 genotype, TMS6SF2 genotype, % DHA enrichment (end of study – baseline % enrichment), baseline serum triglyceride, total fat mass, change in M65 (between end of study and baseline), BMI, diabetes, change in weight, use of orlistat at baseline, use of L thyroxine at baseline and capsule count. R^2=0.54, p<0.0001.

Additionally, PNPLA3 148M/M genotype was independently associated with end of study % liver fat (β=9.5 (95%CI 2.53, 16.39), p=0.008). Overall this model **(Table 9)** accounted for 54% of the variance in end of study % liver fat (R^2=0.54, p<0.0001). The effect of PNPLA3 148M/M genotype on end of study TG concentration was then investigated. From the results of univariate analyses (data not shown), factors included in the model, were age, sex, baseline liver fat %, MRS difference, PNPLA3 genotype, TM6SF2 167E/K+K/K, BMI, diabetes, % EPA enrichment (end of study – baseline % enrichment), total fat mass, change in M65 (between end of study and baseline), TG concentration at baseline, and use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline, and capsule count. Factors that were independently associated with end of study TG concentrations are shown in **Table 10**. In contrast to the data for end of study % liver fat, PNPLA3 148M/M genotype was not associated with end of study TG concentration (β=-0.11 (95%CI -0.64, 0.42), p=0.68). Additionally, % EPA enrichment (β=-0.19 (95%CI -0.31, -0.07), p=0.002) and not % DHA enrichment was independently associated with end of study TG concentration. Factors included in the regression model shown in **Table 10** explained 56% of the variance in end of study TG concentration (R^2=0.56, p<0.0001). There was not relationship between TM6SF2 genotype and MRS liver fat or fasting TG concentrations. However, there was a significant difference in fasting TG at baseline by genotype; for the TM6SF2

167E/K+K/K genotype, TG at baseline were lower (1.5 mmol/L (0.7) (median and IQR)) than for the TM6SF2 167E/E genotype (1.8 mmol/L (1.4) (median and IQR)) (p=0.02).

Since the evidence suggests that PNPLA3 148M/M genotype influences hepatic fat accumulation and liver damage with an additive effect of each 148M allele [209 210], the additive effective of allelic variation for PNPLA3 (I148M) genotype on change in liver fat percentage and change in fasting serum TG was tested between baseline and end of study. Each ANCOVA model **(Table 11)** was adjusted for the same potential confounders as shown in **Tables 9 and 10**. These data show that although there was a change in liver fat % between baseline and end of study in each PNPLA3 genotype, the adjusted mean difference in liver fat % was greater for PNPLA3 148I/I and for PNPLA3 148IM/M (with a decrease in liver fat % between baseline and end of study), compared with the PNPLA3 148M/M group where there was a small increase in liver fat %. For change in TG concentration between baseline and end of study, there was no significant effect of PNPLA3 genotype (in keeping with the results of regression modelling presented in **Table 9)**.

Table 10. Factors independently associated with end of study fasting triglyceride concentration with DHA+EPA treatment.

Variables	Unstandardised B coefficient	95%CI	*p*-value
Age (y)	-0.03	-0.05, -0.02	<0.0001
Being male	0.54	0.13, 0.97	0.011
Baseline triglycerides (mmol/L)	-0.47	-0.60, -0.34	<0.0001
Total body fat mass (kg)	-0.03	-0.05, -0.01	0.003
Capsule count	-0.24	-0.45, -0.03	0.025
Use of beta blockers	0.85	0.07, 1.63	0.034
% EPA enrichment	-0.19	-0.31, -0.07	0.002

Factors included in the model, age, sex, baseline liver fat %, PNPLA3 genotype, TMS6SF2 genotype, % EPA enrichment (end of study – baseline % enrichment), BMI, total fat mass, diabetes, change in M65 (between end of study and baseline), change in weight, triglyceride concentration at baseline, and use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline and capsule count. (N.B. PNPLA3 genotype GG (I148M) (B coefficient -0.02 (95%CI -0.52, 0.49, p=0.95). Final model R^2=0.56, p<0.0001).

Table 11. Adjusted mean differences for change in liver fat percentage, change in serum fasting triglyceride concentration and change in DHA percentage enrichment with DHA+EPA treatment, according to PNPLA3 genotype.

Phenotypes	**Genotypes**		
	CC N=42	**CG N=43**	**GG N=13**
Adjusted mean change in liver fat % (95%CI)	-7.05 (-10.77, -3.33)	-7.30 (-10.75, -3.85)	2.75 (-4.22, 9.73)
*Adjusted mean change in triglyceride (mmol/L) (95%CI)	-0.12 (-0.41, 1.62)	-0.09 (-0.36, 0.17)	-0.20 (-0.71, 0.31)
§Adjusted mean change in DHA (%) (95%CI)	1.84 (1.27, 2.41)	2.06 (1.52, 2.60)	0.75 (-0.23, 1.72)

Change in liver fat %: ANCOVA model adjustments: age, sex, baseline liver fat %, PNPLA3 genotype, TM6SF2, BMI, diabetes, % DHA enrichment (end of study – baseline % enrichment), baseline serum triglyceride, total fat mass, change in M65 (between end of study and baseline), change in weight, use of orlistat at baseline, use of L thyroxine at baseline and capsule count.
Pairwise comparisons C/C v GG, p=0.02 and C/G v GG p=0.012.

*Change in triglycerides: ANCOVA model adjustments: age, sex, baseline liver fat %, PNPLA3 genotype, TM6SF2, BMI, diabetes, % EPA enrichment (end of study – baseline % enrichment), total fat mass, change in M65 (between end of study and baseline), change in weight, triglyceride concentration at baseline, and use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline and capsule count. Pairwise comparisons C/C v GG, p=0.8 and C/G v GG p=0.71

§Change in DHA % enrichment: ANCOVA model adjustments: age, sex, baseline liver fat %, PNPLA3 genotype, TM6SF2, BMI, diabetes, % DHA baseline, baseline serum triglyceride, total fat mass, change in M65 (between end of study and baseline), change in weight, use of orlistat at baseline, use of L thyroxine at baseline and capsule count. Pairwise comparisons C/C v GG, p=0.06 and C/G v GG p=0.023

4.2.5 Discussion

The results of this chapter show that, PNPLA3 148M/M genotype, and not either the TM6SF2 (E167K), TM6SF2 167E/K+K/K or TM6SF2 167E/E genotypes, was associated with an increase in liver fat percentage (+1.2%) at the end of the study regardless of randomisation group. In the treatment group, there were two participants carrying the PNPLA3 148M/M genotype, therefore it was not possible to compare the treatment group with the placebo group. However, in the treatment group there was a trend towards an increase in MRS liver fat percentage at the end of the study. Whereas, in the placebo group there were eight people carrying the PNPLA3 148M/M genotype and at the end of the study there was no change in the MRS liver fat percentage. In contrast, neither PNPLA3 (I148M), nor TM6SF2 (E167K) genotypes were associated with end of study serum TG concentrations. Interestingly, after adjusting for baseline liver fat percentage, baseline body fat mass, and other covariates and confounders, the key independent factors associated with end of study liver fat percentage, were: a) a decrease in weight during the trial, b) baseline body fat, c) an increase in tissue percentage of DHA enrichment, d) PNPLA3 148M/M genotype, and e) baseline liver fat percentage **(Table 9)**.

A meta-analysis of 16 studies (2937 subjects) across different populations with NAFLD assessed the strength of the effect of PNPLA3 148M/M genotype on liver fat. In this meta-analysis, PNPLA3 148M/M genotype was associated with 73% higher liver fat content compared with the 148I/I variant [209]. In keeping with this evidence, in the WELCOME study participants at baseline, median liver fat percentage was 28.5% in subjects with PNPLA3 148M/M compared with 22.6% in subjects with 148I/I or 148I/M. However, to date it is uncertain how PNPLA3 148M/M genotype modifies any response to treatment interventions in NAFLD. Recently it has been suggested that there was a greater reduction in liver fat percentage with a 12 month lifestyle intervention in subjects with the PNPLA3 148M/M genotype [211] than in subjects with 148I/M or 148I/I genotype. However, initial baseline mean liver fat percentage in the intervention group was surprisingly low (i.e. 5.5%) and was only 4.3% in the PNPLA3 148M/M group. As can be seen from these results and from the unstandardized β coefficient in **Table 9**, even after adjusting for baseline liver fat percentage, PNPLA3 148M/M genotype was associated with 10% higher end of study liver fat than seen in subjects with either PNPLA3 148I/I or 148I/M. Importantly, this effect of PNPLA3 148M/M genotype was independent of any benefit conferred by decrease in body weight and increased percentage of DHA enrichment **(Table 9)**.

DHA+EPA treatment produced highly variable inter-individual DHA and EPA tissue enrichment, and importantly this enrichment was independent of compliance and the numbers of capsules

returned unused during the trial. PNPLA3 148M/M was associated with decreased DHA enrichment but was not associated with EPA enrichment. It is known that omega-3 fatty acids are rapidly incorporated into plasma membranes where they affect membrane fluidity and membrane permeability [212] and that measurement of erythrocyte DHA and EPA enrichment is considered a good proxy for omega-3 fatty acid enrichment and bioavailability in liver [213]. However, based the results shown in this chapter, PNPLA3 I148M is involved in DHA/EPA mobilization in the liver, and subjects with PNPLA3 148M/M genotype have lower levels of DHA [214]. Additionally, omega-3 fatty acids decrease the expression of sterol response element binding protein 1c (SREBP1c), a key regulatory factor in hepatic lipogenesis [215], and recently it has been shown that carriers of the PNPLA3 148M allele have decreased *de novo* lipogenesis [216]. Thus, it is possible that the lack of response to DHA+EPA treatment in decreasing liver fat percentage in subjects with PNPLA3 I148M/M could be due to the fact that these subjects already have low levels of *de novo* lipogenesis. That said, it is not known whether PNPLA3 I148M/M genotype affects the incorporation of DHA (or EPA) into the liver lipid droplet, although recent evidence in a small study in children suggests that the PNPLA3 148M/M genotype attenuates the benefit of DHA to decrease liver fat [217]. These data in children are in agreement with, and are extended by, our data, showing PNPLA3 148M/M genotype is associated with lower levels of DHA enrichment. Importantly, PNPLA3 I148M/M genotype is associated with a ~1 SD decrease (i.e. a 1.2% decrease) in erythrocyte DHA enrichment (unstandardized β coefficient -1.02 (95% CI -1.97, -0.07), p=0.036). A 1 SD decrease in percentage of DHA enrichment means that most individuals with the PNPLA3 I148M/M genotype do not achieve the 2% DHA enrichment threshold that was necessary to achieve satisfactory reductions in liver fat in this cohort [218]. Since the results in **Chapter 4.2** show that higher levels of percentage DHA enrichment are associated with lower levels of liver fat [218], it is noteworthy that most patients with PNPLA3 I148M/M genotype do not achieve high levels of percentage DHA enrichment. Similarly, others have shown an effect of PNPLA3 genotype to modify treatment effect and a recent research study by Dongiovanni et al. [219] has shown that statin treatment was associated with a reduction in liver fat content and inflammation in individuals with NAFLD, carrying the PNPLA3 148I/I genotype. The effect was absent in those with the PNPLA3 148M/M genotype. The mechanism by which PNPLA3 I148M/M genotype affects fasting TG concentration is still uncertain. Hyysalo et al. showed in non-obese people with NAFLD that the PNPLA3 I148M/M genotype is associated with hepatic hydrolysis of TG, reducing hepatic VLDL secretion [204] but it is uncertain whether the PNPLA3 I148M/M genotype modifies any change in TG concentrations induced by an intervention. The daily treatment with 4 g of DHA+EPA in this study provided similar amounts of DHA (1520 mg) and EPA (1840 mg) as ethyl esters and the data reported in this chapter suggest that a greater TG-lowering

effect was associated with EPA enrichment rather than DHA enrichment. **Table 9** shows the factors that were associated with end of study fasting serum TG concentrations (adjusting for baseline measurement and other confounders and covariates). These data show that many factors (but not PNPLA3 or TM6SF2 genotypes) were independently associated with TG concentrations and, as expected, compliance (capsule count) was independently associated with end of study TG concentration. This finding is also consistent with licensing data for DHA+EPA for the treatment for high serum TG concentrations, where 4 g per day is the highest licenced dose, and 4 g is more effective in lowering serum TG concentrations than 2 to 3 g/day. It is possible that PNPLA3 I148M genotype only influences VLDL secretion (and thereby fasting TG concentrations), when there are specific hepatic lipids available for incorporation into the secreted VLDL particle [30]. Thus, it is plausible that PNPLA3 I148M genotype may have little or no effect on VLDL levels when there is a modification in the quality of fatty acids and the type of hepatic lipid content [220] (e.g. which may occur after DHA treatment).

This study is the first study to test the effects of the PNPLA3 (I148M) and TM6SF2 (E167K) genotypes on relevant end points in NAFLD as part of an intervention trial testing the effects of high dose DHA+EPA treatment in adults. The data presented in this chapter showed the effects of genotypes on change in liver fat percentage and change in serum TG concentration in NAFLD, rather than testing the effect of genotypes on changes in liver fibrosis biomarkers or other biomarkers of NAFLD severity that are known to be affected by PNPLA3 (I148M) and TM6SF2 (E167K). The reason for this was that these measures did not change between baseline and end of study during the trial.

In conclusion, DHA enrichment and loss of weight during the randomised double-blind placebo controlled trial both independently decreased end of study liver fat percentage, adjusting for baseline liver fat percentage and all other measured covariates and confounders. PNPLA3 148M/M, but not TM6SF2, genotype was strongly and negatively associated with DHA tissue enrichment and was associated with markedly higher (~10%) end of study liver fat levels. In contrast, end of study fasting TG concentrations were strongly associated with percentage of EPA enrichment but not PNPLA3 (I148M) or TM6SF2 (E167K) genotypes.

