The Effect of Omega-3 Fatty Acids on Non-alcoholic Fatty Liver Disease

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Declaration

I, Gail Susan Mary Masterton, hereby declare that the work described herein has been composed by myself, and has not been submitted for any other degree or personal qualification.

Abstract

Non-alcoholic fatty liver disease (NAFLD) may progress to cirrhosis and end-stage liver disease. Worldwide the prevalence is increasing in line with the global obesity epidemic. To date there is no agreed pharmacological therapy for this condition.

Omega-3 fatty acids have been suggested as a treatment for NAFLD. The theoretical rationale, and data from preliminary studies in animals and humans, are reviewed and investigated further. Firstly the effect of omega-3 fatty acids in patients with NAFLD is explored in a placebo-controlled, double-blind randomised trial. This is supplemented by data from a series of cell culture studies using two models of cellular steatosis.

The clinical trial randomised 50 subjects with NAFLD to 4 grams daily of omega-3 fatty acids or placebo for six months. Subjects were reassessed three months after the end of treatment to ascertain if any changes observed had regressed. The primary endpoint of the study was change in ultrasound grade of steatosis at six months. Secondary endpoints included change in serum liver function tests (LFTs) and health-related quality of life scored by WHOQOL-BREF.

The subjects were closely matched at baseline. There was no significant difference between participants who received omega-3 and those who received placebo in change in either the primary or secondary outcome measures i.e. grade of steatosis on ultrasound, serum liver function tests, serum lipids and health related quality of life scores at either six or nine months. This was a negative study.

The role of omega-3 fatty acids was explored *in vitro* using C3Ahepatocytesincubated in standard media and subsequently in two models of cellular steatosis: oleate (a model of 'simple' steatosis) and LPON (a model of steatosis and mitochondrial dysfunction). The optimal concentration of omega-3 (eicosapentaenoic acid (EPA)) was initially determined using dose finding experiments. The effect of EPA on hepatocyte triglyceride content was quantified in standard and steatotic conditions using Oil red O staining. EPA was then evaluated in each model as a prophylactic agent, and as a treatment to cells with established steatosis. Hepatocytes incubated in standard medium with 250μM EPA showed reduced triglyceride content in each experiment and a dose response relationship was observed. In contrast, both oleate and LPON models failed to show a consistent effect.

In summary, although EPA treatment reduced hepatic triglyceride content in cell culture under standard conditions, this was not reproduced in the models of hepatocyte steatosis. The clinical trial findings were consistent with these observations, and overall these studies do not support the use of omega-3 fatty acids as a treatment for NAFLD.

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This thesis is dedicated to the memory

of Dr Mary Gardner.

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List of Abbreviations

Alk Phos/ALK – alkaline phosphatase

ALT – alanine aminotransferase

ANOVA – analysis of variance

AST – aspartate aminotransferase

Bil – bilirubin

BMI – body mass index

ChREBP – carbohydrate regulatory element-binding protein

CTIMP – clinical trial of an investigational medicinal product

DHA – docosahexaenoic acid

DNA – deoxyribonucleic acid

EPA – eicosapentaenoic acid

FXR – farnesoid X receptor

γGT/GGT – gamma glutamyltransferase

G6PdH – glucose-6-phosphate dehydrogenase

HBSS- – Hank's balanced salt solution (without calcium and magnesium)

HBSS+ – Hank's balanced salt solution (supplemented with magnesium and calcium)

HDL – high density lipoprotein

HNF 4α – hepatocyte nuclear factor 4α

HK – hexokinase

HOMA-IR – homeostasis model assessment of insulin resistance

Hr – hour

HRQoL- Health related quality of life

H_2O – water

IL-6 – interleukin 6

LDH – lactate dehydrogenase

LDL – low density lipoprotein

LFT – liver function test

LXR - liver X receptor

MEME- – Minimum Essential Medium Eagle supplemented with penicillin and streptomycin

MEME+ – Minimum Essential Medium Eagle supplemented with FCS

MHRA – Medicines and Healthcare products Regulatory Agency

Min – minute

mRNA – messenger ribonucleic acid

N-3 – omega-3 fatty acids

N3-FA – omega-3 fatty acids

NAFLD – non-alcoholic fatty liver disease

NASH – non-alcoholic steatohepatitis

NFkB – Nuclear factor kappa B

ob/ob mice – a leptin deficient murine model characterised by obese and insulin resistant mice

PPAR – peroxisome proliferator-activated receptor

PUFA – polyunsaturated fatty acid

RIE – Royal Infirmary of Edinburgh

ROS – reactive oxygen species

SD – standard deviation

Sec – second

SEM – standard error of the mean

SREBP-1 – sterol regulatory element binding protein

t-BOOH - tert-butyl hydroperoxide

Trig/TG – triglyceride

TNFα– tissue necrosis factor alpha

VLDL – very low density lipoprotein

WHOQOL – World Health Organisation Quality of Life Score

Aims of Thesis

To address the following questions:

1. Do patients with NAFLD have altered fat content on ultrasound when given omega-3 fatty acid supplements for six months? If yes, are these changes sustained 3 months after treatment has stopped?

2. Does treatment with omega-3 fatty acids alter serum liver function tests in patients with NAFLD?

3. Does treatment with omega-3 fatty acids alter the Health related quality of life scores of patients with NAFLD?

4. Do NAFLD cells in culture have altered lipid content when incubated with omega-3 fatty acids?

Section I

Introduction

1.1 Background

Non-alcoholic fatty liver disease (NAFLD), defined as the pathological accumulation of fat in the liver when no other explanatory disease is present, is a term which encompasses a spectrum of conditions from isolated hepatic steatosis, non-alcoholic steatohepatitis (NASH) and cirrhosis. NAFLD represents a significant health problem both within the UK and worldwide as its prevalence increases in line with the obesity epidemic. The health burden caused by NAFLD is being delineated: a review reported NAFLD affects 10-35% of the adult population globally (1). NAFLD is already the most common cause of abnormal liver function tests in the United States(2) where it is the third commonest reason for referral for liver transplantation.(3) NAFLD also accounts for 11% of referrals to hepatology services.(4)

NAFLD may be considered as the hepatic expression of the metabolic syndrome which consists of hypertension, insulin resistance, obesity and dyslipidaemia.(5) This assertion is based on the observation that the more facets of the metabolic syndrome that are present, the greater the chance of developing NAFLD.(6, 7) Further, the presence of NAFLD predicts the presence or development of other features of the metabolic syndrome.(7, 8)

NAFLD was initially considered a benign condition. It is now recognised that this is not the case. In particular, NASH can progress to fibrosis and cirrhosis.(9, 10)NAFLD is as an increasingly important cause of liver failure and a risk factor for hepatocellular carcinoma. In addition, NASH is independently associated with

increased incidence of cardiovascular events(11) and, in the NHANES-III population based study, increased mortality.(12)

1.2 The Pathogenesis of NAFLD

The pathogenesis of NAFLD remains incompletely understood. Classically it has been considered to be the outcome of ‘two hits’(13)and many still use this as a framework to consider the pathological mechanisms believed to underlie the disease.(14)In this model the first hits are thought to comprise steatosis, primarily in the form of triglyceride accumulation, and insulin resistance.(15) Mitochondrial dysfunction precipitating oxidative stress then occurs. This is thought to trigger an inflammatory and fibrogenic cascade in the primed liver.(13)

More recently it has been proposed that steatosis is an epiphenomenon, and protective mechanism, of oxidative stress.(16, 17). Here substrate excess results in increased stress on the endoplasmic reticulum which, if unresolved, results in de novo lipogenesis.(18) Hepatic inflammation is then a result of increased reactive oxygen species (ROS) generation as the mitochondria adapt to the increased metabolic demands.(19) Increased ROS production not only exacerbates the stress on the endoplasmic reticulum(20) but also increases mitochondrial dysfunction.(19) Then, as with the previous model, oxidative stress result in a profibrotic and proinflammatory cascade through mediators such as nuclear factor kB.(21)

In addition, a full explanation of the pathogenesis of NAFLD requires recognition of the role of adipose tissue with the secretion of pro-inflammatory and pro-thrombotic

adipocytokines, IL-6 and TNFα(22-24) and the reduced production of the adipocytokine adiponectin, a potent anti-inflammatory insulin sensitising agent.(25, 26)

1.3 Current Treatment Strategies for NAFLD

To date there is no effective drug treatment for NAFLD.(27)Various treatments have been tested in animal and human clinic trials. These have derived from an understanding of the pathogenesis of NAFLD which have identified different therapeutic targets. A summary can be found in Table 1.1.

At present the cornerstone of treatment for NAFLD is advice regarding diet and weight loss and the energetic management of any co-existing features of the metabolic syndrome. There is no specific diet which has been shown to be of benefit.(28). Studies of the dietary habits of patients with NAFLD reveal they consume less oily fish, double the quantity of soft drinks and 27% more meat compared with the general population. These dietary differences have been shown to be associated with an increased risk of NAFLD independent of traditional risk factors.(29)

Omega-3 fatty acids have been forwarded as a potential treatment for NAFLD.(30) These essential fatty acids are licenced for the treatment of hypertriglyceridaemia and in the treatment of cardiovascular disease.(31-33) The benefit of omega-3 fatty acids in cardiovascular disease has been questioned and results of recent meta-analyses have failed to clarify this with some continuing to demonstrate benefit and

others suggesting that omega-3 fatty acids are not superior to placebo.(34-36) Despite this interest has grown in their potential as treatments of other myriad conditions ranging from cancer, mood disorders and cognitive disorders.(37-41) There are promising data from both animal and human trials on the use of omega-3s in NAFLD. Here the potential mechanisms through which omega-3 fatty acids may be of benefit in NAFLD and the current data supporting its use will be discussed.

Strategy	*Intervention*	
Weight Loss	Lifestyle measures(42, 43), (44) Bariatric surgery(45-48)	
	Drugs	Orlistat(49-51) Sibutramine(51) Rimonabant(52)
Reduce Insulin Resistance	Metformin(53-57) Thiazolidinediones(58-62)	
Antioxidant	Vitamin E(63-65) Probucol(66) N acetyl cysteine(67)	
Anti-TNF	Pentoxiphylline(68, 69)	
Other	Ursodeoxycholic acid(70) Statins (71) Angiotensin 2 antagonists (72), (73) Betaine(74) Probiotics (75) Yo Jyo Hen Shi Ko(76)	

Table 1.1 Interventions suggested as a treatment for Non-Alcoholic Fatty Liver Disease

1.4 Omega-3 Fatty Acids: Background and Metabolism

Omega-3 (N-3) fatty acids are essential polyunsaturated fatty acids (PUFA), i.e. they cannot be synthesised *in vivo*. Oily fish, flaxseed and some nuts comprise the main dietary sources rich in N-3 PUFAs. Omega-3 fatty acids derive from α linolenic acid and mainly occur as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These both have anti-inflammatory properties (77) but may themselves converted to active metabolites: molecules known as resolvins and protectins. These lipid products have yet to be fully characterised but are believed to have a role in the anti-inflammatory effects of omega-3 fatty acids.(78)This was confirmed in a murine model of NASH where treatment with resolvins reduced hepatic inflammation and steatosis whilst increasing adiponectin levels.(79)

In contrast, the other key group of polyunsaturated fatty acids, omega-6 (N-6) fatty acids, are found predominantly in grain. N-6 PUFAs derive from linolenic acid and their primary metabolite arachadonic acid (AA)which is both proinflammatory and prothrombotic.

N-6 and N-3 fatty acids are competitively metabolised by the same pathways. It is believed that the ratio of N-6 to N-3 should lie at approximately 3:1 however because modern diet is rich in foods containing N-6 PUFAs this ratio can be as high as 15:1.(80, 81)Other diseases, including breast cancer and asthma, have been linked to a high N6:N3 ratio.(82, 83)The importance of this ratio in NAFLD has been explored. In a biopsy based study of NAFLD the N-6:N-3 ratio correlated significantly with the quantity of hepatic triglycerides.(81)However, there is further evidence that the total amount of N-3 fatty acids rather than the N6:N:3 ratio is

important: large trials have shown that reduction in cardiovascular risk is linked to the total amount of N-3 fatty acids rather than the N-6:N-3 ratio.(84)Similarly, a low total N-3 level is found in NAFLD and is associated with steatosis, increased oxidative stress and the development of steatohepatitis (15, 85-88)

1.5 Omega-3 Regulation of Hepatic Gene Expression

Omega-3 fatty acids are key regulators of hepatic gene transcription. Best known of these are peroxisome proliferator-activated receptor alpha (PPARα) and sterol regulatory element binding protein-1 (SREBP-1).Others include PPARγ, hepatocyte nuclear factor 4α (HNF 4α), farnesoid X receptor (FXR)and carbohydrate regulatory element-binding protein (ChREBP). In addition to direct effects on hepatic gene expression, omega-3 fatty acids also may act as hydrophobic hormones: i.e. upon ligand binding and activation they bind to and alter the function of specific response elements in target genes.(89, 90)The diverse effects of omega-3 fatty acids on carbohydrate and lipid metabolism are summarised in Figure 1.1 Each will be considered in turn.

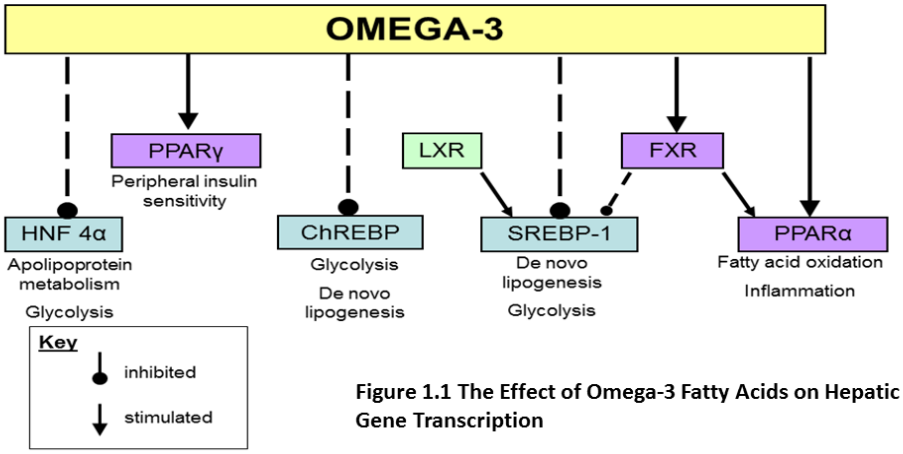


Figure 1.1 The Effect of Omega-3 Fatty Acids on Hepatic Gene Transcription

PPARα

Peroxisome proliferator-activated receptor alpha (PPARα)is a transcription factor whose importance as a therapeutic target for lipid lowering therapy is well recognised as it forms the basis of treatment with fibrates.(91)PPARα also increases mitochondrial beta oxidation.(92)Murine studies of steatohepatitis demonstrated that infusion of a PPARα agonist can not only prevent steatohepatitis, but also reverse established disease.(93, 94) Omega-3 fatty acids are potent activators of PPARα and upregulate several genes that stimulate fatty acid oxidation.(80, 89, 95-98)In addition, and independent to effects on lipid metabolism, PPARα is also thought to mediate an anti-inflammatory effect via suppression of TNFα and IL-6.(97, 99)

SREBP

Sterol regulatory element binding protein (SREBP) exists as three isoforms termed 1a,1c and 2. The latter has a role in cholesterol synthesis.(100). SREBP-1a is a potent activator of all genes under SREBP regulation; and SREBP-1c primarily influences genes involved in fatty acids synthesis. SREBP 1a and 1c will be considered together as SREBP-1.

SREBP-1 is an important regulator of fatty acid synthesis and insulin resistance.(101) Levels of SREBP-1 are elevated in response to high insulin and glucose concentrations by increasing the production of its precursor.(100-102)This precursor undergoes two post-translational processes – proteolytic processing and proteasomal degradation.(92) Following this mature SREBP-1 binds to sterol regulatory elements in the promoter areas of genes (including glucokinase, the intracellular enzyme which is the rate limiting step for glycolysis(102)) and thus stimulates increased de novo lipogenesis(102, 103) and glycolysis.(89)Over-expression of SREBP-1 thus results in hepatic triglyceride accumulation.(104)

Omega-3 fatty acids reduce the amount of mature SREBP-1 available in the nucleus(105) and thereby inhibit the downstream stimulatory effects of insulin.(89) This reduces insulin induced de novo lipogenesis.(106)It is believed that the effects of omega-3 fatty acids on SREBP-1 may be mediated by reducing the effective half-life of SREBP-1 mRNA.(107)Omega-3 has been shown to be effective at suppressing SREBP-1 activity in both *in vitro* in cell culture and *in vivo* in animal models.(103, 108-110)

PPARγ

Omega-3 fatty acids are ligands for PPARγ(90), a nuclear receptor which regulates adipose tissue metabolism and impacts on peripheral insulin sensitivity. The latter is the mechanism of action of thiazolidinediones. Studies to date suggest that omega-3 fatty acids increase fat oxidation and improve peripheral insulin sensitivity(111) but may increase hepatic insulin resistance.(112)

Liver X Receptor

Liver X receptors (LXR) regulate fatty acid and cholesterol transport and metabolism as well as promoting de novo lipogenesis via increased SREBP-1 expression.(113, 114) Whilst it was previously thought that LXR expression is unaffected by omega-3 fatty acids recently a murine model of inflammation demonstrated that derepression of LXR contributed to omega-3 mediated anti-inflammatory and insulin sensitising effects observed.(115-117)

Hepatocyte Nuclear Factor 4α (HNF 4α)

Omega-3 fatty acids are thought to inhibit hepatic lipogenesis through regulation of HNF 4α, a nuclear factor which plays an important role in lipoprotein production and whose activity is inhibited by omega-3 fatty acids.(118)

Farnesoid X Receptor (FXR)

FXR induces PPARα and inhibits SREBP-1 resulting in reduced hepatic triglycerides.(119) Omega-3 fatty acids have been shown to upregulate FXR.(120)

ChREBP

Carbohydrate regulatory element-binding protein (ChREBP), involved in the regulation of glycolysis(89, 121, 121, 122) is implicated in hepatic steatosis and insulin resistance. Animal models of blocking this molecule have improved steatosis and increased insulin sensitivity.(123) Omega-3 fatty acids suppress ChREBP.(124)

1.6 The Effect of Omega-3 on Cell Membrane Composition

Fatty acids are an essential constituent of cell membranes. They modulate the action of membrane-bound transporters and enzymes. Omega-3 fatty acids are membrane stabilisers and can influence cell membrane fluidity.(125) Dietary intake has been shown to correlate with hepatocyte membrane phospholipids composition and function(126)(127)and omega-3 fatty acids have been shown to be readily incorporated into hepatic phospholipids.(128)

Low levels of omega-3 in skeletal muscle phospholipids are associated with insulin resistance.(129) Omega-3 fatty acids also have a role in modifying intracellular messengers and altering intracellular functions.(130) In a cell based study it was shown that EPA did not inhibit lipogenesis but increased the oxidation of endogenous fatty acids, and intracellular CPT-1 (carnitine palmitoyltransferase 1) levels, the latter is an enzyme which catalyses a rate limiting step in the beta oxidation of fatty acids. The study also showed that EPA supplementation altered membrane composition and increased the amount of EPA in the adipocyte mitochondrial membrane.(131)

1.7 Evidence for Omega-3 Fatty Acids as a Treatment for NAFLD from Animal Models

The potentially beneficial effects of omega-3 fatty acids in NAFLD are supported by findings from animal studies using both murine and rat models of NAFLD. These are summarised in Figure 1.2.It should be noted, however that each model has limitations and incompletely replicates the understood pathogenesis and metabolic milieu of NAFLD.(132) A further limitation in the translation of these results to humans is the quantity of omega-3 generally constitute 5-20% of the animal's total dietary intake and far exceeds doses practical for humans.

Steatosis

Omega-3 treatment, as would be expected from cardiovascular studies, reduce plasma lipids in animal models of hepatic steatosis.(133-135) Omega-3 fatty acids have also been shown in murine models of steatosis to reduce established hepatic steatosis(136)(127)and protect the liver against the development of hepatic steatosis when given prophylactically.(137)These findings are confirmed in numerous other studies both for omega-3 mix, and EPA and DHA alone.(133-135, 138-142) One demonstrated improved hepatic steatosis with omega-3 treatment but was associated with a rise in serum transaminases.(143)

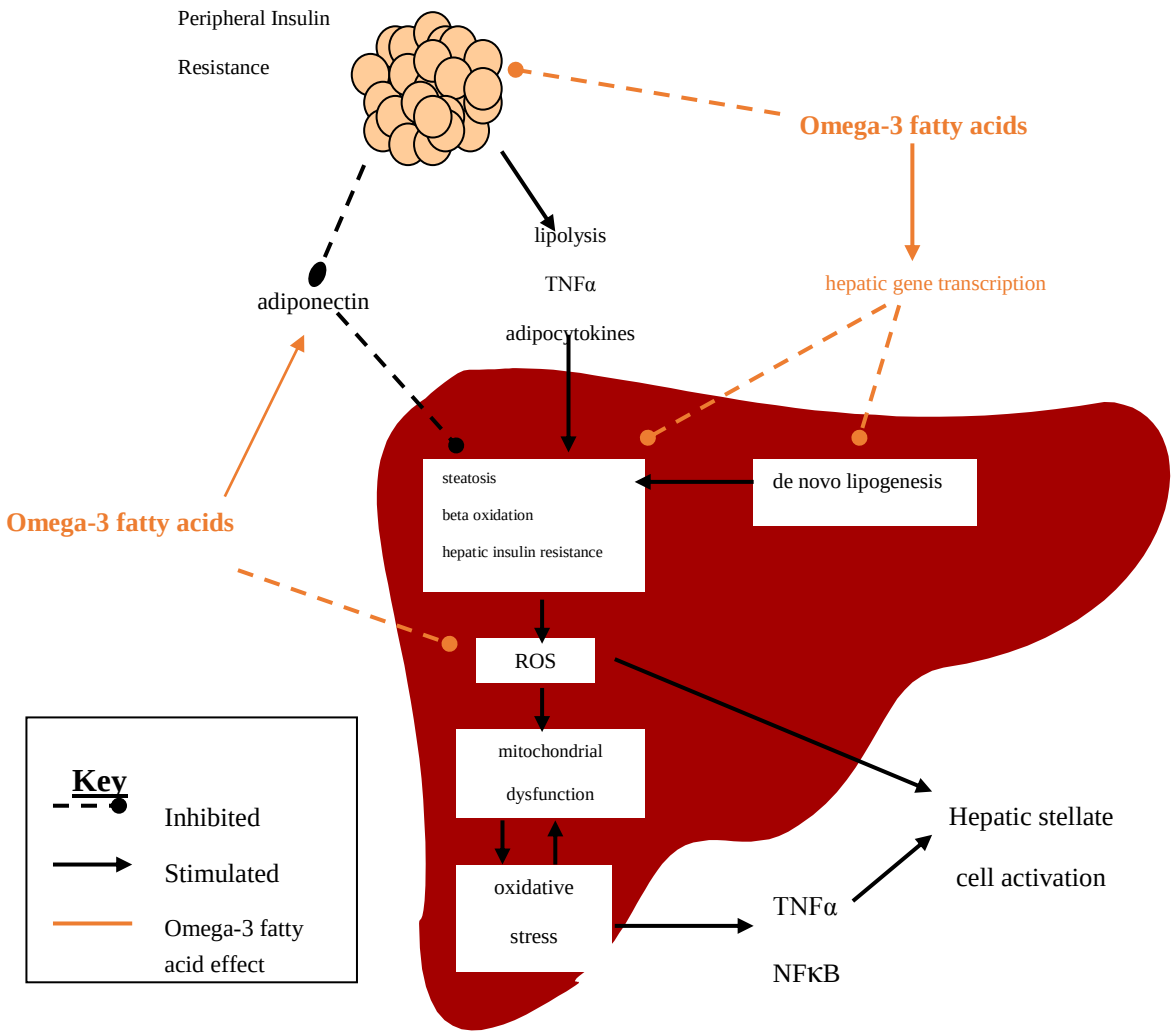


Figure 1.2 Summary of the effects of omega-3 fatty acids on the pathophysiology of NAFLD.

In a murine model of dietary induced NASH DHA was significantly more effective than EPA at reducing hepatic markers of inflammation, oxidative stress and fibrosis, although EPA was also effective.(144)Interestingly, neither EPA nor DHA attenuated hepatic steatosis in this study.

SREBP-1 and PPARα

But is there evidence of an effect on hepatic gene expression? In addition to reduced hepatic steatosis ob/ob mice treated with omega-3s had markedly decreased SREBP-1 levels with consequent reduced expression of lipogenic genes in the liver.(140)The suppression of SREBP-1 and genes involved in lipid metabolism have been confirmed elsewhere.(133)(135)(138)(142)

Further studies in leptin deficient ob/ob mice not only confirmed reduced hepatic triglyceride content with omega-3 supplementation, but also lowered plasma alanine aminotransferase (ALT) levels and improved hyperglycemia and hyperinsulinemia, in a manner hypothesised to be related to PPARα.(140)The increase in PPARα in response to omega-3 supplementation was subsequently confirmed elsewhere.(133)(141)(145)

Insulin Sensitivity

Omega-3s have also been demonstrated to improve insulin sensitivity. In one series of experiments mice treated with omega-3 showed increased expression of insulin sensitising genes in adipose tissue and liver (e.g. PPARγ) and improved insulin sensitivity as well as increased expression of resolvins and protectins. The authors

hypothesised that this might contribute to the mechanism of action of omega-3 fatty acids as, in this study, SREBP-1 expression, TNFα levels and IL-6 were unchanged.(139)In addition, adiponectin, an insulin sensitising agent released from adipocytes, is increased in animals treated with omega-3 fatty acids.(134),(139), (146, 147)

A study of a rat model of NAFLD demonstrated that insulin resistance and central obesity were associated with increased TNFα, decreased PPARα and adiponectin. Animals also demonstrated hepatic insulin resistance with resultant hepatic steatosis and fibrosis. The addition of omega-3 fatty acids restored PPARα and adiponectin levels, reduced TNFα and ameliorated hepatic steatosis and the degree of liver injury.(141)

Oxidative Stress

In theory EPA supplementation may act to increase reactive oxygen species (ROS) production as it can affect not only mitochondrial β-oxidation but also activateω-oxidation and peroxisomal β-oxidation which are known to generate ROS.(148)(149)Mitochondrial beta oxidation is critical for the metabolism of omega-3 fatty acids: a murine model of impaired mitochondrial fatty acid oxidation demonstrated increased hepatic triglyceride accumulation on EPA supplementation.(150, 150) However, increased ROS production and oxidative stress on omega-3 supplementation has not been observed *in vivo.* In a rat model those whose diet was supplemented with fish oil were protected against the severe hepatic steatosis and increased lipid peroxidation seen in a control group.(151)Furthermore, there are suggestions from other studies that omega-3

supplementation actually reduces ROS production.(133, 147)In addition, a study postulated that EPA may attenuate progression of fibrosis in steatohepatitis through reduced ROS production.(152)

Inflammation

Omega-3 fatty acids are thought to possess anti-inflammatory properties but these effects are poorly characterised. One mechanism of action may be as a result of modulation of the inflammatory response as omega-3 fatty acids are associated with reduced levels of TNFα.(134)(141)(147, 153, 154) In addition, in a murine model of NAFLD omega-3 fatty acids reduced leukotriene and prostaglandin levels.(155)

1.8 Evidence for Omega-3 Fatty Acids as a Treatment for NAFLD from Human Trials

There are now a number of clinical trials exploring the use of omega-3s in adults with NAFLD. The optimal composition, dose and duration of omega-3 therapy has not yet been established and so these trials were a heterogenous mix of variable duration and used differing preparations of omega-3 fatty acids. A 2012 systematic review and meta-analysis examined the evidence to date.(156) Nine studies (355 subjects) met the inclusion criteria of adult human trials of omega-3 supplementation in NASH/NAFLD cohorts. Exclusion criteria for this analysis included other causes of liver disease or steatosis including total parenteral nutrition. The dose of omega-3 ranged from0.8-13.7g/day (median 4g/day). Outcomes considered included hepatic fat and serum liver function tests. Six of the seven studies which examined liver fat showed an effect size with benefit. The pooled effect size (ES) showed efficacy in

omega-3 to reduce liver fat (ES=-0.84 p<0.001).(156)This effect remained even when the heterogeneity of studies was considered. Two of seven studies showed a beneficial effect on ALT for omega 3 although another showed benefit for the control group. The pooled effect saw a trend towards omega-3 therapy but this did not reach statistical significance. The methodology of the 7 studies available in English(157-163)are presented in Table 1.2

Since the publication of this meta-analysis two further placebo controlled randomised trials have been published.(164, 165) These, in addition to four of the relevant trials included in the meta-analysis will be examined in more detail.

