Lipidomic profiling of human adipose tissue identifies a pattern of lipids associated with fish oil supplementation

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Abstract

To understand the interaction between diet and health, biomarkers that accurately reflect consumption of foods of perceived health relevance are needed. The aim of this investigation was to use direct infusion-mass spectrometry (DI-MS) lipidomics to determine the effects of fish oil supplementation on lipid profiles of human adipose tissue. Adipose tissue samples from an n-3 polyunsaturated fatty acid (PUFA) supplementation study (n = 66) were analysed to compare the pattern following supplementation equivalent to 0 or 4 portions of oily fish per week. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were incorporated into highly unsaturated (≥ 5 double bonds) triglycerides (TGs), phosphocholines and phosphoethanolamines, as well as being detected directly as the non-esterified fatty acid forms. Multivariate statistics demonstrated that phospholipids were the most accurate and sensitive lipids for the assessing EPA and DHA incorporation into adipose tissue. Potential confounding factors (adiposity, age and sex of the subject) were also considered in the analysis, and adiposity was also associated with an increase in highly unsaturated TGs as a result of incorporation of the n-6 PUFA arachidonic acid. DI-MS provides a high throughput analysis of fatty acid status that can monitor oily fish consumption, suitable for use in cohort studies.

INTRODUCTION

The health benefits of oily fish in the diet and their component long-chain n-3 polyunsaturated fatty acids (PUFAs) have been of interest since the early 1970s when it was observed that native Inuits consuming their traditional diet providing high intakes of long chain n-3 PUFAs had high blood levels of those fatty acids and lower than expected mortality from cardiovascular disease [1, 2]. Within the wider population, higher habitual consumption of fish (oily fish particularly) and long-chain n-3 PUFAs such as docosahexaenoic acid (DHA, C22:6-n3) and eicosapentaenoic acid (EPA, C20:5-n3) has been linked to a decreased risk of cardiovascular disease (CVD) [3, 4], some cancers including colon cancer [5, 6], asthma [7], inflammatory conditions [8] and neurodegenerative diseases such as Alzheimer's disease [9, 10]. However, health outcomes in randomized controlled trials of n-3 PUFA supplementation have been inconsistent [11, 12] raising the question whether observational studies relying on methods such as food frequency questionnaires have adequately assessed consumption of n-3 PUFAs.

Current UK dietary guidelines recommend a minimum consumption of two portions of fish per week, one of which should be oily [13]. However, the average UK fish consumption is estimated to be less than half a portion per week [14]. Increasingly, there is a need for objective and accurate markers of short and long term dietary intake of n-3 PUFAs. These might include the relative levels of n-3 PUFAs in the phospholipid fraction of blood plasma or in red blood cells and in the n-3 PUFA content in adipose tissue as markers of short (plasma phospholipids) and long term (red blood cells; adipose tissue) dietary intakes, respectively. Fatty acids derived from the diet undergo several processes including β-oxidation, conversion to other fatty acids and signalling molecules, storage in the adipose tissue as triacylglycerols (TGs) and incorporation into cell membranes as components of phospholipids. Ingested fatty acids are transported in the bloodstream as TGs in chylomicrons and stored in the adipose tissue rather than being used as an energy source directly. Thus, the fatty acid composition of the adipose tissue is in part determined by that of the diet [15, 16]. However, the composition may also be affected by the type of adipose tissue depot [17], biosynthesis of fatty acids

within the adipose tissue, *de novo* lipogenesis, hormonal effects, age [18, 19], sex [17, 19, 20] and systemic fatty acid metabolism.

Lipidomics has been defined as "the full characterisation of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation" [21]. As such, lipidomics has focused on the identification of perturbations in lipid metabolism and lipid-mediated signalling processes that regulate cellular homeostasis in health and in disease states [22]. The field of lipidomics has been greatly enhanced by advances in mass spectrometry. Direct infusion mass spectrometry (DI-MS) (also referred as shotgun) lipidomics allows the high throughput analysis of a lipidome directly from organic extracts of a crude biological matrix [23]. Lipidomics has been shown to be a powerful investigative tool for the study of lipid profiles in pathophysiological states such as diabetes [24, 25] and Alzheimer's disease [25, 26] as well as being used in studies of the effects of dietary supplementation with long-chain n-3 PUFAs [27].

