Marine omega-3 fatty acid supplementation in non-alcoholic fatty liver disease: Plasma proteomics in the randomized WELCOME* trial

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a liver condition characterised by liver fat accumulation and often considered to be the liver manifestation of metabolic syndrome. The aim of this study was to examine in patients with NAFLD the system-wide effects of treatment with docosahexaenoic acid + eicosapentaenoic acid (DHA+EPA) versus placebo on the plasma proteome.

Methods: Plasma from patients that participated in a 15 to 18 months randomised, double-blind placebo-controlled trial testing the effects of 4 g DHA+EPA daily was analysed using depletion-free quantitative proteomics.

Results: Bioinformatics interpretation of the proteomic analysis showed that DHA+EPA treatment affected pathways involving blood coagulation, immune/inflammatory response and cholesterol metabolism ($p<0.05$). Two key proteins of cardiovascular risk, prothrombin and apolipoprotein B-100, were shown to decrease as a result of DHA+EPA supplementation [Prothrombin: Males DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.13 (0.20) p = 0.05, Females DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.48 (0.35) p = 0.03; Apo B-100: Males DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.24 (0.16) p = 0.01, Females DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.15 (0.05) p = 0.02].

Conclusions: Plasma proteomics applied in a randomised, placebo-controlled trial showed that high dose DHA+EPA treatment in patients with NAFLD affects multiple pathways involved in chronic non-communicable diseases.

WELCOME* = **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD (non-alcoholic fatty liver disease) with **O**Macor th**E**rapy

Keywords: plasma proteomics, omega-3, DHA, EPA, NAFLD, cardiometabolic

Running title: Plasma proteomics of DHA+EPA administration in NAFLD

Abbreviations: NAFLD (non-alcoholic fatty liver disease); CVD (cardiovascular disease); EPA (eicosapentaenoic acid); DHA (docosahexaenoic acid).

Introduction

Non-alcoholic fatty liver disease (NAFLD), a liver condition characterised by liver fat accumulation ≥5%, is often considered to be the liver manifestation of metabolic syndrome and is strongly associated with obesity, type 2 diabetes mellitus and cardiovascular disease (CVD) [1]. We have recently shown that after treatment with high dose (3.36 g/day) marine omega-3 fatty acids [eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)] for 15-18 months in patients with NAFLD, serum fasting triglyceride levels decreased, and that increased tissue enrichment with DHA was associated with decreased liver fat, suggesting that high-dose DHA+EPA treatment may have a favourable effect on cardiovascular risk in patients with NAFLD [2].

Global plasma proteomic analysis can provide unbiased insight into the systemic effects of an intervention. The plasma proteomic profile of patients with NAFLD administered with marine omega-3 fatty acids within the context of a randomised placebo-controlled trial (RCT) has not been studied to date.

Therefore, the aim of the present study was to use a non-targeted quantitative plasma proteomics approach to determine the system-wide effects of high-dose DHA+EPA supplementation in patients with NAFLD who participated in a 15-18 month randomized control trial.

Materials and Methods

Recruitment of participants and intervention

The WELCOME study intervention protocol and inclusion/exclusion criteria have been described in detail previously [3]. This clinical trial was registered at ClinicalTrials.gov (www.clinicalTrials.gov registration number NCT00760513). The study received ethical approval from the Southampton and South West Hampshire local research ethics committee (08/H0502/165). All participants signed informed consent forms and underwent an

assessment of liver fat percentage by magnetic resonance spectroscopy (MRS) at recruitment, to establish the baseline liver fat percentage at entry into the trial, and at follow-up. Briefly, three 20 × 20 × 20 mm^3 spectroscopic volumes of interest (VOI) were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margin of the liver. For the second visit scan, these VOI positions were copied from the first scan, to ensure consistency [2, 3]. Briefly, the inclusion criteria for participation in the study were age > 18 years and: 1) a recent (<3 years) histological diagnosis of non-alcoholic steatosis or steatohepatitis in keeping with NAFLD; or 2) steatosis diagnosed by ultrasound, CT or magnetic resonance imaging in a patient who also had either diabetes and/or features of the metabolic syndrome. All participants underwent an assessment of liver fat percentage by MRS examination at recruitment, to establish the baseline liver fat percentage at entry into the trial. Exclusion criteria included known other causes of liver disease (e.g. hepatitis A, B or C, primary biliary cirrhosis, Wilson's disease, autoimmune hepatitis and haemochromatosis). These conditions were excluded with blood tests. Subjects were also excluded if alcohol consumption was >35 units per week for women and >50 units per week for men. At recruitment, only one man was consuming >21 units of alcohol per week and one woman was consuming >14 units per week. Additional exclusion criteria were: decompensated acute or chronic liver disease; cirrhosis; pregnancy or breast feeding; and hypersensitivity to Omacor, soya or any of the excipients. One-hundred and three participants with NAFLD (sex M/F: 60/43) were randomised to Omacor (DHA+EPA) or placebo (olive oil). Fifty-one (sex M/F: 25/26) participants received 3.36 g daily of DHA+EPA (1 g of Omacor contains 460 mg of EPA and 380 mg of DHA as ethyl esters) and 52 participants (sex M/F: 35/17) received 4g of olive oil. At the end of the study, 95 participants completed the intervention period (DHA+EPA group n=47, sex M/F: 24/23; Placebo group n=48, sex M/F: 32/16). Fasting blood samples at baseline and after the end of the intervention (15-18 months duration) were collected.