The first trial by Capanni et al(157), was an open label study of 56 patients with a clinical diagnosis of NAFLD and fatty liver on ultrasound. 42 patients were treated with 1 gram of omega-3 fatty acids daily. The comparison group comprised 14 patients who declined treatment: they did not receive any other intervention. Groups shared similar baseline clinical, biochemical and ultrasonic characteristics. The primary outcome measure of the study was the appearance of the liver on B mode ultrasound and duplex Doppler (undertaken by one ultrasonographer).

All 56 participants completed the trial. After 12 months of omega-3 supplementation there was a significant reduction in hepatic steatosis determined on ultrasound in the treatment group, with 64% of participants improved (either less steatosis or normal appearances). In contrast, there was no change in the appearances on ultrasound in the comparator group. In addition, there were also significant.

Study	Inclusion Criteria	Treatment arm	Treatment (n)	Control arm	Control (n)	Duration	Main results of treatment
Capanni et al (2006)	Fatty liver on ultrasound	1g PUFA daily	42	Observation only	14	12 months	• Reduced hepatic steatosis on US: PUFA group (baseline vs. after treatment)- 15 severe fatty liver vs. 6 severe; 6 moderate; 3 mild; 19 moderate fatty liver vs. 6 moderate; 8 mild; and 5 no fatty liver; 8 mild fatty liver vs. 3 mild; and 5 no fatty liver. Observation group- At baseline 6 severe; 5 moderate and 3 mild fatty liver. No change at end of study. • Mean change in serum chimistry in PUFA vs Observation group: o LFTs: ALT: -4 IU/L vs. +4 (p<0.002); AST: -2 IU/L vs. +4 (p<0.003); γGT: -4 IU/L vs. +4 (p<0.03) o Triglycerides: -46mg/dL (SD 88) vs. +9 (SD 27) (p<0.02) o Fasting glucose: -6mg/dL (SD 14) vs. +4 (SD 9) (p<0.02)
Spadaro et al (2008)	Fatty liver on ultrasound	2g PUFA daily	18	Dietary advice only	18	6 months	• US findings: steatosis grade (0/1/2/3 (%)). In the PUFA group: 0/0/39/61 at baseline vs. 33/22/44/0 after treatment. In the observation group: 0/0/44/66 at baseline vs. 0/11/50/39 at study end. • Serum biochemistry (baseline vs. after treatment) in the PUFA group: • LFTs: ALT: 56.6 U/L vs. 39.5 (p<0.01); AST: 31.5 U/L vs. 23 (NS); γGT: 39.3 U/L vs 28.0 (p<0.05) • Triglycerides: 147.4 mg/dL (SD 41) vs. 110 (SD 39) (p<0.01) • HOMA-IR: 3.5 (SD 2.0) vs. 2.8 (SD 1.7) (p<0.05) • TNFα: 3.3 pg/ml (SD 0.5) vs. 2.7 (SD 0.5) (p<0.05) • There was no significant change in serum biochemistry (baseline vs. after treatment) in the observation group.
Tanaka et al (2008)	NASH on liver biopsy	2.7g EPA daily	23	No control	N/A	12 months	• Hepatic steatosis grade on US changed from 2.1±0.9 at baseline to 1.6±1.1 after treatment, (p=0.004) • Serum biochemistry (baseline vs. after treatment): • LFTs: ALT: 79 IU/L vs. 50 (p=0.002); AST: 50 IU/L vs. 36 (p=0.001) • Triglycerides: 201 mg/dL (SD 90) vs. 183 (SD 103) (NS) • Total cholesterol: 219 mg/dL (SD 44) vs. 206 (SD 38) (p=0.039) • HOMA-IR: 4.0 (SD 2.0) vs. 3.4 (SD 2.4) (pNS) • Plasma Thioredoxin: 30ng/mL (SD 15) vs. 22 (SD 6) (p=0.036) • Change in histological grade (baseline vs. after treatment): • Steatosis: 2.4 (SD 0.5) vs. 1.7 (SD 0.5); Fibrosis: 1.7 (SD 1.1) vs. 0.7 (SD 0.5); Lobular inflammation: 2.1 (SD 0.7) vs. 1.1 (SD 0.7); Ballooning: 1.6 (SD 0.5) vs. 0.9 (SD 0.4); NAS: 6.1 (SD1.3) vs. 3.7 (SD 1.4)
Vega et al (2008)	Previous elevated liver fat on MRS	9g PUFA daily	16	No control	N/A	8 weeks	• Plasma triglyceride level pre-treatment 117 mg/dL; after PUFA 74 mg/dL. (p<0.03) • Liver fat content 7.9% pre-treatment; 3.0% after PUFA
Zhu et al (2008)	Clinical diagnosis of NAFLD with mixed dyslipidaemi	6g seal oil PUFA	66	Placebo (type not stated)	68	24 weeks	• Ultrasound: PUFA group normal appearances in 19.7% (13/66) and reduction in 53.0% (35/66) vs. control group: normal appearances in 7.35% (5/68) (P = 0.04) and reduction in 35.3% (24/68) had a certain reduction (P = 0.04) • LFTS:. ALT: PUFA group: 62.8 ± 36U/L to 39.3 ± 18U/L vs. control 79.8 ± 51U/L to 42.3 ± 22U/L (p<0.01;. AST PUFA group 38.1.8 ± 21U/L to 30.5 ± 13U/L vs. control 50.1 ± 39U/L to 30.3 ± 14U/L (p=NS). • Trig: PUFA 3.94 ± 2.7mmol/L to 2.08 ± 1.0 vs. control 3.80 ± 2.9mmol/L to 2.33 ± 1.4mmol/L • Total Symptom scores: PUFA group fell from 1.87 ± 1.18 to 0.42 ± 0.72 vs. Control 1.79 ± 1.45 to 0.53 ± 0.97 (p<0.01)
Sofi et al (2010)	Fatty liver on ultrasound and abnormal transaminases	0.83g PUFA in 6.5mls olive oil	6	Olive oil only	5	12 months	• Ultrasound appearances and Doppler perfusion index pre and post treatment in PUFA group after: pre: 0.15 ± 0.03; P < 0.05) vs. 0.19 ± 0.02. No significant changes were seen in controls. • PUFA group: triglycerides pre: 164.5 ± 85.5 mg/dl vs. post: 132.8 ± 63.7; P = 0.04 • Transaminases pre vs. post treatment: ALT: in PUFA group -30.7 vs. control group -23.5 (p=0.03). AST in PUFA group -19.6 vs. -2.2 (p=0.02) • Significant increase of adiponectin levels: pre: 1,143 ± 24.8 µg/ml vs. post: 1,487.9 ± 96.7; P = 0.04
Cussons et al (2009)	Polycystic ovarian syndrome	4g PUFA	25	Olive oil	25	8 weeks	• MR spectroscopy change in liver fat content PUFA 10.2 (1.1) vs. Placebo 8.4 (0.9)% (P = 0.022) • Change in triglycerides: PUFA 1.19 (1.03-1.47) vs. 1.02 (0.93-1.18) mmol/L (P = 0.002)

Table 1.2a Summary of Trial Design and Results

Study	Primary diagnosis	N	Design	Randomised	Sample size calculation	Placebo	Control arm	Primary outcome measures	Histology
Capanni et al (2006)	NAFLD	56	Open label	No Comparison group self-selected	Yes	No	Observation only	Appearance of the liver on ultrasound	No
Spadaro et al (2007)	NAFLD	36	Open label	Yes Random number sampling	Yes	No	Dietary advice only	Appearance of the liver on ultrasound, transaminses	No
Tanaka et al (2008)	NASH	23	Open label	No	No	No	N/A	Appearance of the liver on ultrasound, transaminases, liver histology	Yes
Vega et al (2008)	Fatty liver on MR spectroscopy	16	Open label	No	No	No[1]	N/A	Plasma and hepatic triglycerides	No
Zhu et al (2008)	NAFLD	134	Not stated	Yes	No	Yes	Yes	Symptom scores, transaminases, plasma lipids, appearance of the liver on ultrasound	No
Sofi et al (2010)	NAFLD	11	Not stated	Yes	Yes	Yes	Yes	Appearance of the liver on ultrasound, transaminases	No
Cussons et al (2009)	PCOS	25	Double blind crossover	Yes	Yes	Yes	Crossover	Hepatic fat on MR spectroscopy	No

Table 1.2b Summary of Trial Methodology

improvements in serum liver function tests, fasting blood glucose and serum triglycerides only in the intervention group, while the N6:N3 ratio was reduced. This was the first trial to provide evidence for omega-3s as a potential therapy in NAFLD. However there are significant weaknesses to the study - most notably the absence of blinding and randomisation, and the use for comparison of a self-selected small group consisting of those patients who had declined entry to the treatment arm.

The second trial, by Spadaro et al(158), involved 40 patients with a clinical diagnosis of NAFLD. Participants were assigned to two treatment and control groups on a 1:1 basis using random sampling numbers. The groups were satisfactorily matched at baseline for age, gender, BMI and insulin resistance. Neither participants nor investigators were blinded although the ultrasonographer was. In addition to dietary advice also given to the control group, those in the treatment arm received 2 grams of PUFA daily for six months. Outcomes assessed included: fatty liver as graded by abdominal ultrasound, liver function tests and insulin resistance assessed by HOMA-IR.

Two patients dropped out in each arm resulting in 36 completing the trial. Following 6 months of treatment patients in the omega-3 arm showed improved serum biochemistry with a reduction in plasma triglycerides, γGT and ALT. There was no significant difference in serum biochemistry after six months in the control group. The omega-3 group also demonstrated improved insulin sensitivity and decreased TNFα levels. Ultrasound grading of liver fat improved in 83% of the intervention group with 33% reverting to normal appearances. In contrast, in the control group 72% of steatosis scores were unchanged and none reverted to normal.

Although superior to the study of Capanni et al in that there was an established control group and randomisation there were still design weaknesses - most significantly the lack of a placebo, and the non-blinding of participants and investigators.

A third trial by Tanaka et al(159) included 23 patients with biopsy confirmed NASH who received 2.7grams of EPA daily for 12 months. This pilot trial lacked a control group and no sample size calculation was performed. All patients had previously received dietary advice. Outcome measures were serum liver biochemistry, ultrasonic appearance and liver histology graded using the NAFLD activity score (NAS) (7 participants consented to repeat biopsy at treatment end).

All enrolled patients completed the trial. At 12 months the mean steatosis grade on ultrasound had improved significantly. 6 of the 7 patients who underwent repeat biopsy had reduced steatosis, inflammation and fibrosis on histology. On biochemical testing ALT and AST improved significantly; cholesterol and free fatty acids were significantly reduced. Serum triglycerides, HDLs, fasting blood sugar, adiponectin levels or insulin resistance were unchanged. Serum TNFα improved, but this did not reach statistical significance.(166)

This was the first human study of omega-3 fatty acids to have histological data, generally considered the most valid outcome measure. Whilst this trial adds further evidence of the benefits of using omega-3 fatty acids in NAFLD the absence of randomisation, controls and blinding, the small sample and the lack of statistical power rule out reliable conclusions.

A further small study (Vega et al) involved 17 patients who had previously been enrolled and demonstrated to have elevated hepatic triglycerides on liver MR spectroscopy as part of the Dallas Heart Study.(162)(167) Aetiologies of liver disease other than NAFLD were not excluded and alcohol intake was not reported. Participants then received 8 weeks treatment with 9g of fish oil. One patient withdrew from the trial. Primary outcome measures were plasma and hepatic triglyceride levels as assessed on magnetic resonance spectroscopy (MRS).

This study demonstrated that omega-3 fatty acids supplementation altered the fatty acid constituent of plasma triglycerides, which were themselves significantly reduced but there was no reduction in hepatic triglyceride content. It could be hypothesised that this negative result arose from it being a small, short trial, with an atypical sample (predominantly females and African Americans) whose diagnosis might not have been NAFLD. There was also a skewed baseline hepatic triglyceride content and the dose of PUFA was much higher than those used in other trials.(162)

A recent trial by Sanyal et al(164)(published February 2014) was a phase 2b multi-centre double-blind, randomised, placebo-controlled trial of ethyl-eicosapentaenoic acid (EPA-E; a synthetic polyunsaturated fatty acid)in subjects with non-alcoholic steatohepatitis. 243 subjects from 37 North American sites with biopsy proven NASH were enrolled. Inclusion criteria was a NAFLD activity score ≥4 as well as minimum scores of 1 for steatosis and inflammation, along with either ballooning or at least stage 1a fibrosis on histology. 243 participants were randomised to 3 groups: placebo (n=75), low-dose EPA-E (1800 mg/day; n=82), or high-dose EPA-E (2700 mg/day; n=86) for 12 months. Participants then had a second biopsy within 2

weeks of finishing the trial. The primary endpoint was either total NAFLD activity score ≤ 3 without worsening of fibrosis, or a decrease in NAFLD activity score by ≥2 with contribution from >1 parameter without worsening of fibrosis. All study biopsies were score by 2 pathologists from of a team of 4 with a third adjudicating when there was a discrepancy. Block randomization with a 1:1:1 ratio between the three arms was used.

A total of 181 subjects completed the study and 174 subjects met criteria for a per protocol efficacy evaluable data set. 15 subjects withdrew consent, 14 were lost to follow up, 10 experienced adverse events and 13 a protocol violation. Similar proportions of subjects in each group met the primary endpoint. The groups were comparable at baseline. EPA-E had no significant effect on steatosis, inflammation, ballooning, or fibrosis scores. In addition, there were no significant effects on levels of liver enzymes, insulin resistance, adiponectin, high sensitivity CRP, or hyaluronic acid. High-dose EPA-E reduced levels of triglyceride (–6.5 vs an increase of 12 mg/dl in the placebo group, p=0.03). No change was seen in low dose EPA-E or placebo. However, a reduction in both ALT and AST was significantly greater in the placebo arm than high dose EPA-E (p=0.03 and p=0.04 for ALT and AST respectively).

Adverse events were frequent: 94% of subjects on placebo reported adverse events compared to 79% of subjects on low dose EPA-E and 86% of subjects on high dose EPA-E. There were no treatment-related serious adverse events. The most common adverse events included nausea, diarrhoea and abdominal discomfort.

This study was therefore well designed and executed with rigorous assessment of compliance (both by tablet counting and serum fatty acid levels). One consideration is the dose of EPA used – here two doses were evaluated although even the higher dose was lower than that used in the previous biopsy based NAFLD study. The dose was selected as the treatment dose for hypertriglyceridaemia in the Japanese population. Despite this the effects on serum triglycerides were modest and only seen with high dose EPA. The study experienced a higher dropout than had been expected and placebo response rates were higher. The study population as a whole experienced a high number of adverse events. The reasons for these observations are unclear.

Finally, Dasarathy et al(165) performed a double-blind placebo controlled pilot trial of omega-3 fatty acids in diabetic patients with non-alcoholic steatohepatitis. The initial aim of this study was to obtain data to inform the design of a large, multicentre trial. Here 37 patients with tightly controlled diabetes (HbA1C<8.5%) and a liver biopsy within the 6 months prior to randomisation and were recruited from 2 centres. Subjects were randomised to receive either omega-3 fatty acid tablets containing eicosapentaenoic acid (2160 mg) and docosahexaenoic acid (1440 mg) daily or corn oil. The trial lasted 48 weeks. Liver biopsy was performed at the end of treatment. The study was statistically powered to detect rate of improvement in liver histology of 15% in the placebo arm, 60% in the treatment arm with power of 80% and a type 1 error 0.05 (two tailed).

The two groups were well matched at baseline. The investigators found a significant improvement in hepatic steatosis and the activity score ($p<0.05$) and worsening of

lobular inflammation ($p<0.001$) in the placebo group. There was no change in these parameters in the omega-3 arm. Insulin resistance was worse in the omega-3 arm but unchanged in the placebo arm at the end of the study. During the course of the trial there was no significant chance in liver enzymes, body weight, or body composition in either group.

Similarly to Sanyal et al, this study therefore looked at a subgroup of NAFLD patients: those with biopsy proven non-alcoholic steatohepatitis. This study refined the study group further to only include those with well controlled diabetes. Whilst this is only a subset of the NAFLD population it does allow those included in the study to be well characterised and the placebo and treatment arms to be well matched. Interestingly the authors suggested that the negative result of their study may be accounted for by the patient population (exclusively patients with diabetes) and their outcome measure (liver histology). As may be seen in Table 1.2, the majority of studies to date have used liver fat as graded on ultrasound as the primary outcome measure although the trials that did look at liver histology showed a beneficial effect. It is noteworthy that steatosis as graded by histology improved significantly in the placebo arm rather than the omega 3 arm in this study. This is the first study to show worsening of glycaemic control with omega-3 treatment and this observation might perhaps at least partially account for the lack of effect seen in the omega-3 arm.

1.9 Summary

Non-alcoholic fatty liver disease (NAFLD) is an increasingly common condition and affects 10-35% of the population worldwide. NAFLD is a term which encompasses isolated hepatic steatosis, steatohepatitis and cirrhosis. It can result in end stage liver disease and, if cirrhosis has developed, is a risk factor for hepatocellular carcinoma.

To date there is no consensus on treatment for NAFLD. Weight loss and exercise have been shown to be of benefit but various pharmacotherapies, often targeted to the underlying pathophysiology of the disease, have not withstood the scrutiny of randomised controlled trials.

Omega-3 fatty acids are licenced as a treatment for hypertriglyceridaemia. They are modulators of hepatic gene expression – in particular PPARα, important for fatty acid oxidation and possessing anti-inflammatory properties, and SREBP-1 which regulates de novo lipogenesis.

There are promising data from both animal models and human trials suggesting that omega-3 fatty acids are effective at reducing steatosis and mitochondrial dysfunction in NAFLD. Two more recent trials however did not show benefit. Data are therefore conflicting as to whether omega-3 fatty acids are an effective treatment for adults with non-alcoholic fatty liver disease and further scrutiny is warranted.

Section II

Clinical Trial

2.1 Introduction

Currently, there is no widely accepted, specific pharmacotherapy for NAFLD. There is supportive evidence from animal studies that omega-3 fatty acids may be of benefit in NAFLD. Further, there are trials in adults with NAFLD that support the suggestion of omega-3's as a treatment as they reduced the amount of fat stored in the liver and improved liver function tests in study participants(157-159),although more recent studies have failed to show superiority of omega-3 fatty acids over placebo.(164, 165)This study aims to use the study design of a pilot study which showed positive effects of omega-3 fatty acids and subject this to the rigors of a double-blind, placebo-controlled, randomised clinical trial.

2.2 Methods

2.2.1. Study Design

The aim was to assess the efficacy of omega-3 fatty acids in reducing hepatic steatosis in patients with non-alcoholic fatty liver disease.

This was a single centre, phase 3, double-blind, randomised, placebo-controlled clinical trial.

The study was conducted in accordance with the Declaration of Helsinki and The Medicines for Human Use (Clinical Trials) Amendment Regulations 2006.

Participants attended the trial unit for four visits, each three months apart. At the first visit, participants were randomised to either the study drug or placebo for six months; they then attended for a final visit three months after treatment ended. The schedule of assessments at the four appointments, all of which took place at the Clinical Research Facility at the Royal Infirmary of Edinburgh, is presented in Table 2.1.

Visit Number	Time	Description
1	0 months	**Initial visit**
2	3 months	**Midway through treatment**
3	6 months	**End of treatment**
4	9 months	**End of study**

Table 2.1 Outline of study visits.

2.2.2 Fundings, Approval and Registration

This trial was funded by The University of Edinburgh 'Liver Fund'. No external funding was applied for. No funding or support was received from the pharmaceutical industry,

NHS Lothian and The University of Edinburgh acted as co-sponsors of the study.

This trial was reviewed and approved by the South West Research Ethics Committee (ref no: 09/H0206/15) March 2009.

Submission for approval from the Medicines and Healthcare products Regulatory Agency (MRHA) (EudraCT no: 2008-008275-34) was initially made on the 4th April 2009. Significant delays to study commencement and changes to the study design resulted from this submission. A summary of the timeline from study inception to commencement is displayed in Figure 2.1.

The trial was registered with clinicaltrials.gov (ref: NCT01277237).

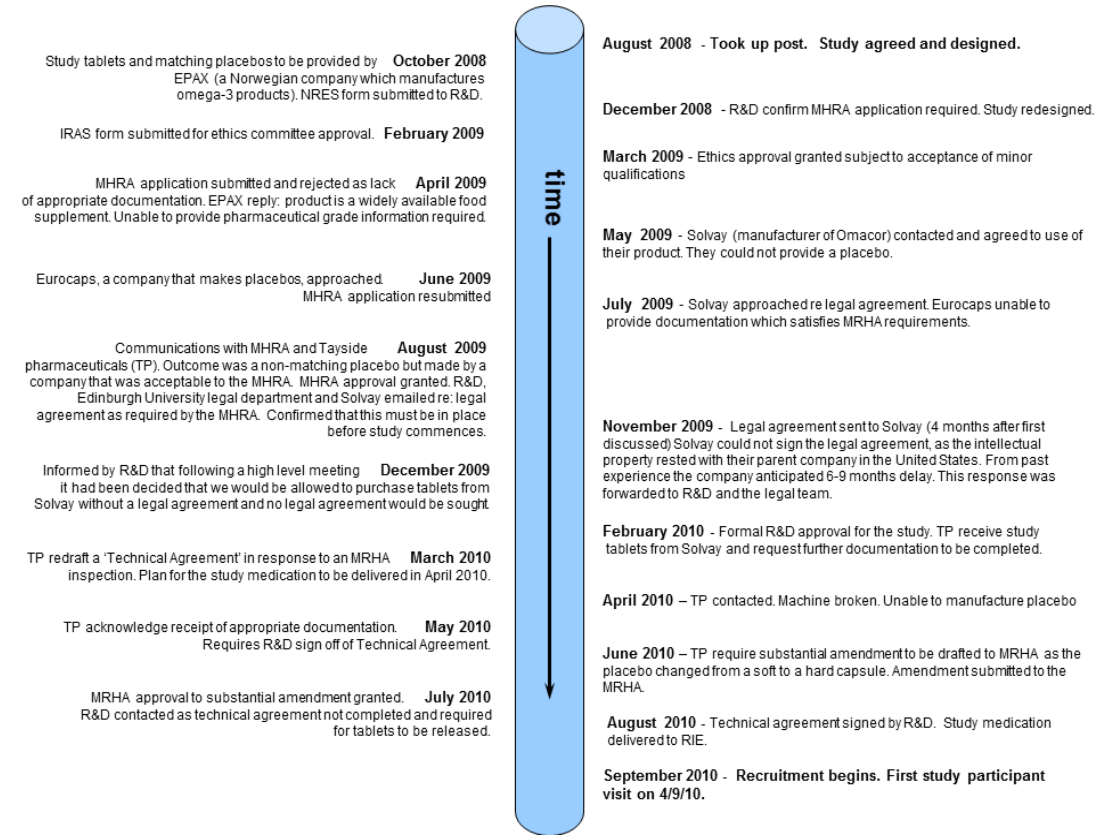


Figure 2.1 Summary of delays to study commencement

2.2.3 Selection of Patients

50 patients with NAFLD attending gastroenterology outpatient clinics at the Royal Infirmary of Edinburgh were enrolled in the study.

Potential participants were identified in one of two ways. Firstly, by screening a database held in the department which contained the names of 248 patients who were attending the specialist NAFLD clinic at November 2009. On initial screening of their casenotes, 61 patients were identified as not being suitable for medical reasons (Table 2.2).

Alternative potential participants were identified from clinic lists for all hepatology/gastroenterology clinics at the Royal Infirmary of Edinburgh. These were screened for the duration of the recruitment period.

A consultant gastroenterologist who knew the patient corroborated that the person was suitable for consideration and agreed to them being approached: no additional patients were excluded at this stage.

Reason	Number
Cirrhosis on ultrasound	35
Alcohol as co-factor	14
Cancer (proven or under investigation)	3
Hepatitis B as co-factor	2
On Warfarin	1
Gallstones refusing treatment as co-factor	1
Poorly controlled Coeliac disease	1
Granulomatous liver disease as co-factor	1
Secondary biliary cirrhosis as co-factor	1
Diagnosis Uncertain	2

Table 2.2 Reasons patients in NAFLD database were deemed unsuitable for inclusion.

Patients were approached in one of two ways. Either sequentially at the outpatient department rather than being randomly chosen from the list because many were on six monthly or annual reviews. Alternatively a letter of invitation was sent to the patient's home address. A letter of invitation was also sent if the subject had been identified as being suitable post hoc from clinic review.

A written information sheet, the wording of which had been approved by the Research Ethics Committee, was given to the patient in clinic or included with the letter of invitation. The patient then contacted the researcher by phone or email to indicate that they were interested in taking part in the study.

Consent was gained at the baseline visit. This was documented in the patient's clinical notes and their GP was informed of their entry into the trial by letter. If study participants were found to have no steatosis on baseline ultrasound (visit 1.) they were not included in the study.

2.2.4 Inclusion and Exclusion Criteria

The inclusion criteria for the study were –

a) a clinical diagnosis of NAFLD made by a gastroenterologist

b) fatty infiltration confirmed on ultrasound

c) abnormal serum liver function tests.

The exclusion criteria of the study were:

- any other established cause of chronic liver disease
- severe heart failure (NYHA class IV)
- the prescription of medication which could cause fatty liver (e.g. tamoxifen)
- on anticoagulants (antiplatelets were permitted)
- current alcohol intake of >20g/day for women, >40g/ day for men
- already taking fish oil
- known allergy to fish oil
- pregnancy/ lactation
- age under 18 years
- unable to provide informed consent

2.2.5 Study Medication

Active Drug

Omacor. (Solvay Healthcare Ltd, Mansbridge Road, Southampton)

Placebo

Lactose tablets (Tayside Pharmaceuticals, Ninewells Hospital, Dundee).

Labelling, packaging and storage

Study medication was packaged, labelled and randomised by Tayside Pharmaceuticals. Study medication was stored and dispensed by the pharmacy department at the Royal Infirmary of Edinburgh.

Dosing regime

4 grams per day taken as two capsules twice daily of either Omacor or placebo. There was no dose alteration during the trial. This dose was selected as it is the maximum dose licenced in the British National Formulary (BNF). The licenced indications for Omacor are: secondary prevention post myocardial infarction; and treatment of hypertriglyceridaemia.

2.2.6 Randomisation

The medication was pre-packed and pack numbers were assigned at random by means of a computer generated list at Tayside Pharmaceuticals. Participants, radiologist, and investigators were blinded but the pharmacist could not be because although the packaging was identical, the tablets were visibly different. Pharmacy also retained a copy of the randomisation list for safety purposes.

As the packs had already been randomised elsewhere, participants were simply allocated treatment by matching their study number to the pack number, so for example patient 12 received pack number 12.

There was no blocking/stratification but rather simple randomisation only.

2.2.7 Study Outcome Measures

Primary outcome measure

There is not an established primary endpoint in treatment trials of NAFLD. It is necessary to use a surrogate outcome as assessment of clinical outcome, i.e. liver related morbidity and mortality would require long-term intervention and follow-up. This is not feasible and surrogate markers are used instead, including serum liver function tests (LFTs), imaging and histology. Whilst an improvement in LFTs is the most obvious primary end point, it is well known that NAFLD can occur with normal LFTs or a purely cholestatic pattern. Moreover, what would be significant? An arbitrary change or normalisation?

Improvement on histology is another option but this requires repeated liver biopsy which would expose the participants to potentially serious complications. Moreover, although liver biopsy is still the current gold standard in liver trials, it is uncomfortable for the patient, has associated risks and it known to be unreliable with a discordance rate of one stage or more between two simultaneous biopsies of 41%.(168)It is only used selectively in clinical practice because of these factors. Further, using histology as the primary end point would have required a pilot trial as there are inadequate data on the effect of omega-3 fatty acids in NAFLD patients using these outcome parameters.

Liver brightness and posterior attenuation on ultrasound have previously been shown to correlate significantly with fat scores on liver biopsy.(169)The pilot trial by

Capanni et al(157) which explored the effect of omega-3 fatty acids in patients with NAFLD assessed these features using a grading system. These data allowed a feasible single centre phase 3 study.

