Short Communication

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

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SUMMARY

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—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.

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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...
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 < 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 < 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 ratios 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 1699 and 2084 proteins were fully quantified in the multiplex experiment of the male and female cohorts respectively. Principal component analysis using the reporter ion log ratios 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 (Fig. 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 (Fig. 1B).
Eleven proteins were analysed with the same trend of differential expression following DHA + EPA supplementation in the male and female cohorts (Fig. 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 log2ratio (SD) = −0.13 (0.20) \( p = 0.05 \), Females DHA + EPA Mean iTRAQ log2ratio (SD) = −0.48 (0.35) \( p = 0.03 \); Apo B-100: Males DHA + EPA Mean iTRAQ log2ratio (SD) = −0.24 (0.16) \( p = 0.01 \), Females DHA + EPA Mean iTRAQ log2ratio (SD) = −0.15 (0.05) \( p = 0.02 \)] as a result of the omega-3 fatty acid 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) (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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

![A. Principal Component Analysis](https://example.com/A.png)

![B. Process Network Analysis](https://example.com/B.png)

![C. Plasma proteins that were analysed to be altered at the end of study vs. baseline as a result of the omega-3 intervention.](https://example.com/C.png)

![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.](https://example.com/D.png)
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.

Conflicts of interest

The authors have no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.07.037.

References