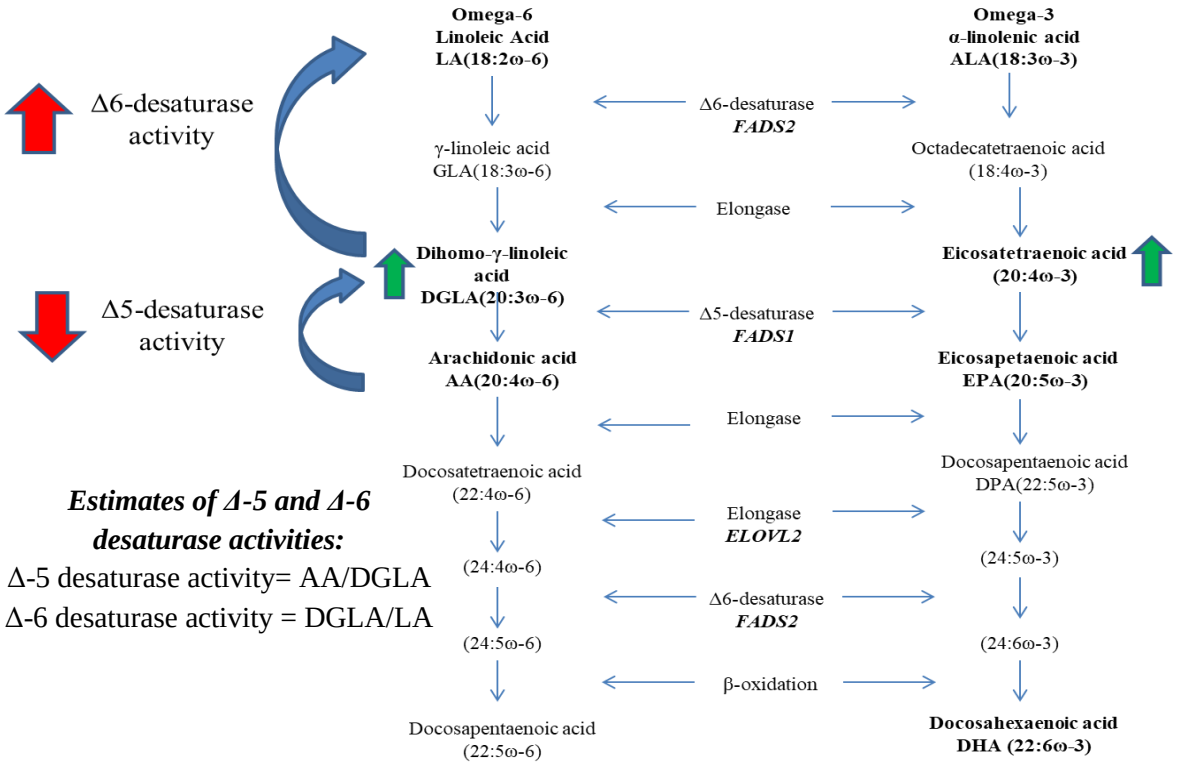
4.3 The effect of Fatty Acid Desaturase (FADS) and Elongase (ELOVL) polymorphisms on indexes of desaturase activity and liver fat in NAFLD.

4.3.1 Introduction

Tissue levels of omega-3 and omega-6 fatty acids are affected by both dietary intake and endogenous synthesis that occurs via desaturation and elongation of fatty acid precursors (ALA and LA). The fatty acid desaturase 1 and fatty acid desaturase 2 (FADS1 and FADS2) genes encode for Δ-5 and Δ-6 desaturases, that 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 [221]. Elongases are encoded by members of the elongation-of-very-long-chain-fatty-acids (ELOVL) gene family located on chromosome 6 [222]. 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 [125,223], NAFLD [224], metabolic syndrome, [225] and dyslipidaemia[125-129,223]. These studies have also shown that low Δ5-desaturase enzyme activity was associated with accumulation of desaturase substrates and low desaturase products [130,131](**See Figure 11**). 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 [226-229]. 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.
The aims of this chapter are to test whether:

a. SNPs of the FADS gene cluster and ELOVL gene family affect:
    i. Δ-5 and Δ-6 desaturase activities as determined by product-to-precursor ratios (see **Figure 11**),
    ii. DHA, EPA and AA percentage in erythrocyte membranes, and
    iii. liver fat percentage measured by MRS;
b. to test whether there is a gene-DHA+EPA interaction affecting
    i. both Δ-5 and Δ-6 desaturase activities,
    ii. DHA, EPA and AA percentage in erythrocyte membranes.

Figure 11. Influence of Δ-5 and Δ-6 desaturase activity on omega-6 and omega-3 fatty acid product/precursor ratio. Influence of Δ-5 and Δ-6 desaturase activities on long-chain polyunsaturated fatty acid synthesis. Erythrocyte fatty acid composition reflects both dietary intake and the endogenous efficiency of elongation and desaturation of their dietary precursors (LA and ALA). **The red arrows** show that the decrease in Δ-5 desaturase activity and increase in Δ-6 desaturase activity result in accumulation of Δ-5 desaturase substrates DGLA and ETA (**green arrows**).



4.3.2 Hypotheses

a. In patients with NAFLD, SNPs of the FADS gene cluster and ELOVL gene family affect
    i. Δ-5 and Δ-6 desaturase activities as determined by product-to-precursor ratios (see **Figure 11**),
    ii. DHA, EPA and AA percentage in erythrocyte membranes, and
    iii. liver fat percentage measured by MRS;
b. to test whether there is a gene-DHA+EPA interaction affecting
    i. both Δ-5 and Δ-6 desaturase activities,
    ii. DHA, EPA and AA percentage in erythrocyte membranes.

4.3.3 Methods

4.3.3.1 Subject and measurements

In 103 WELCOME study participants, sixteen SNPs in FADS1, FADS2 gene cluster and ELOVL gene family were genotyped. All participants had NAFLD and features of metabolic syndrome defined using the International Diabetes Federation criteria [195], the criteria for defining NAFLD were reported previously (Chapter 3.2). MRS of hepatic lipid content was undertaken at the beginning and at the end of the study. Any change in diet during the study was assessed by food frequency questionnaire and a 'prudent diet score' as a healthy diet index was also generated, using principal component analyses as previous described [188].

4.3.3.2 DNA analysis

Sixteen SNPs of the FADS1-FADS2 gene locus (rs174545, rs174546, rs174547, rs174548, rs174550, rs174555, rs174556, rs174549, rs174574, rs174576, rs174577, rs174568, rs1535, rs174581, rs174562, rs174561) and 2 SNPs of the ELOVL2 gene region (rs12195587 and rs4532436) were genotyped with Sequenom MassARRAY iPLEX Gold assays (Sequenom, San Diego, CA).

4.3.3.3 Statistical analysis

All statistical analyses were performed using SPSS for Windows (version 23.0; SPSS). We examined baseline and end of study characteristics in DHA+EPA and placebo groups. Data are reported as means and SDs for normally distributed variables, or as median and interquartile range (IQR) for non-normally distributed variables. Comparisons of means between groups were performed by using t-tests for normally distributed variables and Mann Whitney U for non-normally distributed unpaired variables. Estimates of Δ-5 and Δ-6 desaturase activities were computed using product-to-precursor ratios: (C20:4n-6, arachidonic acid, AA):(C20:3n-6 dihomo-γ-linolenic acid, DGLA), and (C20:3n-6, DGLA):(C18:n-6, linoleic acid, LA), respectively, as previously described [230]. The selected SNPs of the FADS gene cluster (rs174545, rs174546, rs174547, rs174548, rs174550, rs174555, rs174556, rs174549, rs174574, rs174576, rs174577, rs174568, rs1535, rs174581, rs174562, rs174561) and the ELVOLV family (ELOVL2 rs12195587, ELOVL2 rs4532436) were studied. Multivariable linear regression modelling with stepwise elimination approach was undertaken to investigate associations between genotypes and the following outcomes in the baseline data-set: a) Δ-5 and Δ-6 desaturase activity, b) DHA, EPA and AA erythrocyte concentration, and c) MRS liver fat percentage. Additionally, multivariable linear regression

modelling with stepwise elimination was undertaken to test the effect of genotype-DHA+EPA interaction on the following end of study outcomes: a) Δ-5 and Δ-6 desaturase activity, b) DHA, EPA and AA erythrocyte concentration, and c) MRS liver fat percentage. Each of these separate regression models was adjusted for the baseline measurement of the outcome measured in question. Covariates, potential confounders and change in potential confounders between baseline and end of study were included, to test the independence of associations between key exposures and the outcome in question. Explanatory variables that were included in the models were continuous or categorical. The MIXED model was used to test for the effect of the genotype × DHA+EPA treatment interaction for the FADS rs174556 on Δ-5 desaturase activity adjusted for age, sex and BMI. For the selected SNPs within the FADS gene cluster and ELOVL gene family, a binary indicator variable was created and coded per minor allele = 1 and major allele = 0. Dichotomous variables were created for both Δ-5 and Δ-6 desaturase activity to indicate low and high enzymatic activity. Low desaturase activity was defined = 0 (below the median) and high desaturase activity was defined = 1 (above the median). All genotype distributions were tested for deviation from Hardy-Weinberg equilibrium using a χ^2 test with 1 df ($P>0.05$).

4.3.4 Results

4.3.4.1 Baseline association between SNPs of the FADS gene cluster and ELOVL family and Δ-5 and Δ-6 desaturase activities

In 103 WELCOME study participants, sixteen of the SNPs of the FADS gene cluster and two SNPs of the ELOVL family were genotyped. Mean and standard deviation or median and interquartile range for baseline characteristics of biochemical measurements and erythrocyte fatty acids percentage are shown in **Table 12**. Frequencies of the alleles for the selected SNPs within the FADS gene cluster and ELOVL gene family analysed in the WELCOME study cohort are shown in **Table 13**. These SNPs were chosen for the current analysis because of their previous association with omega-3 fatty acid synthesis and desaturase activity [119,129,231]. All genotypes were in Hardy-Weinberg equilibrium.

Table 12. Baseline biochemical variables, anthropometry characteristics and erythrocytes fatty acid percentage.

Variables	Mean –Median	SD-IQR	Minimum-Maximum
Weight (Kg)	94.3	±16.1	59.2-130
Height (cm)	169	±9.5	146-189
Waist circumference	110	±11.7	86-153
BMI (Kg/m2)	33	±5	22.6-50.2
Glucose (mmo/l)*	5.5	1.7	4-19
HbA1c (%tot)*	6.6	±1.6	4.9-13.3
Cholesterol (mmol/l)	4.7	±1.0	2.5-8.1
HDL (mmol/l)	1.1	±0.3	0.5-1.8
LDL (mmol/l)*	2.9	±0.9	1.1-5.5
Chol/HDL ratio	4.6	±1.4	2.3-8.7
TG (mmol/l)*	1.6	1.0	0.6-3.7
ALT (iu/l)*	56	46	19-206
AST (iu/l)*	41	37	18-254
GGT (U/l)*	50	73	17-1377
Hyaluronic acid (ug/l)*	23	29	10-253
P3 N terminal peptide (ug/l)*	5.4	2.8	2.3-23.3
MRS average	21	26	1.7-85
EPA [20:5(n-3)]	0.9	±0.4	0.3-2.5
DHA [22:6(n-3)]	4	±1.4	0.9-8.5
ARA [20:4(n-4)]	12.7	±2.7	5-17.6
GLA [20:3n-6]	1.9	0.6	0.9-3.4
ALA [20:3n-6]	0.23	0.16	0.04-0.9

Normally distributed data are expressed as mean ± standard deviation (SD).

*Non-normally distributed data are expressed as median ± inter-quartile range (IQR).

Table 13. Characteristics of SNPs within the FADS gene cluster and ELOVL gene family.

SNP	Minor/major allele %	MAF
FADS1		
rs174545	G / C	39
rs174546	T / C	41
rs174547	C / T	39
rs174548	G/ C	34
rs174549	A / G	33
rs174550	C / T	39
rs174555	C/ T	33
rs174556	T/ C	33
FADS2		
rs1535	G/ A	40
rs174568	T/ C	39
rs174574	A/C	41
rs174576	A/C	40
rs174577	A/C	40
rs174581	A/G	40
rs174561*	C/ T	33
rs174562*	C/T	35
ELOVL2		
rs12195587	A/G	17
rs4532436	G/C	56

Distribution of the minor and major allele for the FADS1, FADS2 and ELOVL genes in the WELOCME study population. The minor allele frequency and Hardy-Weinberg equilibrium was calculated using http://oege.org/software/hwe-mr-calc.shtml

4.3.4.2 Associations between baseline Δ-5 and Δ-6 desaturase activities and SNPs within the FADS gene cluster and ELOVL gene family

In order to study associations between estimates of Δ-5 and Δ-6 desaturase activities and minor allele of the SNPs within the FADS gene cluster and ELOVL gene family, a stepwise linear regression model using baseline Δ-5 or Δ-6 desaturase activities as outcome and age, sex, BMI and all SNPs for the FADS gene cluster as the explanatory variables, two SNPs FADS1 rs174556 (β=-1.4; 95% CI -1.9, 0.7; p<0.0001) and FADS2 rs1535 (β=-1.4; 95% CI -2, -0.8; p<0.0001) were inversely associated with baseline Δ-5 desaturase activity. These inverse associations were suggesting that carriers of the minor alleles of the above SNPs (rs174556 and rs1535) had lower Δ-5 desaturase activity compared with common homozygotes. Besides, one SNP of FADS1 rs174546 (β=1.3; 95% CI 0.6, 2.1; p<0.0001), was found to be positively associated with baseline Δ-5 desaturase activity suggesting that there was an increase in desaturase activity in the presence of the minor allele. With regard to Δ-6 desaturase activity, the minor allele of the FADS1 rs174556 (β=0.05; 95% CI 0.01, 0.1; p=0.02) and FADS2 rs1535 (β=0.02; 95% CI 0.009, 0.04; *p*=0.002) were associated with an increased Δ-6 desaturase activity **(Table 14)**.

4.3.4.3 Association between Δ-5 and Δ-6 desaturase activities, FADS1, FADS2 and MRS liver fat percentage

Table 14 A, B, and C describes the baseline characteristics of the cohort stratified by FADS2 rs1535, rs174546 and rs174556 genotype. There were between group differences for Δ-5 and Δ-6 desaturase activities and for upstream fatty acid erythrocytes percentage. Δ-5 desaturase activity was lower (p≤0.0001) and Δ-6 desaturase activity was higher (p=0.02) in people carrying the minor allele compared with people carrying the homozygote-dominant. This combination of low Δ-5 desaturase activity and high Δ-6 desaturase activity resulted in an increase in the upstream fatty acid products (DGLA p≤0.0001, and ETA p= 0.005) in the group of people carrying the minor allele compared with people carrying the homozygote dominant. Moreover, there was a trend towards an increase in MRS liver fat percentage in the FADS rs1535 minor allele group compared with the homozygote-dominant. In order to understand the association between MRS liver fat, Δ-5 and Δ-6 desaturase activities, and FADS rs1535 genotype, three linear regression models were designed. The first regression model was designed using MRS liver fat at baseline as outcome and age, sex, BMI and Δ-5, and Δ-6 desaturase activities as explanatory variables. From this regression model, only Δ-5 desaturase activity (β=-0.2; 95% CI -0.3, -0.09; p=0.001) was significantly and inversely associated with baseline MRS liver fat accumulation. These data suggest that low Δ-5 desaturase activity was associated with high percentage MRS liver fat. Interestingly, in the second

regression model adjusted for age, sex, BMI and SNPs of the FADS gene cluster and ELOVL family and with MRS liver fat at baseline as outcome, only FADS2 rs1535 was independently and positively associated with MRS liver fat percentage at baseline (β=0.7 95% CI 0.08, 1.3; *p*=0.03).