Therefore the primary outcome was selected to be the change in the amount of liver fat content, graded by ultrasound assessment, following six months of treatment with either omega-3 or placebo.

A single consultant radiologist performed or reviewed all of the liver ultrasound scans of the trials. He evaluated the liver echotexture against the right kidney cortical echogenicity. Liver brightness and posterior attenuation on ultrasound has been shown to correlate significantly with fat scores on liver biopsy and, using the same adaptation of this grading system as previous studies (Table 2.3), this was selected to be the primary outcome measure. (157)(169)

Grade 0	**No steatosis**
Grade 1	**Mild steatosis** • lightly and homogeneously increased liver echotexture • patent intra-hepatic vascular pattern • posterior attenuation absent
Grade 2	**Moderate steatosis** • moderate increase of liver echotexture • partial dimming of the vessels • early posterior attenuation
Grade 3	**Severe steatosis** • diffuse increase of liver echotexture • intra-hepatic vessels not visible • heavy posterior attenuation

Table 2.3 Grading of Ultrasound Appearance.

Secondary outcome measures

Secondary outcome measures evaluated the effects of six months treatment with omega-3 fatty acids on

Serum liver function tests (LFTs)

Serum samples were taken to evaluate liver function including bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT) and alkaline phosphatase (ALK)

Health related quality of life (HRQoL)

The World Health Organisation (WHOQOL-Bref) is a brief, validated research tool that subjectively assesses four quality of life domains - physical health, psychological health, social relationships and environment.(170) Study participants were asked to complete this 26 item scale at each attendance.

Additional information

In addition to the primary and secondary outcome measures data was also gathered relating to weight, height, waist circumference and hip circumference, pulse and blood pressure. Blood samples were also taken to assess serum lipids, and serum glucose. See Table 2.4.

Visit Number	Month	Height	Weight	Waist:Hip ratio	Pulse & BP	Abdominal ultrasound	Blood tests	HR-QOL questionnaire	Study Tablets
1	0	✓	✓	✓	✓	✓	✓	✓	✓
2	3	X	✓	✓	✓	X	✓	✓	✓
3	6	X	✓	✓	✓	✓	✓	✓	x
4	9	X	✓	✓	✓	✓	✓	✓	x

HR-QoL – health related quality of life

Table 2.4 Schedule of Trial Visits

2.3 Statistical Analysis

2.3.1 Sample size calculation

The sample size calculation for the study was based on the results of the pilot trial of omega-3 fatty acids in non-alcoholic fatty liver disease by Capanni et al.(157) In the Capanni paper when subjects with NAFLD were treated with omega-3 supplements there was 64% improvement in the ultrasound appearances in the treatment group and 0% in placebo. More conservative figures of 0.5 improvement in treatment group and 0.1 in placebo group were chosen. Power=0.8, P=0.05. Sample size was calculated by Dr G Masterton (GM) using the Gore-Altman nomogram. Dr S Lewis (SL), independent statistician, then undertook a calculation using Fisher's exact test. These agreed that group sizes of 25 patients in each arm would adequately power the study, allowing for a 5% drop out rate (there had been no drop-outs in the Capanni study). See Appendix 1 & 2 for GM and SL power calculations.

2.3.2 Statistical Analysis of Results

The statistical analyses of results of the clinical trial were performed by a qualified statistician at the Clinical Research Facility, Western General Hospital, Edinburgh (Mrs Sharon Tuck) in conjunction with Dr G Masterton.

Patients were randomised in a 1:1 allocation ratio to either active drug or placebo. All randomised patients were followed up and included for analysis in their allocated

treatment groups regardless of the treatment actually received. Patients with missing outcome data were omitted from analysis.

Overall compliance (%) was assessed by comparing the number of tablets actually taken between Visit 1 and Visit 3 compared to the number of tablets supposed to be taken.

Binary outcome measures, including the primary outcome of change in the ultrasound appearance of the liver at 6 months compared to baseline was examined using risk differences plus 95% confidence intervals (Fisher's Exact Test if numbers were small). This method was also used to compare change in ultrasound appearance between 6 months and 9 months (i.e. to assess any change in appearance once the intervention is stopped).

Secondary outcome measures included change (i.e. change vs. no change) in liver function tests (i.e. Bilirubin, ALT, AST, Alkaline phosphatase and GGT) at 6 months compared to baseline were also be reported using risk differences plus 95% confidence intervals. A 'change' was considered a $\geq$10% difference (increase or decrease) between 6 month outcome measures compared with baseline. The change between 6 months and 9 months was also considered.

Continuous outcome measures, including change in weight, waist and hip circumference at 6 months compared to baseline was reported using two-sample t-tests (non-parametric as appropriate).

Another secondary outcome measure was the WHOQOL-BREF score and it had been planned to examine the QoL data over all visits using repeated measures ANOVA. However, because fewer patients had QoL data recorded at the 3 month visit this was not appropriate and therefore two-sample t-tests (or non-parametric where appropriate) was used to compare QoL data at 6 months with baseline.

2.4 Results

2.4.1 Study Recruitment

50 patients were recruited to the study. The first participant was recruited to the study in September 2010 and the last September 2011.

184 letters of invitation were sent. 39 patients responded. 3 responded to decline (1 no reason was given, 1 inconvenience, 1 concern re: blood sugars). 5 patients were already taking supplementary omega-3 treatment. Of the 31 who responded expressing interest in the study: 3 failed to attend the first study visit and so were not recruited and 2 did not have steatosis on baseline ultrasound. A total of 26 patients (of the total of 50) were thus recruited by letter. The remainder of the study participants were recruited at clinic attendance.

Of the 57 who attended for the baseline visit 7 potential participants were excluded: 5 patients did not have steatosis on ultrasound at the baseline visit; 1 patient was not recruited as alcohol intake was in excess of that allowed by the study and the other took tamoxifen (contraindicated in the study).

The rate of recruitment to the omega-3 study was faster than had been predicted. These data are displayed in the cumulative and quarterly recruitment plots (Figure 2.2).

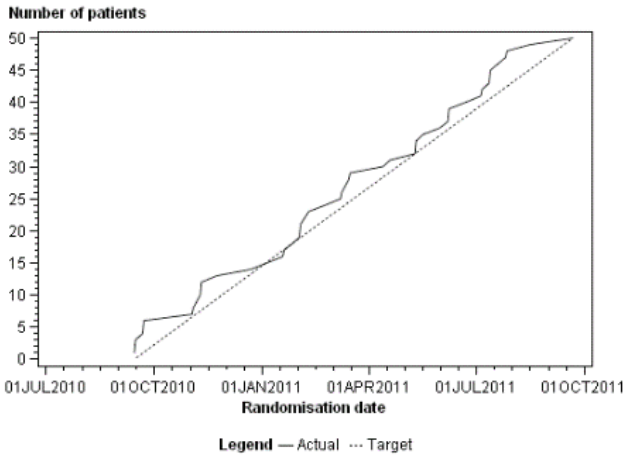


Figure 2.2a Cumulative Recruitment Plot

Figure 2.2b Quarterly Recruitment Plot

2.4.2 Study Withdrawals

50 participants were recruited to the study. 1 patient was initially randomised to the placebo group but at baseline ultrasound no steatosis was found. This patient was withdrawn from the study before attending any further visits. This patient has not been included in any of the analyses in this report. A further study participant had evidence of a dilated common bile duct (CBD) on the initial study ultrasound in addition to steatosis. This participant went on to have an ERCP and a choledochal stone removed. This participant was not withdrawn from the study as the presence of a CBD stone would not affect the primary outcome measure however their data were not included in the analysis of serum liver function tests.

84% (19 placebo; 22 omega-3) attended visit 3 – the primary endpoint of the study. One patient (Subject ID 17) did not attend visit 3 due to developing a stroke but managed to attend visit 4. With the exception of this patient, the participants who did not attend a visit then did not attended further visits.

A summary of study attendance at each visit by treatment group is shown in Table 2.5

4 participants in the placebo arm withdrew from the study at Visit 2 with a further 2 withdrawals in the omega-3 arm. The latter withdrew due to diarrhoea and severe back pain. In the placebo arm 1 patient was withdrawn as they had commenced a drug which met exclusion criteria (warfarin). In the other 3 instances no reason was given.

2 further study participants did not attend visit 3. Both were in the placebo arm of the study. One did not attend as she had had a stroke. The other did not give a reason. At visit 4 one further patient in each arm of the study failed to attend but in each case no reason was given.

	Group				All	
	Placebo		Omega-3			
	N	%	N	%	N	%
Total	25	100	24	100	49	100
Attended Visit 1 (baseline)						
Yes	25	100	24	100	49	100
Attended Visit 2 (3 mths)						
No	4	16	2	8	6	12
Yes	21	84	22	92	43	88
Attended Visit 3 (6 mths)						
No	6	24	2	8	8	16
Yes	19	76	22	92	41	84
Attended Visit 4 (9 mths)						
No	6	24	3	13	9	18
Yes	19	76	21	88	40	82

Table 2.5 Summary of study attendance by treatment group

2.4.3 Participant compliance

Adherence to study medication was ascertained by self-reported number of remaining tablets or finishing date of the tablets by the study participants or, where tablets were returned, by tablet counting by the pharmacist.

Compliance data are available for 48% of participants in the placebo arm (20% self-reported finish time; 28% tablet count). In the omega-3 arm 77% of participants have compliance data – here 38% have tablet return data; 29% self-reported finishing time. 6 participants (3 in each arm of the study) attended for visit 2 after the time the study tablets would've run out. The compliance data are corrected for this.

The median %compliance in patients randomised to omega-3 was 94% (Q1 88.2; Q3 95.4). In the placebo arm the median compliance was 80% (Q1 75.2; Q3 91.8). These data are shown in Figure 2.3. The mean number of days tablets should've been taken for was longer in placebo arm (mean 197days) than in the omega-3 arm (188 days) which might account for some of the difference in the observed compliance rates (Figure 2.4).

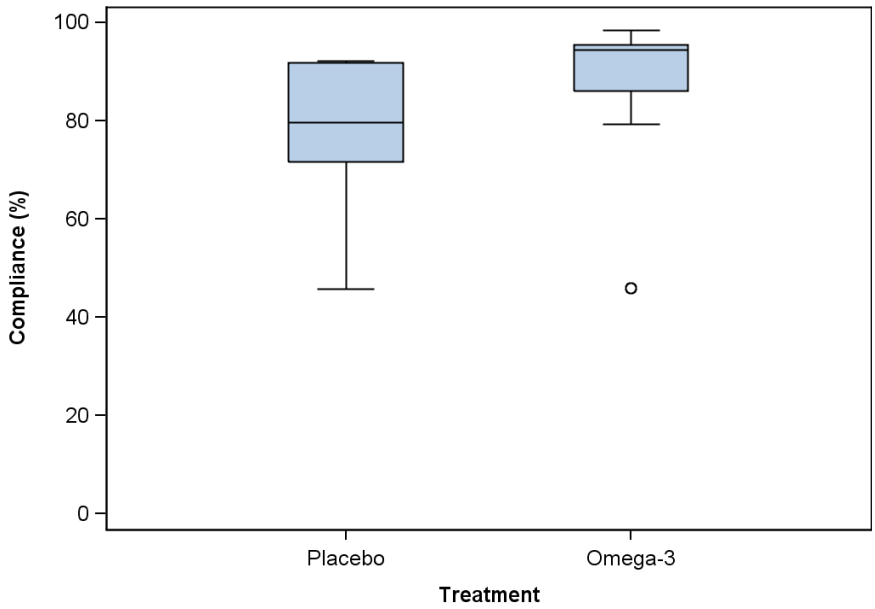


Figure 2.3 %Compliance by treatment arm

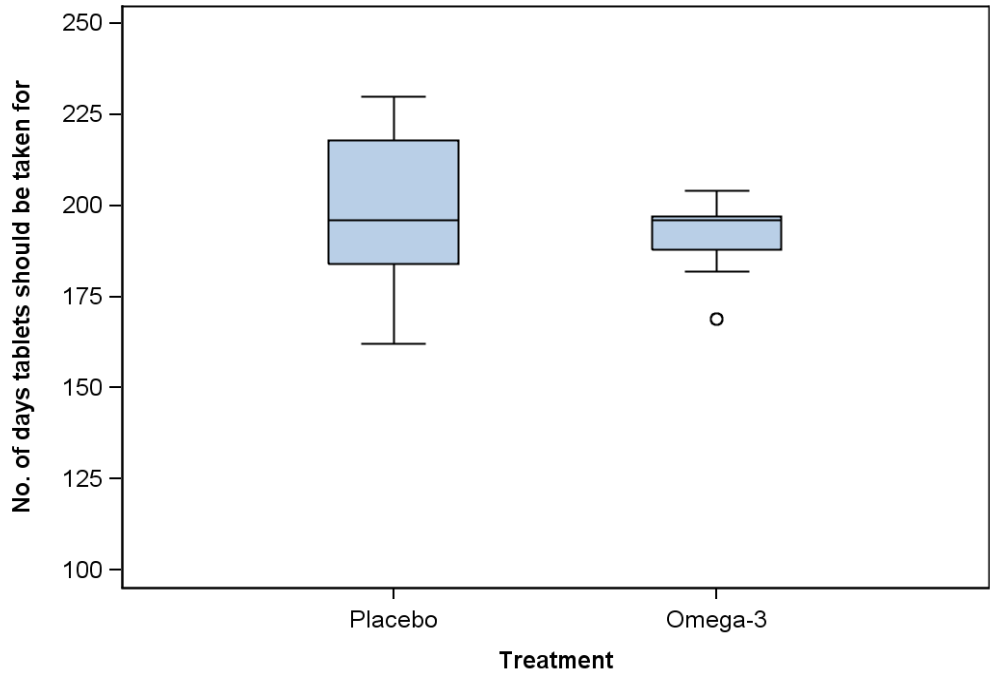


Figure 2.4 Number of days study medication should have been taken

2.4.4 Baseline Characteristics

In all 26 study participants were male, 23 female. 16 of the participants had a diagnosis of diabetes (3 with insulin dependent diabetes); 23 hypertension and 28 hyperlipidaemia.

Baseline demographic and clinical data are displayed in Table 2.6. There was no significant difference in age, gender or weight between the two groups at baseline. Participants in the placebo arm had a larger waist circumference, but not hip circumference and higher diastolic blood pressure but not systolic blood pressure at baseline.

There was no significant difference between groups in either ultrasound grades of steatosis, serum liver function tests or domain of WHOQOL score at baseline.

Parameter	Placebo N=25	Omega-3 N=24	p-value
Patient Demographics			
Age (yrs)	54.2 ± 12.2	52.5 ± 12.1	**0.63**
Female N (%)	10 (40)	13 (54)	**0.32**
Weight (Kg)	96.4 ± 13.9	92.4 ± 13.2	**0.31**
Waist circumference (cm)	107.4 ± 9.6	100.2 ± 10.3	**0.02**
Hip circumference (cm)	112.7 ± 10.1	109.6 ± 10.9	**0.32**
Pulse (BPM)	77.4 ± 10.3	76.3 ±12.1	**0.74**
Systolic BP	145.5 ± 22.1	138.3 ± 16.2	**0.21**
Diastolic BP	93.2 ± 11.4	86.5 ± 10.3	**0.04**
Liver Function Tests			
Bilirubin	10.0 (9, 13)	9.0 (7, 13)	**0.53**
ALT	50.0 (38, 69)	50.0 (31, 67)	**0.91**
AST	36.0 (31, 42)	34.0 (25, 47)	**0.66**
Alk Phos	85.0 (72, 111)	95.0 (73, 128)	**0.98**
GGT	80.0 (54, 125)	69.0 (51, 135)	**0.65**
Glucose	5.3 (4.9, 5.5)	5.2 (5.0, 5.5)	**0.92**
Triglycerides	1.9 (1.4, 3.5)	1.7 (1.1, 2.0)	**0.10**
WHOQOL-BREF Scores			
Physical Health	12.5 ± 1.8	12.9 ± 1.6	**0.39**
Psychological Health	13.2 ± 2.3	13.8 ± 2.1	**0.37**
Social Relationships	14.5 ± 4.1	15.4 ± 3.2	**0.43**
Environment	15.6 ± 3.1	16.3 ± 2.0	**0.37**
Ultrasound Grade			
1 N(%)	19 (76)	18 (75)	
2 N(%)	5 (20)	6 (25)	
3 N(%)	1 (4)		**>0.99**

Table 2.6 Baseline characteristics.

Patient demographics and WHOQOL scores are presented as mean ± SD. Biochemical (LFT, lipids and glucose) data are presented as median (25th percentile, 75th percentile). Gender and ultrasound scores at baseline are presented as number (percentage).

2.4.5 Primary Outcome Measure

Change in grade of steatosis on ultrasound assessment of the liver at 6 months was the primary outcome measure of the study. The scores at baseline and 6 months by treatment group are displayed in Table 2.7 and Figure 2.5.

In the omega-3 arm of the study 7 subjects had a change in ultrasound grade of steatosis at 6 months: 3 (14%) subjects had increased grade of steatosis whilst 4 (18%) had decreased steatosis. The remaining 15 (68%) were unchanged. Similarly 5 changed in the placebo group with 3 (16%) decreased; 2 (11%) increased and 14 (74%) were unchanged. On statistical analysis, there was no significant difference between omega-3 and placebo in change in ultrasound assessment grade of the liver at 6 months compared with baseline (difference 5.5% (99%CI -22.3 – 33.3); p=0.70).

Ultrasound assessment of steatosis was repeated at 9 months. In the placebo group 19 participants attended both 6 and 9 months scans. There was no change in grade of steatosis in any patient in the placebo group between 6 and 9 months. Three patients in the omega-3 group had a change in ultrasound grade at 9 months compared with 6 month visit: in 2 study participants the grade of steatosis increased (from 0 to 1 and 1 to 2); and in 1 patient the grade of steatosis decreased (from grade 1 steatosis to grade 0). Due to the small numbers no formal statistical analysis was performed on these results.

		Placebo Ultrasound Grade (6 months)				Omega-3 Ultrasound Grade (6 months)			
Ultrasound Grade (Baseline)		Missing	0	1	2	Missing	0	1	2
	Total	6	1	14	4	2	2	14	6
1	**N**	4	1	12	2	1	2	12	3
	%	*21*	*5*	*63*	*11*	*6*	*11*	*67*	*17*
2	**N**	2	.	1	2	1	.	2	3
	%	*40*	*.*	*20*	*40*	*17*	*.*	*33*	*50*
3	**N**	.	.	1	.				
	%	*.*	*.*	*100*	*.*				

Table 2.7 Ultrasound grade of steatosis at baseline and 6 months by treatment arm

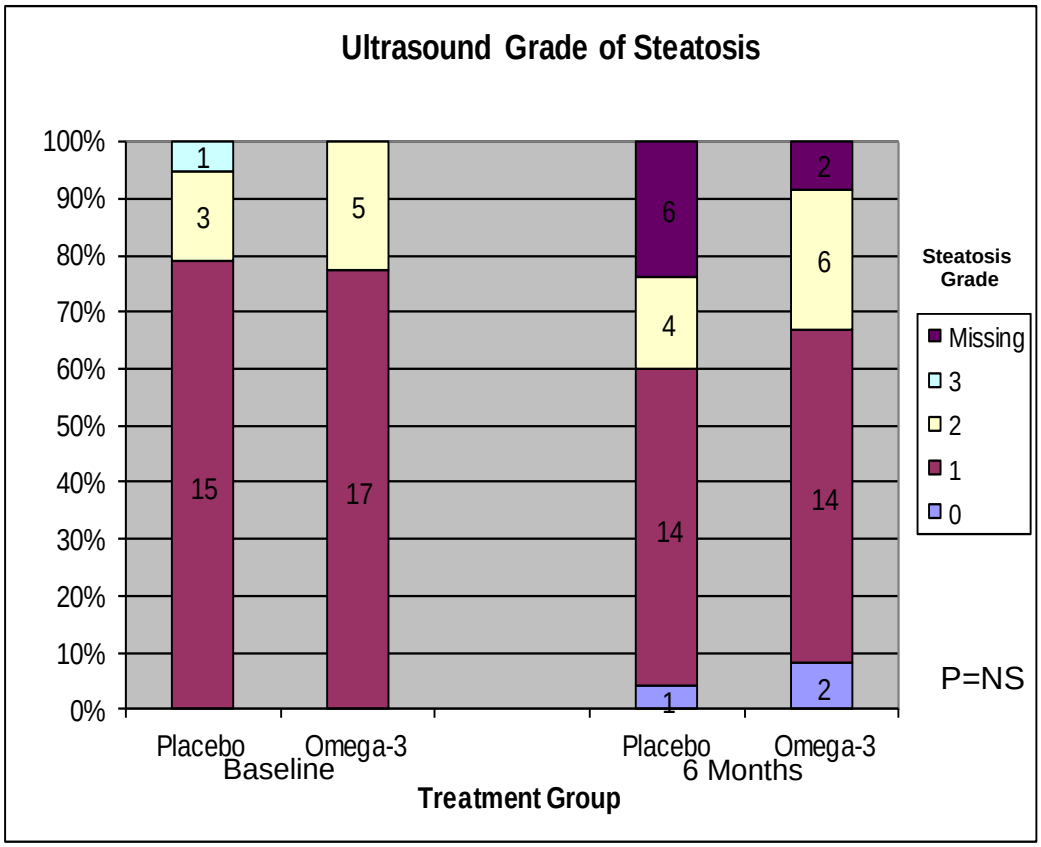


Figure 2.5 Ultrasound grade of steatosis at baseline and 6 months by treatment arm.

2.4.6 Secondary Outcome Measures (i) Serum Liver Function Tests (LFTs)

Change in serum liver function tests at 6 months was assessed. A change between six months (treatment end) and 9 months (study end) was also considered. In this study a change in serum liver function tests was pre-defined as a ≥10% difference (increase or decrease) in values.

Baseline-6 months

There was no significant difference in change in any serum liver function test in those who had omega-3 compared to placebo between baseline and 6 months. These data are displayed in Table 2.8

Line plots are presented to illustrate individual alterations in LFT for each individual study participant throughout the study. These are displayed in Figure 2.6.

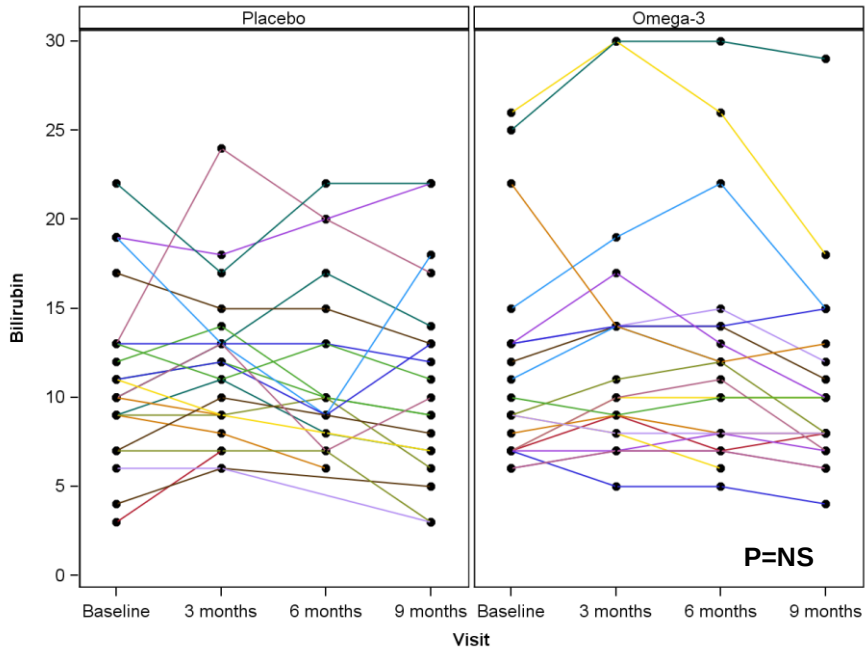
6-9 months

Similarly, at 9 months compared with 6 there was no significant difference between omega-3 and placebo in the change of bilirubin (p=0.14); ALT (p=0.32); AST (p=0.10); Alkaline phosphatase (p=0.68) or GGT (p= 0.73).

	Placebo					Omega-3						
	Total Number	No. with decrease	No. with same	No. with increase	% with change (95% CI)	**Total Number**	No. with decrease	No. with same	No. with increase	% with change (95% CI)	Difference of % change	p value
Bilirubin (%)	17	7 (41)	6 (35)	4 (24)	64.7 (42.0-87.4)	21	4 (19)	8 (38)	9 (43)	61.9 (41.1-82.7)	-2.8	0.86
ALT (%)	17	10 (59)	5 (29)	2 (12)	70.6 (48.9-92.2)	21	6 (29)	6 (29)	9 (43)	71.4 (52.1-90.8)	0.8	0.95
AST (%)	18	9 (50)	7 (39)	2 (11)	61.1 (38.6 -83.6)	20	5 (25)	10 (50)	5 (25)	50.0 (28.1-71.9)	-11.1	0.49
Alk Phos (%)	17	3 (18)	12 (71)	2 (12)	29.4 (7.8-51.1)	21	7 (33)	13 (62)	1 (5)	38.1 (17.3-58.9)	8.7	0.57
GGT (%)	18	8 (44)	3 (17)	7 (39)	83.3 (66.1-100)	21	6 (29)	3 (14)	12 (57)	85.7 (70.7-100)	2.4	1.00

Table 2.8 Number of patients with a change in serum liver function tests at six months by treatment arm

(a) Bilirubin



(b) ALT

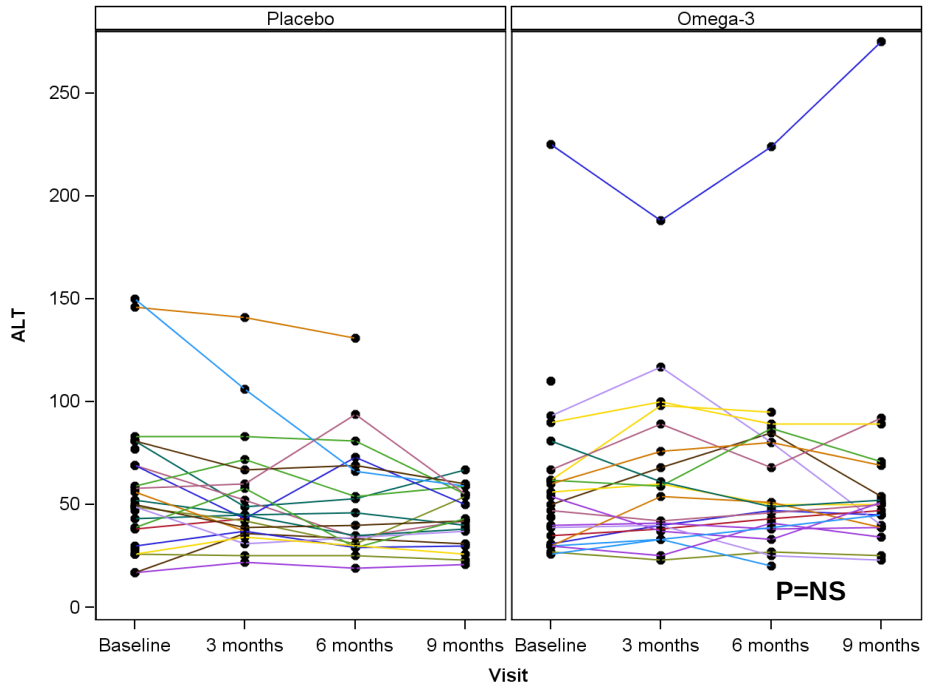
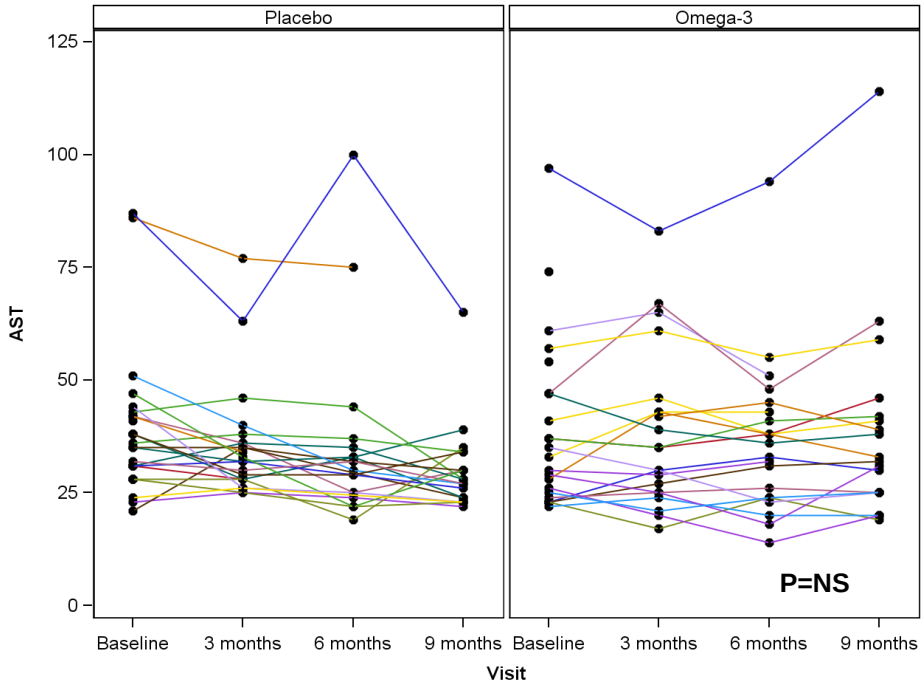
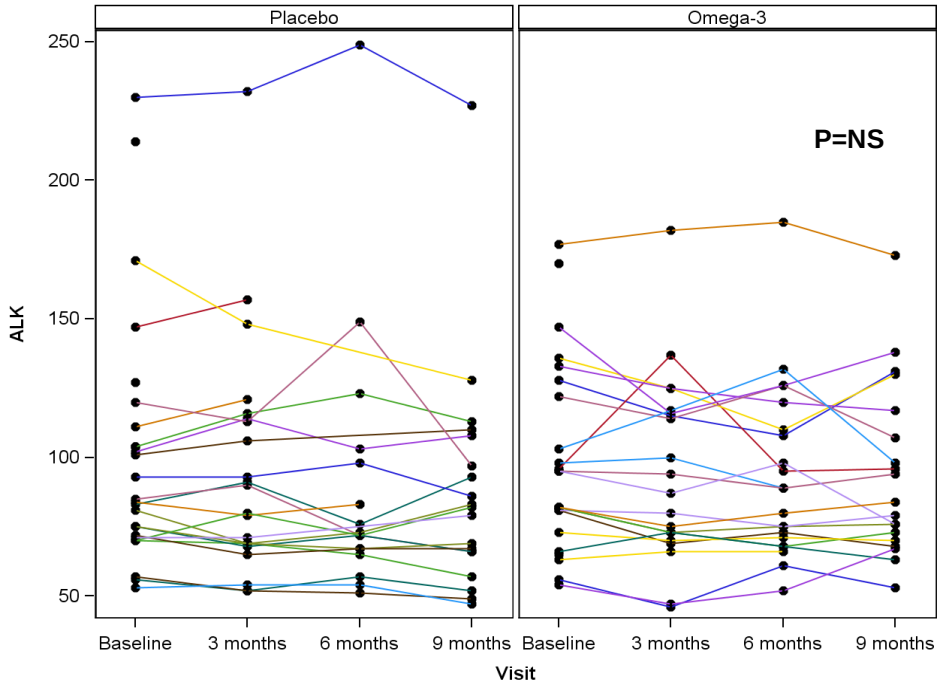