Plasma procurement and proteomic analysis

Two multiplex experiments were performed for men and women participants respectively, to correct for potential baseline sex-specific plasma proteome differences and sex-dependent effects of the omega-3 intervention. Only plasma from patients who completed the intervention was used for the proteomic analysis (n=95; DHA+EPA group n=47; Placebo group n=48). Individual 50 µL aliquots from male participants in the DHA+EPA and placebo groups were randomly pooled using the randomization function in Microsoft Excel (version 15.11.1) at baseline and after the end of the intervention to form two biological replicates per time-point and treatment group [Males Baseline DHA+EPA 1 (n=12), Males Baseline DHA+EPA 2 (n=12), Males Baseline Placebo 1 (n=16), Males Baseline Placebo 2 (n=16), Males End-of-Study DHA+EPA 1 (n=12), Males End-of-Study DHA+EPA 2 (n=12), Males End-of-Study Placebo 1 (n=16), Males End-of-Study Placebo 2 (n=16)]. The same pooling scheme was applied for female participants [Females Baseline DHA+EPA 1 (n=11), Females Baseline DHA+EPA 2 (n=12), Females Baseline Placebo 1 (n=8), Females Baseline Placebo 2 (n=8), Females End-of-Study DHA+EPA 1 (n=12), Females End-of-Study DHA+EPA 2 (n=11), Females End-of-Study Placebo 1 (n=8), Females End-of-Study Placebo 2 (n=8)]. Unprocessed plasma was subjected to depletion-free proteomic analysis as reported elsewhere [4].

Database searching and statistics

Unprocessed raw files were submitted to Proteome Discoverer 1.4 for target decoy searching against the SwissProt homo sapiens database (v2015-11-11) as reported previously[4]. Proteins reported were analysed with a peptide level FDR $p < 0.05$. All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD003760.

A two-tailed unpaired T-Test was used to identify proteins that changed (end-of-study vs. baseline) differentially between the DHA+EPA and placebo groups of each sex. A value for $p \leq 0.05$ was considered significant. According to the Paris Publication Guidelines (http://www.mcponline.org/site/misc/ParisReport_Final.xhtml), only proteins identified with at least two unique peptides were considered.

Bioinformatics analysis

Principal component analysis using the log_2ratios of the fully quantified proteins in each experiment (males and females respectively) was performed using the online tool ClustVis (http://biit.cs.ut.ee/clustvis/). MetaCore (GeneGo, St. Joseph, MI, USA) and Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany) were applied to identify biological processes and protein networks significantly enriched in the plasma proteins altered as a result of the omega-3 fatty acid intervention. In all analyses, a false discovery rate (FDR) corrected p-value < 0.05 was considered significant.

Results

The participants' baseline characteristics have been reported previously [3] and are also presented in **Supplementary Table 1**. A total of 1,699 and 2,084 proteins were fully quantified in the multiplex experiment of the male and female cohorts respectively. Principal component analysis using the reporter ion log_2ratios of all profiled proteins showed that the DHA+EPA group clustered separately from the placebo group for both male and female cohorts, indicating that change (end-of-study vs. baseline) in plasma proteins was different between DHA+EPA and placebo groups (**Figure 1A**). In the male and female DHA+EPA group compared to placebo, 221 and 213 proteins respectively were significantly altered at the end-of-study vs. baseline (**Supplementary Tables 2** and **3** respectively for male and female cohorts).