Since lipidomics will typically generate a large amount of data, visualisation of such data has often employed chemometric methods including principal components analysis (PCA), hierarchical clustering analysis (HCA), partial least squares projection to latent structures (PLS) and orthogonal PLS (OPLS). OPLS is a powerful chemometric tool for investigation of -omic data particularly in clinical studies where there are many, sometimes confounding, sources of variation [28]. Applying OPLS results in reduced model complexity whilst maintaining the model's predictive capability [29] and potentially improving the visualisation and discrimination of potential biomarkers [30]. The assessment of the sensitivity and specificity of potential biomarkers for a given physiological or pathophysiological state can be achieved by use of receiver operator characteristic (ROC) curves to summarise the impact of a single biomarker [31] or of multiple biomarkers [32]. In the present study, we have assessed the use of a lipidomic approach combined with ROC curve and logistic regression analysis [33] to determine the multiple lipid patterns in adipose tissue associated with supplemental n-3 PUFA consumption and the nature of confounding effects such as natural variation associated with percentage body fat, sex and age of subject. We present a lipidomic method which yields better objective markers for long term habitual n-3 PUFA intake than gas chromatography (GC)-based

analysis of total fatty acid content in adipose tissue for a fraction of the cost and analytical time per sample.

EXPERIMENTAL PROCEDURES

Study design and sample details

A subset of human adipose tissue samples (n = 68) from a study examining fatty acid responses to 12 months EPA and DHA supplementation, in doses equivalent to 0 to 4 portions of oily fish per week, was analysed [34]. Participants were given supplements containing the equivalent EPA and DHA to a portion of oily fish on 0-4 random days each week, with placebo tablets containing high oleic sunflower oil on intervening days. Samples collected at three timepoints (0 months (baseline), 6 and 12 months) were analysed. Full details of the original study may be found elsewhere [34].

The original study was conducted in line with the Declaration of Helsinki, approved by the Suffolk Local Research Ethics Committee (approval 05/Q0102/181) and was registered at www.controlledtrials.com (Trial Registration: ISRCTN4839526). Written informed consent was obtained from all participants. Metadata from the original study were included in the analyses including age, sex, BMI, waist circumference, percentage body fat and individual fatty acid measurements (as percentage of the total fatty acids (%TFA) in the adipose tissue) determined by GC analysis of the total fatty acid content using methyl esterification in the original study. The full details of the lipid extraction, derivatisation of fatty acids to form methyl esters and GC analysis have been described previously [34]. For this study, the following samples were used: 0 month, control = 23, 6 months, control = 16, 12 months, control = 9, 6 months, fish oil = 15, 12 months, fish oil = 5. All subjects who received fish oil capsules received the equivalent of 4 portions per week. For the analyses, no time differences within the control samples considered as a group or the fish oil supplement samples considered as a group were found to be more significant than the other effects described in the paper (data not shown) and so the groups were considered as control (n=48) and fish oil (n=20) (**Supplementary Table S1** for key characteristics of the subjects).

Preparation of adipose tissue lipid extracts for DIMS analysis

Adipose tissue samples were prepared for DI-MS analysis using a modified Folch extraction method [35]. 10 mg of adipose tissue was homogenised in 400 μL of deionised water using a Qiagen Tissue

Lyser II (Qiagen, GmbH D-40724, Hilden) at 30 Hz for 30 s. The homogenised tissue was transferred to a 5 mL culture tube containing 1 mL chloroform/methanol (2:1 v/v) and mixed thoroughly for 20-30 s. The sample was centrifuged at 3000 g for 10 min and 500 µL of the lower organic layer was retained. A second extraction was performed as described above and 800 µL of the lower organic layer was retained and combined with that from the first extraction. The combined organic layer was dried under a stream of nitrogen and reconstituted in 300 µL of chloroform/methanol (1:1, v/v). 150 µL of internal standard mixture in HPLC grade methanol (containing 5 µM 1,2-di-O-octadecyl-sn-glycero-3-phosphocholine (**DOC**), 10 µM 1,2-di-O-phytanyl-sn-glycero-3-phosphoethanolamine (**DPG**), 5 µM C8-ceramide (**C8**), 5 µM N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (**NHD**), 50 µM undecanoic Acid (**DEC**), 5 µM trilaurin (**TRI**) and 10 µM β-sitosterol acetate (**BSA**)) was diluted 1:1 (v/v) with chloroform and added to each lipid extract. 25 µL of each extract was added to the appropriate well on a glass-coated 96-well plate and 90 µL of 7.5 mM ammonium acetate in isopropanol: methanol (2:1, v/v) was added to each well. The plate also contained eight samples each of pooled samples (10 µL of each lipid extract combined to form the pool samples), quality controls (human (male) serum, Sera Laboratories International Ltd, UK) and internal standard blanks. Samples were placed on the plate in a randomised but balanced pattern for treatment group and sex descriptors.