Figure 2.6 Individual change in serum liver function tests: (a) bilirubin (b) ALT (c) AST (d) alkaline phosphatase (Alk Phos) (e) GGT

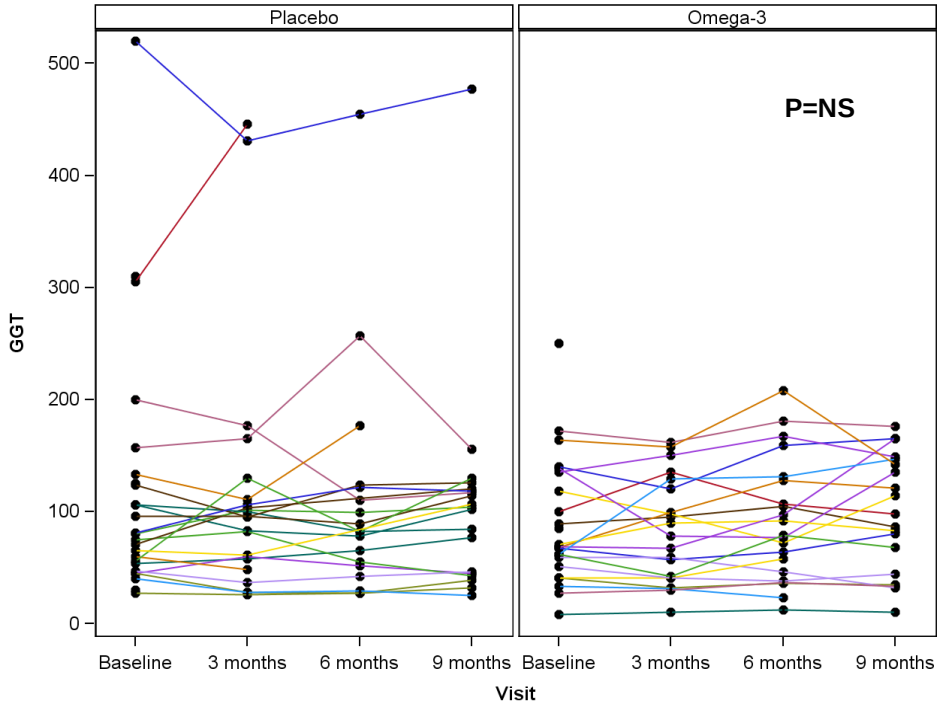
(c) AST



(d) Alkaline Phosphatase



(e) GGT


Placebo
Omega-3
P=NS
GGT
500
400
300
200
100
0
Baseline
3 months
6 months
9 months
Visit

2.4.7 Secondary Outcome Measures (ii) Health-Related Quality of Life Scores

The effect of omega-3 supplementation on health related quality of life (HRQoL) compared to those on placebo was assessed.

The data of 3 subjects were discarded as more than 20% of the questionnaire data were missing. Each of the 4 domains of the WHOQOL-Bref score were considered in turn. There was no significant difference in any domain of the HRQoL score in those who were randomised to omega-3 compared to placebo between baseline and 6 months. Results are found in Table 2.9.

		Placebo			Omega-3			p-value
		N	Missing	Score	N	Missing	Score	
Physical Health	**Baseline**	22	3	12.5 (1.8)	21	3	12.9 (1.6)	
	6 months	16	9	13.0 (2.1)	18	6	12.8 (1.8)	0.99
Psychological Health	**Baseline**	22	3	13.2 (2.3)	21	3	13.8 (2.1)	
	6 months	16	9	13.7 (1.7)	18	6	13.8 (1.8)	0.54
Social Relationships	**Baseline**	22	3	14.5 (4.1)	20	4	15.4 (3.2)	
	6 months	16	9	15.3 (3.2)	17	7	14.6 (3.8)	0.35
Environment	**Baseline**	22	3	15.6 (3.1)	21	3	16.3 (2.0)	
	6 months	16	9	16.6 (1.9)	18	6	16.6 (1.9)	0.47

Table 2.9 WHOQOL-Bref Scores at baseline and 6 months.

Results expressed as Mean (SD)

2.4.8 Additional Results: Anthropomorphic Data, Serum Lipids and Glucose

Waist:Hip Ratio

There was no significant change in waist:hip ratio in omega-3 group compared with placebo after six months of treatment (+0.01 vs. -0.02; p=0.2). These data are displayed in a box-and-whisker plot below (Figure 2.7).

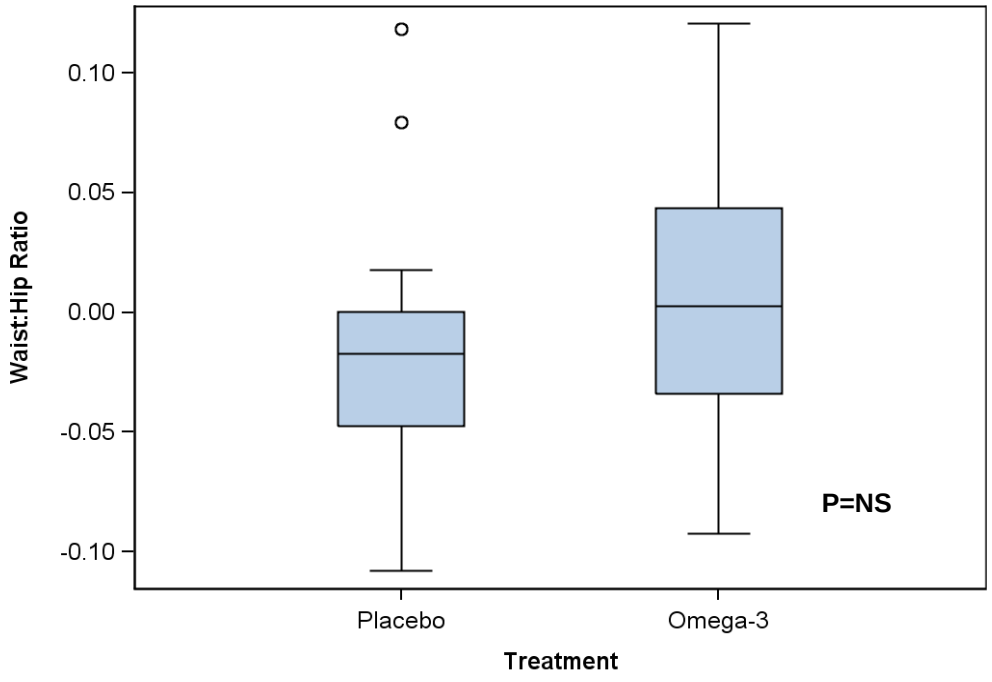


Figure 2.7 Change in Waist:hip circumference at 6 months compared with baseline

Weight

Subjects in the omega-3 arm of the trial gained a median 0.9Kg (Q1 -0.8; Q3 2.9) between baseline and 6 month whilst in the placebo arm subjects lost a median 0.9Kg (Q1 -2.5; Q3 0.2). This reached statistical significance: p=0.048.This result is displayed in Figure 2.8.

This result was also calculated as % change in weight between baseline and six months and similar results were obtained: omega-3+1.2% (Q1-0.9; Q3 2.9) vs placebo -1.1 (Q1-2.9; Q3 0.2); p=0.042.

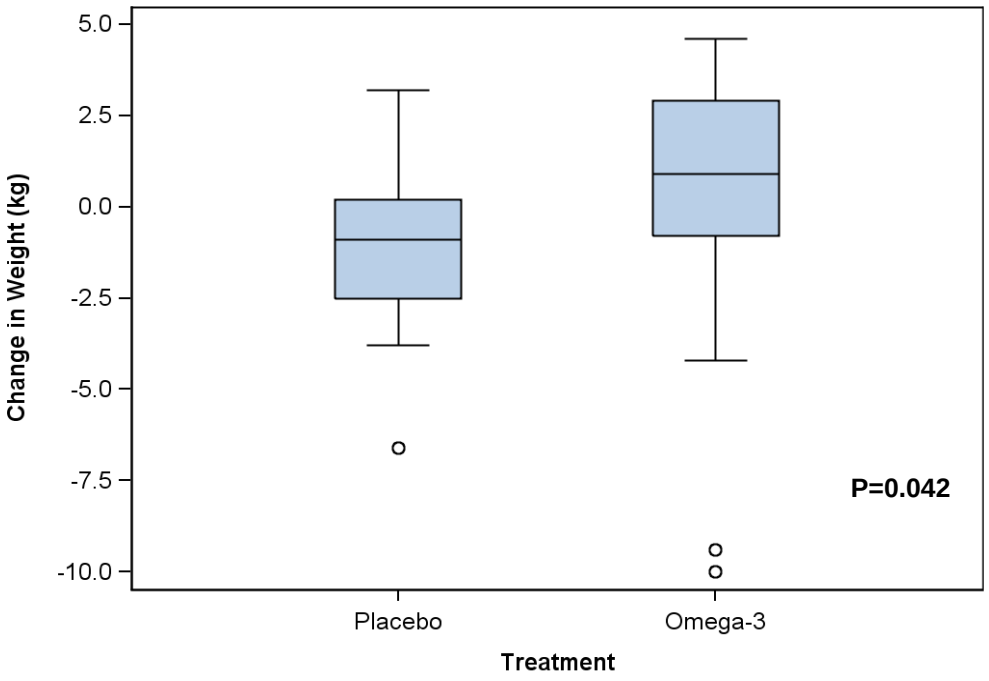


Figure 2.8 Change in weight at 6 months compared with baseline

Lipids

Change in serum lipids, defined as levels changed by >10% from baseline, was assessed following six months supplementation with omega-3 or placebo. There was no significant difference in change in lipids in those who omega-3 compared to placebo between baseline and 6 months. The results are summarised in Table 2.10.

Change in serum triglycerides by treatment group was plotted on a box plot and confirmed no significant change in serum triglycerides level between baseline and 6 months in those who received with omega-3 compared with those on placebo (p=0.34).These results are expressed in a box plot (Figure 2.9).

	Placebo				Omega-3				p-value
	N	Decreased	Same	Increased	N	Decreased	Same	Increased	
Triglycerides	17	8 (47)	4 (24)	5 (29)	22	7 (32)	6 (27)	9 (41)	1.00
Cholesterol	17	5 (29)	9 (53)	3 (18)	22	1 (5)	18 (82)	3 (14)	0.08
LDL	17	4 (27)	5 (33)	6 (40)	22	3 (14)	12 (57)	6 (29)	0.14
HDL	17	2 (12)	12 (71)	3 (18)	22	1 (5)	16 (73)	5 (23)	0.88

Table 2.10 Number of patients with a change in serum lipids at six months by treatment arm

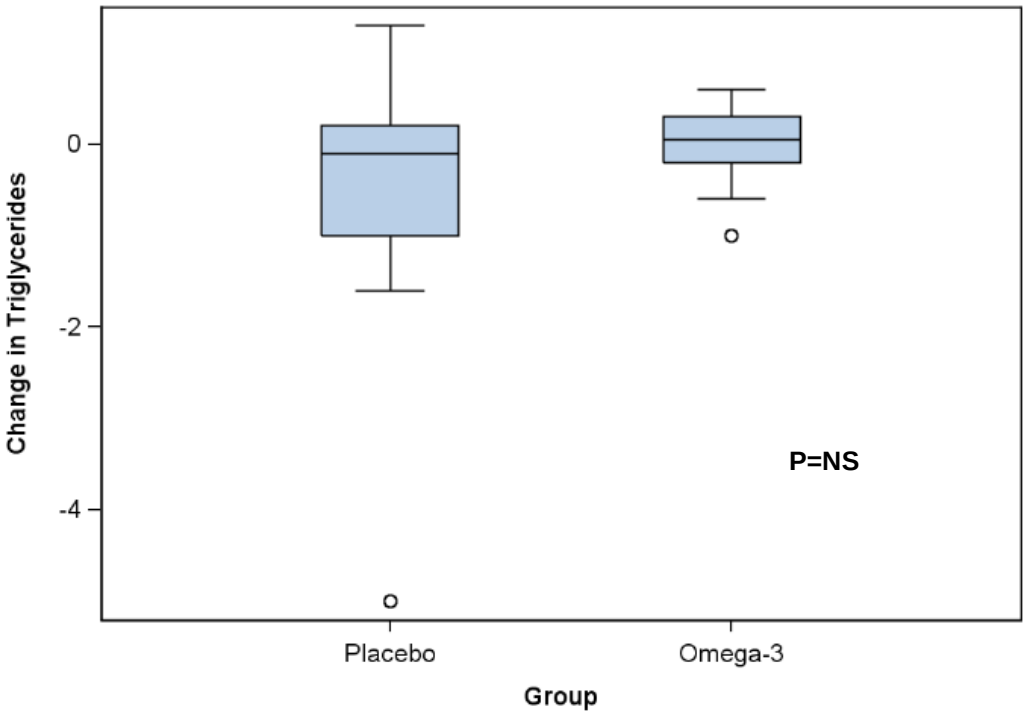


Figure 2.9 Change in Serum Triglycerides at 6 months compared with baseline (mmol)

Glucose

Glucose levels were only assessed in study participants without insulin dependent diabetes mellitus. Serum glucose levels changed by >10% from baseline in 5 of the 15 patients in the omega-3 group (1 decreased; 4 increased) and 5 of the 13 patients in the placebo arm (again, 1 decreased; 4 increased). Figure 2.10 shows individual results. There was no difference in change in serum glucose between omega-3 and placebo between baseline and 6 months (p=0.78). In addition, there was no difference in change in glucose in those treated with omega-3 and those treated with placebo between 6 and 9 months (p=0.91).

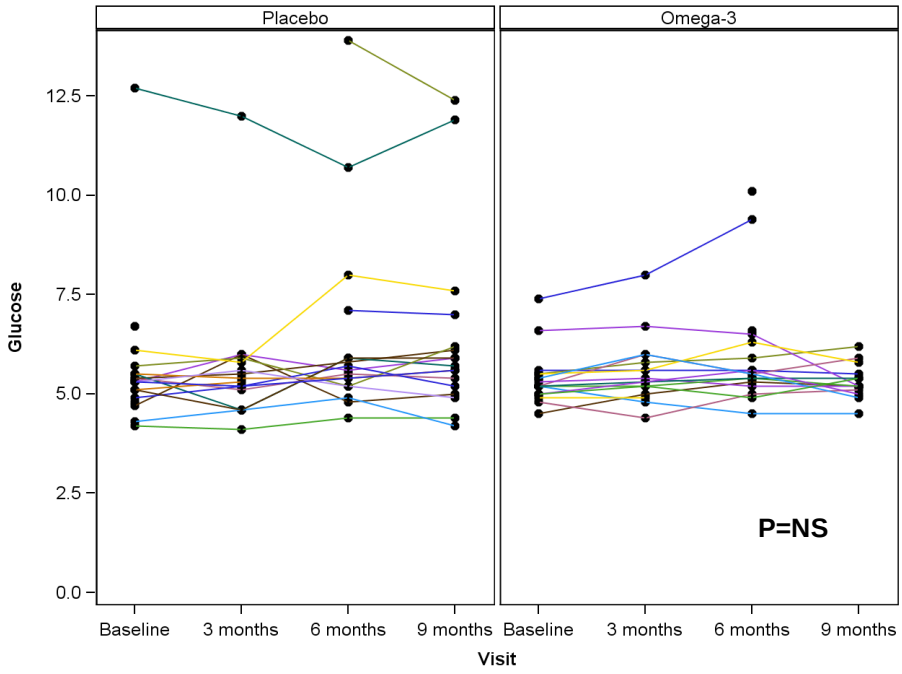


Figure 2.10 Individual change in serum glucose

2.4.9 Significant Adverse Events

2 significant adverse events were encountered during the study. Both were within the placebo treatment group. One participant was admitted to hospital with a stroke, the other admitted with atrial flutter (2:1 block) in the context of a viral illness. The former subject missed visit 3 due to her admission but attended visit 4. The other participant was withdrawn as he commenced warfarin (contraindicated in the study protocol). Neither was deemed expected or to be related to the trial. Both were reported to trial monitors.

2.4.10 Adverse Events

Symptoms were recorded at each study attendance. 10 patients in the placebo group reported symptoms at the 3 months visit and 4 at the 6 months clinic. Omega-3 fared better initially with 8 patients at 3 months but then 13 subjects reported adverse events at visit 6 months.

Most symptoms reported were mild and not deemed to be related to the study medication (e.g. hayfever, bad back, dental issues). At 3 months 4 patients in omega-3 reported side-effects deemed to be possibly related to the study medication: bad skin (n=1) and GI disturbance (n=3). 1 patient in the placebo arm also described GI disturbance.

At six months 5 patients in omega-3 arm reported GI disturbance and 1 a fishy taste to the tablets. No patients in the placebo arm had GI upset.

2.5 Summary of Results

This is the first phase 3 randomised control trial which uses ultrasound as its primary endpoint and is powered by a published pilot trial comparing omega-3 fatty acids to placebo in patients with non-alcoholic fatty liver disease. Omega-3 fatty acids were not shown to be beneficial either in reducing steatosis as graded on ultrasound, serum liver function tests or health related quality of life scores. Omega-3 fatty acids cannot be recommended as a treatment for non-alcoholic fatty liver disease based on these results. These results are in keeping with recent published trials, but are in contrast to the findings of previous studies and the pooled data from a 2009 meta-analysis. The results are discussed in full in Section IV.

Section III

Cell Culture Studies

3.1 Materials and Methods

3.1.1 Cell Lines

Human C3A cell lines were used. These were the property of the Department of Hepatology, University of Edinburgh. The C3A cell line is clonally derived from the human hepablastoma cell line HepG2. It is the cell line used in the majority of clinical trials of liver assist devices and is felt to be a good model both in terms of hepatocyte function and morphology.(171)(172)

3.1.2 Cell Culture Materials

Sigma Aldrich (St Louis): Dulbecco's phosphate buffered saline (PBS); Minimum essential medium eagle (MEME): 1g/L glucose, 0.292g/L glutamine(M0268); Hank's balanced salt solution (HBSS); 1% penicillin and streptomycin; Sodium bicarbonate; Trypsin; Dimethyl sulfoxide(DMSO); Tert-butyl hydroperoxide (t-BOOH); Eicosapentaenoic acid (EPA) (E2011); Octanoic acid (C2875); Lactate (L7022); Sodium pyruvate; Ammonium chloride; Glycine; Hydrazine hydrate; *N*-morpholino-propanesulfonic sodium salt (Mops sodium salt); EDTA disodium; ***Invitrogen (USA):*** 10% foetal calf serum (FCS); ***BDH Chemicals Ltd (UK):*** Paraformaldehyde; *N*-morpholino-propanesulfonic acid (Mops free acid); ***Roche (USA):*** NAD (free acid, approx 98%); NADH (disodium salt approx 98%); ***Roche (Germany):***L-Lactate dehydrogenase; 3-Hydroxybutyrate dehydrogenase; Hexokinase; Glucose–6–phosphate dehydrogenase; ***Boehringer Mannheim (Germany):***ATP; ***Randox Laboratories (UK):*** AST Kit ;***Sentinel diagnostics (Italy):*** Triglyceride kit (ref 17624); Lactate dehydrogenase kit (ref 17294); **Fluka (*Switzerland*):** Albumin blue 580 (AB580)

3.1.3 Machines and Equipment

- Cobas-Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK)
- Sanyo MSE Soniprep 150 (Sanyo MSE, London, UK)
- Unicam UV1 spectrophotometer (Unicam Ltd, U.K)
- Nanodrop ND-1000 spectrophotometer (Labtech International)
- T75 flasks (Corning Inc, USA)
- Costar 3516 6 well plates (9.5 cm^2) (Corning Inc, USA)
- Sarstedt Cuvettes (Sarstedt, Germany)

3.1.4 Cell Culture Techniques

Cell Culture

The C3A cells were stored in liquid nitrogen until use. When required the cells were defrosted and then passaged. Cells were then grown in a T75 flask in 10-12mls of MEME supplemented with 10% foetal calf serum, 1% streptomycin and penicillin and sodium bicarbonate (2.2g/L). Cells were incubated at 37^oC in a 5% CO_2-air humidified atmosphere and grown to confluence. The media were changed every 48-72 hours and cells passaged every 7-10 days.

Cell Passage

Cell passage (also known as splitting) refers to the trypsinisation and division of cells grown in cell culture. This process permits a small number of cells to be transferred to a new medium where they are then allowed to grow to confluence. Cell passage prevents cellular senescence, which is associated with a prolonged

period spent at confluence. Each time a cell line has been split and replated '1' is added to the passage number. Cell lines are discarded after 20 passages.

Cell passage was performed in sterile conditions. The old media were aspirated from the flasks and cells washed with HBSS. 5mls of trypsin were added to the flasks which were then incubated for 5-8mins at 37^{o}C in a 5% CO_2-air humidified atmosphere.

The flasks were then agitated to dislodge the cells. 5mls of MEME+ was added to halt the trypsin reaction. The resultant suspension was transferred to a sterile universal container and centrifuged at 600 revs per minute for 2 minutes to form a pellet. The supernatant was aspirated and discarded. 1ml of fresh media was added and the cells gently mixed with solution. Further media was added (amount dependant on number of flasks/plates required: typically 3-5mls), and the new suspension split into new flasks/ plates and incubation media added as required.

Preparation of Incubation Media

Preparation of Oleate Solution

4 grams of bovine serum albumin was mixed with PBS+ to a volume of 20mls. Oleate (molecular weight 304.45) was made in solution of 18mM. Therefore 109.6mg of oleate was added to 20mls of the prepared solution (0.25mM). This was mixed in an ice bath until clear.

Preparation of LPON Solution

In order to make 20mls of LPON: 200μL of lactate/ pyruvate mixture (11mg of pyruvate Sigma Sodium pyruvate 100084543 is added to 1ml aliquot of 1M lactate) is added to 400μL of octanoate, (0.1M: 865.2mg/60mls H_20 pH 7.0-7.4) and 80μL of ammonium chloride (53mg NH_4Cl in 1mls PBS –ve) were added to 19.34mls of MEME under sterile conditions. This was then filtered before use. LPON solution was prepared fresh for each experiment.

Preparation of EPA solution

Eicosapentaenoic acid (EPA) was purchased from Sigma Aldrich (St Louis) as 1M solution. Following manufacturer's instructions, this was diluted with PBS under sterile conditions and, following filtering, was stored as 0.5ml aliquots of 5mM solution at -20^{o}C.

Preconditioning

Preconditioning is the process of incubating cells in culture with the test media prior to samples being collected for analysis.

Confluent C3A cells were divided into the required number of groups in 6 well plates and/or chamber slides and the culture medium replaced by the test and control solutions. Cells were usually harvested after 72 hours.

Cell Harvesting

Cells were grown in a single layer in standard 6 well plates to confluence. The supernatant was aspirated and stored in labelled Eppendorf tubes. Cells were then washed twice with PBS before being harvested by scraping with 0.5 or 1mls of PBS and decanting the resultant solution into labelled Eppendorf tubes. The efficiency of scraping was assessed by visual inspection. Following this the solutions were stored at -20^{o}C. For assessment, the cells were thawed and underwent sonication to induce cell lysis for 3 x 10 seconds on Sanyo MSE Soniprep 150 (Sanyo MSE, London, UK).

3.1.5 Assays

Background

Glucose, betahydroxybutyrate and, acetoacetate flux in the supernatant were calculated on Unicam UV1 spectrophotometer (Unicam Ltd, U.K) according to the methods of Bergmeyer.(173) Triglyceride concentration, lactate dehydrogenase (LDH) and total protein were calculated on Cobas-Fara centrifugal analyser (Roche diagnostics UK).

Glucose

Principle:

$$\text{Glucose + ATP} \xrightarrow{\text{(Hexokinase (HK))}} \text{Glucose – 6 –phosphate}$$

$$\text{Glucose–6–phosphate + NAD} \xrightarrow{\text{(G6PdH)}} \text{Gluconolactone 6–phosphate + NADH}$$

The assay detects the concentration of NADH produced by the two reactions above. This corresponds to the concentration of glucose in the cuvette.

Method

The glucose buffer is prepared by mixing NAD (17mg), ATP (125μL), G6PdH (12.5μL) HK (5μL) and diluting in 50mls of PBS+.200μL of each sample was pipetted into a cuvette and 1ml of glucose buffer added. This was then homogenised by gently inverting the cuvette 3-5 times. A blank well was prepared with 200μL of

H_2O and 1ml of buffer to act as a control. The samples were then left at room temperature for 60 minutes. Following this absorbance was read at 340nm wavelength for each cuvette and blank on Unicam UV1 spectrophotometer (Unicam Ltd, U.K). Glucose flux was then calculated according to the methods of Bergmeyer.(173)

Beta-hydroxybutyrate

Principle:

(betahydoxybutyrate dehydrogenase)

Betahydroxybutyrate + NAD ↔ acetoacetate + NADH

The assay detected the concentration of NADH produced by the reaction which corresponds to the concentration of betahydroxybutyrate in the sample. Two readings were taken as the changes in absorbance were small.

Method

A betahydroxybutyrate buffer was made by mixing NAD (50mg), glycin (3g), hydrate hydrazine (2ml). This was diluted in 100mls sterile water. For the reaction 200μL of sample was added to each cuvette. 200μL of sterile water was added to an additional cuvette to act as control. 1ml of betahydroxybutyrate buffer was then added to each sample and this was homogenised by gentle inversion. A reading was then made at 340nm wavelength on Unicam UV1 spectrophotometer (Unicam Ltd, U.K). A solution of betahydroxybutyrate dehydrogenase was then prepared by diluting 200μL of betahydroxybutyrate dehydrogenase in 1ml of sterile water. 10μL

of this solution was added to each cuvette and the sample homogenised. Samples were left for 1 hour at room temperature before a repeat reading was made.