Process Network Analysis using MetaCore showed that blood coagulation, immune response and inflammatory response were significantly enriched networks in the plasma

proteins of both sexes that were altered as a result of the omega-3 intervention (**Figure 1B**). Eleven proteins were analysed with the same trend of differential expression following DHA+EPA supplementation in the male and female cohorts (**Figure 1C**). Of the eleven proteins that were found to be affected by DHA+EPA supplementation in a sex-independent manner, two nodal proteins participating in key pathways influencing vascular disease were prothrombin and apolipoprotein B-100, affecting blood coagulation and cholesterol transport from the liver to the tissue respectively. Levels of prothrombin and apolipoprotein B-100 were found to decrease [Prothrombin: Males DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.13 (0.20) p = 0.05, Females DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.48 (0.35) p = 0.03; Apo B-100: Males DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.24 (0.16) p = 0.01, Females DHA+EPA Mean iTRAQ log_2ratio (SD) = -0.15 (0.05) p = 0.02] as a result of the omega-3 supplementation compared to placebo. Ingenuity Pathway Analysis showed that "molecular transport, lipid metabolism and small molecule biochemistry" was a significantly enriched network in the plasma proteins affected by the omega-3 intervention in males and females (score = 30, focus molecules = 18 in males; score = 42, focus molecules = 23 in females) (**Figure 1D**).

Discussion

This systems biology plasma proteomics study in patients with NAFLD provides novel insight into the effects of marine omega-3 fatty acid supplementation on plasma proteins related to CVD and other non-communicable diseases. The study results show that proteins involved in blood coagulation, inflammatory/immune responses and lipid metabolism were significantly altered in the male and female groups following omega-3 supplementation. Interestingly, levels of prothrombin and apolipoprotein B-100 were found to decrease as a result of the omega-3 intervention (**Figure 1C**).

Studies have shown that marine omega-3 fatty acids reduce hypercoagulability, a major risk factor for vascular disease, without increasing the risk of bleeding [5]. Our results show that the blood coagulation pathway was significantly enriched in the plasma proteins

altered as a result of the omega-3 fatty acid supplementation (**Figure 1B**). Prothrombin, a nodal protein in the blood coagulation pathway, has been reported as a surrogate marker of cardiovascular risk [6] whereas reduction in plasma prothrombin levels has been associated with decreased risk of arterial and venous thrombosis [7].

In vitro experiments and animal studies support an anti-inflammatory and immunomodulatory role for omega-3 fatty acids [8]. However, evidence from human randomized control trials is more equivocal. Our study results show that proteins involved in immune and inflammatory responses are affected by omega-3 supplementation (**Figure 1B**).

Our results show that DHA+EPA treatment decreases concentrations of apo-B100. Marine omega-3 fatty acid supplementation is effective in decreasing very-low density lipoprotein (VLDL) concentrations [9]. Apo B-100 is a major apolipoprotein of the VLDL particles and apoB-100 concentration is a stronger cardiovascular disease risk factor, compared to total cholesterol, LDL-c and VLDL-c levels [10].

The strengths of this study include its RCT design, its long duration and robust sample size and the application of a global, untargeted plasma proteomics methodology. One potential limitation is the sample pooling strategy used, which did not permit the assessment of the anticipated inter-individual heterogeneity in protein expression levels. Although our approach may have limited the sensitivity of the methodology to find differences in treatment effects between individuals, a strength of our approach is that we are able to provide summary effects of DHA+EPA treatment for patients with NAFLD and consequently, any differences found are likely to be large and therefore potentially more clinically relevant.

Conclusions

Plasma proteomics applied in a randomised, placebo-controlled trial, lasting between 15 and 18 months, showed that high dose DHA+EPA treatment in patients with NAFLD affects multiple pathways involved in chronic non-communicable diseases.

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Figure legend

Figure 1. **A**. Principal Component Analysis using the reporter ion log_2ratios of all analysed proteins showed that DHA+EPA treatment had a distinct effect on the plasma proteomic profile compared to placebo in both male and female cohorts. **B**. Process Network Analysis using MetaCore showed that blood coagulation, immune response and inflammatory response were significantly enriched processes in the plasma proteins that were altered as a result of the omega-3 intervention. **C**. Plasma proteins that were analysed to be altered at the end of study vs. baseline as a result of the omega-3 intervention. **D**. Ingenuity Pathway Analysis showed that “Lipid Metabolism, Small Molecule Biochemistry” was significantly enriched in the plasma proteins altered as a result of the omega-3 intervention in both sexes.

Supplementary Table legends

Supplementary Table 1. Baseline variables in placebo and DHA+EPA groups at randomisation

Supplementary Table 2. Proteins significantly altered between the DHA+EPA and placebo groups of the male cohort (T-Test P <0 .05).

Supplementary Table 3. Proteins significantly altered between the DHA+EPA and placebo groups of the female cohort (T-Test P <0 .05).