Direct Infusion Mass Spectrometry (DI-MS) analysis of human adipose tissue

An open-profiling DI-MS approach was performed on the lipid extracts using chip-based nanoelectrospray with an Advion TriVersa NanoMate® (Advion, Ltd., Harlow, UK) interfaced to a Thermo Exactive Orbitrap (Thermo Scientific, Hemel Hempstead, UK) [36]. From the plate, 5 µl was aspirated by the NanoMate using a fresh tip, followed by 1.5 µl of air to prevent blockage of the tip opening. The tip was then placed against the chip on a new nozzle, the sample was infused using 1.2 psi of N_2, and a stable ionization spray was obtained using 1.2 kV. After 20 s of infusion the NanoMate started the acquisition mode of the Exactive in positive mode. After 1.2 min of acquisition, the system switched to negative mode and spectra acquired for a further 1.1 min. A mass acquisition

window ranging from m/z = 200 to 2000, with an acquisition rate of 1 Hz was used in the sample analysis. Data processing was performed using the XCMS platform [37] within R (RStudio (version 2.15.2, RStudio Inc., The R Foundation for Statistical Computing, 2012) to perform peak detection and alignment of the ions. The raw data were converted to the .mzXML format using MSConvert (ProteoWizard, http://proteowizard.sourceforge.net/news.shtml) [38] and the script performed peak picking in the m/z domain using the area under curve at full-width-half-maximum (FWHM) using a pre-determined list of target lipids and target m/z values for positive (m/z = 200-1200 for a retention window of 20-60 s) and negative (m/z = 180-1200 for a retention window of 80-120 s) modes separately. Lipid masses were assigned using data from LIPID MAPS [39] and the Human Metabolome Database (HMDB) [40].

LCMS/MS experiments for the determination of the chain composition of selected intact triglycerides

LCMS/MS experiments were used to determine the fatty acid composition of selected intact TGs in fifteen adipose tissue extracts. A 10 µL aliquot of the lipid extracts described above was diluted with 100 µL of 2:1:1 (v/v/v) of HPLC-grade isopropanol/acetonitrile/water. The instrumentation comprised of a Thermo Scientific Accela Autosampler coupled to a Thermo Scientific Elite™ Iontrap-Orbitrap Hybrid mass spectrometer with a heated electrospray ionisation (HESI) source (Thermo Scientific, Hemel Hempstead Hertfordshire, UK). Separation of the TG lipid species was achieved by injecting 5 µL of each sample onto a Waters Acquity BEH (C18, 50 x 2.1 mm, 1.7 µm) column (Waters Ltd., Elstree, Hertfordshire, UK) maintained at 55°C. Mobile phase A consisted of 10 mM ammonium formate in acetonitrile:water (6:4), whilst mobile phase B contained 10 mM ammonium formate in isopropanol:acetonitrile (9:1). The concentration of mobile phase B was increased from 40 to 99% in 8 min and then equilibrated at 40% for 1.5 min with a flow rate of 0.5 mL/min. The HESI source was operated in positive ion mode with the source temperature maintained at 375°C, the desolvation gas temperature was 380 °C and the gas flow rate was set at 40 arbitary units. Mass spectrometric data were collected in the range m/z = 100–2000. The data were processed in Xcalibur™ software

(Version Thermo Scientific, Hemel Hempstead, Hertfordshire, UK) for each pre-selected m/z value subtracting each fragment mass from the parent ion (see supplementary data).

Multivariate Data Analysis

Multivariate data analysis (MVDA) of the DI-MS data/metadata matrix was performed in SIMCA (version 14, Umetrics AB, Umeå, Sweden). The positive and negative mode DI-MS data (X-matrix) were considered separately and were initially analysed using PCA and HCA to investigate the inherent variation in the data. Subsequent groupings in the data were probed further using OPLS-DA class models. OPLS models were constructed using the DI-MS data (X-matrix) alongside metadata descriptors (Y-matrix) of continuous variables (age, BMI, waist circumference and percentage body fat) with the category variables of sex and diet ('control' and 'fish oil'). Predicted metadata descriptors (Ypred) were calculated from the X-matrix data and compared to the original 'observed' data using simple linear regression for the continuous variables and assignment of each sample to a class above a limit (Ypred >0.5) for the category variables[28].

Two-class OPLS-DA models ('control' and 'fish oil') were constructed to investigate the impact of EPA and DHA incorporation into intact lipids and models were constructed using different combinations of lipid variables: all lipids (detected in positive (M1) or negative (M5) mode), lipids with VIP >1 (positive (M2) or negative (M6) mode), TGs (M3), phosphocholines (PCs) and sphingomyelins (SM) (M4), non-esterified fatty acids (NEFAs) (M7) and phosphoethanolamines (PEs) (M8). These models were used to predict class membership, assigning each sample to a class above a limit (determined by ROC curve analysis described below). The robustness of all MVDA models was assessed using the R^2 ('goodness of fit') and Q^2 ('predictability'), values with R^2 >0.5 and Q^2 >0.4 considered a robust model. Further validation of the models was performed by dividing the data into training and test sets (⅔ and ⅓, respectively). OPLS-DA models were constructed on the training set and used to predict the class membership of the test set samples. Validation was repeated three times on different combinations of samples to form test sets. Lipids associated with either groups/classes or metadata descriptors were determined from the Variable Importance on Projection

(VIP) data (lipids with a VIP score greater than 1 in the model were considered to have the greatest contribution to the variation in the data) and S-plot p(corr) values [30] showing the correlation (positive or negative) with each group/class.