It is noteworthy that, when FADS2 rs1535 was added to the third model comprising MRS liver fat as the outcome, and age, sex, BMI, Δ-5 desaturase, and Δ-6 desaturase as explanatory variables, only Δ-5 desaturase (β=-4.2; 95% CI -7.2, -1.2; *p*=0.006) and BMI (β=0.8; 95% CI 0.04, 1.5; *p*=0.04) were independently associated with MRS liver fat, and these two explanatory factors together explained 12% of the variance in liver fat percentage **(Figure 12)**.

Table 14 A. Baseline characteristics according to FADS rs1535 genotype.

Variables	**FADS2 rs1535**		***p*-value**
	Major (n=41)	Minor (n=52)	
Cholesterol (mmol/l)*	4.6 (0.9)	4.7 (1.4)	0.5
HDL (mmol/l)	1.0 (±0.3)	1.1 (±0.3)	0.4
LDL (mmol/l)	2.9 (±0.7)	2.9 (±0.9)	0.8
Chol/HDL	4.7 (2.4)	4.8 (2.2)	0.7
TG (mmol/l)*	1.6 (1.0)	1.7 (1.2)	0.9
AST (iu/l)*	42 (26)	38 (21)	0.5
ALT (iu/l)*	57 (48)	50 (39)	0.3
HA (ug/l)*	23 (32)	21 (27)	0.5
PIIINP (ug/l)*	6 (2.2)	5 (2.7)	0.1
MRS (%)*	18.3 (23.5)	25 (27)	0.2
Δ-5 desaturase (%)*	**7.7 (1.7)**	**6 (1.5)**	**<0.0001**
Δ-6 desaturase (%)	**0.1 (0.03)**	**0.2 (0.05)**	**0.02**
EPA (20:5n-3) (%)	0.8 (±0.3)	0.9 (±0.4)	0.2
DHA (22:6n-3) (%)	3.9 (±1.2)	4.1 (±1.5)	0.4
AA (20:4n-6) (%)*	14 (2.3)	13 (3.1)	0.07
LA (18:2n-6) (%)	12.5 (±2.1)	13.2 (±2)	0.1
GLA (18:3n-6) (%)*	0.2 (0.1)	0.14 (0.07)	0.05
ALA (18:3n-3) (%)*	0.24 (0.2)	0.25 (0.1)	0.5
DGLA (20:3n-6) (%)	**1.7 (±0.3)**	**2.1 (±0.4)**	**<0.0001**
ETA (20:4n3) (%)	**0.08 (±0.03)**	**0.1 (±0.04)**	**0.005**
DPA (22:5n-6) (%)*	2.3 (0.8)	2.4 (0.5)	0.3

Variables that are normally distributed are expressed as mean ± standard deviation (SD). *Variables that are non-normally distributed are expressed as median ± inter-quartile range (IQR).

Table 14 B. Baseline characteristics according to FADS rs174546 genotype.

Variables	rs174546		***p*-value**
	Major	Minor	
BMI	31.1 (8.4)	32 (7.2)	0.7
Cholesterol*	4.6 (1.4)	4.5 (1.3)	0.3
HDL	1.0 (0.3)	1.0 (0.2)	0.6
LDL	2.9 (0.8)	2.8 (0.9)	0.9
Chol/HDL	4.9 (1.5)	4.7 (1.5)	0.3
Triglycerides*	1.6 (1.0)	1.7 (1.3)	0.3
AST*	38 (21)	39 (22)	0.9
ALT*	51 (40)	54 (35)	0.6
HA*	17.5 (34)	21.5 (24)	0.4
PIIINP*	5.4 (2.2)	5.0 (2.4)	0.4
MRS*	22.6 (2.2)	20.8 (2.2)	0.9
D5D*	0.5 (1.8)	0.5 (1.2)	0.1
D6D	0.007 (0.03)	0.006 (0.03)	0.9
EPA	0.8 (0.2)	0.9 (0.4)	0.3
DHA	3.8 (0.9)	4 (1.5)	0.5
Arachidonic acid*	13.4 (2.5)	12.4 (5.3)	0.06
Linoleic acid	12.4 (2.1)	13.2 (2)	0.1
Alpha Linolenic acid*	0.3 (0.1)	0.3 (0.2)	0.9
DiHomo g Linolenic acid	1.7 (0.3)	2.0 (0.4)	<0.0001
Eicosatetraenoic acid	0.08 (±0.03)	0.11 (±0.04)	<0.0001

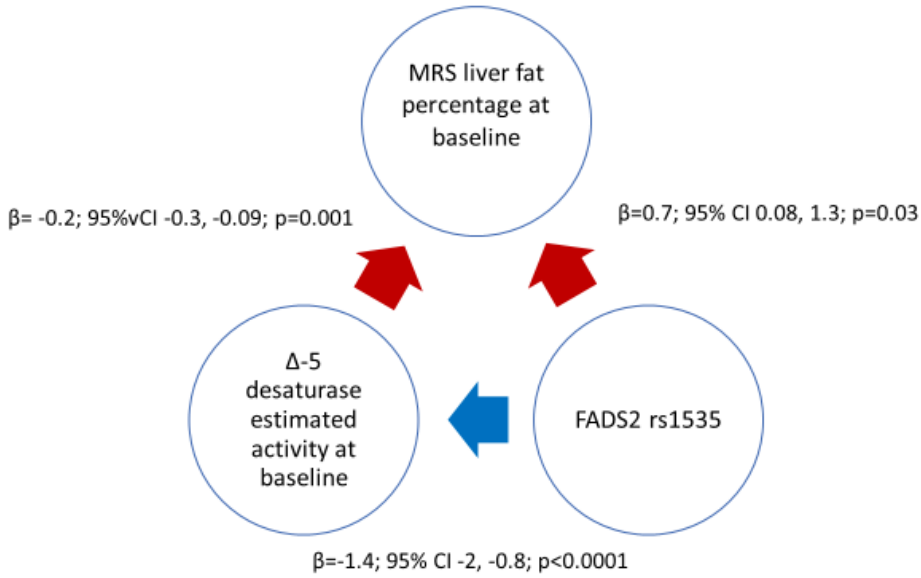
Variables that are normally distributed are expressed as mean ± standard deviation (SD). *Variables that are non-normally distributed are expressed as median ± inter-quartile range (IQR).

Table 14 C. Baseline characteristics according to FADS rs174556 genotype.

Variables	rs174556		***p*-value**
	Major (n=41)	Minor (n=52)	
BMI	33 (7.6)	32.6 (6.8)	0.7
Cholesterol *	4.5 (1.4)	4.7 (1.3)	0.2
HDL	1.0 (0.4)	1.1 (0.4)	0.4
LDL	2.8 (±0.8)	2.9 (±0.9)	0.6
Chol/HDL	4.7 (2.2)	4.4 (2)	0.9
Triglycerides*	1.5 (0.7)	1.8 (1.2)	0.4
AST*	41 (23)	38 (27)	0.7
ALT*	56 (53)	53 (45)	0.3
HA*	24 (31)	22 (27)	0.9
PIIINP*	6 (1.9)	5.3 (3.4)	0.2
MRS*	20.6 (30)	22.1 (26.3)	0.9
D5D*	0.6 (2.0)	0.4 (1.2)	0.1
D6D	0.007 (0.02)	0.006 (0.03)	0.9
EPA	0.8 (0.4)	1.0 (0.5)	0.01
DHA	3.8 (1.2)	4.2 (1.5)	0.1
Arachidonic acid*	13.7 (2.6)	13.1 (2.5)	0.2
Linoleic acid	12.5 (2.2)	13.2 (1.9)	0.08
Alpha Linolenic acid*	0.26 (0.1)	0.29 (0.2)	0.4
DiHomo g Linolenic acid	1.7 (0.4)	2.1 (0.4)	<0.0001
Eicosatetraenoic acid	0.1 (0.04)	0.08 (0.03)	<0.0001

Variables that are normally distributed are expressed as mean ± standard deviation (SD). *Variables that are non-normally distributed are expressed as median ± inter-quartile range (IQR).

Figure 12. Proposed direction of the associations between FADS rs1535, Δ-5 desaturase activity, and MRS liver fat percentage (schematic developed from the results of regression modelling data. Carriers of the minor allele for the SNP rs1535 have low Δ-5 desaturase activity that in turn leads to an increase accumulation of liver fat.



4.3.4.4 Association between baseline desaturase activity, SNPs of the FADS gene cluster and ELOVL family, and DHA, EPA and AA erythrocyte percentage

In order to study associations between baseline erythrocyte percentage of DHA, EPA or AA and estimates of baseline Δ-5 and Δ-6 desaturase activities, and minor allele of the SNPs within the FADS gene cluster and ELOVL gene family, three regression models were built using DHA, EPA or AA as outcomes and age, sex BMI, Δ-5 and Δ-6 desaturase activity, and SNPs of the FADS gene cluster and ELOVL family as explanatory variables.

The results from the first regression model show that baseline DHA erythrocyte percentage was independently and positively associated with baseline Δ-5 desaturase activity (β=0.3; 95% CI 0.2, 0.5; p<0.0001), FADS2 rs1535 (β=0.8 95% CI 0.2, 1.5; p=0.009) and FADS rs174546 (β=2.1; 95% CI 0.1, 3.9; *p*=0.04). The second regression model shown that baseline AA erythrocyte percentage was independently and positively associated with Δ-5 desaturase activity (β=1.3; 95% CI 1.0, 1.5; p<0.0001) and FADS rs174546 (β=0.2; 95% CI 0.04, 0.34; *p*=0.01) **(Table 15)**.

In the third regression model EPA erythrocyte percentage was positively associated with Δ-5 desaturase activity (β=0.05; 95% CI 0.006, 0.1; p=0.03), FADS2 rs1535 (β=0.2; 95% CI 0.005, 0.4; p=0.04) and FADS1 rs174556 (β=0.2; 95% CI 0.01, 0.3; p=0.034) suggesting that carriers of this specific minor allele were characterised with an accumulation of Δ-5 desaturase substrate in the omega-3 series.

There was no significant interaction between the SNPs of the ELOVL2 gene family and baseline Δ-5 desaturase substrate, MRS liver fat, DHA, EPA or AA erythrocyte concentration. Furthermore, in light of the observed associations with omega-3 and omega-6 fatty acids downstream products, the associations of SNPs of the FADS gene cluster or ELOVL family with concentrations of total cholesterol, LDL-C, HDL-C, TG, and total cholesterol/HDL-C ratio, were studied; however, none were significant (all p > 0.05).

Table 15. Fatty acids percentage according to Δ-5 and Δ-6 desaturase activities.

	Low Δ-5 desaturase activity	**High Δ-5 desaturase activity**	***P* values**	**Low Δ-6 desaturase activity**	**High Δ-6 desaturase activity**	***P* values**
DHA%	3.6 (±1.4)	4.4 (±1.3)	0.1	3.9 (±1.5)	4 (±1.3)	0.4
EPA%*	0.8 (0.4)	1 (0.4)	0.2	0.8 (0.4)	0.9 (0.4)	0.6
AA%	11.6 (±2.6)	14 (±2.1)	<0.0001	12.5 (±2.7)	13.3 (±2.5)	0.018
LA%	13.5 (±2.4)	12.6 (±1.7)	0.025	13.7 (±2)	12.1 (±1.7)	<0.0001
ALA%*	0.25 (0.2)	0.23 (0.13)	0.3	0.25 (0.24)	0.23 (0.1)	0.2
DGLA%*	2.1 (0.4)	1.7 (0.3)	<0.0001	1.7 (0.49)	2.2 (0.4)	<0.0001
ETA%	0.1 (±0.04)	0.09 (±0.03)	0.009	0.089 (±0.037)	0.1 (±0.03)	0.001
MRS%*	29.1 (35.6)	17.6 (20.6)	0.05	21 (27.9)	18.4 (26.5)	0.9

Normally distributed data are expressed as mean ± standard deviation (SD).

*Non-normally distributed data are expressed as median ± inter-quartile range (IQR).

4.3.4.5 Gene-DHA+EPA interaction between DHA+EPA treatment and FADS genes

Table 15 shows the changes between baseline and end-of-trial measurements, stratified by randomization group, for the main erythrocytes fatty acids associated with Δ-5 and Δ-6 desaturase activities. After 15-18 months of 4 g of DHA+EPA treatment daily, Δ-5 desaturase activity significantly increased in both placebo and active group, whereas Δ-6 desaturase activity increased only in the placebo group **(Table 16)**. These results had different effects on polyunsaturated fatty acid metabolism: in the placebo group, the increase in Δ-5 and Δ-6 desaturase activity was probably due to the 4Kg weight loss and it was associated with 1.3% increase in erythrocyte AA enrichment; whereas, in the DHA+EPA group the increase in Δ-5 desaturase activity was associated with no change in ETA and significantly decrease AA erythrocyte percentage **(Table 16)**.

Moreover, treatment with DHA+EPA resulted in a gene-diet effect on some of the SNPs of the FADS gene cluster. In a MIXED model for repeated measures including the effects of time (supplementation), genotype and the interaction [genotype (FADS rs174556) × supplementation (DHA+EPA enrichment)] adjusted for age sex and BMI, gene-DHA+EPA interaction [genotype (FADS rs174556) × supplementation (DHA+EPA enrichment)] was associated with estimates of Δ-5 desaturase activity difference. There was a significant main effect of the FADS rs174556 × DHA+EPA interaction on Δ-5 desaturase activity: $F(1, 87) = 16.5$, $p<0.0001$. The gene-DHA+EPA effect was confirmed with the regression model using Δ-5 desaturase activity difference as outcome and age, sex, BMI difference, Δ-5 desaturase activity at baseline and FADS rs174556 as explanatory variables. The results of the regression model showed that, carriers of the minor allele of the FADS rs174556 (β=-0.8; 95% CI -1.3, -0.2; p=0.007) had 0.6% increase in Δ-5 desaturase activity compared with baseline. Interestingly, when the same linear regression model for MRS was repeated using MRS difference as outcome, and age, sex, DHA erythrocyte enrichment, BMI difference, Δ-5 desaturase difference, Δ-6 desaturase difference, rs1535 and MRS at baseline as explanatory variables, both Δ-5 desaturase difference (β=-2.3; 95% CI -4.3, -0.3; p=0.03) and BMI difference (β=2.6; 95% CI 1.1, 4.1; p=0.001) were again the only two variables that were independently associated with MRS difference. At the end of the study, delta 5 desaturase activity increased in both groups. In order to understand the increase in delta-5 desaturase activity in placebo and treatment group, two simple regression models were built using delta-5 desaturase difference as outcome and weight difference or DHA and EPA enrichment as explanatory variables. In the first model, delta 5 desaturase difference was the outcome and weight difference was the explanatory variable. With regard to the placebo group, the unstandardized β coefficient for weight difference was 2.2 (95% CI 1.1, 4.3; p=0.03). Whereas,

in the DHA+EPA group, there was no significant association between weight loss and increase in delta 5 desaturase activity at the end of the study (p=0.4). In the second model, delta 5 desaturase difference was the outcome and DHA and EPA enrichment were the explanatory variables. In the placebo group there was a significant association between DHA enrichment and increase in delta 5 desaturase activity (β=3 95% CI 1.3, 7.9; p=0.01). In addition, in the treatment group, there was a significant association between DHA enrichment and increase in delta 5 desaturase activity (β=1.9 95% CI 1.03, 3.5; p=0.03). However, when the analyses were repeated without the protocol violators in the placebo group, the association between DHA enrichment and delta 5 desaturase activity was not significant (p=0.98). There was no association between EPA enrichment and increase in delta 5 desaturase activity.