Acetoacetate

Principle:

(betahydoxybutyrate dehydrogenase)

Betahydroxybutyrate + NAD ↔ acetoacetate + NADH

The reaction determined the concentration of NADH, which corresponds to the concentration of acetoacetate in the sample. Hence 2 readings – the first to determine how much acetoacetate is in the cuvette, the second to see how much is used by the reaction.

Method

The acetoacetate buffer was made by diluting 12mg NADH in 100mls of PBS+. The absorbance of the buffer was checked at 340nm wavelength on Unicam UV1 spectrophotometer (Unicam Ltd, U.K) to ensure it was within the range 0.800-1.200. For the reaction 200μL of sample was added to each cuvette. 200μL of sterile water was added to an additional cuvette to act as control. 1ml of acetoacetate buffer was then added to each sample and this was homogenised by gentle inversion. A first reading was then made at 340nm wavelength on Unicam UV1 spectrophotometer (Unicam Ltd, U.K). A solution of betahydroxybutyrate dehydrogenase was then prepared by diluting 200μL betahydroxybutyrate dehydrogenase in 1ml of sterile water. 10μL of this solution was then added to each cuvette and the sample

homogenised. The samples were then left for 1hr at room temperature before a repeat reading was made.

Lactate Dehydrogenase (LDH) Assay

Background

The contrasting intracellular: extracellular LDH concentration was used to assess cell viability. This assay is based on the procedure of Gay et al.(174) Here the LDH concentration in the cell lysates and supernatant were determined by following the rate at which NAD is reduced to NADH measured as a increase in absorbance at 340nm in the presence of lactate using a LDH kit method (Sentinel Diagnostics, Italy) modified for use on the Cobas-Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). The rate of decrease in absorbance at 340nm, measured at 37^{o}C, is directly proportional to LDH activity in the sample.

Results were expressed as % LDH released calculated as follows – intracellular LDH/ (extracellular LDH + Intracellular LDH) x 100

Method

Reagents were premade in Sentinel kits. Samples were collected as described above. Precinorm U (a commercially produced 'universal control serum') was used for quality control for each assay. LDH calculated on neat supernatants and on diluted sonicated (no detergent) cell extracts. The change in absorbance at 340nm wavelength was measured in 25μL of sample in a final volume of 1500μL of the reagent. The sample and reagent were pipetted into the cuvettes and incubated for

30 seconds. The change in absorbance of the reaction mixture was read at 0.5sec.and was then read every 5 sec for a total of 20 readings. One unit of activity per litre was calculated as follows: Activity in U/l = Delta absorbance/minxfactor

Where the factor = total reaction volume (ml) x 1000/6.3 x sample volume (ml) x cuvette pathlength (cm)

The LDH activity was expressed as units per litre (U/L) as determined by kinetic analysis.

Total Protein Assay

Background

Total protein content was determined in PBS-cell suspension (no detergent) after sonication, using the dye-binding Bradford assay technique modified for a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK).(175)

Method

The Bradford reagent was prepared by dissolving 100mg Coomassie Brilliant Blue G-250 in 50ml 95% ethanol. Then 100ml 85% (w/v) phosphoric acid was added and the mixture stirred for 30 mins. The resulting solution was diluted with distilled water to a final volume of 1000mls, filtered through Whatman Grade 1 filter paper and stored at room temperature in a closed bottle. A standard curve was constructed using bovine serum albumin as standard and distilled water as a diluent covering the range 0-100mg/L.Bradford reagent (256μL) was added to each cuvette which was incubated for 100sec at 37ºC prior to sample addition, with an initial absorbance reading (595nm) taken at 95 sec. Following the addition of 25μL sample plus 50μL

distilled water (diluent) to the cuvettes a further incubation took place for 180sec at 37^{o}C. A final absorbance was then read at 595nm. The difference between the final and initial absorbencies was calculated and a standard curve plotted.

The protein concentration of the samples were interpolated from the standard curve. Samples were diluted with distilled water as required to fall in the middle portion of the standard curve and a quality control (QC) was run with every rotor to assess the reproducibility of the results.

Triglyceride Quantification

Background

The triglyceride assay is based on the method of Wako and the modifications by McGowan et al and Fossari et al. (176)(177)

The assays run according to the following reactions:

$$\text{Triglyceride} + H_2O \rightarrow \text{Glycerol} + \text{FFAs}$$

$$\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \rightarrow \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{3.5DHBS} \rightarrow \text{quinine imine dye plus } H_2O$$

In the first step in the reaction triglycerides are converted to glycerol and fatty acids in the presence of water. Next, glycerol in the presence of adenosine triphosphate (ATP) is converted to glycerol-3-phosphate and adenosine diphosphate (ADP). In the third step, glycerol-3-phosphate is oxidised to from dihydroxyacetone phosphate (DAP) and hydrogen peroxide. In the final step of the reaction the hydrogen peroxide, 4 aminoantipyrine (4AAP) and 3,5 dichloro 2 hydroxybenzene sulphate

(DHBS) are converted to a red coloured dye with water. The concentration of triglycerides in the sample is proportional to the dye absorbance.

Method

Triglyceride measurements were made using a commercial kit (Sentinel diagnostics, Italy) adapted for use on the Cobas Fara centrifugal analyser. Precinorm (1.2mmol/L) and a standard (0.7mmol/L) were used for quality control for every assay run.120μL of neat sonicated samples were transferred into the Cobas cups for triglyceride measurement. The change in absorbance at a wavelength of 500nm was measured in 3μL sample in a final volume of 303μL (3μL sample 300μL reagent). The sample and reagent were pipetted into the cuvettes and incubated at 37^{o}C for 5 minutes. The triglyceride concentration was expressed as mM as determined by kinetic analysis.

AST

AST was determined by a commercial kit (Randox Laboratories, UK) adapted for use on the Cobas-Fara centrifugal analyser (Roche Diagnostic Ltd, Welwyn Garden City, UK). α-oxogluterate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The indicator reaction utilises the oxaloacetate for a kinetic determination of NADH consumption. Within run precision was CV<4% while intra-batch precision was CV <5%.

Albumin Assay

Background

The albumin assay is a fluorescent assay based upon the principles of Kessler et al. (178)

Method

Albumin working solution was made up from powdered albumin (Albumin blue 580. This was made into solution with isopropanolol (3mg/100mls isopropanolol) to give concentration 30mg/L. The absorbance of the solution was read at 580nm and the solution was diluted such that the OD was 1.00. The solution was diluted with a buffer comprising 0.6g *N*-morpholino-propanesulfonic acid (Mops free acid), 1.8g Mops sodium salt, 2.4g sodium chloride, 0.2g ethylene-diaminetetraacetic acid and disodium salt (EDTA disodium), 200 mL distilled water, and 20 mL isopropanol. The pH of the resulting solution is 7.4.

This solution was then diluted with buffer to create standards with albumin concentration 2.5, 5.0, 10, 20, 40, 50, 75, 100, 150, 200 μg/mL. 80μL of each standard or sample was added in duplicate to wells in a microtitre place. 160μL of dye was added to each well. The plate was then shaken for 30seconds before the fluorescence was read (excitation 590nm, emission 645nm) on Cytofluor Series 4000 (PerSeptive Biosystems). A standard curve was created by inputting the data to Microsoft excel. Test values were then calculated from the standard curve.

3.1.6 DNA Quantification

Background

Nucleic acid quantification was determine using the Beer-Lambert equation modified for use on the NanoDrop 1000 Spectrophotometer.

The equation used was: **c = (A x e)/b**

Where:

c = the nucleic acid concentration in ng/microliter; **A =** absorbance in AU; **e =** the wavelength-dependent extinction coefficient in **ng-cm/microliter** (Nb. the generally accepted extinction coefficient for double-stranded DNA is 50 ng-cm/μl); **b =** path length in cm (here path lengths of 1.0mm and 0.2mm).

Method

DNA quantification was assessed on cell lysates using the NanoDrop 1000 Spectrophotometer. 'Nucleic Acid' application module was selected. The machine was calibrated with sterile water. 2μl of sample was then pipetted onto the measuring point such that it was completely covered and the arm was lowered. The sample absorbance was then read at 260 nm. This process was repeated for each sample. After a set of 10 samples the measuring point was cleaned with sterile water.

3.1.7 Oil Red O Staining

Background

This is a method for staining fat in cells. In the resultant slides unsaturated hydrophobic lipids and mineral oils are stained red. The method has been described by Green and Kehinde.(179)

Method

Stock solution was prepared by dissolving 0.5g of Oil red O in 60% alcohol. The solution was warmed to 56-60^{o}C for at least 1hr then cooled and stored at room temperature. Working solution was then prepared immediately before use by adding 4 parts distilled water to 6 parts stock solution. The working solution was then vortexed and left for 10mins. Finally it was filtered through a fine paper. Cells were cultured on chamber slides for the purpose of staining. After completion of the experiment cells were fixed with 10% formalin for 10mins and washed in 60% alcohol. Oil red O staining was conducted for 15 minutes. The cells then washed in 60% alcohol. After that they were washed in distilled water and counterstained in HARRIS Haematoxylin, differentiate in 1% Acid Alcohol followed by Scott's tap water substitute. Washing was repeated with distilled water and the slides examined microscopically. Cells were then rinsed in distilled water and mounted in glycerine jelly or Aquamount equivalent.

3.1.8 Statistical Analysis

Outliers

Outliers were pre-defined as either (a) one value being more than double or less than half the average of the other two values if these values are within 25% of each other or (b) where the results in triplicate are not within 25% of each other than a value shall only be discarded if there is a tenfold difference between it and either of the other two. If a value is deemed to be an outlier and is discarded it shall be replaced by the average of the other two results in that triplicate.

Statistical Tests

The main considerations when selecting the statistical tests were:

(1) Independence. As the cells came from a single source they could not be considered independent.

(2) Distribution. Within the limit of the small sample sizes the results were felt to be normally distributed. Parametric tests are therefore suitable.

(3) Outcome. The outcome measure to be tested was a difference between all groups, rather than hypothesis defined *a priori*.

As a result of the above Repeated measure ANOVA with Tukey post-test (comparing each column to others) on SPSS version 16.0 and GraphPad Prism Version 5.0 was used. The above was discussed with a statistician (Ms C Graham) at the Wellcome Trust Clinical Research Facility at the Western General Hospital, Edinburgh.

3.2 Experiment 1. Delineating the optimal concentration of EPA to use in cell culture experiments

3.2.1 Introduction

Omega-3 fatty acids (N3-PUFA's) have been suggested as a novel therapy for the treatment of non-alcoholic fatty liver disease (NAFLD).(30) N3-PUFA's are known to reduce serum triglyceride levels and probably hepatic triglycerides too.(133, 134, 157)Eicosapentaenoic acid (EPA) is one of the major physiologically active constituents of N3-PUFA's(159).

The aim of these experiments was to assess the efficacy of EPA in cell culture, a validated technique for assessing toxicity and delineating the metabolic effects of substances.(180)

Two models of cellular steatosis were used in these experiments. The first, developed at the University of Edinburgh and termed the LPON model, is a model of hepatocyte steatosis, mitochondrial dysfunction, increased reactive oxygen species and glucose dysregulation(17). In contrast the second model, oleic acid (oleate), is a widely used cell culture model for inducing hepatic steatosis. It is not known to be associated with mitochondrial dysfunction, glucose dysregulation or increased

reactive oxygen species production. Oleate is therefore a cellular model of isolated cellular steatosis and LPON a model of steatosis and mitochondrial dysfunction.

The concentration of EPA used in cell culture experiments in the published literature range from 1μM to 1mM in untreated hepatocytes.(181)(182) The optimal concentration of eicosapentaenoic acid (EPA) in the LPON model is not known and as the LPON model of fatty liver is new its viability and validity for assessing the therapeutic effects of EPA needs to be established. Cell viability may be assessed by measuring LDH retention in the hepatocytes compared with LDH concentration in the supernatant(183).

Glucose metabolism is significantly altered with LPON (gluconeogenesis is increased by 3 fold with 7 days incubation)(172). Endogenous glucose production was selected as the primary outcome to assess efficacy as this can be rapidly assessed by spectrophotometry with a validated technique and is known to be altered by incubation with LPON. There is conflicting evidence in the published literature about the effect of N3-PUFA's on hepatocyte glucose metabolism. If efficacy is proven using glucose as an outcome measure future studies could delineate the effect of EPA on glucose homeostasis using the LPON model but this is out with the remit of this project.

3.2.2 Aim

The aim of this experiment was to establish the optimal dose of EPA to use in cell culture experiments by assessing (i) cell viability and (ii) efficacy. The former was assessed by LDH leakage from the hepatocytes into the supernatent, and the latter by comparing endogenous glucose production the LPON model with different concentrations of EPA.

3.2.3 Methods

C3A cells were grown in T75 flasks as described in Methods (Section 3.1). Cells were passaged into six well plates (35mm) and grown in confluence in a standard medium (MEME). This was then replaced by the test media in triplicate (i.e. three wells of each) and incubated for 72 hours at 37^{o}C. Supernatant and cells were then harvested, and LDH and metabolic outcomes determined. This procedure was repeated four times as the second run had to be discarded following an incubator malfunction. Therefore the results are obtained from nine repetitions.

In this experiment the following media were compared:

(I) MEME Control (standard control also termed untreated cells)

(II) LPON Control

(III) LPON + EPA 10μM

(IV) LPON + EPA 50μM

(V) LPON + EPA 100μM

(VI) LPON + EPA 250μM

(VII) LPON + EPA 500μM

Statistical Analysis

Repeated measure ANOVA with Tukey post-test was used where stated to compare groups. p values <0.05 were deemed significant.

3.2.4 Results

Cell viability

The data are presented in Table 3.1 as % of LDH in the supernatant/ total LDH. The results are also presented in Figure 3.1 as means and standard error of the mean (SEM).

Analysis demonstrated that only 500μM EPA had a significantly elevated LDH leakage when compared with LPON control (p=<0.001). This equates to a 76% increase in LDH leakage (95% CI 40-112%) when compared to LPON alone. LDH leakage with 500μM EPA was also significantly elevated compared to all other concentrations of EPA.

On post hoc analysis a linear relationship between EPA concentration and LDH concentration was demonstrated with repeated ANOVA for trend (p=<0.001).

Efficacy

To assess further whether the LPON model is valid to assess the effects of EPA endogenous glucose production was measured. The results are displayed in Table 3.2. The data are also displayed in Figure 3.2.

All concentrations of EPA were effective at significantly altering endogenous glucose production in the LPON model. These alterations are significant: incubation with 250μM EPA reduces the mean endogenous glucose production by 30% (95% CI 11-

49%) and to levels similar to that of the MEME control. There is a relationship between glucose flux and increasing EPA concentration as confirmed on ANOVA post hoc test for trend (p=<0.0001).

The efficacy of EPA in the LPON model is confirmed in ketone body production: see Appendix 3.

	Untreated Cells	LPON Control	LPON + 10µM EPA	LPON + 50µM EPA	LPON + 100µM EPA	LPON + 250µM EPA	LPON + 500µM EPA
%LDH[(a)]	11.36	13.99	15.79	13.35	16.94	17.58	25.69*
Std. Deviation	6.18	3.53	3.63	3.24	3.77	8.31	9.99
Std. Error	2.06	1.18	1.21	1.08	1.26	2.77	3.33
Lower 95% CI	6.61	11.28	12.99	10.86	14.04	11.20	18.01
Upper 95% CI	16.11	16.70	18.58	15.84	19.83	23.97	33.36

Table 3.1 Summary of the effect of increasing concentration of EPA on LDH leakage.

[(a)]Results presented as % of LDH supernatant/ total LDH.

* p=<0.001 compared with LPON control

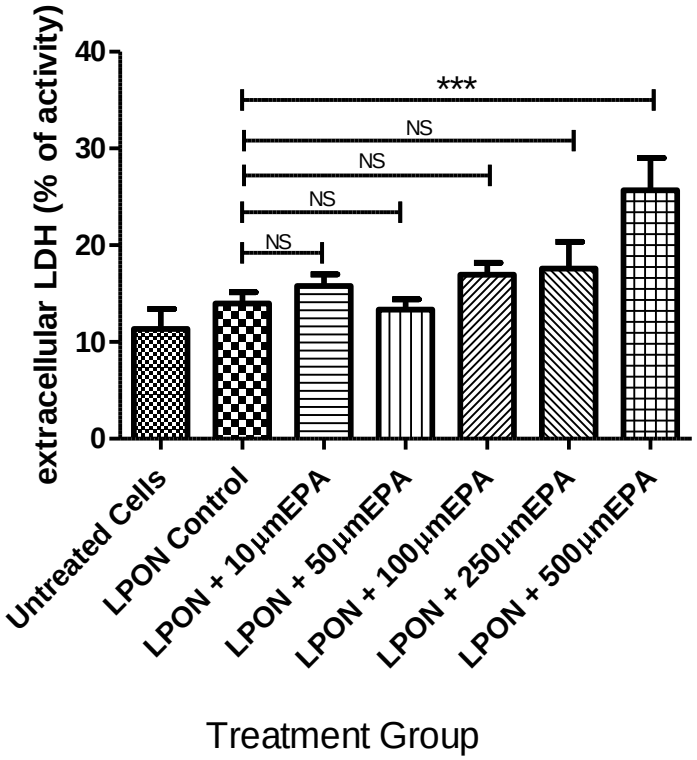


Figure 3.1 The effect of increasing concentrations of EPA on LDH leakage in the LPON model.

Results presented as % of LDH supernatant/ total LDH (i.e. LDH leakage) and expressed as mean and standard error of the mean (SEM). LDH leakage was significantly increased compared to LPON control cells when cells were incubated with 500µM EPA in the LPON model.

	Untreated Cells	LPON Control	LPON + 10μM EPA	LPON + 50μM EPA	LPON + 100μM EPA	LPON + 250μM EPA	LPON + 500μM EPA
Glucose Production	335.1	445.2	366.2*	303.6*	276.9*	312.0*	310.8*
Std. Deviation	21.32	115.2	84.95	100.8	64.62	72.27	108.9
Std. Error	7.11	38.39	28.32	33.60	21.54	24.09	36.29
Lower 95% CI	356.6	300.9	226.1	227.2	256.4	227.1	356.6
Upper 95% CI	533.7	431.5	381.1	326.6	367.5	394.5	533.7

Table 3.2 Summary of the effect of increasing concentration of EPA on Endogenous Glucose Production.

NB. Glucose production measured in mmol.h-1.gTP-1

* p=<0.001 compared with LPON control

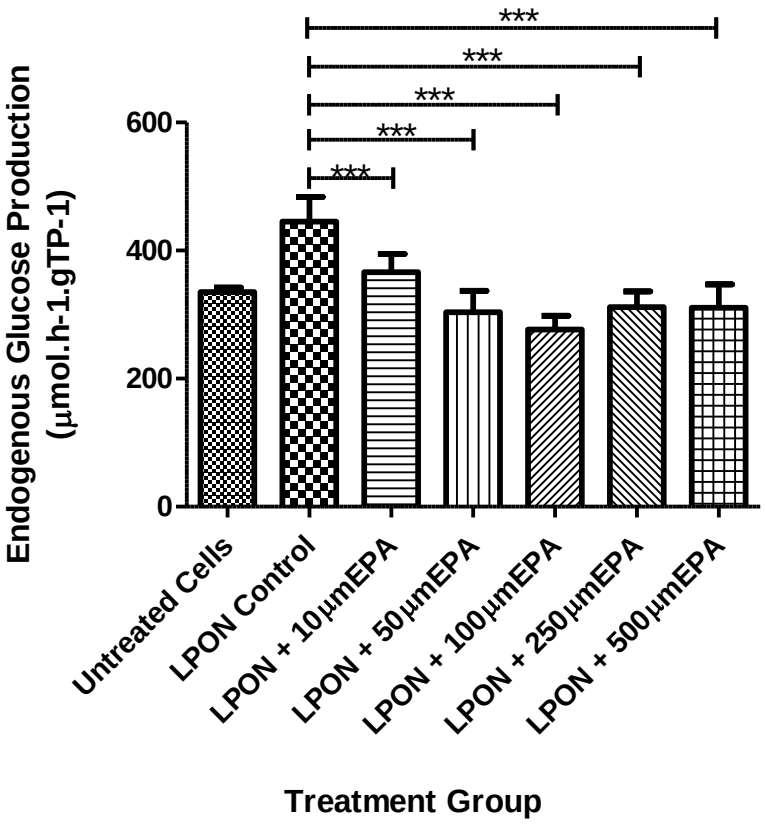


Figure 3.2 The effect of EPA on endogenous glucose production in the LPON model.

Where endogenous glucose production is measured in mmol.h-1.gTP-1 and results expressed as mean and SEM. The endogenous glucose production of LPON control is significantly higher than untreated cells. All concentrations of EPA significantly reduce endogenous glucose control in the LPON model to levels not statistically different to untreated cells.

3.2.5 Summary of Results

These results support the use of the LPON model for assessing the efficacy of eicosapentaenoic acid (EPA) in NAFLD cells. The findings indicate that 500μM EPA results in a significant increase in cell death as shown by increased LDH leakage, and is therefore unsuitable. All concentrations of EPA are effective at altering endogenous glucose production in the LPON model.

Based on these results it appears reasonable to use 10μM, 50μM, 100μM or 250μM EPA in LPON cell culture experiments, and given the association between increasing EPA concentration and both greater efficacy and greater cell death, using both 50μM and 250μM represent a trade-off between these wanted and unwanted outcomes.

3.3 Experiment 2. The effect of eicosapentaenoic acid on hepatocyte triglyceride content of C3A cells incubated in different test conditions

3.3.1 Introduction

There is evidence that eicosapentaenoic acid (EPA) reduces intrahepatic triglycerides in vivo, although findings are inconsistent.(157, 158, 162) Experiments were designed to assess if incubation with eicosapentaenoic acid (EPA) can prevent or reverse the accumulation of excess hepatocyte triglycerides in three different cell culture models: (1) C3A hepatocytes cultured in standard media (MEME) (termed untreated cells) (2) hepatocytes cultured with oleate (a model of cellular steatosis) and (3) hepatocytes cultured with LPON (a model of cellular steatosis and mitochondrial dysfunction).

The study to assess whether EPA can reduce or reverse hepatocyte triglyceride accumulation was conducted in three parts: (a) quantification using Oil Red O Staining (b) the prevention of intrahepatic triglyceride accumulation and (c) the effectiveness of EPA in reversing established intrahepatic steatosis.

(a) **Oil Red O Staining**

Oil Red O staining is a valid technique for assessing lipid accumulation in cell culture.(184)It is used to confirm the presence of intrahepatic triglycerides and of assessing the effect of EPA on triglyceride content although, as the dye can have variable penetrance and there may be sampling error, lipid quantification with Oil red O should be followed up with triglyceride assessment on cell lysates.

(b) **'Prophylactic'**.

In these experiments, as is standard protocol, the effect of EPA on intrahepatic triglycerides was ascertained when the cells were pre-treated with the test culture. Cells were harvested at 3 days as is usual, but also at 7 days which is not. The prolonged incubation allowed confirmation of 3 day incubation results and assessment of ongoing lipid lowering effects beyond those seen at day 3.

(c) **'Treatment'**.

In these experiments, once cells were grown to 80% confluence in standard media, the media were changed to the test media without EPA for 72 hours. Cells thus became fatty. Following this the media were changed to include EPA in the test media for a further 72 hours. The treatment experiments were designed to represent the capacity of EPA to ameliorate or reverse established hepatocyte steatosis in the context of ongoing poor environment.

3.3.2 Methods

In each experiment C3A cells were grown in T75 flasks and passaged into six well plates before being grown to confluence in standard media (MEME) as described in Section 3.1. Wells were then incubated with 50 and 250μM concentrations of EPA under the protocols as described. Following treatment supernatant and cells were harvested and outcomes determined as per described in Section 3.1. Each prophylaxis and treatment experiment was performed in triplicate and repeated 3 times, each value therefore represents the average of nine results.

For each experiment cells were also prepared for Oil Red O staining. Cells were plated onto chamber slides and grown to confluence in standard media (MEME). The media were then changed to the test media and cells incubated for a further 72hrs. Cells were prepared and stained as described in Methods (Section 3.1). Following this images of the stained hepatocytes were taken by a single operator (GM) using standardised image capture settings. Images were taken at random and the operator was blind to the treatment group. Pixel counts from at least 25 images were obtained in each case using Adobe Photoshop 5 software.

Analysis

Repeated measure ANOVA with Tukey post-test was used to compare groups. p values <0.05 were deemed significant.

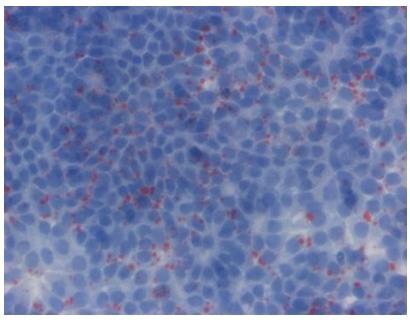
3.3.3 Hepatocytes Cultured In Standard Media (MEME)

Aim

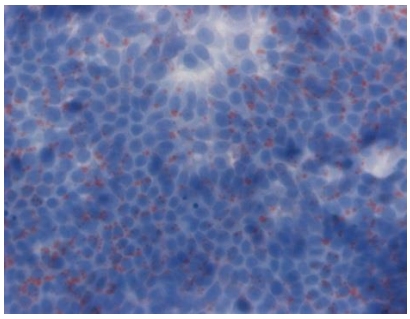
To assess the effects of EPA on the accumulation of hepatic triglycerides in C3A cells incubated in MEME (standard media). Here the test media were: (i) MEME (standard control also termed untreated cells); (ii) MEME + EPA 50μM or (iii) MEME + EPA 250μM.

Experiment (a): Oil Red O Staining

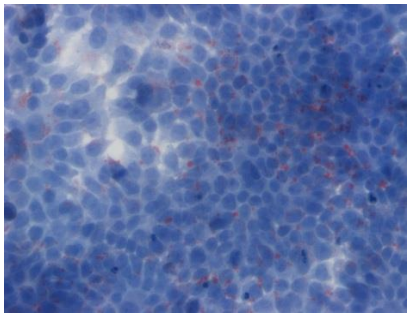
Slides are displayed in Figure 3.3. Data are summarised in Table 3.3, the results are also presented in Figure 3.4. Oil Red O staining confirmed the presence of intrahepatic triglycerides in the standard model. The number of red pixels, denoting Oil red O staining and therefore lipids, were significant reduced when cells were incubated in standard media (MEME) containing 250μM EPA ($p=<0.001$) but not 50μM EPA (NS). This represents a 73.1% reduction (95% CI 63-83%) when incubated with 250μM EPA compared with untreated cells. A linear trend between increasing EPA concentration and reduced Oil red O staining was confirmed on post-test analysis in the MEME model ($p<0.0001$).



MEME Control (i.e. Untreated)



MEME + 50μM EPA



MEME + 250μM EPA

Figure 3.3 Oil Red O Staining in the MEME model

	MEME Control (i.e. Untreated cells)	MEME + 50μM EPA	MEME + 250μM EPA
Mean number of red pixels	28564	31526	7659*
Std. Deviation	14693	14981	7902
Std. Error	2639	2691	1419
Lower 95% CI	23174	26031	4760
Upper 95% CI	33953	37021	10558

Table 3.3 Summary of the effect of EPA on intrahepatic triglyceride accumulation as measured by the number of pixels stained red as a result of Oil Red O Staining in the MEME model.

* p=<0.001 compared with MEME control

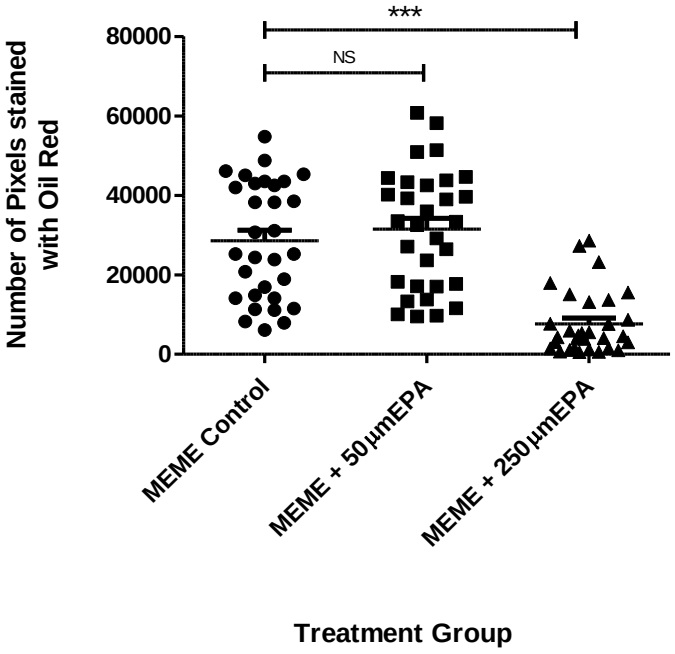


Figure 3.4 Scatterplot of the effect of EPA on intrahepatic triglyceride accumulation as measured by the number of pixels stained red as a result of Oil Red O Staining in the MEME model. Means are displayed in each group.