Univariate Statistical Data Analysis

Univariate statistical analyses were performed in GraphPad Prism (version 6.05, 2014 GraphPad Software Inc., San Diego, California, USA) and Microsoft® Excel® (Microsoft Office Professional Plus 2013) on lipids selected by MVDA. Data were initially stratified by both diet ('control' and 'fish oil' groups) and the degree of obesity ('lean': BMI ≤25 kg/m^2 and percentage body fat ≤25% (male) and ≤32% (female), 'overweight': BMI >25 kg/m^2 and percentage body fat >25% (male) and >32% (female)) and 2-way ANOVA (significance level P <0.05) performed on selected lipid levels to determine the nature of any interaction of diet and adiposity. Subsequent analysis focused on a comparison of stratification by diet alone ('control' (n = 47) and 'fish oil' (n = 19) groups) using the non-parametric, two-tailed Mann-Whitney U Test with a significance level of P <0.05.

Receiver Operator Characteristic (ROC) curve analysis of single and multivariate lipids associated with EPA and DHA incorporation into intact lipids

ROC curve analysis was performed in GraphPad Prism (version 6.05, 2014 GraphPad Software Inc., San Diego, California, USA) for the Ypred values for each dietary OPLS-DA model (M1-8) and for each single lipid determined by MVDA and univariate analysis to be increased in the 'fish oil' group. For each ROC curve, the accuracy, sensitivity and specificity were determined and the area under the curve (AUC) reported, with AUC values > 0.8 considered to be indicative of a robust model [28-30]. For each potential Ypred or concentration (% TCS) threshold the d^2 value was calculated using the equation:

$$d^2 = (1 - Sensitivity)^2 + (1 - Specificity)^2$$

The threshold at the minimum *d^2* value represented the optimal sensitivity and specificity for each ROC curve (above which a sample could be considered a 'true positive'). Multivariate ROC curves were validated using the training/test set procedures described above.

Logistic Regression and Linear Regression Analysis

Logistic and linear regression analysis were performed in RStudio (version 2.15.2, RStudio Inc., The R Foundation for Statistical Computing, 2012) using the Generalised Linear Models (glm) and Linear Models functions, respectively. For the logistic regression, models of single lipids and groups of lipids (by lipid class; TG, PC, NEFA and PE) were constructed and from the intercept (β_0) and coefficient (β_i) values it was possible to calculate the probability (p) of a given sample originating from the 'fish oil' group by the equation:

$$p = \frac{1}{1 + e^{-(\beta_0 + \beta_1 + \beta_2 \ldots + \beta_i)}}$$

The logistic regression models were evaluated using the Akaike Information Criterion (AIC) value with lower AIC values being indicative of a more robust model [33]. Multivariate linear regression models were used to examine the relationship between the GC-MS FAME measures of the total concentrations of EPA and DHA with combinations of lipids for each lipid class.

RESULTS

Multivariate statistics models inter individual variation as the dominant effect in the dataset

DIMS analysis coupled with an XCMS-based peak picking algorithm highlighted the presence of several lipid species including TGs (n=102), PCs (n=21) and SMs (n=14) in positive mode (**Figure 1**) and NEFAs (n=38) and PEs (n = 15) in negative mode. The concentrations of TGs were approximately 100-fold higher than those of PCs and the concentrations of PEs were comparable to those of NEFAs. Assignments were confirmed by fragmentation during LC-MS/MS (**Figure S1**).
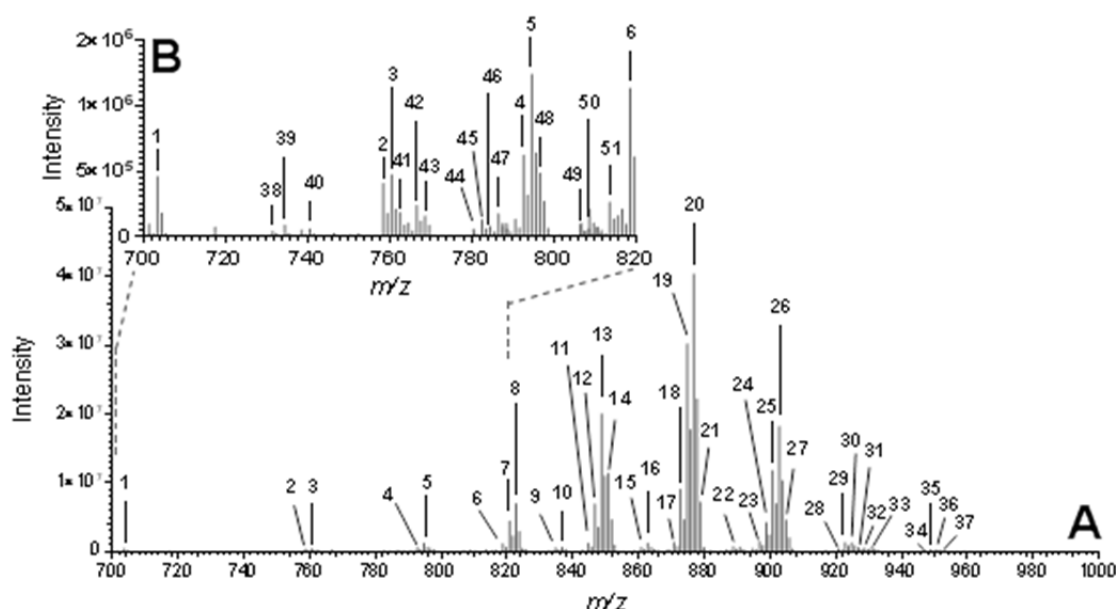