Table 16. Characteristics of fatty acid erythrocytes percentage and desaturase activity at baseline and end of study according to randomisation group.

	Placebo			DHA+EPA		
Variables	**Baseline**	**End of study**	***p*-value**	**Baseline**	**End of study**	***p*-value**
Plasma desaturase activity (%)						
Δ-5 desaturase activity (20:4n-6/20:3n-6) *	6.5 (2)	7.2 (1.8)	<0.0001	6.9 (2.3)	7.5 (1.7)	<0.0001
Δ-6 desaturase activity (20:3n-6/18:2n-6) *	0.14 (0.03)	0.16 (0.03)	<0.0001	0.14 (0.06)	0.15 (0.04)	0.3
Omega-3 fatty acids (erythrocyte %)						
Eicosatetraenoic acid (20:4n-3) ETA	0.1 (±0.04)	0.11 (±0.05)	0.5	0.1 (±0.04)	0.11 (±0.04)	0.1
Eicosapentaenoic acid (20:5n-3) EPA*	0.9 (0.4)	1.0 (0.2)	0.17	0.8 (0.3)	2.4 (1.8)	< 0.0001
Docosahexaenoic acid (22:6n-3) DHA	4.1(±1.6)	5.0 (±1)	0.002	3.8 (±1.2)	7.1 (±1.3)	< 0.0001
Omega-6 fatty acids (erythrocyte %)						
Linoleic acid (18:2n-6) LA	13 (±2.4)	12 (±2.4)	0.001	13 (±2.8)	11.6 (±2.1)	P<0.0001
DiHomo γ Linolenic acid (20:3n-6) DGLA	1.9 (±0.6)	1.9 (±0.5)	0.13	1.9 (±0.4)	1.7 (±0.3)	P<0.0001
Arachidonic acid (20:4n-6)* AA	13.3 (1.8)	14.6 (2.2)	<0.0001	13.7 (3.1)	12.4 (2.9)	P<0.03

Variables that are normally distributed are expressed as mean ± standard deviation (SD).
Variables that are non-normally distributed* are expressed as median ± inter-quartile range (IQR).

4.3.5 Discussion

4.3.5.1 Baseline results

The baseline results analysed in this chapter investigating the effects of 4 g of DHA+EPA treatment daily show three significant findings.

First, the minor alleles for the FADS2 rs1535 and FADS1 rs174556 were associated with low Δ-5 desaturase activity and high Δ-6 desaturase activity. This is consistent with other research studies showing that patients carrying the minor allele of the FADS genes had low Δ-5 desaturase activity, due to a decrease in the transcriptional levels of the genes encoding for Δ-5 desaturase activity [127].

This combination of low Δ-5 desaturase activity and high Δ-6 desaturase activity has been shown to have effects on both upstream and downstream products of the omega-3 and omega-6 fatty acid series[127,229] . Specifically, it causes accumulation of Δ-5 desaturase substrates di-homo-γ-linolenic acid (DGLA, 20:3n-6) and eicosatetraenoic acid (ETA, 20:4n3), and the reduction of Δ-5 desaturase downstream products in the omega-3 and omega-6 fatty acid series (**See Figure 11**). These changes in the desaturase enzymatic activity due to SNPs on the FADS1 and FADS2 genes can affect endogenous synthesis of EPA, DHA, and AA from essential fatty acids, ALA, and LA. In line with the literature, in the WELCOME study cohort, Δ-5 and Δ-6 desaturase activity levels were found to be independently associated with DHA, EPA and AA percentage in erythrocyte membranes. Namely, subjects with low Δ-5 and Δ-6 desaturase activity levels had lower DHA, EPA (downstream products) and significantly lower AA percentage in erythrocyte membranes compared with subjects with high Δ-5 and Δ-6 desaturase activity levels **(Table 15)**.

Interestingly, low Δ-5 desaturase activity was also associated with high MRS percentage of liver fat compared with subjects with high Δ-5 desaturase activity. This association between Δ-5 desaturase activity and high MRS percentage liver fat was also confirmed in the adjusted regression model. This is consistent with several previous studies in which both Δ-5 and Δ-6 desaturase enzymes were found to be lower in the liver of patients with obesity and NAFLD compared to healthy subjects [122,223,232].

When patients carrying the minor allele of the FADS2 rs1535 were compared with patients with the common homozygotes **(Table 14)**, MRS liver fat was found to be higher in the presence of the minor allele. However, when this association was studied in a regression model adjusted for different confounders, only low Δ-5 desaturase activity was associated with high MRS liver fat percentage.

Secondly, the minor alleles of the FADS2 rs1535 were correlated with high ETA erythrocyte percentage. This finding was consistent with the aforementioned results showing an accumulation of Δ-5 desaturase substrates, and low Δ-5 desaturase downstream products.

Thirdly, the minor allele for the FADS rs174546 was correlated with high DHA and AA percentage on erythrocyte membranes. This was consistent with the result showing that carriers of the minor allele for the FADS rs174546 were also associated with high Δ-5 desaturase activity explaining the high levels of downstream products DHA and AA.

At the end of the study, there was a significant increase in delta 5 desaturase activity in both placebo and treatment group. This increase was significantly associated with weight loss in the placebo group and with DHA enrichment in the treatment group. Each 1Kg of weight loss was associated with 2.2% increase in delta 5 desaturase activity, whereas in the treatment group, DHA enrichment but not weight loss was significantly associated with an increase in delta 5 desaturase activity. EPA was not associated with delta 5 desaturase activity

Interestingly, a gene-DHA+EPA interaction was found between DHA+EPA intake and FADS1 rs174556 in people with NAFLD. The effect of this gene-DHA+EPA interaction was a reduction of the negative influence of the minor allele of the FADS1 rs174556 on Δ-5 desaturase activity. At the end of this study, carriers of the minor allele of the FADS1 rs174556 had 0.6% increase in Δ-5 desaturase activity compared with baseline.

Previous studies described the effect of omega-3 fatty acids on Δ-5 desaturase and Δ-6 desaturase activity. Data reported in this chapter did not show a beneficial effect of DHA+EPA on Δ-6 desaturase activity. However, the effect of the gene-DHA+EPA interaction in increasing Δ-5 desaturase activity was not in line with a previous study by Cormier et al. In this study, they showed that supplementation with 2 g of EPA plus 1 g of DHA daily for 6 weeks in 210 healthy people subjects increased Δ-5 desaturase activity and decreased Δ-6 desaturase activity, affecting omega-3 and omega-6 fatty acid plasma levels [129].

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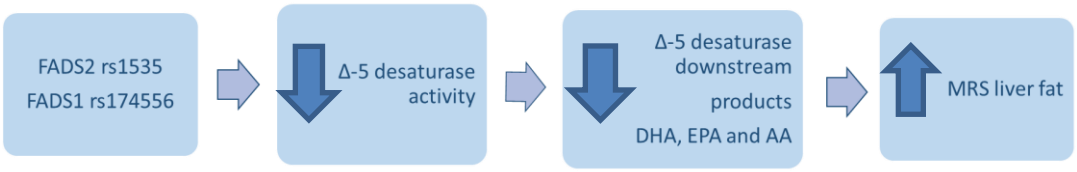
Figure 13. Suggested sequence of events linking FADS rs1535 and FADS rs174556 genotypes with Δ-5 desaturase activity and MRS liver fat percentage.

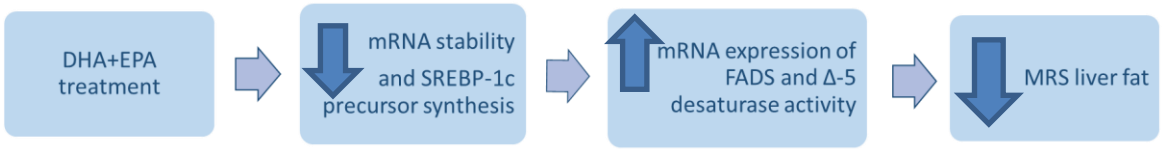


4.3.5.2 Possible explanation for the DHA+EPA gene-diet effect

The chromosome region associated with FADS1 and FADS2 gene expression overlaps with a conserved region containing predicted binding sites for SREBP-1. This was shown by Reardon et al. who found that the minor haplotype was associated with enhanced response to SREBP-1c in a binding site with shared regulatory activity for both FADS1 and FADS2 [233]. In people with NAFLD SREBP-1c is upregulated increasing *de novo* fatty acid synthesis in the liver that in turn leads to liver fat accumulation [21,66].

There are two mechanisms that might explain this gene-DHA+EPA interaction. First, omega-3 fatty acids can influence Δ-5 and Δ-6 desaturase activity levels through inhibiting the proteolytic release of mature SREBP-1c from its membrane-bound precursor. This in turn reduces SREBP-1c mRNA stability diminishing SREBP-1c precursor synthesis [122,234]. In NAFLD patients, the reduction in liver Δ-5 and Δ-6 desaturase activities may result in omega-3 fatty acids depletion induced by SREBP-1c expression and upregulation of lipogenic genes, leading to reduced desaturase synthesis and activity [235]. Secondly, high intake of EPA and DHA increases mRNA expression of FADS1 gene in the liver, and consequently increases Δ-5 desaturase activity, resulting in an increase of endogenous production of EPA and DHA (**Figure 14**).

Figure 14. Suggested sequence of events linking DHA+EPA treatment, FADS 1 genotype and desaturase with MRS liver fat.



4.3.5.3 Limitations

The analysis conducted in the WELCOME study cohort did not show any effect of the ELOVL gene family on the omega-3 and omega-6 fatty acid metabolism. Moreover, Δ-5 and Δ-6 desaturase activity levels had no influence on the lipid profile. This is inconsistent with findings in the literature [226-229]. This lack of significant results might be due to the small number of WELCOME study participants and consequent lack of power, as all the research studies discussed in this chapter included larger number of subjects [236].

4.3.5.4 Conclusions

The baseline data from the WELCOME study participants showed that the combination of low Δ-5 desaturase activity and high BMI was associated with high MRS liver fat. Moreover, the minor alleles for the SNPs FADS1 rs174556 and FADS2 rs1535 were associated with low Δ-5 desaturase activity, resulting in low erythrocytes DHA, EPA and AA fatty acid percentage.

The baseline data from the WELCOME study participants showed that SNPs of the FADS gene cluster (FADS1 rs174556, FADS2 rs1535 and FADS rs174546) and not ELOVL gene family were associated with alteration in Δ-5 and Δ-6 desaturase activity. This in turn was affecting the variability in erythrocyte fatty acid composition and MRS liver fat percentage. Moreover, the combination of low Δ-5 desaturase activity and high BMI was associated with high MRS liver fat.

In addition, at the end of the study, there was a gene-DHA+EPA interaction between treatment with and FADS1 rs174556 and Δ-5 desaturase activity. Namely, treatment with DHA+EPA was associated with a 0.6% increase in Δ-5 desaturase activity in people carrying the minor allele for the FADS1 rs174556 compared with baseline.

In conclusion, this chapter describes the association between SNPs of the FADS gene cluster and omega-3 and omega-6 fatty acid metabolism with consequent implication for the production of AA, DHA and EPA. Moreover, the increase in delta 5 desaturase activity was significantly associated with weight loss in the placebo group and with DHA enrichment in the treatment group. Each 1Kg of weight loss was associated with 2.2% increase in delta 5 desaturase activity, whereas in the treatment group, DHA enrichment but not weight loss was significantly associated with an increase in delta 5 desaturase activity. EPA was not associated with delta 5 desaturase activity.

5. Discussion

5.1 Overview of the WELCOME study

The WELCOME study was the first randomized, double-blind, placebo-controlled trial to test the efficacy of a 15-18 months treatment with a high dose (4 g daily) of highly purified DHA+EPA on a quantitative measurement of liver fat in NAFLD and relate changes in erythrocyte DHA+EPA enrichment to changes in liver fat percentage.

5.2 Rationale underpinning the WELCOME study

The WELCOME study was designed based on the previous published literature, at a time when there was controversy as to whether omega-3 fatty acids had a beneficial effect on NAFLD.

The WELCOME study differed markedly from previous studies in five cardinal areas: (1) duration of the treatment, (2) dosage of omega-3 fatty acids, (3) composition of the omega-3 fatty acid treatment, (4) lack of testing for both adherence to the omega-3 intervention and for contamination with omega-3 fatty acids obtained from other readily available sources, using DHA and EPA measurements, and (5) the semi-quantitative measures for assessing changes in liver fat percentage.

Hereafter, I will discuss these five unique characteristics of the WELCOME study.

1. **Duration**

In the literature, there was evidence of omega-3 fatty acid interventions with markedly different durations [190]. The duration of the WELCOME study treatment was chosen based on a recent meta-analysis by He et al. showing 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 [237-240].

2. **Dosage**

The dosage of 4 g of DHA+EPA daily was selected for two reasons. First, in light of its proven effectiveness in hypertriglyceridemia [241]. Secondly, it was hypothesised that this dosage would raise the erythrocyte level of EPA and DHA above the pre-specified level of 0.7% and 2% respectively. This level is deemed to be the minimum increase for improving the DHA+EPA sum after treatment to a value proposed to result in significantly decreased risk of CVD [200].

3. **Composition and chemical purity of WELCOME study treatment**

The WELCOME study treatment differed from all previous studies both for its composition and chemical purity.