Experiment (b): 'Prophylaxis'

Data are summarised in Table 3.4, the results are also presented in Figure 3.5. There was a statistically significant reduction in hepatocyte triglyceride content when cells were incubated in standard media (MEME) containing 250μM EPA at both 3 ($p<0.05$) and 7 days ($p<0.05$) compared with untreated cells. These equate to 21.9% (95%CI 9-35%) and 23.1% (95%CI 5-41%) reduction in triglycerides respectively. Incubation with 50μM had no effect on hepatocyte triglyceride content. A linear trend between increasing EPA concentration and reduced hepatocyte triglyceride content was confirmed on post-test analysis in the MEME model for both day 3 ($p=0.005$) and day 7 ($p=0.006$). This effect was confirmed when correcting hepatocyte triglyceride content for DNA (Appendix 4).

	DAY 3			DAY 7		
	MEME Control (Untreated cells)	**MEME + 50µM EPA**	**MEME + 250µM EPA**	**MEME Control (Untreated cells)**	**MEME + 50µM EPA**	**MEME + 250µM EPA**
Mean Trig Concentration (mmol/gTP)	94.90	89.84	74.12*	80.94	78.92	62.23*
Std. Deviation	21.81	6.13	16.09	19.96	19.43	19.27
Std. Error	7.27	2.04	5.36	6.65	6.48	6.42
Lower 95% CI	78.13	85.13	61.75	65.60	63.99	47.42
Upper 95% CI	111.7	94.55	86.49	96.29	93.86	77.04

Table 3.4The effect of different concentrations EPA on intrahepatic triglyceride concentration in cells treated with standard media (MEME) with 3 and 7 days incubation. Each value is measured in mmol and is corrected for gram of total protein.

* p=<0.05 compared with MEME control

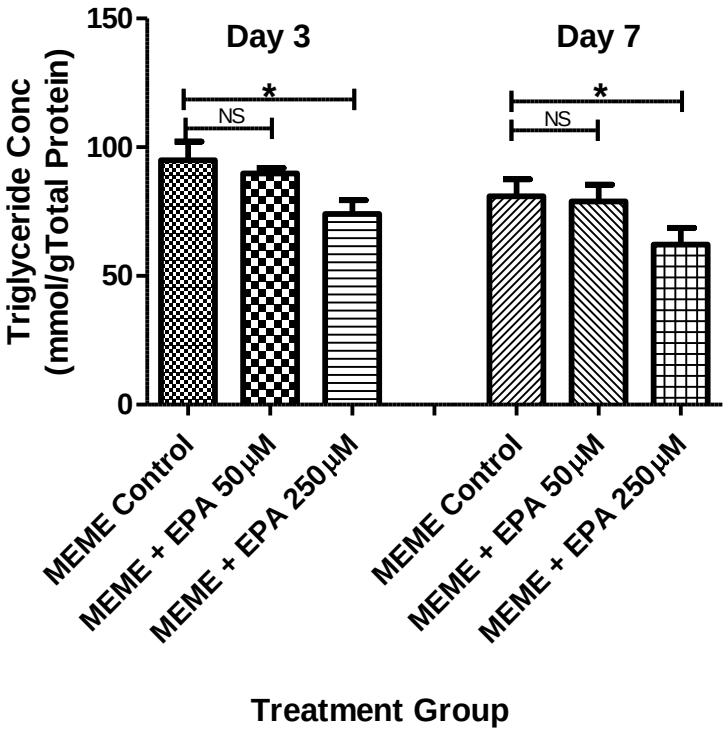


Figure 3.5 The effect of different concentrations EPA on intrahepatic triglyceride concentration in cells treated with standard media (MEME) with 3 and 7 days incubation. Each value is measured in mmol and is corrected for gram of total protein and results are expressed as mean and standard error of the mean (SEM).

Experiment (c): 'Treatment'

Data are summarised in Table 3.5 and Figure 3.6. There was a statistically significant reduction in hepatocyte triglyceride content when cells were incubated in standard media (MEME) containing 50μM EPA ($p<0.05$) and 250μM EPA ($p<0.001$). The latter equates to a 49.9% (95%CI 38-62%) reduction in hepatocyte triglyceride content. A linear trend between increasing EPA concentration and reduced triglyceride content was confirmed on post-test analysis ($p=0.0007$).

	MEME Control	MEME + 50µM EPA	MEME + 250µM EPA
Mean Trig Concentration (mmol/gTP)	97.49	64.47*	48.87**
Std. Deviation	48.78	9.30	15.28
Std. Error	16.26	3.10	5.10
Lower 95% CI	59.99	57.32	37.12
Upper 95% CI	135.0	71.61	60.62

Table 3.5 Summary of the effect of different concentrations EPA on reversing established intrahepatic triglycerides in cells treated with standard media (MEME) with 3 days incubation. Each value is measured in mmol and is corrected for gram of total protein.

* p=<0.05 compared with MEME control

** p=<0.001 compared with MEME control

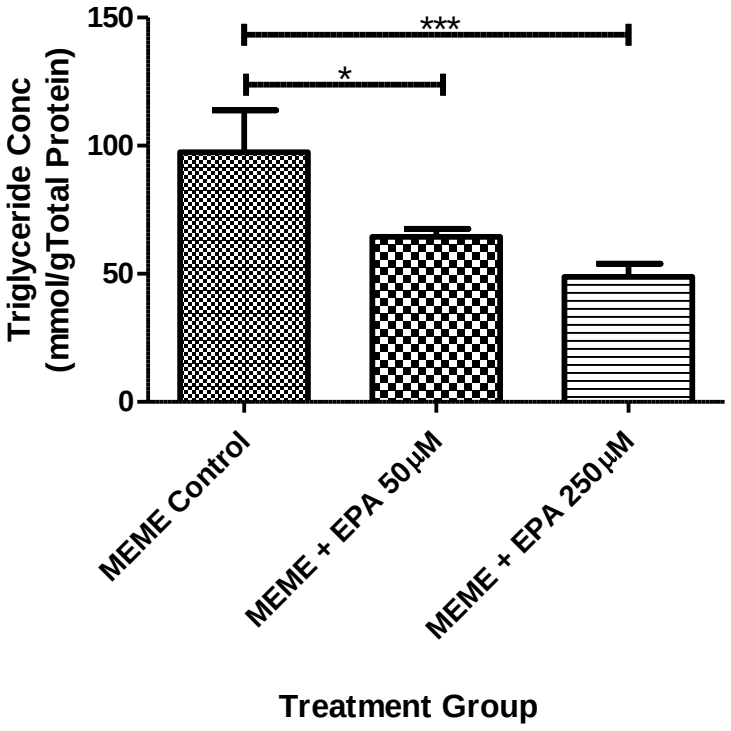


Figure 3.6 Summary of the effect of different concentrations EPA on reversing established intrahepatic triglycerides in cells treated with standard media (MEME) with 3 days incubation. Each value is measured in mmol and is corrected for gram of total protein. Results are expressed as mean and standard error of the mean (SEM).

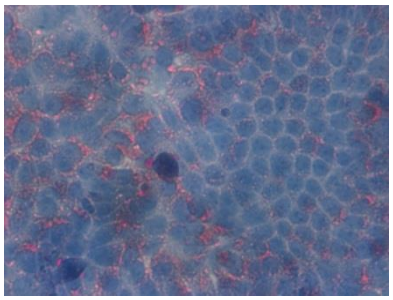
3.3.4 Hepatocytes cultured in the oleate model of cellular steatosis

Aim

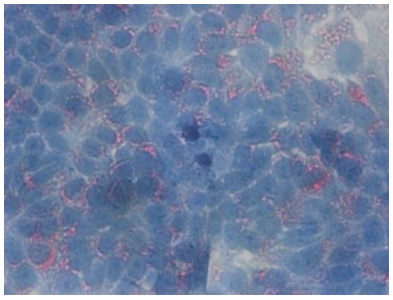
To assess the effects of EPA on the accumulation of hepatic triglycerides in C3A cells incubated with oleate in a model of simple steatosis. Here the three test media were: (i) Oleate (simple steatosis control); (ii) Oleate + EPA 50μM; or (iii) Oleate + EPA 250μM.

Experiment (a): Oil Red O Staining

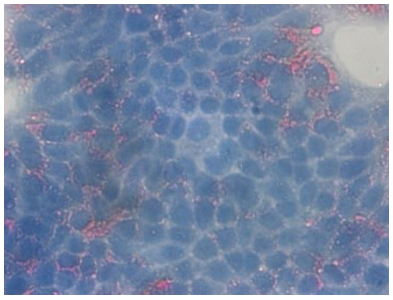
Slides are displayed in Figure 3.7. Data are summarised in Table 3.6, the results are also presented in Figure 3.8. There was a significant reduction in Oil Red O staining following incubation with both 50μM EPA ($p<0.01$) and 250μM EPA ($p<0.05$). These equate to reductions of 27.6% (95% CI 16-39%) and 22.5% (95% CI 9-36%). On post hoc analysis, there was an association between increasing EPA concentration and the amount of triglyceride as quantified by Oil red O staining ($p=0.0071$).



Oleate Control



Oleate + 50μM EPA



Oleate + 250μM EPA

Figure 3.7 Oil Red O Staining in the Oleate model

	Oleate Control	Oleate + 50μM EPA	Oleate + 250μM EPA
Mean number of red pixels	120469	87189**	93353*
Std. Deviation	28135	36210	42502
Std. Error	5137	6611	7760
Lower 95% CI	109963	73668	77483
Upper 95% CI	130975	100710	109224

Table 3.6 Summary of the effect of EPA on intrahepatic triglyceride accumulation as measured by the number of pixels stained red as a result of Oil Red O staining in the oleate model of cellular steatosis.

* p=<0.01 compared with Oleate control

** p=<0.05 compared with Oleate control

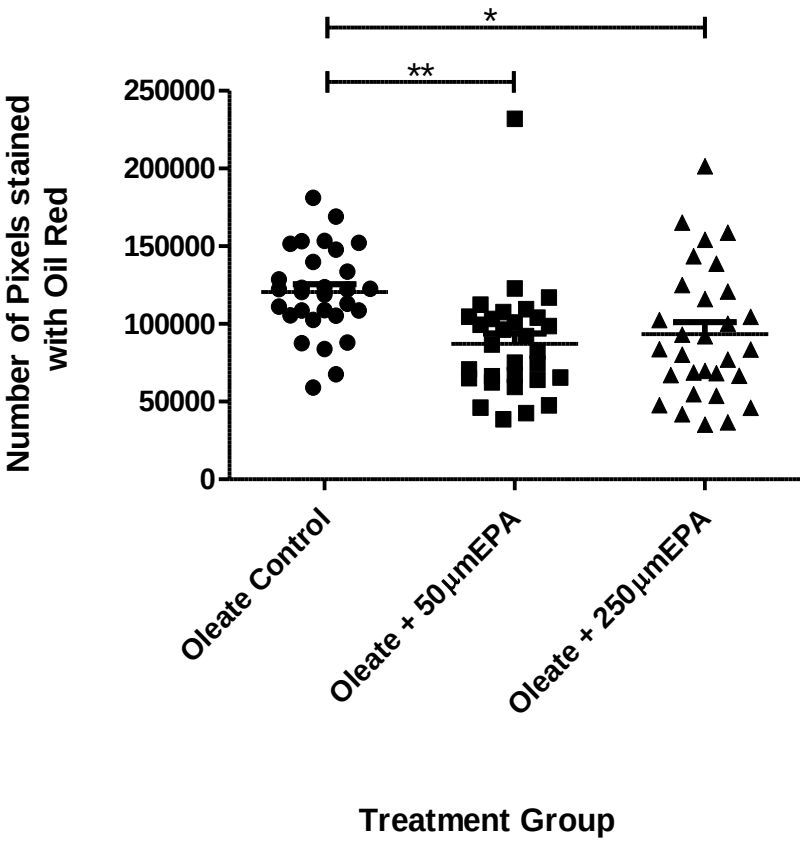


Figure. 3.8 Scatterplot of the effect of EPA on intrahepatic triglyceride accumulation as measured by the number of pixels stained as a result of Oil Red O staining in the oleate model of cellular steatosis. Means are displayed in each group.

Experiment (b): 'Prophylaxis'

Data are summarised in Table 3.7. The results are also presented in Figure 3.9. By this method, there is no statistically significant reduction in hepatocyte triglyceride content when hepatocytes are incubated in the oleate model of cellular steatosis and EPA. On post-test analysis, a linear trend between increasing EPA concentration and reduced hepatocyte triglyceride content trended to significance on day 3 ($p=0.08$), and was significant on analysis of day 7 data ($p=0.04$).

	DAY 3			DAY 7		
	Oleate Control	**Oleate + 50µM EPA**	**Oleate + 250µM EPA**	**Oleate Control**	**Oleate + 50µM EPA**	**Oleate + 250µM EPA**
Mean Trig Concentration (mmol/gTP)	108.1	104.7	91.24	216.2	204.6	189.2
Std. Deviation	19.56	26.24	29.87	43.11	24.91	36.74
Std. Error	6.52	8.75	9.96	14.37	8.30	12.25
Lower 95% CI	93.08	84.49	68.29	183.0	185.5	161.0
Upper 95% CI	123.2	124.8	114.2	249.3	223.8	217.5

Table 3.7. Summary of the effect of different concentrations EPA on intrahepatic triglyceride concentration in the oleate model of cellular steatosis with 3 and 7 days incubation. Each value is measured in mmol and is corrected for gram of total protein.

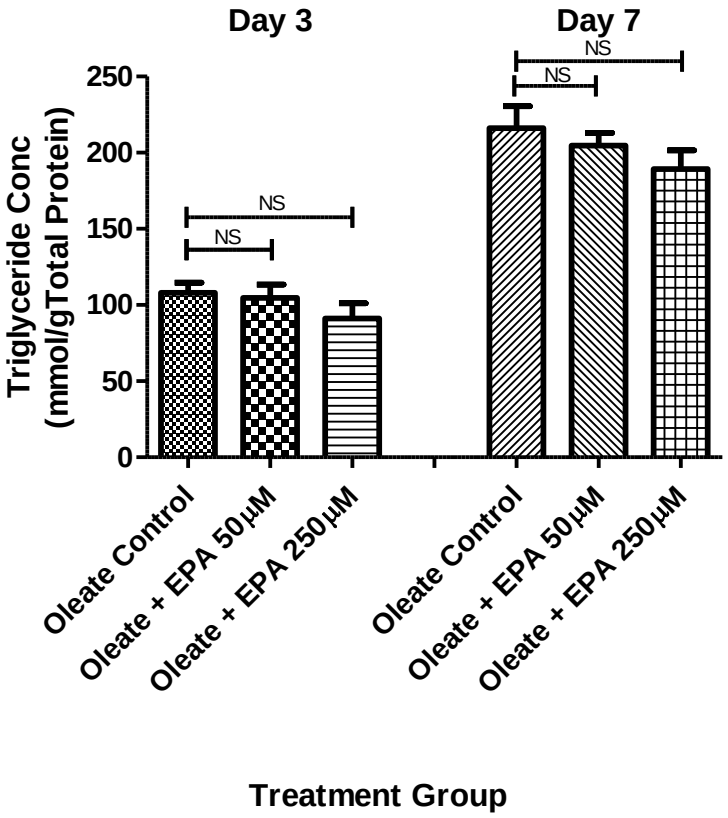


Figure 3.9The effect of EPA on hepatocyte triglyceride content in the Oleate Model of cellular steatosis with 3 and 7 days of treatment. Results are expressed as mean and standard error of the mean (SEM).

Experiment 1(c): 'Treatment'

Data are summarised in Table 3.8 and Figure 3.10. There was no significant change in hepatocyte triglyceride content when cells were incubated with oleate and EPA in this experiment. On post-test analysis there was not a trend between EPA concentration and hepatocyte triglyceride content (p=0.16).

	Oleate Control	Oleate + 50μM EPA	Oleate + 250μM EPA
Mean Trig Concentration (mmol/gTP)	176.7	169.2	162.8
Std. Deviation	23.81	23.95	22.36
Std. Error	7.94	7.98	7.45
Lower 95% CI	158.4	150.7	145.6
Upper 95% CI	195.0	187.6	180.0

Table 3.8 Summary of the effect of different concentrations EPA on established intrahepatic triglycerides in the oleate model of cellular steatosis after 3 days incubation. Each value is measured in mmol and is corrected for gram of total protein.

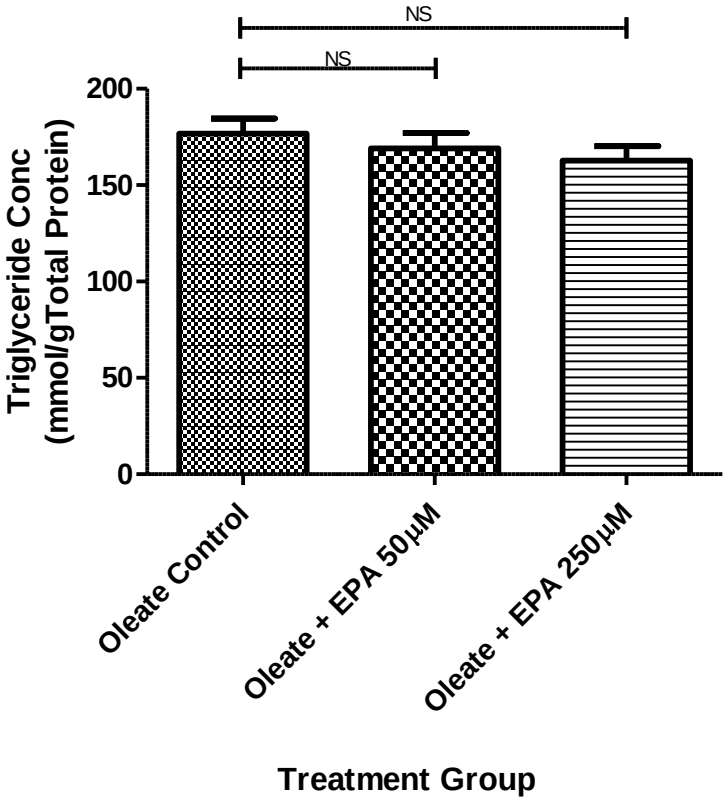


Figure 3.10 The effect of different concentrations of EPA on established intrahepatic triglycerides in the oleate model of cellular steatosis after 3 days incubation. Results are expressed as mean and standard error of the mean (SEM). There was no significant difference between groups.

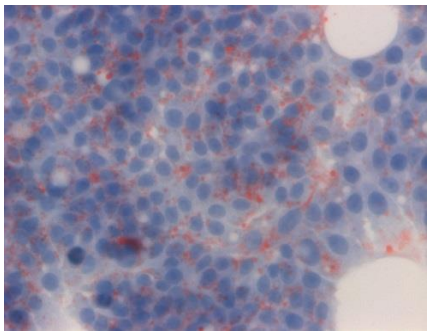
3.3.5 Hepatocytes cultured with LPON

Aim

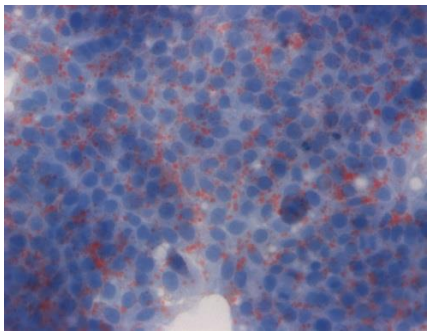
To assess the effects of EPA on the accumulation of hepatic triglycerides in C3Ahepatocytes incubated with LPON (a model of cellular steatosis with mitochondrial dysfunction). For these experiments the test media therefore were:(i) LPON; (ii) LPON + EPA 50μM; or (iii) LPON + EPA 250μM.

Experiment (a): Oil Red O Staining

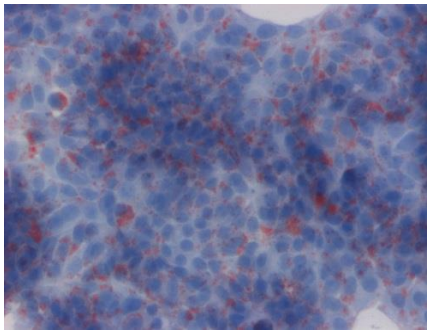
Slides are displayed in Figure 3.11. Data are summarised in Table 3.9, the results are also presented in Figure 3.12. The number of pixels stained with oil red was significantly reduced when LPON cells were incubated with 250μM EPA ($p<0.05$) but not 50μM EPA. A linear trend between EPA concentration and the number of pixels stained with oil red was significant ($p=0.027$).



LPON Control



LPON + 50μM EPA



LPON + 250μM EPA

Figure 3.11 Oil Red O Staining in the LPON model

	LPON Control	LPON + 50μM EPA	LPON + 250μM EPA
Mean number of pixels stained with oil red	79219	77096	60308*
Std. Deviation	35861	26564	20222
Std. Error	6659	4933	3755
Lower 95% CI	65578	66992	52616
Upper 95% CI	92859	87201	68000

Table 3.9 Summary of the effect of EPA on intrahepatic triglyceride accumulation as measured by the number of pixels stained red as a result of Oil Red O staining in the LPON model of cellular steatosis and mitochondrial dysfunction.

* p=<0.05 compared with LPON control

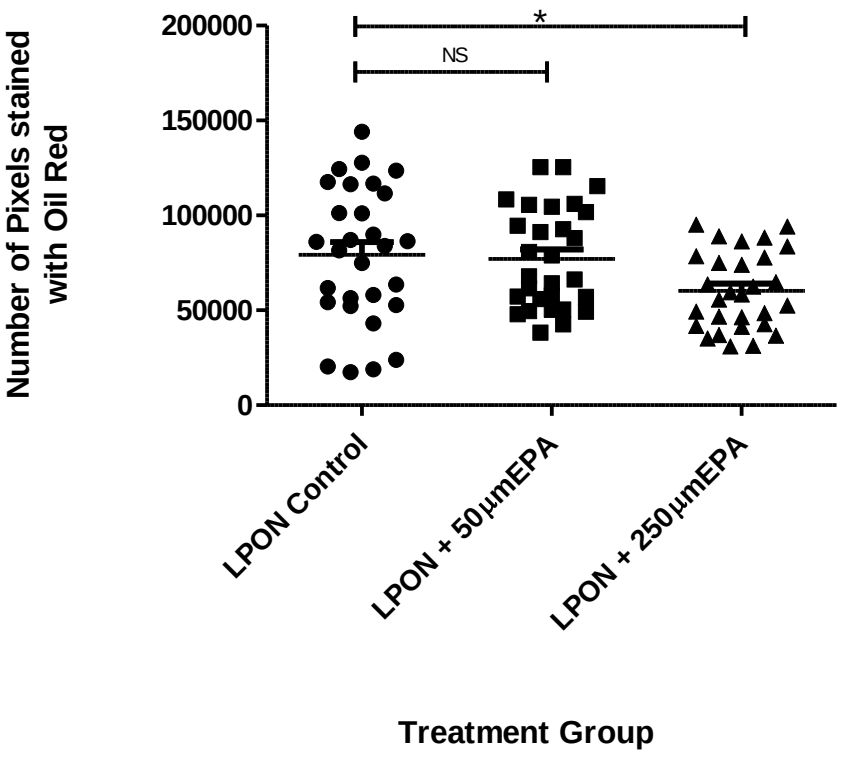


Figure 3.12 Scatterplot of the effect of EPA on intrahepatic triglyceride accumulation as measured by the number of pixels stained as a result of Oil Red O staining in the LPON model of cellular steatosis and mitochondrial dysfunction. Means are displayed in each group.

Experiment (b): 'Prophylaxis'

Data are summarised in Table 3.10 and Figure 6.13. There was no reduction in hepatocyte triglyceride content when hepatocytes are incubated with LPON and EPA either at a concentration 50μM or 250μM in this experiment. Post hoc analysis did not show a trend between EPA concentration and hepatocyte triglyceride content.

	DAY 3			DAY 7		
	LPON Control	LPON + 50µM EPA	LPON + 250µM EPA	LPON Control	LPON + 50µM EPA	LPON + 250µM EPA
Mean Trig Concentration (mmol/gTP)	255.1	236.1	245.9	262.0	274.8	270.5
Std. Deviation	22.47	25.78	25.14	114.8	102.2	90.46
Std. Error	7.49	8.59	8.38	38.26	34.06	30.15
Lower 95% CI	237.8	216.3	226.5	173.7	196.3	201.0
Upper 95% CI	272.4	256.0	265.2	350.2	353.4	340.1

Table 3.10 Summary of the effect of different concentrations EPA on intrahepatic triglyceride concentration In the LPON model of cellular steatosis and mitochondrial dysfunction with 3 and 7 days incubation. Each value is measured in mmol and is corrected for gram of total protein.

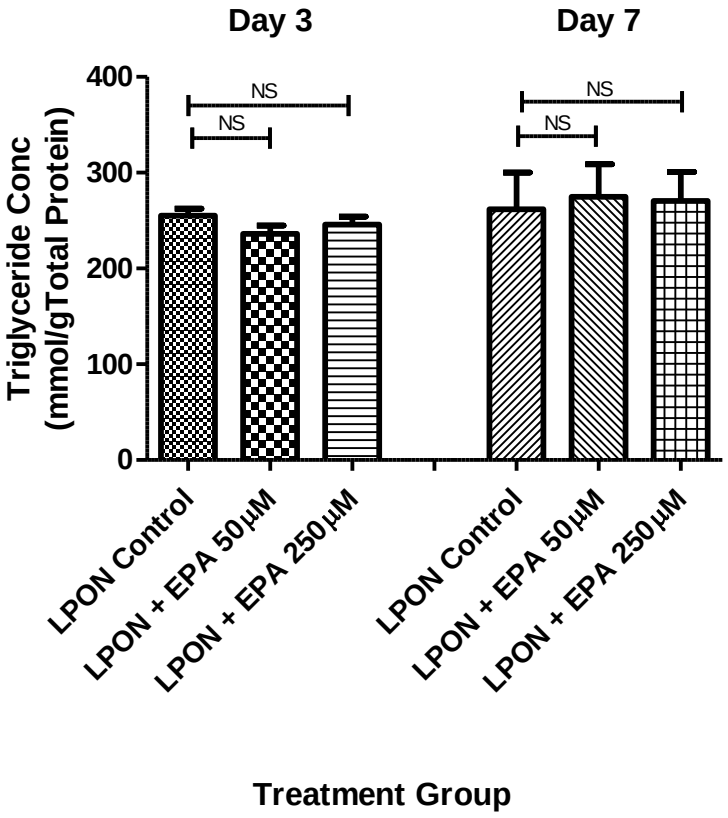


Figure 3.13The effect of EPA on hepatocyte triglyceride content in the LPON Model of cellular steatosis and mitochondrial dysfunction with 3 and 7 days of treatment. Results are expressed as mean and standard error of the mean (SEM).

Experiment (c): 'Treatment'

Data are summarised in Table 3.11 and presented in Figure 3.14. There was no significant reduction in hepatocyte triglyceride content when cells were incubated with EPA in the LPON model in this experiment.

	LPON Control	LPON + 50μM EPA	LPON + 250μM EPA
Mean Trig Concentration (mmol/gTP)	294.2	294.7	288.5
Std. Deviation	32.67	27.63	23.27
Std. Error	10.89	9.21	7.76
Lower 95% CI	269.1	273.5	270.6
Upper 95% CI	319.3	316.0	306.4

Table 3.11 Summary of the effect of different concentrations EPA on established intrahepatic triglycerides in the LPON model of cellular steatosis and mitochondrial dysfunction with 3 days incubation. Each value is measured in mmol and is corrected for gram of total protein.