Figure 1: DIMS spectrum (positive mode, 0.2-1.0 min) of human adipose tissue (male subject, age 59 y, BMI 25.2 kg/m^2) collected at 12 months after commencing EPA+DHA capsules equivalent to 4-portions of oily fish per week showing (A) the region m/z = 700-1000 showing assignment of triacylglycerols (TGs) and (B) expansion of the region m/z = 700-820 showing assignment of phosphocholines (PCs) and sphingomyelins (SMs).

Lipid assignments: In each case the dominant ion is labelled with a number for each identified lipid. Minor isotopologues of given ions are not labelled. **1** – SM(34:1); **2** – PC(34:2); **3** – PC(34:1); **4** –

TG(46:2); **5** – TG(46:1); **6** – TG(48:3), **7** – TG(48:2); **8** – TG(48:1); **9** – TG49:2; **10** – TG(49:1), **11** –

TG(50:4); **12** – TG(50:3); **13** – TG(50:2); **14** – TG(50:1); **15** – TG(51:3); **16** – TG(51:2); **17** –

TG(52:5); **18** – TG(52:4); **19** – TG(52:3); **20** – TG(52:2); **21** – TG(52:1); **22** – TG(53:3); **23** –

TG(54:6); **24** – TG(54:5); **25** – TG(54:4); **26** – TG(54:3); **27** – TG(54:2); **28** – TG(54:1); **29** –

TG(56:8); **30** – TG(56:7); **31** – TG(56:6); **32** – TG(56:5); **33** – TG(56:4); **34** – TG(56:3); **35** –

TG(58:9), **36** – TG(58:7); **37** – TG(58:6); **38** – PC(32:1); **39** – PC(32:0), **40** – TG(42:0); **41** –

TG(44:3), **42** – TG(44:1); **43** – TG(44:0); **44** – PC(36:5), **45** – PC(36:4); **46** – PC(36:3); **47** –

PC(36:2); **48** – TG(46:0), **49** – PC(38:6); **50** – PC(38:5), **51** – SM(42:2).