The composition of the WELCOME study treatment was 380 mg DHA and 460 mg EPA per g of oil. Notably, contrary to fish oil preparations used in other studies no lipid-soluble vitamins A and D were present [190]. 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''[242]. 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 [243,244]. Moreover, DHA also inhibits the secretion of apoB-100 by promoting its autophagic degradation causing a reduction in VLDL synthesis in the liver [66].

With regard to the chemical purity of the WELCOME study treatment, the conscientious decision was made to select the most highly purified DHA and EPA available on the market. 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.

4. Compliance

Compliance was monitored with capsule count at every visit and with measurement of DHA and EPA on erythrocytes membrane using gas chromatography before and after the study. At the end of the study, enrichment of DHA and EPA was measured as the difference between end of study and baseline. Moreover, participants were asked if they had side effects or adverse events. A few people had difficult digest the capsules, but this was resolved by splitting the dose in correspondence of the three main meals during the day. In previous studies in the literature, compliance was never assessed through a rigorous procedure bur merely through subjective interactions.

5. Quantification of liver fat

In the WELCOME study, liver fat was assessed by way of two independent, yet synergistic, methods: magnetic resonance spectroscopy (MRS) and non-invasive markers of liver function, and NAFLD disease severity. This was a unique feature and a substantial departure from the traditional approach of carrying out this measurement solely through liver biopsy.

The choice to use MRS over liver biopsy was based on multiple reasons. First, liver biopsy is invasive, expensive, and subject to sampling variability [183-187], 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 [245,246] with a coefficient of variance of only 8%, and liver fat signals of only 0.2% are clearly evident above the noise level [246].

With regard to non-invasive markers of liver function, and NAFLD disease severity, the WELCOME study adopted algorithms that have been developed to generate a score (ELF score) utilizing HA and PIIINP, in combination with another marker TIMP-1 [171]. It has been suggested that this approach can engender an increase in specificity and sensitivity for diagnosing NAFLD[247]. The liver unit in Southampton is routinely using these non-invasive markers in routine clinical practice and our experience replicates the published literature. Whilst other non-invasive approaches also include measurement of liver stiffness using the ultrasound technique 'fibroscan', this methodology has limited applicability in groups who are obese as obtaining reproducible data with this technique can be a problem [248].

5.3 Findings of the WELCOME study

The findings of the WELCOME study can be schematically described as follows.

First, as analysed in chapter 4.2, after 15-18 months of daily DHA+EPA treatment there was a beneficial effect of erythrocyte DHA enrichment (≥2%) in reducing liver fat. Specifically, 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. Moreover, there was an improvement of the genotype effect on Δ-5 desaturase activity. The beneficial effect of DHA on liver fat found in the WELCOME study was consistent with previous published literature [201,249-252]. 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 [201,250]. 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) and epicardial adipose tissue was reduced by 14.2% (95% CI 0-28.2%; p= 0.01) in the DHA group.

Secondly, as described in chapter 4.4, that low Δ-5 desaturase activity affected erythrocyte fatty acid composition, consequently reducing DHA and EPA erythrocyte concentration. Interestingly, at the end of the study there was a gene-DHA+EPA interaction. Namely, the negative effect of FADS1 rs174556 on Δ-5 desaturase activity was reduced by 0.6% and this was confirmed by the MIXED model for repeated measures including the effect of time (supplementation).

Thirdly, as shown in chapters 4.3 and 4.4, polymorphisms for PNPLA3 and the FADS1 and FADS2 gene clusters were contributing to the variability of erythrocytes fatty acid composition and therefore to the variability of DHA erythrocyte enrichment.

Fourthly, as discussed in chapter 4.3, PNPLA3 148M/M genotype, and not either the TM6SF2 (E167K), TM6SF2 167E/K+K/K or TM6SF2 167E/E genotypes, was associated with an increase in liver fat percentage (+1.2%) at the end of the study regardless of randomisation group. In the treatment group, there were two participants carrying the PNPLA3 148M/M genotype, therefore it was not possible to compare the treatment group with the placebo group. However, in the treatment group there was a trend towards an increase in MRS liver fat percentage at the end of the study. Whereas, in the placebo group there were eight people carrying the PNPLA3 148M/M genotype and at the end of the study there was no change in the MRS liver fat percentage. 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 (chemical composition of WELCOME study capsules) to TGs.

Sixthly, as analysed in chapter 4.4, FADS genotypes regulate Δ-5 and Δ-6 desaturase activity affecting erythrocytes fatty acid composition. FADS2 rs1535 and FADS1 rs174556 were associated with low Δ-5 desaturase activity and high Δ-6 desaturase activity. This combination of low Δ-5 desaturase activity and high Δ-6 desaturase activity has been shown to have effects on both upstream and downstream products of the omega-3 and omega-6 fatty acid series. This combination results in an accumulation of di-homo-γ-linolenic acid (DGLA, 20:3n-6) and eicosatetraenoic acid (ETA, 20:4n3), and a reduction of Δ-5 downstream products in the omega-3 fatty acid series (DHA and EPA). Moreover, there was an influence of Δ-5 desaturase activity on baseline level of MRS liver fat.

5.4 Limitations

The WELCOME study was designed to avoid the limitations of many previous studies, such as the non-blinding of participants and investigators, and the lack of a placebo for the control group. Despite this effort, the WELCOME study does have two limitations.

First, small sample size. However, this limitation is not substantial, as this was a proof-of-concept study to test efficacy of the DHA+EPA intervention and DHA and EPA enrichment to decrease liver fat percentage and liver fibrosis scores.

Secondly, treatment duration. The choice to limit treatment duration to 15-18 months was based on the hypothesis that this would be an adequate time frame to assess variation in liver fat caused by a DHA+EPA intervention. A longer treatment duration would have been required to assess whether DHA+EPA have a positive effect on fibrosis and NASH, as these are more advanced stages and NAFLD the reversal of which likely requires lengthier interventions.

5.5 NICE NAFLD guidelines: present state and possible future evolution

At present, the NICE NAFLD guidelines (ng49) do not recommend omega-3 fatty acids to adults with NAFLD because there is not enough evidence of their beneficial effect. They recommend pioglitazone or vitamin E for adults with advanced liver fibrosis, whether they have diabetes or not. These treatments are prescribed only in secondary and tertiary care settings because of their side-effects and concerns about long term safety [193,194].

However, there is increasing evidence that the paradigm on which these guidelines were originally based is shifting. On one hand, the results of trials testing treatment with thiazolidinediones [36] and anti-oxidants such as vitamin E [38], that have focused primarily on modifying pathways affecting insulin resistance [46], oxidative stress [38] and lipid metabolism [190] have produced variable results. These relatively small trials have also generated controversy, not least because any positive effects were limited by side-effects and concerns about long term safety of glitazones [193] and the high dose of vitamin E required [194].

On the other, there is increasing support for the hypothesis that omega-3 fatty acids treatment might have a beneficial effect on NAFLD, with minimal side effects [66]. 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) [66]. Secondly, a recent systematic review and meta-analysis has shown a benefit of omega-3 fatty acid treatment on liver fat in NAFLD [190]. This body of evidence is not diminished by a recent trial in which treatment involving high doses of EPA failed to show an improvement in NAFLD histological score [253]. There is considerable evidence that EPA and DHA have different biological effects: in men, the conversion of EPA to DHA is <1%, whereas in women is up to 9% [254-258 259]. In light of these physiological effects, it is not surprising that Sanyal et al. did not find that EPA treatment on its own had a beneficial effect on NAFLD. Results of the WELCOME trial suggest that DHA would be more effective than EPA.

The WELCOME study lends additional support to the hypothesis that omega-3 fatty acids treatment might have a beneficial effect on NAFLD, with minimal side effects. Although there was no beneficial effect of DHA+EPA in reducing liver fat, data presented in Chapter 4.1.4.4 showed that there was a significant association between in DHA erythrocyte enrichment and decrease in liver fat percentage.

Before suggesting a revision of the extant NICE NAFLD guidelines further studies are required. In particular it is necessary to consider different factors that might affect DHA erythrocyte enrichment and thus its beneficial effect on liver that accumulation.

5.6 Conclusions

The novel data presented in this thesis show that erythrocyte DHA enrichment after 4 g of DHA+EPA treatment daily, was independently associated with a decrease in MRS liver fat percentage. Moreover, at the end of the study, there was a gene-DHA+EPA interaction between treatment with DHA+EPA and FADS1 rs174556 resulting in a 0.6% increase in Δ-5 desaturase activity in people carrying the minor allele for the FADS1 rs174556, compared with baseline. The results presented in this thesis also show several important genetic factors that might influence the therapeutic effect of DHA erythrocyte enrichment to decrease liver fat. For example, the PNPLA3 148M/M variant was associated with low DHA enrichment with DHA+EPA treatment and the FADS2 rs1535 and FADS1 rs174556 SNPs were associated with low Δ-5 desaturase activity and low DHA enrichment.

Based on the results presented in my thesis, my recommendations are that new randomised placebo controlled trials are needed in patients who have the early stages of NAFLD, to test the effect of DHA treatment at a dose of >1520 mg/day (dosage based on the use of 4 g of daily DHA+EPA treatment with Omacor in the WELCOME study) for 15 months. Moreover, these trials should stratify patients according to PNPLA3 I148M genotype and FADS rs1535 polymorphism as

these are important gene variants that might influence the therapeutic effect of DHA enrichment and thereby affect responses to treatment in NAFLD.

6. Reference List

1. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 2012;142:1592-609.
2. Calzadilla Bertot L, Adams LA. The Natural Course of Non-Alcoholic Fatty Liver Disease. International journal of molecular sciences 2016;17.
3. de Alwis NM, Day CP. Non-alcoholic fatty liver disease: the mist gradually clears. Journal of hepatology 2008;48 Suppl 1:S104-12.
4. Angulo P. Nonalcoholic fatty liver disease. The New England journal of medicine 2002;346:1221-31.
5. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism: clinical and experimental 2016.
6. Byrne CD. Fatty liver: role of inflammation and fatty acid nutrition. Prostaglandins, leukotrienes, and essential fatty acids 2010;82:265-71.
7. Angulo P, Hui JM, Marchesini G, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology (Baltimore, Md) 2007;45:846-54.
8. Rinella ME. Nonalcoholic fatty liver disease: a systematic review. Jama 2015;313:2263-73.
9. Sherif ZA, Saeed A, Ghavimi S, et al. Global Epidemiology of Nonalcoholic Fatty Liver Disease and Perspectives on US Minority Populations. Digestive diseases and sciences 2016;61:1214-25.
10. Dulai PS, Singh S, Patel J, et al. Increased risk of mortality by fibrosis stage in non-alcoholic fatty liver disease: Systematic Review and Meta-analysis. Hepatology (Baltimore, Md) 2017.
11. Whalley S, Puvanachandra P, Desai A, Kennedy H. Hepatology outpatient service provision in secondary care: a study of liver disease incidence and resource costs. Clinical medicine (London, England) 2007;7:119-24.
12. Agrawal S, Duseja AK. Non-alcoholic Fatty Liver Disease: East Versus West. Journal of clinical and experimental hepatology 2012;2:122-34.
13. Levene AP, Goldin RD. The epidemiology, pathogenesis and histopathology of fatty liver disease. Histopathology 2012;61:141-52.
14. Shen J, Chan HL, Wong GL, et al. Assessment of non-alcoholic fatty liver disease using serum total cell death and apoptosis markers. Alimentary pharmacology & therapeutics 2012;36:1057-66.
15. Targher G, Byrne CD. Clinical Review: Nonalcoholic fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications. The Journal of clinical endocrinology and metabolism 2013;98:483-95.
16. Goh GB, McCullough AJ. Natural History of Nonalcoholic Fatty Liver Disease. Digestive diseases and sciences 2016;61:1226-33.
17. Lonardo A, Ballestri S, Marchesini G, Angulo P, Loria P. Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver 2015;47:181-90.
18. Ballestri S, Zona S, Targher G, et al. Nonalcoholic fatty liver disease is associated with an almost twofold increased risk of incident type 2 diabetes and metabolic syndrome.

Evidence from a systematic review and meta-analysis. Journal of gastroenterology and hepatology 2016;31:936-44.
19. Perry RJ, Samuel VT, Petersen KF, Shulman GI. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. Nature 2014;510:84-91.
20. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? European heart journal 2012;33:1190-200.
21. Scorletti E, Calder PC, Byrne CD. Non-alcoholic fatty liver disease and cardiovascular risk: metabolic aspects and novel treatments. Endocrine 2011;40:332-43.
22. Scorletti EB, C.D. Extrahepatic diseases and NAFLD: The triangular relationship between NAFLD, type 2-diabetes and dysbiosis

Digestive diseases and sciences 2016;Accepted.
23. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science (New York, NY) 2011;332:1519-23.
24. Byrne CD, Targher G. NAFLD: a multisystem disease. Journal of hepatology 2015;62:S47-64.
25. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. The New England journal of medicine 2010;363:1341-50.
26. Targher G, Byrne CD, Lonardo A, Zoppini G, Barbui C. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: A meta-analysis. Journal of hepatology 2016;65:589-600.
27. Byrne CD, Targher G. Ectopic fat, insulin resistance, and nonalcoholic fatty liver disease: implications for cardiovascular disease. Arteriosclerosis, thrombosis, and vascular biology 2014;34:1155-61.
28. Scorletti E, West AL, Bhatia L, et al. Treating liver fat and serum triglyceride levels in NAFLD, effects of PNPLA3 and TM6SF2 genotypes: Results from the WELCOME trial. Journal of hepatology 2015;63:1476-83.
29. Veena J, Muragundla A, Sidgiddi S, Subramaniam S. Non-alcoholic fatty liver disease: need for a balanced nutritional source. The British journal of nutrition 2014;112:1858-72.
30. Palmer CN, Maglio C, Pirazzi C, et al. Paradoxical lower serum triglyceride levels and higher type 2 diabetes mellitus susceptibility in obese individuals with the PNPLA3 148M variant. PloS one 2012;7:e39362.
31. Musso G, Gambino R, De Michieli F, et al. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology (Baltimore, Md) 2003;37:909-16.
32. Softic S, Cohen DE, Kahn CR. Role of Dietary Fructose and Hepatic De Novo Lipogenesis in Fatty Liver Disease. Digestive diseases and sciences 2016;61:1282-93.
33. Leung C, Rivera L, Furness JB, Angus PW. The role of the gut microbiota in NAFLD. Nature reviews Gastroenterology & hepatology 2016;13:412-25.
34. Henao-Mejia J, Elinav E, Thaiss CA, Licona-Limon P, Flavell RA. Role of the intestinal microbiome in liver disease. Journal of autoimmunity 2013;46:66-73.
35. Yu J, Marsh S, Hu J, Feng W, Wu C. The Pathogenesis of Nonalcoholic Fatty Liver Disease: Interplay between Diet, Gut Microbiota, and Genetic Background. Gastroenterology research and practice 2016;2016:2862173.
36. Ratziu V, Giral P, Jacqueminet S, et al. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. Gastroenterology 2008;135:100-10.