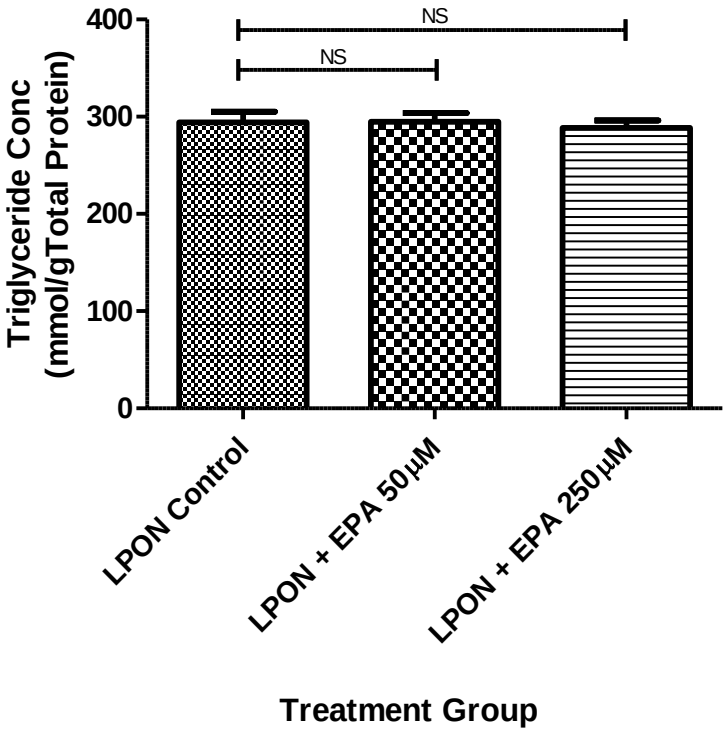


Figure 3.14 The effect of EPA on established intrahepatocyte triglyceride content in the LPON Model of cellular steatosis and mitochondrial dysfunction with 3 days of treatment. Results are expressed as mean and standard error of the mean (SEM).

3.3.6 Summary of Results

These results (summarised in Table 3.12) show that eicosapentaenoic acid (EPA) is effective at reducing intrahepatic triglyceride concentrations in untreated (non-fatty) hepatocytes but EPA is not effective at reducing hepatocyte triglyceride content in steatotic cells in either oleate or LPON models.

	Oil Red O Staining	'Prophylaxis' Experiment 3 days Incubation	'Prophylaxis' Experiment 7 day Incubation	'Treatment' Experiment
MEME (i.e. Untreated cells)				
MEME + 50μM EPA	No Change	No change	No change	Significant reduction (p<0.05)
MEME + 250μM EPA	Significant Reduction (p=<0.001)	Significant reduction (p<0.05)	Significant reduction (p<0.05)	Significant Reduction (p=<0.001)
Dose response relationship Observed?	Yes	Yes	Yes	Yes
Oleate				
Oleate + 50μM EPA	Significant Reduction (p=<0.01)	No Change	No Change	No Change
Oleate + 250μM EPA	Significant Reduction (p=<0.05)	No Change	No Change	No Change
Dose response relationship Observed?	Yes	No	Yes	No
LPON				
LPON + 50μM EPA	No Change	No Change	No Change	No Change
LPON + 250μM EPA	Significant Reduction (p=<0.05)	No Change	No Change	No Change
Dose response relationship Observed?	Yes	No	No	No

Table 3.12 Summary of results of the experiments of the effect of eicosapentaenoic acid on hepatocyte triglyceride content of C3A cells incubated in different test conditions

3.4 Experiment 3: Further investigation into the effects of eicosapentaenoic acid on C3A hepatocytes

3.4.1 Introduction

Whilst EPA is effective at reducing fat in untreated hepatocytes, it does not follow that this will help the cell in other ways. In order to be an effective treatment EPA must also reduce cellular inflammation whilst having a neutral or beneficial effect on cell function. The former may be roughly quantified by hepatic transaminases, the latter by quantifying albumin synthesis. In addition, what is the effect on ketone body production, a marker of beta oxidation?

3.4.2 Methods

The supernatants of the cells were analysed in the experiments as described in Section 3.1 for hepatic transaminases (AST and ALT) and ketone bodies (acetoacetate and betahydroxybutyrate). Albumin concentration was also calculated. N.B. Alanine transaminase (ALT) levels were too low to be quantified by the above methods and therefore only the results for aspartate transaminase (AST) are presented.

Statistical Analysis

Results are expressed as mean and standard error of the mean (SEM). Repeated measure ANOVA with Tukey post-test was used to compare groups. p values <0.05 were deemed significant.

3.4.3 Results: Hepatic transaminases

Standard (MEME) Model

There was no significant alteration in AST concentration following incubation with EPA in the MEME model. Although this just missed significance (p=0.06) and equates to a 25% reduction in AST level when incubated with 250μM (95%CI 5-45%). There was a significant linear trend between increasing EPA concentration and reduced AST levels in the supernatant (p=0.03). (Figure 3.15)

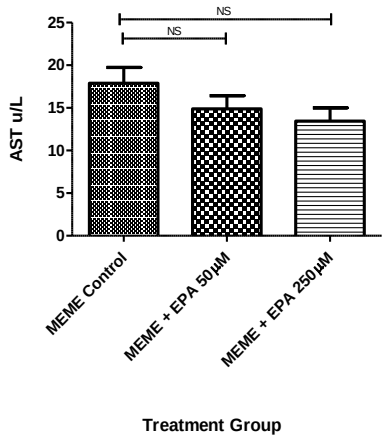


Figure 3.15 The effect of EPA on AST level in the standard (MEME) model. Results are expressed as mean and standard error of the mean (SEM).

Oleate

There was no significant change in AST concentration with incubation with EPA in the oleate model. (Figure 3.16)

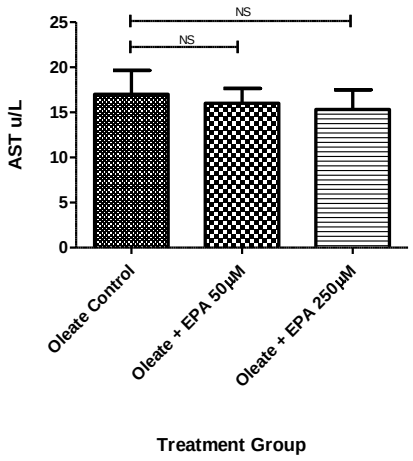


Figure 3.16 The effect of EPA on AST level in the oleate model. Results are expressed as mean and standard error of the mean (SEM).

LPON

There was no significant alteration in AST levels following incubation with EPA in the LPON model. (Figure 3.17)

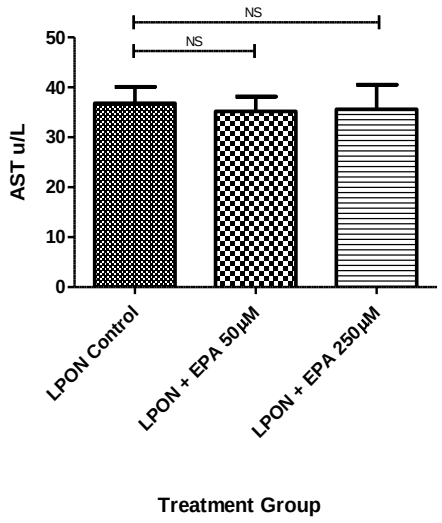


Figure 3.17 The effect of EPA on AST level in the LPON model. Results are expressed as mean and standard error of the mean (SEM).

3.4.4 Results: Albumin Synthesis

Standard (MEME) Model

There was a significant increase in albumin concentration with incubation with 250μM compared with untreated cells (p=<0.05) following incubation in the standard model (MEME) (Figure 3.18).

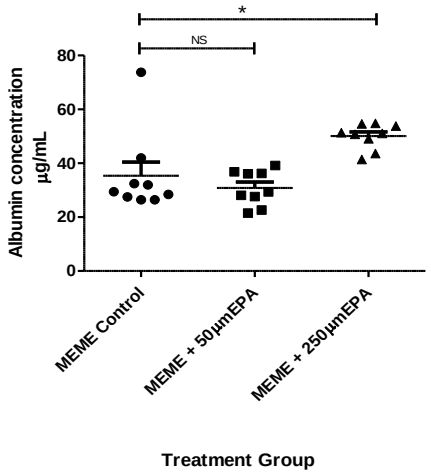


Figure 3.18 The effect of EPA on albumin level in the standard (MEME) model. Results are expressed as mean and standard error of the mean (SEM).

Oleate

Albumin concentration in the supernatant was decreased following incubation with 50μM in the oleate model of cellular steatosis. This is likely to the result of an outlier in the control cells. No change was observed in albumin concentration following incubation with 250μM EPA. These results are displayed in Figure 3.19.

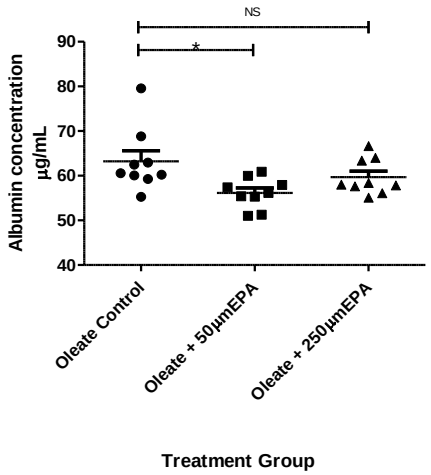


Figure 3.19 The effect of EPA on albumin levels in the oleate model. Results are expressed as mean and standard error of the mean (SEM).

LPON

There was no significant difference in albumin concentration in LPON treated cells. (Figure 3.20)

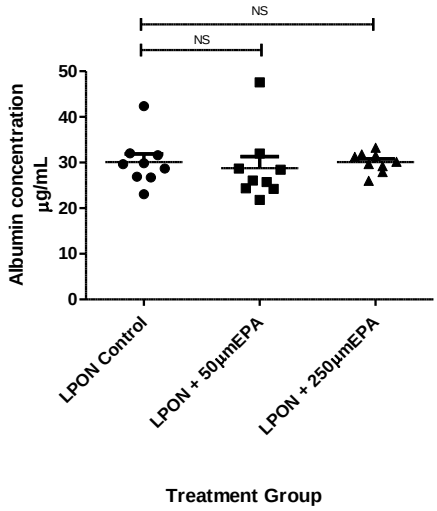


Figure 3.20 The effect of EPA on supernatant albumin levels in the LPON model. Results are expressed as mean and standard error of the mean (SEM).

3.4.5 Results: Ketone body production

Standard (MEME) Model

There was no significant change in ketone body production (measured as the sum of acetoacetate and betahydroxybutyrate) in cells incubated with EPA in the standard model. There was also no trend to efficacy with EPA treatment in this model. These data are displayed in Figure 3.21.

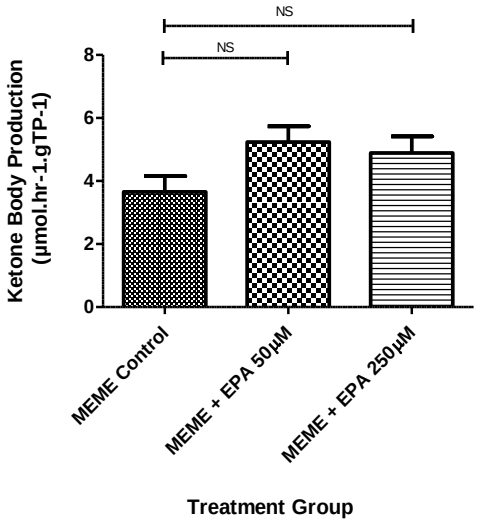


Figure 3.21 The effect of EPA on ketone body (acetoacetate + betahydroxybutyrate) production in the standard (MEME) model. Results are expressed as mean and standard error of the mean (SEM).

Oleate

There was no significant change in ketone body production (measured as the sum of acetoacetate and betahydroxybutyrate) in cells incubated with EPA in the

standard model. There was also no trend to efficacy with EPA treatment in this model. See Figure 3.22.

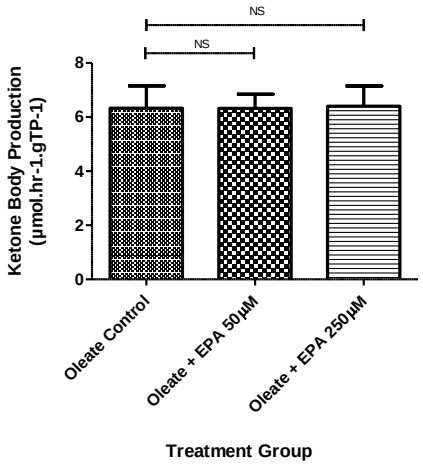


Figure 3.22 The effect of EPA on ketone body (acetoacetate + betahydroxybutyrate) production in the oleate model. Results are expressed as mean and standard error of the mean (SEM).

LPON

There was a significant reduction in the concentration of ketone bodies (measured as the sum of acetoacetate and betahydroxybutyrate) detected in the supernatant in the LPON model following incubation with both 50µM ($p<0.05$) and 250µM EPA ($p<0.01$) compared with untreated cells. This was associated with a linear trend to reduced ketone body production with increasing concentration of EPA in this model ($p=0.0027$). These data are displayed in Figure 3.23.

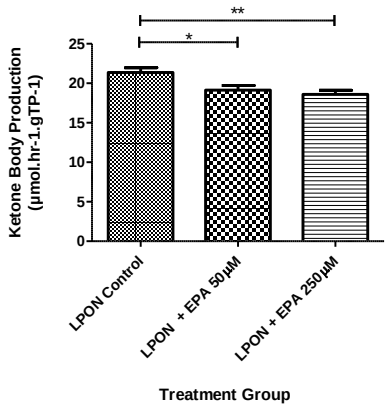


Figure 3.23 The effect of EPA on ketone body (acetoacetate + betahydroxybutyrate) production in the LPON model. Results are expressed as mean and standard error of the mean (SEM).

3.4.6 Summary of Results

These results that supernatant transaminase levels are unaffected by incubation with EPA both in the standard model and in the oleate and LPON models of cellular steatosis although there was a trend to efficacy with increasing dose in the standard model and, perhaps, with higher doses an effect may have been elicited. Increased albumin synthesis with EPA was confirmed in the standard model although no effect was seen in LPON. An observed decreased in the oleate model is likely to be spurious. Ketone body production was unchanged in the standard model and oleate model but was reduced in the LPON model.

3.5 Experiment 4: The Effect of Adding a Reactive Oxygen Species Donor

3.5.1 Introduction

Non-alcoholic steatohepatitis (NASH) is associated with increased oxidative stress and reactive oxygen species (ROS)(13). The role of reactive oxygen species in the pathogenesis of NASH is poorly understood. The LPON cell culture model is associated with increased ROS, whereas the oleate and MEME models are not(17). A possible explanation for lack of effect of EPA in the LPON model could be that the increased ROS prevent its lipid lowering effects. Therefore this experiment was designed to explore the effect of EPA on intrahepatic triglycerides when a reactive oxygen species donor is added to EPA in the standard model and the oleate model of cellular steatosis.

Tert-butylhydroxyperoxide (tBOOH) causes oxidative stress in a manner similar to that of endogenous lipid hydroperoxides *in vivo*(185, 186). A dose of 100μM has been used in cell culture experiments as a non-lethal dose of ROS donor(185). As the LPON model already contains increased ROS this was not examined in this experiment.

3.5.2 Methods

C3A cells were grown in T75 flasks as described in Methods (Section 3.1). Cells were passaged into six well plates (35mm) and grown in confluence in a standard medium (MEME). This was then replaced by the test media (MEME or oleate) with, or without, a reactive oxygen species donor (100μM tBOOH) in triplicate and incubated for 72 hours at 37^{o}C. Supernatant and cells were then harvested and LDH and triglyceride levels determined as per Methods (Section 3.1).

3.5.3 Results

Standard (MEME) Model

The data are presented in Table 3.13 and Figure 3.24. The presence of a reactive oxygen species donor did not prevent the reduction in hepatocyte triglycerides in the MEME model: both with and without a ROS donor there was a significant reduction in hepatocyte triglyceride content ($p<0.001$). Again a significant trend was seen between increasing EPA concentration and hepatocyte triglyceride content ($p <0.0001$).

Oleate

Data are shown in Table 3.14 and Figure 3.25. There was not significant change in hepatocyte triglyceride content when cells were incubated in media containing oleate, EPA and tBOOH. A trend in the oleate model between increasing EPA concentration and hepatocyte triglyceride content just failed to reach significance ($p=0.053$).

(iii) Lactate Dehydrogenase

LDH levels in the supernatant were low in both the MEME and Oleate models suggesting there was not excessive cell death as a result of tBOOH incubation.

	Without ROS Donor			With ROS Donor		
	MEME Control	**MEME + 50µM EPA**	**MEME + 250µM EPA**	**MEME Control + tBOOH**	**MEME + tBOOH + 50µM EPA**	**MEME + tBOOH + 250µM EPA**
Mean Trig Concentration (mmol/gTP)	151.8	146.7	104.7*	125.2	107.4	87.64**
Std. Deviation	18.41	20.36	7.18	36.06	26.85	23.81
Std. Error	6.14	6.79	2.39	12.02	8.95	7.94
Lower 95% CI	137.7	131.0	99.21	97.46	86.76	69.34
Upper 95% CI	166.0	162.3	110.3	152.9	128.0	105.9

Table 3.13 Summary of the effect of EPA on triglyceride Concentration in the MEME model in the presence of a reactive oxygen species donor.

* p=<0.001 compared with MEME control

** p=<0.001 compared with MEME control + tBOOH

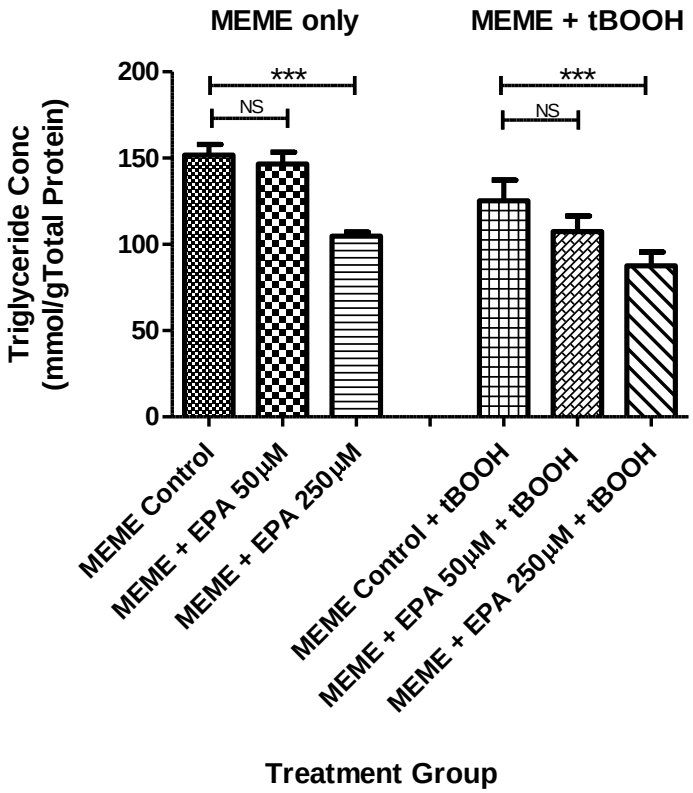


Figure 3.24 Summary of the effect of EPA on triglyceride concentration in the MEME model in the presence of a reactive oxygen species donor.

	Without ROS Donor			With ROS Donor		
	Oleate Control	**Oleate + 50μM EPA**	**Oleate + 250μM EPA**	**Oleate Control + tBOOH**	**Oleate + tBOOH + 50μM EPA**	**Oleate + tBOOH + 250μM EPA**
Mean Trig Concentration (mmol/gTP)	172.8	183.0	160.2	156.2	167.4	140.3
Std. Deviation	22.03	21.16	24.71	12.60	26.79	18.86
Std. Error	7.34	7.05	8.24	4.20	8.93	6.29
Lower 95% CI	155.9	166.7	141.2	146.5	146.8	125.8
Upper 95% CI	189.7	199.2	179.2	165.9	188.0	154.8

Table 3.14 Summary of the effect of EPA on triglyceride concentration in the oleate model in the presence of a reactive oxygen species donor.

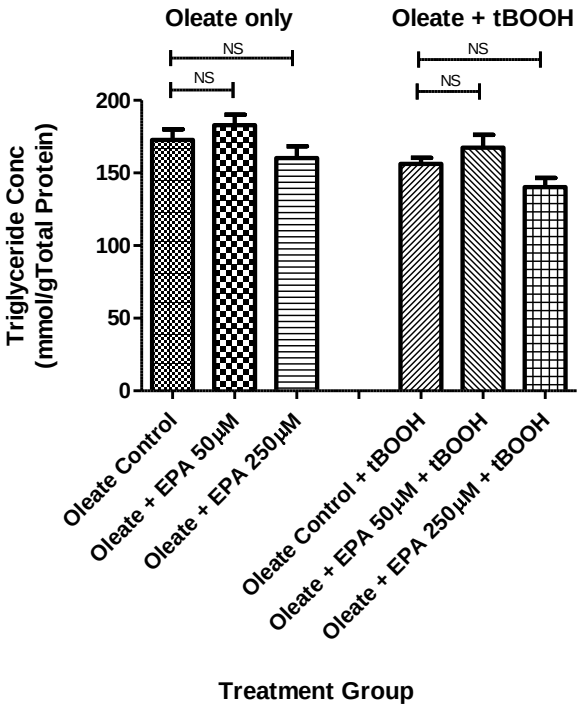


Figure3.25 Summary of the effect of EPA on triglyceride concentration in the oleate model in the presence of a reactive oxygen species donor.

3.5.4 Summary of Results

Eicosapentaenoic acid (EPA) remains effective at reducing intrahepatocyte triglyceride content in the standard model even in the presence of a reactive oxygen species donor. Cells in the oleate model had no significant change in hepatocyte triglyceride content with EPA either in the presence or absence of a reactive oxygen species donor.

3.6 Discussion and Conclusion

Eicosapentaenoic acid (EPA) was consistently effective at a dose of 250μM at reducing intrahepatic triglyceride content in the standard model ('healthy cells') when quantified using oil red, and in the prophylaxis and treatment experiments. This effect was confirmed when results were corrected for DNA content rather than total protein (Appendix 4). The reductions in hepatocyte triglyceride content were significant – between 21% and 73% in the different experiments. In addition, a linear trend between reducing hepatocyte triglyceride content and increasing EPA concentration was consistently seen, demonstrating a dose relationship.

In the oleate model there was reduced intrahepatic triglyceride content as quantified by Oil red O staining although this was not confirmed in the cell culture experiments. However, a trend to reduced hepatocyte triglycerides following 7 days incubation with EPA in the oleate model of simple steatosis was demonstrated. It is possible, therefore, that with higher concentrations of prolonged incubation an effect may be seen.

Although incubation with 250μM EPA reduced hepatocyte triglyceride content in the LPON model when quantified with Oil red O staining, this was not confirmed in cell culture experiments either in the treatment or prophylaxis protocols. On post hoc analysis no trend was demonstrated between EPA concentration and triglyceride content suggesting that this lack of effect is a genuine finding.

A further series of experiments aimed to examine the effect of EPA on other cellular functions including hepatic transaminase production, albumin synthesis and ketogenesis.

The results showed that AST was not significantly altered in any of the models examined, although a trend to reduced AST level with increasing EPA concentrations in the standard model only just missed statistical significance. Ketone body production was unchanged in the standard model and oleate model but was reduced in the LPON model. These data confirm results from the Experiment 1 (shown in Appendix3).

An increase in albumin synthesis with co-incubation with EPA was confirmed in the standard model. This would support the suggestion that EPA has beneficial effects on the cell beyond just reduced hepatocyte triglyceride content. There was no change in albumin synthesis with the addition of EPA in the LPON model and the change observed with 50μM in the oleate model is likely due to a statistical error.

This chapter also explored the effect of adding a ROS donor to the standard and oleate models with EPA. It was observed that co-incubation with a reactive oxygen species donor did not mitigate the triglyceride lowering effect seen when healthy hepatocytes were incubated with EPA. This would suggest that the ineffectiveness of EPA in the LPON model is not purely as a result of the increased ROS in this model.

Section IV

Discussion and Conclusion

4.1 Overview

This thesis comprised studies to investigate the effect of omega-3 fatty acids in non-alcoholic fatty liver disease. The first of these was a placebo-controlled, double-blind randomised trial. The second series of studies comprised cell culture experiments using different models of cellular steatosis.

In this section the results of each of these will be discussed. Possible explanation for these results will be considered and the strengths and limitations of the studies, reviewed. Firstly, however, the development of the clinical trial design and changes made in the evolution of this will be discussed.

4.2 Discussion of the Development of the Study Design

Several changes to the study design were made during its inception and have had an impact, both positive and negative, on the study. These, and the reasons behind them, shall now be reviewed.

Original study design

Initially, a pilot trial with a primary outcome measure of improvement in serum liver function tests after 6 months of omega-3 fatty acids treatment was proposed. Ultrasound appearances formed a secondary endpoint. While undoubtedly inferior to the eventual design, the aim had been to enable completion within the scheduled two year research fellowship.

Original study drug

A Norwegian pharmaceutical company, EPAX, had agreed to provide the study drug (containing 430mg DHA and 90mg EPA) and matched placebo (corn oil) free of charge. This would have been delivered to the Royal Infirmary of Edinburgh for packaging, labelling and randomisation.

Participants would have been randomised to either study drug (4 grams of omega-3 fatty acids - EPAX1050TG) or placebo (4 grams of corn oil) for six months.

Strengths of original design

The main attraction was the matching placebo. Corn oil would have reproduced the oily taste associated with omega-3 fatty acids and so would have been harder to distinguish by the participant and investigator, while the pharmacist would have been blinded too. Corn oil would also have been supplemented with Vitamin E, which is necessary to stabilize omega-3 preparations. This was theoretically useful because Vitamin E may have an independent effect on liver function.

Changes necessitated by the MHRA

Study drug

EPAX manufacture a widely available over the counter omega-3 product as a health food supplement which had been used in clinical trials in Norway and Iran (187, 188) but the product did not possess the necessary documentation (a summary of product characteristics (SmPC) or investigational medicinal product dossier (IMPD)) to be acceptable to the MHRA for its use in a clinical trial in the UK. The MHRA was unwilling to accept that the nature of the investigational product did not necessitate these requirements, so EPAX's support for the study had to be abandoned.

Solvay, which manufactures Omacor, an omega-3 drug which has a British product licence for treatment of hyperlipidaemia, was contacted. Solvay agreed to use of their product (purchased) in the study and to provide the information required by the MHRA. Funding for the trial was diverted to purchase Omacor.

Placebo

A new source for the placebo also had to be found. Solvay was the obvious choice, but it was unwilling to provide a placebo for this study because the company was not a sponsor and it was not under the aegis of its clinical trials unit. Eurocaps, a company that specialises in making placebos, was then approached and agreed to make matching placebo for the trial using olive oil. MHRA submission was made for this placebo. The MHRA required a manufacturer's authorisation from Eurocaps which it did not have. Again, this was a mandatory requirement, so another source had to be found. Tayside pharmaceuticals, which make placebos, was contacted regarding the MHRA application and a suitable placebo. The outcome was a non-matching, lactose-based capsule as the placebo but made by a company that was acceptable to the MHRA, and the MHRA determined that the study remained adequately blinded. MHRA approval was then granted.

Study Design

When the requirements and delays occasioned by the MHRA became apparent a decision was made to upgrade the study design from a pilot to a phase 3 trial. Essentially, it was agreed that all the work entailed to get the trial off the ground justified undertaking a definitive trial rather than another preliminary project. In the course of this, the study primary endpoint was redefined to power a full study based upon the publication of Capanni et al (10). Professional statisticians were involved in the redesign and subsequent result analysis.

Isotope breath test

The IRIS breath test machine is a non-radioactive, non-invasive dynamic test of hepatic function. This involves the ingestion of C^{13} labelled isotope of octanoate, a substance metabolised by hepatocyte mitochondrial beta-oxidation. Breath samples are then taken at standard time intervals. The ratio of C^{13}/C^{12} isotopes of CO_2 in the breath is calculated and compared to healthy controls. This is a safe and accepted way of measuring hepatocyte mitochondrial function.(189) Because of financial and time constraints this test was dropped from the study.

Consequences of the changes in trial design on the study

Positive Effects

These changes had several positive effects on the study design, not the least of these was upgrading from a pilot project to a definitive, robust trial. This in turn resulted in a change of primary endpoint to a direct measure of liver fat with a validated scoring system. Health-related quality of life assessment was also added to the study. Finally, Omacor contains a higher concentration of EPA than EPAX 1050TG with similar DHA concentrations and is therefore preferable as a study drug.