An initial PCA model of the normalised (%TCS) data-matrix from the positive mode revealed inter-individual variation between samples in the study cohort whereby samples from the same individual mapped in the same score space irrespective of diet (control or fish oil supplementation) **(Figure 2A)**. Subsequent HCA of the score data from the first 4 components (R^2 = 0.83, Q^2 = 0.67 where 1 represents a perfect model and 0 represents a model no better than chance classification) showed two distinct groups of adipose tissue samples **(Figures 2B)** as confirmed by an OPLS-DA class model (R^2 = 0.68, Q^2 = 0.63, **Figure 2C**). These two groups related to the balance between C18:2 (Group 1) and C16:0/C16:1 (Group 2) containing TGs, with Group 1 showing relatively higher concentrations of TG(52:3), TG(52:4), TG(54:3), TG(54:4), TG(54:5), TG(54:6) and TG(56:4) compared with relatively higher concentrations of TG(48:1), TG(48:2), TG(50:1), TG(50:2), TG(50:3), TG(52:1) and TG(52:2) in Group 2 **(Figure 2D)**. A similar PCA/HCA approach for the negative mode data using the first 5 components (R^2 = 0.89, Q^2 = 0.63) showed two clusters related to the balance between the highest concentration free fatty acids with one group containing relatively higher concentrations of the saturated fatty acids (SFAs) C16:0 and C18:0 compared to higher concentrations of the unsaturated fatty acids C18:1, C16:1 and C18:2 in the second group. However, unlike the positive mode, the variation could not be directly attributed to inter-individual variation or other factors such as age or sex. Having identified the dominant trends in the dataset we next used supervised multivariate statistics to model diet and the other metadata associated with individuals.
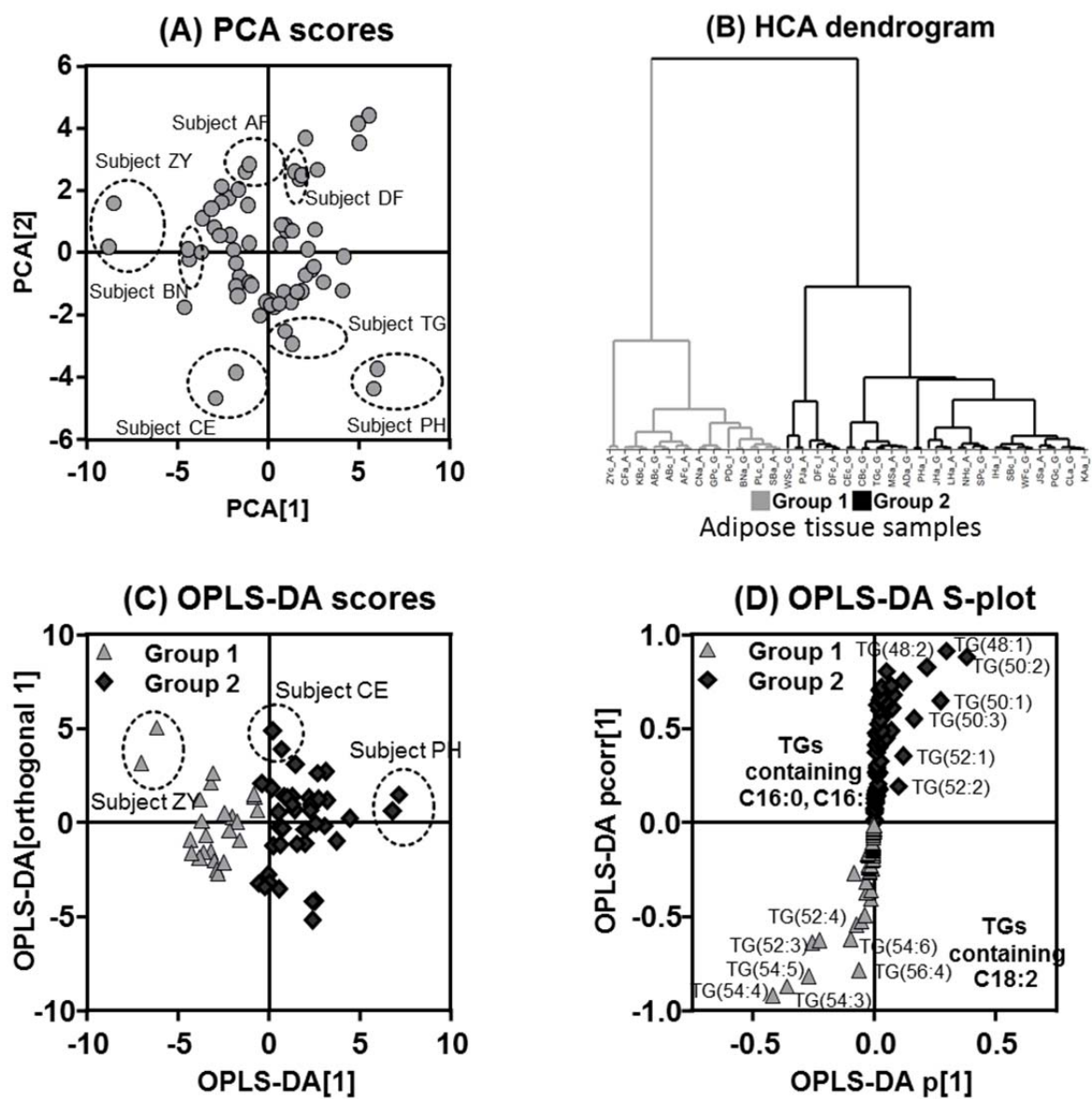
Figure 2: The results of initial multivariate data analysis (MVDA) of positive mode DIMS data to investigate the inter-individual variation in human adipose tissue showing (A) PCA scores map highlighting selected subjects with (B) corresponding HCA dendrogram constructed from the first four principal components revealing two inherent groupings in the data and the results of OPLS-DA group analysis showing (C) clustering of the two groups on the OPLS-DA score

map with triangles and diamonds referencing the two different clusters (D) corresponding S-plot showing TG lipids responsible for the groupings.