37. Ratziu V, Charlotte F, Bernhardt C, et al. Long-term efficacy of rosiglitazone in nonalcoholic steatohepatitis: results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. Hepatology (Baltimore, Md) 2010;51:445-53.
38. Sanyal AJ, Chalasani N, Kowdley KV, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. The New England journal of medicine 2010;362:1675-85.
39. Caldwell S. NASH Therapy: omega 3 supplementation, vitamin E, insulin sensitizers and statin drugs. Clinical and molecular hepatology 2017;23:103-8.
40. Dyson J, Day C. Treatment of non-alcoholic fatty liver disease. Digestive diseases (Basel, Switzerland) 2014;32:597-604.
41. Bugianesi E, Gentilcore E, Manini R, et al. A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. The American journal of gastroenterology 2005;100:1082-90.
42. Haukeland JW, Konopski Z, Eggesbo HB, et al. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. Scandinavian journal of gastroenterology 2009;44:853-60.
43. Loomba R, Lutchman G, Kleiner DE, et al. Clinical trial: pilot study of metformin for the treatment of non-alcoholic steatohepatitis. Alimentary pharmacology & therapeutics 2009;29:172-82.
44. Shargorodsky M, Omelchenko E, Matas Z, Boaz M, Gavish D. Relation between augmentation index and adiponectin during one-year metformin treatment for nonalcoholic steatohepatosis: effects beyond glucose lowering? Cardiovascular diabetology 2012;11:61.
45. Razavizade M, Jamali R, Arj A, Matini SM, Moraveji A, Taherkhani E. The effect of pioglitazone and metformin on liver function tests, insulin resistance, and liver fat content in nonalcoholic Fatty liver disease: a randomized double blinded clinical trial. Hepatitis monthly 2013;13:e9270.
46. Lomonaco R, Ortiz-Lopez C, Orsak B, et al. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. Hepatology (Baltimore, Md) 2012;55:1389-97.
47. Nakade Y, Murotani K, Inoue T, et al. Ezetimibe for the treatment of non-alcoholic fatty liver disease: A meta-analysis. Hepatology research : the official journal of the Japan Society of Hepatology 2017.
48. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. Diabetologia 2016;59:1121-40.
49. Klein EA, Thompson IM, Jr., Tangen CM, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Jama 2011;306:1549-56.
50. Schurks M, Glynn RJ, Rist PM, Tzourio C, Kurth T. Effects of vitamin E on stroke subtypes: meta-analysis of randomised controlled trials. BMJ (Clinical research ed) 2010;341:c5702.
51. Zelber-Sagi S, Kessler A, Brazowsky E, et al. A double-blind randomized placebo-controlled trial of orlistat for the treatment of nonalcoholic fatty liver disease. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 2006;4:639-44.
52. Nelson A, Torres DM, Morgan AE, Fincke C, Harrison SA. A pilot study using simvastatin in the treatment of nonalcoholic steatohepatitis: A randomized placebo-controlled trial. Journal of clinical gastroenterology 2009;43:990-4.
53. Lazo M, Solga SF, Horska A, et al. Effect of a 12-month intensive lifestyle intervention on hepatic steatosis in adults with type 2 diabetes. Diabetes care 2010;33:2156-63.
54. Patel NS, Doycheva I, Peterson MR, et al. Effect of weight loss on magnetic resonance imaging estimation of liver fat and volume in patients with nonalcoholic

steatohepatitis. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 2015;13:561-8.e1.
55. Sevastianova K, Kotronen A, Gastaldelli A, et al. Genetic variation in PNPLA3 (adiponutrin) confers sensitivity to weight loss-induced decrease in liver fat in humans. The American journal of clinical nutrition 2011;94:104-11.
56. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. Gastroenterology 2015;149:367-78.e5; quiz e14-5.
57. St George A, Bauman A, Johnston A, Farrell G, Chey T, George J. Independent effects of physical activity in patients with nonalcoholic fatty liver disease. Hepatology (Baltimore, Md) 2009;50:68-76.
58. Bellentani S, Dalle Grave R, Suppini A, Marchesini G. Behavior therapy for nonalcoholic fatty liver disease: The need for a multidisciplinary approach. Hepatology (Baltimore, Md) 2008;47:746-54.
59. Eslamparast T, Tandon P, Raman M. Dietary Composition Independent of Weight Loss in the Management of Non-Alcoholic Fatty Liver Disease. Nutrients 2017;9.
60. Promrat K, Kleiner DE, Niemeier HM, et al. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. Hepatology (Baltimore, Md) 2010;51:121-9.
61. Steven S, Hollingsworth KG, Al-Mrabeh A, et al. Very Low-Calorie Diet and 6 Months of Weight Stability in Type 2 Diabetes: Pathophysiological Changes in Responders and Nonresponders. Diabetes care 2016;39:808-15.
62. Lim EL, Hollingsworth KG, Aribisala BS, Chen MJ, Mathers JC, Taylor R. Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. Diabetologia 2011;54:2506-14.
63. Kirk E, Reeds DN, Finck BN, Mayurranjan SM, Patterson BW, Klein S. Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction. Gastroenterology 2009;136:1552-60.
64. Browning JD, Baker JA, Rogers T, Davis J, Satapati S, Burgess SC. Short-term weight loss and hepatic triglyceride reduction: evidence of a metabolic advantage with dietary carbohydrate restriction. The American journal of clinical nutrition 2011;93:1048-52.
65. Bjermo H, Iggman D, Kullberg J, et al. Effects of n-6 PUFAs compared with SFAs on liver fat, lipoproteins, and inflammation in abdominal obesity: a randomized controlled trial. The American journal of clinical nutrition 2012;95:1003-12.
66. Scorletti E, Byrne CD. Omega-3 fatty acids, hepatic lipid metabolism, and nonalcoholic fatty liver disease. Annual review of nutrition 2013;33:231-48.
67. Mirmiran P, Hosseinpour-Niazi S, Naderi Z, Bahadoran Z, Sadeghi M, Azizi F. Association between interaction and ratio of omega-3 and omega-6 polyunsaturated fatty acid and the metabolic syndrome in adults. Nutrition (Burbank, Los Angeles County, Calif) 2012;28:856-63.
68. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental biology and medicine (Maywood, NJ) 2008;233:674-88.
69. Pachikian BD, Neyrinck AM, Cani PD, et al. Hepatic steatosis in n-3 fatty acid depleted mice: focus on metabolic alterations related to tissue fatty acid composition. BMC physiology 2008;8:21.
70. Marsman HA, Heger M, Kloek JJ, et al. Reversal of hepatic steatosis by omega-3 fatty acids measured non-invasively by (1) H-magnetic resonance spectroscopy in a rat model. Journal of gastroenterology and hepatology 2011;26:356-63.

71. Ryan MC, Itsiopoulos C, Thodis T, et al. The Mediterranean diet improves hepatic steatosis and insulin sensitivity in individuals with non-alcoholic fatty liver disease. Journal of hepatology 2013;59:138-43.
72. Keating SE, Hackett DA, George J, Johnson NA. Exercise and non-alcoholic fatty liver disease: a systematic review and meta-analysis. Journal of hepatology 2012;57:157-66.
73. Bacchi E, Negri C, Targher G, et al. Both resistance training and aerobic training reduce hepatic fat content in type 2 diabetic subjects with nonalcoholic fatty liver disease (the RAED2 Randomized Trial). Hepatology (Baltimore, Md) 2013;58:1287-95.
74. Montesi L, Caselli C, Centis E, et al. Physical activity support or weight loss counseling for nonalcoholic fatty liver disease? World journal of gastroenterology 2014;20:10128-36.
75. Hallsworth K, Fattakhova G, Hollingsworth KG, et al. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. Gut 2011;60:1278-83.
76. Houghton D, Thoma C, Hallsworth K, et al. Exercise Reduces Liver Lipids and Visceral Adiposity in Patients With Nonalcoholic Steatohepatitis in a Randomized Controlled Trial. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 2017;15:96-102.e3.
77. McCarthy EM, Rinella ME. The role of diet and nutrient composition in nonalcoholic Fatty liver disease. Journal of the Academy of Nutrition and Dietetics 2012;112:401-9.
78. Basaranoglu M, Basaranoglu G, Bugianesi E. Carbohydrate intake and nonalcoholic fatty liver disease: fructose as a weapon of mass destruction. Hepatobiliary surgery and nutrition 2015;4:109-16.
79. Berlanga A, Guiu-Jurado E, Porras JA, Auguet T. Molecular pathways in non-alcoholic fatty liver disease. Clinical and experimental gastroenterology 2014;7:221-39.
80. Jones JG. Hepatic glucose and lipid metabolism. Diabetologia 2016;59:1098-103.
81. Mayes PA. Intermediary metabolism of fructose. The American journal of clinical nutrition 1993;58:754s-65s.
82. Tappy L, Le KA. Metabolic effects of fructose and the worldwide increase in obesity. Physiological reviews 2010;90:23-46.
83. Kushiyama A, Nakatsu Y, Matsunaga Y, et al. Role of Uric Acid Metabolism-Related Inflammation in the Pathogenesis of Metabolic Syndrome Components Such as Atherosclerosis and Nonalcoholic Steatohepatitis. Mediators of inflammation 2016;2016:8603164.
84. Abdelmalek MF, Lazo M, Horska A, et al. Higher dietary fructose is associated with impaired hepatic adenosine triphosphate homeostasis in obese individuals with type 2 diabetes. Hepatology (Baltimore, Md) 2012;56:952-60.
85. Mosca A, Nobili V, De Vito R, et al. Serum uric acid concentrations and fructose consumption are independently associated with NASH in children and adolescents. Journal of hepatology 2017.
86. Abdelmalek MF, Suzuki A, Guy C, et al. Increased fructose consumption is associated with fibrosis severity in patients with nonalcoholic fatty liver disease. Hepatology (Baltimore, Md) 2010;51:1961-71.
87. Spruss A, Bergheim I. Dietary fructose and intestinal barrier: potential risk factor in the pathogenesis of nonalcoholic fatty liver disease. The Journal of nutritional biochemistry 2009;20:657-62.
88. Chassaing B, Etienne-Mesmin L, Gewirtz AT. Microbiota-liver axis in hepatic disease. Hepatology (Baltimore, Md) 2014;59:328-39.
89. Sellmann C, Priebs J, Landmann M, et al. Diets rich in fructose, fat or fructose and fat alter intestinal barrier function and lead to the development of nonalcoholic fatty liver disease over time. The Journal of nutritional biochemistry 2015;26:1183-92.

90. Redgrave TG. Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. The Journal of clinical investigation 1970;49:465-71.
91. Nassir F, Rector RS, Hammoud GM, Ibdah JA. Pathogenesis and Prevention of Hepatic Steatosis. Gastroenterology & hepatology 2015;11:167-75.
92. Hodson L, Frayn KN. Hepatic fatty acid partitioning. Current opinion in lipidology 2011;22:216-24.
93. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. The Journal of clinical investigation 2005;115:1343-51.
94. Byrne CD. Ectopic fat, insulin resistance and non-alcoholic fatty liver disease. The Proceedings of the Nutrition Society 2013;72:412-9.
95. Chainani-Wu N, Weidner G, Purnell DM, et al. Changes in emerging cardiac biomarkers after an intensive lifestyle intervention. The American journal of cardiology 2011;108:498-507.
96. Ueno T, Sugawara H, Sujaku K, et al. Therapeutic effects of restricted diet and exercise in obese patients with fatty liver. Journal of hepatology 1997;27:103-7.
97. Sreenivasa Baba C, Alexander G, Kalyani B, et al. Effect of exercise and dietary modification on serum aminotransferase levels in patients with nonalcoholic steatohepatitis. Journal of gastroenterology and hepatology 2006;21:191-8.
98. Lawlor DA, Sattar N, Smith GD, Ebrahim S. The associations of physical activity and adiposity with alanine aminotransferase and gamma-glutamyltransferase. American journal of epidemiology 2005;161:1081-8.
99. Pan X, Hussain MM. Gut triglyceride production. Biochimica et biophysica acta 2012;1821:727-35.
100. Petersen KF, Dufour S, Hariri A, et al. Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. The New England journal of medicine 2010;362:1082-9.
101. Rodriguez-Cuenca S, Pellegrinelli V, Campbell M, Oresic M, Vidal-Puig A. Sphingolipids and glycerophospholipids - The "ying and yang" of lipotoxicity in metabolic diseases. Progress in lipid research 2017;66:14-29.
102. Merrill AH, Jr., Lingrell S, Wang E, Nikolova-Karakashian M, Vales TR, Vance DE. Sphingolipid biosynthesis de novo by rat hepatocytes in culture. Ceramide and sphingomyelin are associated with, but not required for, very low density lipoprotein secretion. The Journal of biological chemistry 1995;270:13834-41.
103. Gatt S, Bierman EL. Sphingomyelin suppresses the binding and utilization of low density lipoproteins by skin fibroblasts. The Journal of biological chemistry 1980;255:3371-6.
104. Pingitore P, Pirazzi C, Mancina RM, et al. Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function. Biochimica et biophysica acta 2014;1841:574-80.
105. Trepo E, Romeo S, Zucman-Rossi J, Nahon P. PNPLA3 gene in liver diseases. Journal of hepatology 2016.
106. Iacono A, Raso GM, Canani RB, Calignano A, Meli R. Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms. The Journal of nutritional biochemistry 2011;22:699-711.
107. Arab JP, Karpen SJ, Dawson PA, Arrese M, Trauner M. Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives. Hepatology (Baltimore, Md) 2017;65:350-62.
108. Clayton PT. Disorders of bile acid synthesis. Journal of inherited metabolic disease 2011;34:593-604.
109. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiological reviews 2009;89:147-91.