Negative Effects

The change of the placebo from corn oil to lactose tablets compromised the study design. The study drug and placebo were now non-matching, which meant the pharmacist had to be unblinded (as she had to count out the tablets when dispensing). Further, because of the absence of an oily taste participants could, and

investigators probably would, be able to hazard an informed guess as to whether the patient was on fish oil or placebo. However, the use of independently assessed biological endpoints made the effect of this partial unblinding most likely to be confined to the quality of life assessment, albeit there remained an outside possibility that unmatched vitamin E content might affect liver function results.

The other negative effect arose from the additional costs incurred in having to purchase study medication and placebos. The consequence was there was less money available for undertaking all the assessments originally proposed: specifically the breath test could no longer be used to give insight into the effect of omega-3 fatty acids on hepatic mitochondrial function, serum fatty acids assessment to assess compliance, repeat ultrasound at 3 months (visit 2) and a plan to undertake MRI scanning had to be shelved.

4.3 Discussion of the Results of the Clinical Trial

In 2012a meta-analysis of nine clinical trials found omega-3 fatty acids superior to placebo in reducing hepatic steatosis as graded on ultrasound and the transaminase AST(156). This thesis contains the first phase 3 randomised placebo controlled trial powered to a change in liver fat as graded on ultrasound as the primary endpoint. No benefit of omega-3 fatty acids over placebo in reducing steatosis as graded on ultrasound, serum liver function tests or health-related quality of life scores was found. This trial was negative and was in keeping with the lack of efficacy of omega-3 in NAFLD demonstrated in the two similar trials published recently which used liver histology as their primary endpoint.(164, 165) Interestingly, recent meta-analyses have similarly questioned the efficacy of omega-3 fatty acids in cardiovascular trials.(35, 36)although others continue to find a beneficial effect.(34)

However, the rationale for using omega-3 fatty acids in NAFLD is well founded.(30) There are promising data in both human and animal studies.(139, 147, 152, 157-159)Could this result be false negative? In order to answer this question the study design and results will be reviewed.

Let us first consider who took part in the study. This was a single-centre study. Patients who attended a gastroenterology clinic at the Royal Infirmary of Edinburgh were invited to take part. Patients were considered for inclusion if they had a clinical diagnosis of NAFLD made by a consultant gastroenterologist – a biopsy was not considered necessary. This mirrors clinical practice as biopsy is not routinely used

to confirm the diagnosis of NAFLD. Alcohol intake was, by necessity, self-reported and was confirmed at the time of randomisation. Indeed, one patient who attended for the first visit was excluded due to alcohol intake above that permitted by the trial. Subjects were thought to not be cirrhotic and this was confirmed clinically and radiologically prior to inclusion in the study. Confounding conditions were screened for in line with previous studies. The study population, therefore, was felt to be as representative as possible of the patients seen in clinical practice.

Those who consented to the study were randomised to either omega-3 fatty acids (Omacor) or placebo (lactose tablets). The participants of these two groups were well matched at baseline for gender, serum liver function tests, serum lipid levels and weight. Participants in the placebo arm had larger waist circumference and higher diastolic blood pressure at baseline compared with those in the omega-3 arm of the study but this is not likely to be of clinical significance. The omega-3 and placebo arms were also well matched at baseline in relation to hepatic steatosis as graded on ultrasound. 75% of participants in this study had Grade 1 (mild) steatosis. This is in contrast to omega-3 arm of the pilot trial by Capanni et al where 19% had Grade 1 steatosis, 45% Grade 2 and 36% Grade 3.(157)It must be considered, therefore, whether the skewed nature of our study population to mild grades of steatosis explains the apparent lack of efficacy. However, in the trial by Capanni et al 63%of subjects with Grade 1 steatosis had complete resolution of their steatosis at the end of the trial period. Omega-3 fatty acids were therefore effective even at low grades of steatosis. Therefore we would still expect an effect to be seen.

Participants within the treatment arm were shown to have gained weight over the six months of the study in excess of those in the placebo arm:0.9Kg (Q1 -0.8; Q3 2.9) gained vs 0.9Kg (Q1 -2.5; Q3 0) lost respectively. Could this confound the results? It is recognised that weight loss is associated with improvement of liver histology in patients with NAFLD.(42) Although in that study, by Huang et al, the mean weight loss was in 2.9Kg and thus in excess of the changes in weight seen here. It is possible, therefore, that although the change in weight between placebo and omega-3 arms was statistically significant it may not be clinically significant. It should also be noted that another marker of obesity, namely waist:hip ratio, remained unchanged between placebo and omega-3 arms throughout the course of the study.

Let us now consider the study design. Was the study adequately powered? Both the investigator (Dr G Masterton) and a qualified statistician (Dr S Lewis) performed a power calculation based upon the data of Capanni et al(157)(although using slightly more conservative figures than found it that study) and to achieve significance level 0.05; Power 0.8 each found that 50 patients in total (25 in each arm) were required. This accepted a 5% dropout rate. Unfortunately more study participants withdrew than had been anticipated with 41 patients reaching the primary endpoint. More patients withdrew from the placebo group (n=6) than omega-3 fatty acid group (n=2) and although in excess than had been anticipated the number of withdrawals were less than the 25% seen in a recent trial of omega-3 in NASH.(164) As a result of these withdrawals the study was technically underpowered. However, although a type 2 error is possible as a result of this, similar primary endpoint outcome of the omega-3 arm to the placebo arm would suggest that the addition of more subjects would be unlikely to change the outcome of the study.

Next we shall consider what impact the choice of study medication might have had. Omacor, comprising460mg eicosapentaenoic acid (EPA) and 380mg docosahexaenoic acid (DHA) per gram, was selected as the omega-3 supplement for this trial as it was able to fulfil the MHRA application requirements. Omacor is licensed in the British National Formulary (BNF) and the dose selected for use in the trial was the maximum licensed for use in the UK. Although in the midrange of the doses used in previous published clinical trials of omega-3 in NAFLD, and in excess of those used by Capanni et al, it is possible that this dose was too low to observe an effect. This may account for the lack of reduction in serum triglycerides was seen in the omega-3 group in this trial.

The effect of a non-matching placebo and active drug must also be acknowledged. This was a result of the requirements of the MRHA and was approved by them. Despite this the researchers, radiologist and participants remained blinded as only pharmacists were exposed to both tablets. In theory this should therefore be unlikely to have acted as a confounder upon the results.

Patient compliance is available for around half of patients on placebo and three quarters of patients in the omega-3 arm. Both have adequate compliance: 94% and 80% in the omega-3 arms and placebo arms respectively. These data however are incomplete and we cannot reject the hypothesis that participants in the placebo arm realised that they were not receiving the active drug and may not have been blind to their study arm. The lactose tablets were hard capsules, rather than soft, and would by necessity lack a ‘fishy taste’. The latter had been reported as a side-effect by a participant in the omega-3 group. This should not be the case with the participants in

the omega-3 arm of the trial – they were not exposed to the placebo tablets and therefore should have remained blinded to which treatment they received.

Some issues and flaws in the running of the study only became apparent as the clinical trial unfolded – initially there was a delay in receiving the ultrasound reports and this led to a subject being randomised who did not have steatosis on their baseline ultrasound (although steatosis had been present on a previous ultrasound). Following the withdrawal of this subject the running of the study was changed to ensure that the result was available prior to randomisation. Similarly it was recognised that return of documentation given to subjects between study visits was inconsistently returned (WHOQOL-Bref). The study was altered to ask patients to complete the WHOQOL-Bref during the study visit wherever possible.

The clinical trial also contained several strengths in its design. The study was appropriately powered at the outset based on the data of a published pilot trial. The trial was independent of industry or pharmaceutical backing. The study groups were well matched at baseline. All of the ultrasounds in the trial were reviewed by a single radiologist and the primary endpoint of the study was objective. Only two researchers (Dr G Masterton and Dr A Shams) were involved in the day to day running of the trial ensuring uniformity of approach. Moreover the outcome of the trial is clear. There is no trend towards a positive result that was just failed to reach the level of statistical significance – it was clearly negative.

In conclusion, in this study omega-3 fatty acids were not effective at reducing hepatic steatosis as graded on ultrasound. These findings are in keeping with two

recently published, well designed placebo controlled clinical trials in patients with biopsy confirmed NASH(164, 165). Taken together, whilst a beneficial effect of omega-3 fatty acids independent of effects on hepatic fat content or serum liver function tests cannot be excluded, omega-3 fatty acids as a treatment for NAFLD cannot be recommended on these results.

4.4 Discussion of the Results of the Cell Culture Experiments

In parallel to the clinical trial in adults with NAFLD outlined above a series of cell culture experiments examined the effect of the omega-3 fatty acid eicosapentaenoic acid (EPA) *in vitro*. C3A hepatocytes (well differentiated hepatoblastoma cells) were incubated in three different media to explore these effects. The first was standard media; here hepatocytes would mimic the 'healthy liver'. The second, oleate, is an established model in cell culture experiments which demonstrates increased fat accumulation without oxidative stress. The clinical equivalent here is 'simple steatosis'.(17) The third model, termed LPON, was designed at the University of Edinburgh and has been shown to more closely mimic the metabolic milieu of non-alcoholic steatohepatitis with increased steatosis, impaired mitochondrial function and increased oxidative stress.(17)

The first experiments were designed to establish the optimal concentration of eicosapentaenoic acid (EPA) to use in these experiments. Two markers were evaluated: LDH leakage was used to assess toxicity and cell death, and glucose production to reflect efficacy. The results showed that EPA was effective at altering endogenous glucose production in the LPON model but that large, i.e. 500μM, doses of EPA resulted in a significant increase in cell death. With these results 50μM and 250μM doses were chosen as they represented a balance of desirable and undesirable effects as well as allowing a dose-response relationship to be demonstrated.

The next experiments looked at the effect of these two different doses of EPA on the lipid content of cells incubated with standard media, oleate and LPON. Lipid content was assessed both by quantification of Oil red O staining, and hepatocyte triglyceride content (corrected for both protein and DNA content). These studies found that when C3A hepatocytes were incubated in standard media EPA was consistently effective at a dose of 250μM at reducing hepatocyte triglyceride content. This effect was confirmed with Oil red O staining and on quantification of hepatocyte DNA content. Furthermore the reductions in triglyceride content were significant – equating to 21-73% in the different experiments. In addition, a dose relationship was reliably observed with a linear trend between reducing hepatocyte triglyceride content and increasing EPA concentration supporting the suggestion of the efficacy of EPA on these cells is genuine.

In contrast, in the oleate model hepatic triglyceride content was reduced when hepatocytes were incubated in EPA and oleate when quantified by Oil red O staining but not when triglyceride content was quantified by more reliable methods on cell lysates. However, in both the Oil red O experiment a linear trend to reduced hepatocyte triglycerides following incubation with increasing concentrations of EPA.

LPON with the addition of 250μM EPA showed a reduced hepatocyte triglyceride content when quantified with Oil Red O staining, but, similar to the oleate model, this was not confirmed in cell culture experiments either in the treatment or prophylaxis protocols and here no trend was demonstrated between EPA concentration and triglyceride content. Therefore we can conclude that eicosapentaenoic acid does not affect hepatocyte triglyceride content in the LPON model.

To summarise these results: although effective in normal cells when cells were modelled to be steatotic no reduction in triglyceride content was seen with EPA treatment. This result is significant as, after all, these are the models of the patients in the liver clinic with NAFLD.

Several additional experiments were designed to explore and explain these results. First considered was whether these results could be explained by LPON related inhibition on cell growth as this is known to be affected.(190) No difference was observed in total protein levels between cells incubated in standard media, oleate and LPON suggesting that this is not the case. These data are presented in Appendix5.

Could the negative results in the oleate and LPON groups be related to time? In order to clarify this cells were incubated for 7 days with no change in the results although these was now a trend to efficacy in the oleate model. It is possible therefore, that prolonged incubations at higher doses of EPA an effect may have been seen in the oleate model although of course toxicity would also need to be assessed.

It was also considered whether the lack of response in the LPON group could be a result of the increased reactive oxygen species seen in this model. Cells were therefore co-incubated with a non-lethal dose of a reactive oxygen species donor (tBOOH). The effect of this was explored in cells in standard media and in the oleate

steatosis model. EPA continued to have an effect in cells in the standard media but there was still no significant change in the oleate group when incubated with EPA and tBOOH. Co-incubation with a reactive oxygen species (ROS) donor did not, therefore, mitigate the triglyceride lowering effect seen when untreated hepatocytes were incubated with EPA. This suggests that the ineffectiveness of EPA in the LPON model to lower triglyceride content is not purely as a result of the increased ROS in this model.

How might these results be explained? EPA is thought to mediate PPAR-α oxidative pathways(89, 95, 96, 98). Free fatty acids are oxidised to acetyl coA and then further metabolised by the tricarboxylic acid (TCA) cycle or, as happens in times of substrate excess, undergoes *de novo* lipogenesis. Both oleate and LPON are models of substrate excess and therefore it is possible that the addition of the polyunsaturated fatty acid eicosapentaenoic acid further overwhelms the TCA cycle and results in enhanced *de novo lipogenesis*. It is also recognised the EPA is incorporated in cellular membranes (and possibly lipid droplet membranes) the effect of this in models of steatosis and increased oxidative stress are unclear. Also unknown is the effect on reactive oxygen species (ROS) production. It is now recognised that omega-3 fatty acids may increase ROS production by activation of PPARα mediated oxidative pathways including ω-oxidation and peroxisomal β-oxidation.(148, 149) The omega-3 fatty acids DHA has been shown to increase lipid peroxidation.(191)Assessing ROS production was outwith the scope of these studies.

Interestingly, one study examined the effect of EPA in a murine model of mitochondrial dysfunction and impaired mitochondrial β-oxidation and demonstrated increased hepatic triglyceride accumulation.(150)This, perhaps, helps explain the results of the LPON studies and that the beneficial effects of EPA require intact mitochondrial function although these results may further refine this by clarifying that it is not just increased ROS *per se* which would account for this.

4.5 Conclusion

Unfortunately, despite a robust theoretical rationale and promising data from preliminary studies in both animals and humans this phase 3 randomised clinical trial exploring the effectiveness of omega-3 fatty acids in non-alcoholic fatty liver disease was negative. Importantly it was not just a lack of efficacy in reducing hepatic steatosis, there was also no effect on serum liver function tests or health-related quality of life scores.

The data from the cell culture studies may give some insight into these results. In these studies omega-3 fatty acids (EPA) were effective at reducing fat in hepatocytes incubated in standard media but was ineffective in models of fat loading, one of which also includes increased reactive oxygen species (ROS). Interestingly, the addition of a reactive oxygen species donor did not ameliorate the triglyceride lowering effects of EPA seen in untreated cells. Future work could further explore tease out these results. Work including lipidomics and assessment of hepatic mitochondrial function and ROS synthesis would be of particular interest.

Several studies registered on clinical trial registers also examining the effectiveness of omega-3 fatty acids in adults with non-alcoholic fatty liver disease are ongoing. Based on these results further clinical trials cannot be recommended. Meanwhile an effective treatment for non-alcoholic fatty liver disease remains elusive.

Section V

Appendices

Appendix S1. Power Calculation (1)

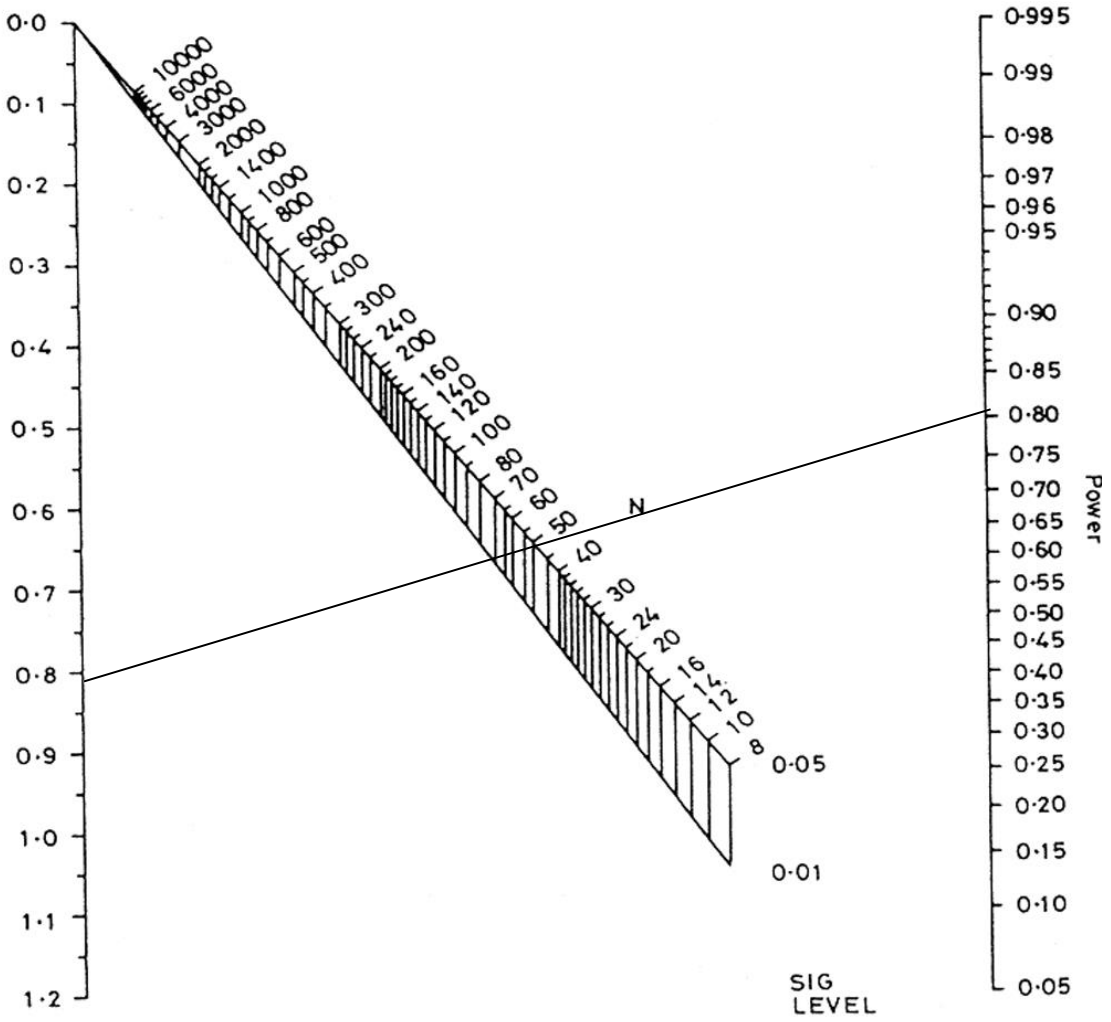


Figure S1.1 Gore Altman Nomogram(192) **with Sample Size Calculation.**

Based upon the figures in Capanni et al(157) and to achieve significance level 0.05; Power 0.8:

Standardised difference (SD) = p1-p2/ √p (1-p)

Where p1= improvement in treatment group i.e. 0.5

p2 = improvement in placebo group 0.1

(Placebo showed no improvement in Capanni et al(157) however given value 0.1 to allow a margin of error/ drop out)

p = (p1 + p2) /2 0.55

Therefore: SD = p1- p2/ √p (1-p)

= 0.5 –0.1/ √0.55 (0.45)

= 0.4/0.497

= 0.805

When entered into the nomogram this gives a sample size of 50 (i.e. 25 in each group)

Dr G Masterton

January 2009

Appendix S2. Power Calculation (2)

Sample size estimate

Name of trial: The effect of omega-3 fatty acids on non-alcoholic fatty liver disease

Chief Investigator: Prof PC Hayes, Dr Gail Masterton

CI Contact details: Dr GSM Masterton, Clinical Research Fellow, Department of Hepatology, Edinburgh Royal Infirmary

Trial design: 2 arm parallel group, treatment = omega-3 capsules, placebo = corn oil capsules

Primary outcome: Liver function on ultrasound.

Likely proportion and causes of missing outcome data: unknown

Power: 80%

Details: Using SAS Proc power, Fishers exact test option.

Group 1 proportion = 0.5, Group 2 proportion = 0.1

(Effect more conservative than that found in Capanni et al, Alimentary Pharmacology and Therapeutics 2006;23:1143-51 which was 0.64 vs 0)

Number of patients per arm = 24

Allowing for 5% drop out = **25 per group**

Statistician: Steff Lewis

Stats contact details: Public Health Sciences; University of Edinburgh Medical School; Teviot Place, Edinburgh. Email: steff.lewis@ed.ac.uk

Date sample size estimate run: 2 February 2009

Appendix S3. Ketone Body Production

Method

Cells were grown and pre-treated and the supernatant analysed for beta hydroxybutyrate (BOH) and acetoacetate (AA) as described in Methods page (Section 3.1)

Results

On post hoc analysis there were statistically significant differences in ketone body production in all cells incubated with EPA compared to LPON Control. On post hoc analysis, there was a significant trend between increasing EPA concentration and ketone body production ($p < 0.0001$).

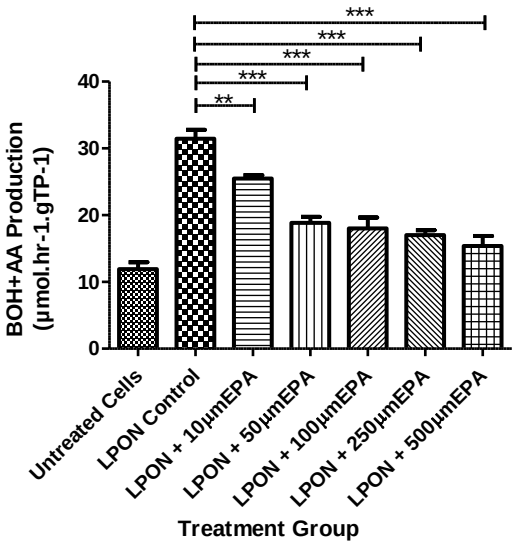


Figure S3.1 The effect of increasing concentrations of EPA on ketone body production in the LPON model.

Results are expressed as mean and standard error of the mean (SEM).

Appendix S4. DNA content

Aim

To confirm the observed effects of EPA on hepatocyte triglyceride content when corrected for DNA rather than total protein count.

Methods

Cells were grown, pre-treated for 7 days and harvested as described in Section 3.1. DNA was quantified as described in Methods when incubated in the (i) Standard (MEME); (ii) Oleate and (iii) LPON Models.

Results

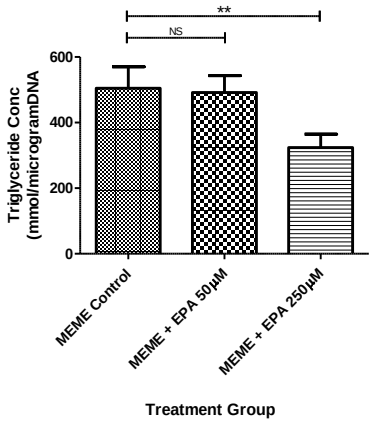
Standard (MEME) Model

A significant reduction in hepatocyte triglyceride content was confirmed with incubation with 250μM EPA ($p < 0.01$). On post hoc analysis there was a significant dose response relationship ($p = 0.0009$). Results are displayed in Table S4.1 and Figure S4.1

	MEME Control	MEME + 50µM EPA	MEME + 250µM EPA
Mean Trig Concentration (mmol/ngDNA)	504.8	491.6	323.6*
Std. Deviation	196.2	155.4	123.4
Std. Error	65.39	51.79	41.12
Lower 95% CI	354.0	372.2	228.8
Upper 95% CI	655.6	611.1	418.4

TableS4.1 The effect of EPA on hepatocyte triglyceride content in the MEME Model corrected for DNA.

* p=<0.01 compared with MEME control



FigureS4.1 The effect of EPA on hepatocyte triglyceride content in the MEME Model corrected for DNA.

Results are expressed as mean and standard error of the mean (SEM).

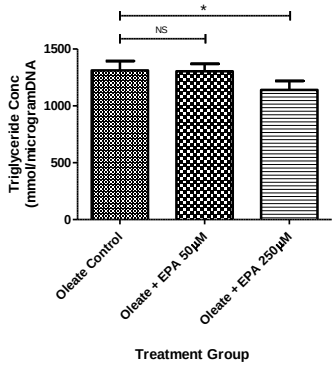
Oleate

A significant reduction in hepatocyte triglyceride content was confirmed with incubation with 250μM EPA ($p<0.05$). On post hoc analysis there was a significant dose response relationship ($p= 0.01$). These results are displayed in Table S4.2 and Figure S4.2.

	Oleate Control	Oleate + 50μM EPA	Oleate + 250μM EPA
Mean Trig Concentration (mmol/μgDNA)	1312	1305	1141*
Std. Deviation	248.6	193.1	236.2
Std. Error	82.85	64.38	78.72
Lower 95% CI	1121	1156	959.6
Upper 95% CI	1503	1453	1323

TableS4.2 The effect of EPA on hepatocyte triglyceride content in the Oleate Model corrected for DNA.

* p=<0.05 compared with MEME control



FigureS4.2 The effect of EPA on hepatocyte triglyceride content in the Oleate Model corrected for DNA.

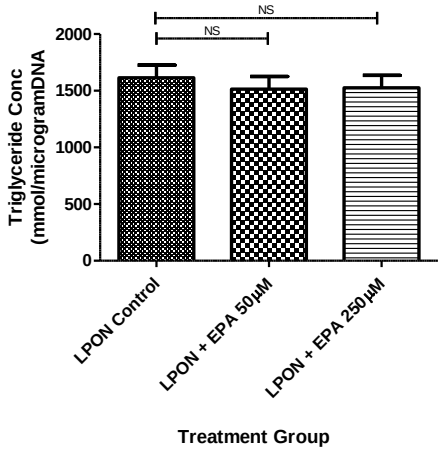
Results are expressed as mean and standard error of the mean (SEM).

LPON

There was no significant difference in hepatocyte triglyceride content following incubation with either 50μM EPA or 250μM EPA. On post hoc analysis there was not a significant dose response relationship. These data are displayed in Table S4.3 and Figure S4.3.

	LPON Control	LPON + 50μM EPA	LPON + 250μM EPA
Mean Trig Concentration (mmol/μgDNA)	1614	1513	1526
Std. Deviation	338.2	336.7	327.9
Std. Error	112.7	112.2	109.3
Lower 95% CI	1354	1255	1274
Upper 95% CI	1874	1772	1778

TableS4.3 The effect of EPA on hepatocyte triglyceride content in the LPON Model corrected for DNA.



FigureS4.3The effect of EPA on hepatocyte triglyceride content in the LPON Model corrected for DNA.

Results are expressed as mean and standard error of the mean (SEM).

Appendix S5. Total Protein

Background and Aim

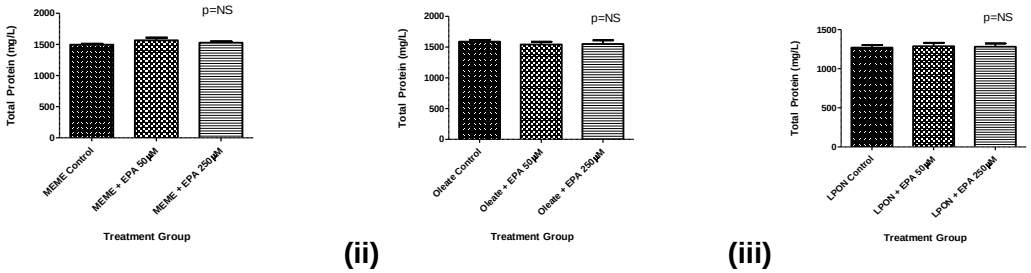
A previous study suggested that there was a significant reduction in cell growth, as quantified on total protein content, following incubation with LPON.(190) Total Protein content was therefore compared using ANOVA to ensure that this did not confound the results.

Methods

Cells were grown and treated as described in Section 3.1. Total protein was quantified as described in methods.

Results

No difference in total protein content was observed with incubation with 50μM and 250μM EPA in either the MEME, Oleate or LPON models.



(i) **(ii)** **(iii)**

Graph S4.1. The effect of EPA on hepatocyte total protein content in (i) the MEME Model (ii) Oleate (iii) LPON

Results are expressed as mean and standard error of the mean (SEM).

Published Paper

Review article: omega-3 fatty acids - a promising novel therapy for non-alcoholic fatty liver disease.

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