OPLS modelling of lipidomic data and metadata descriptors identified patterns of lipids associated with age, sex, percentage body fat and fish oil consumption

An OPLS model ($R^2 = 0.71$, $Q^2 = 0.47$) constructed using positive mode DIMS data modelled alongside metadata descriptors including continuous measurements of BMI, age, waist circumference and percentage body fat with sex and diet category descriptors, revealed patterns of lipids associated with each of these meta-data descriptors (note multivariate analyses naturally taken into account collinearities of variables). Lipids associated with increasing age were revealed in the first OPLS component (OPLS(1)) and included TGs containing odd chain fatty acids including TG(47:2), TG(49:3) and TG(51:2). The second OPLS component (OPLS(2)) reflected TGs associated with increasing BMI including TG(52:3) but also in concentrations of 56-chain TGs with a higher degree of unsaturation such as TG(56:6) and TG(56:7). These latter 56-chain TGs were also increased with dietary fish oil consumption detected in the third OPLS component (OPLS(3)). 2-way ANOVA analysis performed on these 56-chain TGs with $\geq$5 double bonds **(Table 1)** determined that the less unsaturated TGs with 5 or 6 carbon-carbon double bonds (incorporating C20:4n-6 as determined by MS/MS (see supplementary data)) were associated with increased BMI. The same analysis also confirmed that the more unsaturated 56-chain TGs with 7, 8 and 9 carbon-carbon double bonds (incorporating C20:5n-3 and C22:6n-3) were predominantly associated with fish oil consumption. Furthermore, there was no interaction between the degree of obesity and fish oil consumption on the concentrations of these TGs.

Table 1: Results of 2-way ANOVA analysis examining the influence of the degree of obesity ('lean'/'overweight') and diet ('control'/'fish oil') on the chain composition of 56-chain triglyceride lipids containing highly unsaturated PUFA.

TG lipid	mz $[M+NH_4]^{1+}$	Chain composition determined by LCMS/MS	2-way ANOVA P values
TG(56:5)	926.817	TG(*C20:4*/C18:1/C18:0)	Interaction: 0.61[NS] *Obesity: 0.0032*** Diet: 0.082[NS]
TG(56:6)	924.801	TG(C22:5/C18:1/C16:0) TG(*C20:4*/C18:1/C18:1)	Interaction: 0.18[NS] *Obesity: 0.028** Diet: 0.18[NS]
TG(56:7)	922.785	TG(**C22:6**/C18:1/C16:0) TG(C22:5/C18:2/C16:1) TG(**C20:5**/C18:1/C18:1) TG(*C20:4*/C18:2/C18:2)	Interaction: 0.39[NS] *Obesity: 0.023** **Diet: <0.0001******
TG(56:8)	920.770	TG(**C22:6**/C18:2/C16:0) TG(**C20:5**/C18:2/C18:1) TG(*C20:4*/C18:2/C18:2)	Interaction: 0.62[NS] *Obesity: 0.049** **Diet: <0.0001******
TG(56:9)[1]	918.755	TG(**C22:6**/C18:2/C16:1) TG(**C20:5**/C18:2/C18:2)	Interaction: 0.42[NS] Obesity: 0.94[NS] **Diet: 0.0023****

[1]Chain composition of TG(56:9) assigned from the LIPID MAPS database [39].

Key: C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C20:4 (arachidonic acid, AA), C20:5 (eicosapentaenoic acid, EPA), C22:5 (docosapentaenoic acid, DPA), C22:6 (docosahexaenoic acid, DHA)