110. Smelt AH. Triglycerides and gallstone formation. Clinica chimica acta; international journal of clinical chemistry 2010;411:1625-31.
111. Li T, Matozel M, Boehme S, et al. Overexpression of cholesterol 7alpha-hydroxylase promotes hepatic bile acid synthesis and secretion and maintains cholesterol homeostasis. Hepatology (Baltimore, Md) 2011;53:996-1006.
112. Iannelli F, Collino A, Sinha S, et al. Massive gene amplification drives paediatric hepatocellular carcinoma caused by bile salt export pump deficiency. Nature communications 2014;5:3850.
113. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet 2015;385:956-65.
114. Kamisako T, Tanaka Y, Ikeda T, Yamamoto K, Ogawa H. Dietary fish oil regulates gene expression of cholesterol and bile acid transporters in mice. Hepatology research : the official journal of the Japan Society of Hepatology 2012;42:321-6.
115. Kalaany NY, Mangelsdorf DJ. LXRS and FXR: the yin and yang of cholesterol and fat metabolism. Annual review of physiology 2006;68:159-91.
116. Zhao A, Yu J, Lew JL, Huang L, Wright SD, Cui J. Polyunsaturated fatty acids are FXR ligands and differentially regulate expression of FXR targets. DNA and cell biology 2004;23:519-26.
117. Kim EH, Bae JS, Hahm KB, Cha JY. Endogenously synthesized n-3 polyunsaturated fatty acids in fat-1 mice ameliorate high-fat diet-induced non-alcoholic fatty liver disease. Biochemical pharmacology 2012;84:1359-65.
118. Portolesi R, Powell BC, Gibson RA. Delta6 desaturase mRNA abundance in HepG2 cells is suppressed by unsaturated fatty acids. Lipids 2008;43:91-5.
119. Glaser C, Heinrich J, Koletzko B. Role of FADS1 and FADS2 polymorphisms in polyunsaturated fatty acid metabolism. Metabolism: clinical and experimental 2010;59:993-9.
120. Burdge GC. Metabolism of alpha-linolenic acid in humans. Prostaglandins, leukotrienes, and essential fatty acids 2006;75:161-8.
121. Calder PC, Yaqoob P. Understanding omega-3 polyunsaturated fatty acids. Postgraduate medicine 2009;121:148-57.
122. Araya J, Rodrigo R, Pettinelli P, Araya AV, Poniachik J, Videla LA. Decreased liver fatty acid delta-6 and delta-5 desaturase activity in obese patients. Obesity (Silver Spring, Md) 2010;18:1460-3.
123. Cordain L, Eaton SB, Sebastian A, et al. Origins and evolution of the Western diet: health implications for the 21st century. The American journal of clinical nutrition 2005;81:341-54.
124. Calder PC. Omega-3 fatty acids and inflammatory processes. Nutrients 2010;2:355-74.
125. Lemaitre RN, Tanaka T, Tang W, et al. Genetic loci associated with plasma phospholipid n-3 fatty acids: a meta-analysis of genome-wide association studies from the CHARGE Consortium. PLoS genetics 2011;7:e1002193.
126. Tanaka T, Shen J, Abecasis GR, et al. Genome-wide association study of plasma polyunsaturated fatty acids in the InCHIANTI Study. PLoS genetics 2009;5:e1000338.
127. Rzehak P, Heinrich J, Klopp N, et al. Evidence for an association between genetic variants of the fatty acid desaturase 1 fatty acid desaturase 2 (FADS1 FADS2) gene cluster and the fatty acid composition of erythrocyte membranes. The British journal of nutrition 2009;101:20-6.
128. Malerba G, Schaeffer L, Xumerle L, et al. SNPs of the FADS gene cluster are associated with polyunsaturated fatty acids in a cohort of patients with cardiovascular disease. Lipids 2008;43:289-99.

129. Cormier H, Rudkowska I, Lemieux S, Couture P, Julien P, Vohl MC. Effects of FADS and ELOVL polymorphisms on indexes of desaturase and elongase activities: results from a pre-post fish oil supplementation. Genes & nutrition 2014;9:437.
130. Schaeffer L, Gohlke H, Muller M, et al. Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. Human molecular genetics 2006;15:1745-56.
131. Lattka E, Illig T, Heinrich J, Koletzko B. Do FADS genotypes enhance our knowledge about fatty acid related phenotypes? Clinical nutrition (Edinburgh, Scotland) 2010;29:277-87.
132. Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. Progress in lipid research 2008;47:147-55.
133. Chan DC, Watts GF, Mori TA, Barrett PH, Redgrave TG, Beilin LJ. Randomized controlled trial of the effect of n-3 fatty acid supplementation on the metabolism of apolipoprotein B-100 and chylomicron remnants in men with visceral obesity. The American journal of clinical nutrition 2003;77:300-7.
134. Pan M, Maitin V, Parathath S, et al. Presecretory oxidation, aggregation, and autophagic destruction of apoprotein-B: a pathway for late-stage quality control. Proceedings of the National Academy of Sciences of the United States of America 2008;105:5862-7.
135. Caviglia JM, Gayet C, Ota T, et al. Different fatty acids inhibit apoB100 secretion by different pathways: unique roles for ER stress, ceramide, and autophagy. Journal of lipid research 2011;52:1636-51.
136. Olivieri O, Martinelli N, Sandri M, et al. Apolipoprotein C-III, n-3 polyunsaturated fatty acids, and "insulin-resistant" T-455C APOC3 gene polymorphism in heart disease patients: example of gene-diet interaction. Clinical chemistry 2005;51:360-7.
137. Papamandjaris AA, MacDougall DE, Jones PJ. Medium chain fatty acid metabolism and energy expenditure: obesity treatment implications. Life sciences 1998;62:1203-15.
138. Frangioudakis G, Garrard J, Raddatz K, Nadler JL, Mitchell TW, Schmitz-Peiffer C. Saturated- and n-6 polyunsaturated-fat diets each induce ceramide accumulation in mouse skeletal muscle: reversal and improvement of glucose tolerance by lipid metabolism inhibitors. Endocrinology 2010;151:4187-96.
139. Boini KM, Zhang C, Xia M, Poklis JL, Li PL. Role of sphingolipid mediator ceramide in obesity and renal injury in mice fed a high-fat diet. The Journal of pharmacology and experimental therapeutics 2010;334:839-46.
140. Pagadala M, Kasumov T, McCullough AJ, Zein NN, Kirwan JP. Role of ceramides in nonalcoholic fatty liver disease. Trends in endocrinology and metabolism: TEM 2012;23:365-71.
141. Moles A, Tarrats N, Morales A, et al. Acidic sphingomyelinase controls hepatic stellate cell activation and in vivo liver fibrogenesis. The American journal of pathology 2010;177:1214-24.
142. Haus JM, Kashyap SR, Kasumov T, et al. Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance. Diabetes 2009;58:337-43.
143. D'Alessio A, Al-Lamki RS, Bradley JR, Pober JS. Caveolae participate in tumor necrosis factor receptor 1 signaling and internalization in a human endothelial cell line. The American journal of pathology 2005;166:1273-82.
144. Ma DW, Seo J, Davidson LA, et al. n-3 PUFA alter caveolae lipid composition and resident protein localization in mouse colon. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2004;18:1040-2.
145. Opreanu M, Lydic TA, Reid GE, McSorley KM, Esselman WJ, Busik JV. Inhibition of cytokine signaling in human retinal endothelial cells through downregulation of

sphingomyelinases by docosahexaenoic acid. Investigative ophthalmology & visual science 2010;51:3253-63.
146. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). Progress in lipid research 2009;48:1-26.
147. Shapiro H, Tehilla M, Attal-Singer J, Bruck R, Luzzatti R, Singer P. The therapeutic potential of long-chain omega-3 fatty acids in nonalcoholic fatty liver disease. Clinical nutrition (Edinburgh, Scotland) 2011;30:6-19.
148. Zuniga J, Cancino M, Medina F, et al. N-3 PUFA supplementation triggers PPAR-alpha activation and PPAR-alpha/NF-kappaB interaction: anti-inflammatory implications in liver ischemia-reperfusion injury. PloS one 2011;6:e28502.
149. Chen G, Liang G, Ou J, Goldstein JL, Brown MS. Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. Proceedings of the National Academy of Sciences of the United States of America 2004;101:11245-50.
150. Ikegami T, Hyogo H, Honda A, et al. Increased serum liver X receptor ligand oxysterols in patients with non-alcoholic fatty liver disease. Journal of gastroenterology 2012;47:1257-66.
151. Pannu PS, Allahverdian S, Francis GA. Oxysterol generation and liver X receptor-dependent reverse cholesterol transport: not all roads lead to Rome. Molecular and cellular endocrinology 2013;368:99-107.
152. Takeuchi Y, Yahagi N, Izumida Y, et al. Polyunsaturated fatty acids selectively suppress sterol regulatory element-binding protein-1 through proteolytic processing and autoloop regulatory circuit. The Journal of biological chemistry 2010;285:11681-91.
153. Neschen S, Morino K, Dong J, et al. n-3 Fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner. Diabetes 2007;56:1034-41.
154. Novak EM, Keller BO, Innis SM. Metabolic development in the liver and the implications of the n-3 fatty acid supply. American journal of physiology Gastrointestinal and liver physiology 2012;302:G250-9.
155. Shearer GC, Savinova OV, Harris WS. Fish oil -- how does it reduce plasma triglycerides? Biochimica et biophysica acta 2012;1821:843-51.
156. Jump DB, Botolin D, Wang Y, Xu J, Demeure O, Christian B. Docosahexaenoic acid (DHA) and hepatic gene transcription. Chemistry and physics of lipids 2008;153:3-13.
157. Botolin D, Wang Y, Christian B, Jump DB. Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways. Journal of lipid research 2006;47:181-92.
158. Uyeda K, Yamashita H, Kawaguchi T. Carbohydrate responsive element-binding protein (ChREBP): a key regulator of glucose metabolism and fat storage. Biochemical pharmacology 2002;63:2075-80.
159. Kabashima T, Kawaguchi T, Wadzinski BE, Uyeda K. Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat liver. Proceedings of the National Academy of Sciences of the United States of America 2003;100:5107-12.
160. Ishii S, Iizuka K, Miller BC, Uyeda K. Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. Proceedings of the National Academy of Sciences of the United States of America 2004;101:15597-602.
161. Xu J, Christian B, Jump DB. Regulation of rat hepatic L-pyruvate kinase promoter composition and activity by glucose, n-3 polyunsaturated fatty acids, and peroxisome proliferator-activated receptor-alpha agonist. The Journal of biological chemistry 2006;281:18351-62.

162. Sung KC, Jeong WS, Wild SH, Byrne CD. Combined influence of insulin resistance, overweight/obesity, and fatty liver as risk factors for type 2 diabetes. Diabetes care 2012;35:717-22.
163. Bugianesi E, McCullough AJ, Marchesini G. Insulin resistance: a metabolic pathway to chronic liver disease. Hepatology (Baltimore, Md) 2005;42:987-1000.
164. Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. The Journal of clinical investigation 2008;118:829-38.
165. Lorente-Cebrian S, Bustos M, Marti A, Martinez JA, Moreno-Aliaga MJ. Eicosapentaenoic acid stimulates AMP-activated protein kinase and increases visfatin secretion in cultured murine adipocytes. Clinical science (London, England : 1979) 2009;117:243-9.
166. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. The Journal of clinical investigation 2003;112:1821-30.
167. Cai L, Wang Z, Ji A, Meyer JM, van der Westhuyzen DR. Scavenger receptor CD36 expression contributes to adipose tissue inflammation and cell death in diet-induced obesity. PloS one 2012;7:e36785.
168. Madonna R, Salerni S, Schiavone D, Glatz JF, Geng YJ, De Caterina R. Omega-3 fatty acids attenuate constitutive and insulin-induced CD36 expression through a suppression of PPAR alpha/gamma activity in microvascular endothelial cells. Thrombosis and haemostasis 2011;106:500-10.
169. Puglisi MJ, Hasty AH, Saraswathi V. The role of adipose tissue in mediating the beneficial effects of dietary fish oil. The Journal of nutritional biochemistry 2011;22:101-8.
170. Claria J, Gonzalez-Periz A, Lopez-Vicario C, Rius B, Titos E. New insights into the role of macrophages in adipose tissue inflammation and Fatty liver disease: modulation by endogenous omega-3 Fatty Acid-derived lipid mediators. Frontiers in immunology 2011;2:49.
171. Guha IN, Parkes J, Roderick P, et al. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. Hepatology (Baltimore, Md) 2008;47:455-60.
172. Scorletti E, Bhatia L, McCormick KG, et al. Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chainomega-3 fatty acid treatment in non-alcoholic fatty liver disease [corrected]. Contemporary clinical trials 2014;37:301-11.
173. Cussons AJ, Watts GF, Mori TA, Stuckey BG. Omega-3 fatty acid supplementation decreases liver fat content in polycystic ovary syndrome: a randomized controlled trial employing proton magnetic resonance spectroscopy. The Journal of clinical endocrinology and metabolism 2009;94:3842-8.
174. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology (Baltimore, Md) 2005;41:1313-21.
175. Duffy JC. Alcohol consumption and all-cause mortality. International journal of epidemiology 1995;24:100-5.
176. Menon BK, Smith EE, Modi J, et al. Regional leptomeningeal score on CT angiography predicts clinical and imaging outcomes in patients with acute anterior circulation occlusions. AJNR American journal of neuroradiology 2011;32:1640-5.
177. Elizondo A, Araya J, Rodrigo R, et al. Effects of weight loss on liver and erythrocyte polyunsaturated fatty acid pattern and oxidative stress status in obese patients with non-alcoholic fatty liver disease. Biological research 2008;41:59-68.

178. Barros KV, Carvalho PO, Cassulino AP, et al. Fatty acids in plasma, white and red blood cells, and tissues after oral or intravenous administration of fish oil in rats. Clinical nutrition (Edinburgh, Scotland) 2013;32:993-8.
179. Bannas P, Kramer H, Hernando D, et al. Quantitative magnetic resonance imaging of hepatic steatosis: Validation in ex vivo human livers. Hepatology (Baltimore, Md) 2015;62:1444-55.
180. Reeder SB, Cruite I, Hamilton G, Sirlin CB. Quantitative assessment of liver fat with magnetic resonance imaging and spectroscopy. Journal of magnetic resonance imaging : JMRI 2011;34:729-49.
181. Nasr P, Forsgren MF, Ignatova S, et al. Using a 3% Proton Density Fat Fraction as a Cut-Off Value Increases Sensitivity of Detection of Hepatic Steatosis, Based on Results From Histopathology Analysis. Gastroenterology 2017;153:53-5.e7.
182. Byrne CD, Targher G. Time to Replace Assessment of Liver Histology With MR-Based Imaging Tests to Assess Efficacy of Interventions for Nonalcoholic Fatty Liver Disease. Gastroenterology 2016;150:7-10.
183. Bedossa P, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology (Baltimore, Md) 2003;38:1449-57.
184. Colloredo G, Guido M, Sonzogni A, Leandro G. Impact of liver biopsy size on histological evaluation of chronic viral hepatitis: the smaller the sample, the milder the disease. Journal of hepatology 2003;39:239-44.
185. Ratziu V, Charlotte F, Heurtier A, et al. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology 2005;128:1898-906.
186. Vuppalanchi R, Unalp A, Van Natta ML, et al. Effects of liver biopsy sample length and number of readings on sampling variability in nonalcoholic Fatty liver disease. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 2009;7:481-6.
187. Kleiner DE, Bedossa P. Liver histology and clinical trials for nonalcoholic steatohepatitis-perspectives from 2 pathologists. Gastroenterology 2015;149:1305-8.
188. Robinson S, Syddall H, Jameson K, et al. Current patterns of diet in community-dwelling older men and women: results from the Hertfordshire Cohort Study. Age and ageing 2009;38:594-9.
189. Mignault D, St-Onge M, Karelis AD, Allison DB, Rabasa-Lhoret R. Evaluation of the Portable HealthWear Armband: a device to measure total daily energy expenditure in free-living type 2 diabetic individuals. Diabetes care 2005;28:225-7.
190. Parker HM, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. Journal of hepatology 2012;56:944-51.
191. Targher G, Byrne CD. Nonalcoholic Fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications. JClinEndocrinolMetab 2013;98:483-95.
192. Lam B, Younossi ZM. Treatment options for nonalcoholic fatty liver disease. Therapeutic advances in gastroenterology 2010;3:121-37.
193. Murphy CE, Rodgers PT. Effects of thiazolidinediones on bone loss and fracture. The Annals of pharmacotherapy 2007;41:2014-8.
194. Dietrich M, Jacques PF, Pencina MJ, et al. Vitamin E supplement use and the incidence of cardiovascular disease and all-cause mortality in the Framingham Heart Study: Does the underlying health status play a role? Atherosclerosis 2009;205:549-53.
195. Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 2009;120:1640-5.

196. Cusi K, Chang Z, Harrison S, et al. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. Journal of hepatology 2014;60:167-74.
197. Holt HB, Wild SH, Wareham N, et al. Differential effects of fatness, fitness and physical activity energy expenditure on whole-body, liver and fat insulin sensitivity. Diabetologia 2007;50:1698-706.
198. Browning LM, Walker CG, Mander AP, et al. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. The American journal of clinical nutrition 2012;96:748-58.
199. Slee EL, McLennan PL, Owen AJ, Theiss ML. Low dietary fish-oil threshold for myocardial membrane n-3 PUFA enrichment independent of n-6 PUFA intake in rats. Journal of lipid research 2010;51:1841-8.
200. Harris WS, Von Schacky C. The Omega-3 Index: a new risk factor for death from coronary heart disease? Preventive medicine 2004;39:212-20.
201. Nobili V, Bedogni G, Alisi A, et al. Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial. Archives of disease in childhood 2011;96:350-3.
202. Romeo S, Kozlitina J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nature genetics 2008;40:1461-5.
203. Mashek DG, Greenberg AS. Serum TAG analysis differentiates between genetic and obesity-associated NAFLD. Diabetes 2014;63:42-4.
204. Hyysalo J, Gopalacharyulu P, Bian H, et al. Circulating triacylglycerol signatures in nonalcoholic fatty liver disease associated with the I148M variant in PNPLA3 and with obesity. Diabetes 2014;63:312-22.
205. Pirazzi C, Adiels M, Burza MA, et al. Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro. Journal of hepatology 2012;57:1276-82.
206. Kahali B, Liu YL, Daly AK, Day CP, Anstee QM, Speliotes EK. TM6SF2: catch-22 in the fight against nonalcoholic fatty liver disease and cardiovascular disease? Gastroenterology 2015;148:679-84.
207. Milano M, Aghemo A, Mancina RM, et al. Transmembrane 6 superfamily member 2 gene E167K variant impacts on steatosis and liver damage in chronic hepatitis C patients. Hepatology (Baltimore, Md) 2015;62:111-7.
208. Dongiovanni P, Petta S, Maglio C, et al. Transmembrane 6 superfamily member 2 gene variant disentangles nonalcoholic steatohepatitis from cardiovascular disease. Hepatology (Baltimore, Md) 2015;61:506-14.
209. Sookoian S, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. Hepatology (Baltimore, Md) 2011;53:1883-94.
210. Dongiovanni P, Donati B, Fares R, et al. PNPLA3 I148M polymorphism and progressive liver disease. World journal of gastroenterology 2013;19:6969-78.
211. Shen J, Wong GL, Chan HL, et al. PNPLA3 gene polymorphism and response to lifestyle modification in patients with nonalcoholic fatty liver disease. Journal of gastroenterology and hepatology 2015;30:139-46.
212. Stillwell W, Wassall SR. Docosahexaenoic acid: membrane properties of a unique fatty acid. Chemistry and physics of lipids 2003;126:1-27.
213. Tan ZS, Harris WS, Beiser AS, et al. Red blood cell omega-3 fatty acid levels and markers of accelerated brain aging. Neurology 2012;78:658-64.

214. Santoro N, Savoye M, Kim G, et al. Hepatic fat accumulation is modulated by the interaction between the rs738409 variant in the PNPLA3 gene and the dietary omega6/omega3 PUFA intake. PloS one 2012;7:e37827.
215. Wojcik C, Lohe K, Kuang C, Xiao Y, Jouni Z, Poels E. Modulation of adipocyte differentiation by omega-3 polyunsaturated fatty acids involves the ubiquitin-proteasome system. Journal of cellular and molecular medicine 2014;18:590-9.
216. Mancina RM, Matikainen N, Maglio C, et al. Paradoxical dissociation between hepatic fat content and de novo lipogenesis due to PNPLA3 sequence variant. The Journal of clinical endocrinology and metabolism 2015;100:E821-5.
217. Nobili V, Bedogni G, Donati B, Alisi A, Valenti L. The I148M variant of PNPLA3 reduces the response to docosahexaenoic acid in children with non-alcoholic fatty liver disease. Journal of medicinal food 2013;16:957-60.
218. Scorletti E, Bhatia L, McCormick KG, et al. Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology (Baltimore, Md) 2014;60:1211-21.
219. Dongiovanni P, Petta S, Mannisto V, et al. Statin use and non-alcoholic steatohepatitis in at risk individuals. Journal of hepatology 2015;63:705-12.
220. Ruhanen H, Perttila J, Holtta-Vuori M, et al. PNPLA3 mediates hepatocyte triacylglycerol remodeling. Journal of lipid research 2014;55:739-46.
221. Leonard AE, Pereira SL, Sprecher H, Huang YS. Elongation of long-chain fatty acids. Progress in lipid research 2004;43:36-54.
222. Alsaleh A, Maniou Z, Lewis FJ, Hall WL, Sanders TA, O'Dell SD. ELOVL2 gene polymorphisms are associated with increases in plasma eicosapentaenoic and docosahexaenoic acid proportions after fish oil supplement. Genes & nutrition 2014;9:362.
223. Al-Hilal M, Alsaleh A, Maniou Z, et al. Genetic variation at the FADS1-FADS2 gene locus influences delta-5 desaturase activity and LC-PUFA proportions after fish oil supplement. Journal of lipid research 2013;54:542-51.
224. Wang L, Athinarayanan S, Jiang G, Chalasani N, Zhang M, Liu W. Fatty acid desaturase 1 gene polymorphisms control human hepatic lipid composition. Hepatology (Baltimore, Md) 2015;61:119-28.
225. Truong H, DiBello JR, Ruiz-Narvaez E, Kraft P, Campos H, Baylin A. Does genetic variation in the Delta6-desaturase promoter modify the association between alpha-linolenic acid and the prevalence of metabolic syndrome? The American journal of clinical nutrition 2009;89:920-5.
226. Aulchenko YS, Ripatti S, Lindqvist I, et al. Loci influencing lipid levels and coronary heart disease risk in 16 European population cohorts. Nature genetics 2009;41:47-55.
227. Sabatti C, Service SK, Hartikainen AL, et al. Genome-wide association analysis of metabolic traits in a birth cohort from a founder population. Nature genetics 2009;41:35-46.
228. Kathiresan S, Willer CJ, Peloso GM, et al. Common variants at 30 loci contribute to polygenic dyslipidemia. Nature genetics 2009;41:56-65.
229. Nakayama K, Bayasgalan T, Tazoe F, et al. A single nucleotide polymorphism in the FADS1/FADS2 gene is associated with plasma lipid profiles in two genetically similar Asian ethnic groups with distinctive differences in lifestyle. Human genetics 2010;127:685-90.
230. Bokor S, Dumont J, Spinneker A, et al. Single nucleotide polymorphisms in the FADS gene cluster are associated with delta-5 and delta-6 desaturase activities estimated by serum fatty acid ratios. Journal of lipid research 2010;51:2325-33.
231. Takkunen MJ, de Mello VD, Schwab US, et al. Gene-diet interaction of a common FADS1 variant with marine polyunsaturated fatty acids for fatty acid composition in plasma and erythrocytes among men. Molecular nutrition & food research 2016;60:381-9.

232. Walle P, Takkunen M, Mannisto V, et al. Fatty acid metabolism is altered in non-alcoholic steatohepatitis independent of obesity. Metabolism: clinical and experimental 2016;65:655-66.
233. Reardon HT, Zhang J, Kothapalli KS, Kim AJ, Park WJ, Brenna JT. Insertion-deletions in a FADS2 intron 1 conserved regulatory locus control expression of fatty acid desaturases 1 and 2 and modulate response to simvastatin. Prostaglandins, leukotrienes, and essential fatty acids 2012;87:25-33.
234. Nakamura MT, Nara TY. Essential fatty acid synthesis and its regulation in mammals. Prostaglandins, leukotrienes, and essential fatty acids 2003;68:145-50.
235. Matsuzaka T, Shimano H, Yahagi N, et al. Dual regulation of mouse Delta(5)- and Delta(6)-desaturase gene expression by SREBP-1 and PPARalpha. Journal of lipid research 2002;43:107-14.
236. Lu Y, Feskens EJ, Dolle ME, et al. Dietary n-3 and n-6 polyunsaturated fatty acid intake interacts with FADS1 genetic variation to affect total and HDL-cholesterol concentrations in the Doetinchem Cohort Study. The American journal of clinical nutrition 2010;92:258-65.
237. He XX, Wu XL, Chen RP, et al. Effectiveness of Omega-3 Polyunsaturated Fatty Acids in Non-Alcoholic Fatty Liver Disease: A Meta-Analysis of Randomized Controlled Trials. PloS one 2016;11:e0162368.
238. Capanni M, Calella F, Biagini MR, et al. Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study. Alimentary pharmacology & therapeutics 2006;23:1143-51.
239. Sofi F, Giangrandi I, Cesari F, et al. Effects of a 1-year dietary intervention with n-3 polyunsaturated fatty acid-enriched olive oil on non-alcoholic fatty liver disease patients: a preliminary study. International journal of food sciences and nutrition 2010;61:792-802.
240. Spadaro L, Magliocco O, Spampinato D, et al. Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver 2008;40:194-9.
241. Kris-Etherton PM, Harris WS, Appel LJ. Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. Arteriosclerosis, thrombosis, and vascular biology 2003;23:151-2.
242. Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. The American journal of clinical nutrition 2006;83:1505s-19s.
243. Rius B, Titos E, Moran-Salvador E, et al. Resolvin D1 primes the resolution process initiated by calorie restriction in obesity-induced steatohepatitis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2014;28:836-48.
244. Nobili V, Alisi A, Musso G, Scorletti E, Calder PC, Byrne CD. Omega-3 fatty acids: Mechanisms of benefit and therapeutic effects in pediatric and adult NAFLD. Critical reviews in clinical laboratory sciences 2016;53:106-20.
245. Szczepaniak LS, Nurenberg P, Leonard D, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. American journal of physiology Endocrinology and metabolism 2005;288:E462-8.
246. Machann J, Thamer C, Schnoedt B, et al. Hepatic lipid accumulation in healthy subjects: a comparative study using spectral fat-selective MRI and volume-localized 1H-MR spectroscopy. Magnetic resonance in medicine 2006;55:913-7.
247. Rosenberg WM, Voelker M, Thiel R, et al. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology 2004;127:1704-13.

248. Kettaneh A, Marcellin P, Douvin C, et al. Features associated with success rate and performance of FibroScan measurements for the diagnosis of cirrhosis in HCV patients: a prospective study of 935 patients. Journal of hepatology 2007;46:628-34.
249. Pacifico L, Bonci E, Di Martino M, et al. A double-blind, placebo-controlled randomized trial to evaluate the efficacy of docosahexaenoic acid supplementation on hepatic fat and associated cardiovascular risk factors in overweight children with nonalcoholic fatty liver disease. Nutrition, metabolism, and cardiovascular diseases : NMCD 2015;25:734-41.
250. Nobili V, Alisi A, Della Corte C, et al. Docosahexaenoic acid for the treatment of fatty liver: randomised controlled trial in children. Nutrition, metabolism, and cardiovascular diseases : NMCD 2013;23:1066-70.
251. Della Corte C, Carpino G, De Vito R, et al. Docosahexanoic Acid Plus Vitamin D Treatment Improves Features of NAFLD in Children with Serum Vitamin D Deficiency: Results from a Single Centre Trial. PloS one 2016;11:e0168216.
252. de Castro GS, Calder PC. Non-alcoholic fatty liver disease and its treatment with n-3 polyunsaturated fatty acids. Clinical nutrition (Edinburgh, Scotland) 2017.
253. Sanyal AJ, Abdelmalek MF, Suzuki A, Cummings OW, Chojkier M. No significant effects of ethyl-eicosapentanoic acid on histologic features of nonalcoholic steatohepatitis in a phase 2 trial. Gastroenterology 2014;147:377-84.e1.
254. Mori TA, Woodman RJ. The independent effects of eicosapentaenoic acid and docosahexaenoic acid on cardiovascular risk factors in humans. Current opinion in clinical nutrition and metabolic care 2006;9:95-104.
255. Mori TA, Burke V, Puddey IB, et al. Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. The American journal of clinical nutrition 2000;71:1085-94.
256. Woodman RJ, Mori TA, Burke V, et al. Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. Atherosclerosis 2003;166:85-93.
257. Mesa MD, Buckley R, Minihane AM, Yaqoob P. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on the oxidizability and thrombogenicity of low-density lipoprotein. Atherosclerosis 2004;175:333-43.
258. Leigh-Firbank EC, Minihane AM, Leake DS, et al. Eicosapentaenoic acid and docosahexaenoic acid from fish oils: differential associations with lipid responses. The British journal of nutrition 2002;87:435-45.
259. Baker EJ, Miles EA, Burdge GC, Yaqoob P, Calder PC. Metabolism and functional effects of plant-derived omega-3 fatty acids in humans. Progress in lipid research 2016;64:30-56.