P values: NS non-significant, * <0.05, ** <0.01, *** <0.001, **** <0.0001

Lipids associated with sex were confounded by other meta-data descriptors including age and percentage body fat and could not be detected in a single OPLS component (the female descriptor mapped in the same OPLS(1)/OPLS(2) score space as these two descriptors – **Figure 3A**). For this particular sub group of the study cohort, percentage body fat was higher in females compared to males and tended to increase with age for males only (2-way ANOVA: sex $P<0.0001$, age $P=0.204$, interaction $P=0.0618$). This OPLS model was used to determine predictions of the meta-data parameters from the lipidomic data and predicted/observed plots showed that for the continuous variables age **(Figure 3B)**, BMI **(Figure 3C)**, waist circumference **(Figure 3D)** and percentage body fat **(Figure 3E)** there was good agreement between the observed and predicted values for each variable ($R^2 \approx 0.7$). For the class descriptor variables of sex **(Figure 3F)** and diet **(Figure 3G),** the predicted class membership was 92% for both sex and diet (which decreased to 85% and 82%, respectively, with validation). For negative mode dataset, the OPLS model did not meet the cross-validation and permutation test results considered to be robust.
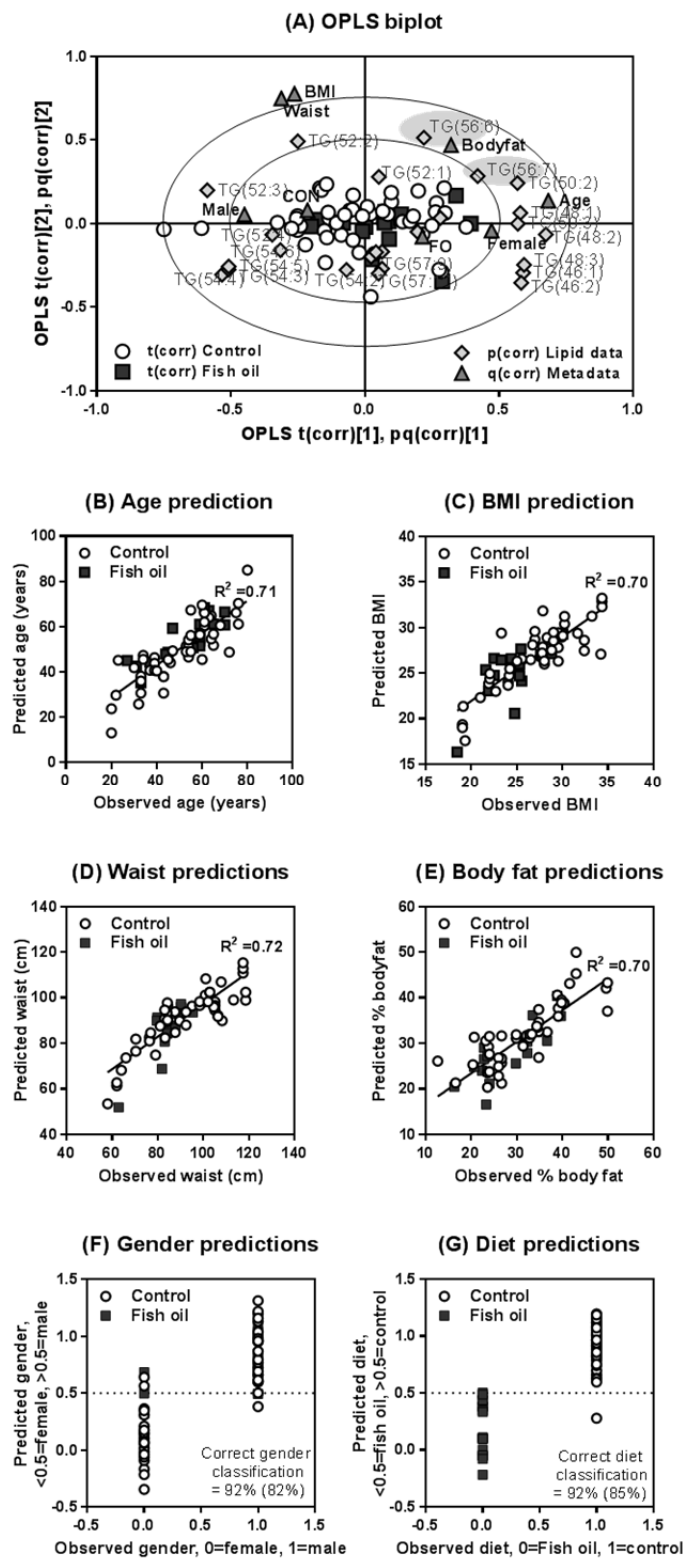
Figure 3: The results of OPLS modelling of positive mode DI-MS data with metadata showing

(A) OPLS biplot[1] with simultaneous mapping of t(corr) scores and pq(corr) loadings and OPLS

observed/predicted plots for (B) age, (C) BMI, (D) waist circumference, (E) percentage body fat (F) sex and (G) dietary intervention.

[1]For the purposes of visualisation only lipids with VIP >1 are included on the biplot

OPLS-DA modelling coupled with ROC curve analysis characterised the lipid profile associated with fish oil consumption

Eight OPLS-DA models were constructed to investigate the influence of supplemental n-3 PUFAs on the lipid profiles incorporating several combinations of lipid variables in either positive or negative mode (**Figure 4, Table 2**). All models were robust (Q^2 >0.4) and showed clear distinction of 'control' and 'fish oil' profiles. Subsequent ROC curve analysis of each model using the predicted class membership value (Ypred) confirmed the robustness of the models with AUC values >0.95 demonstrating excellent classification given the complexity of the data and the fact that inter-individual variation dominated. The accuracy of the models in predicting diet was high with >85% for the training set models which lowered to >75% once the models were validated. The specificity (true negative rate or control group) was high for all models (>70% for validated models) whereas the sensitivity (true positive rate or 'fish oil' group) was lower (53%) for the TG only model (M3) once validated.
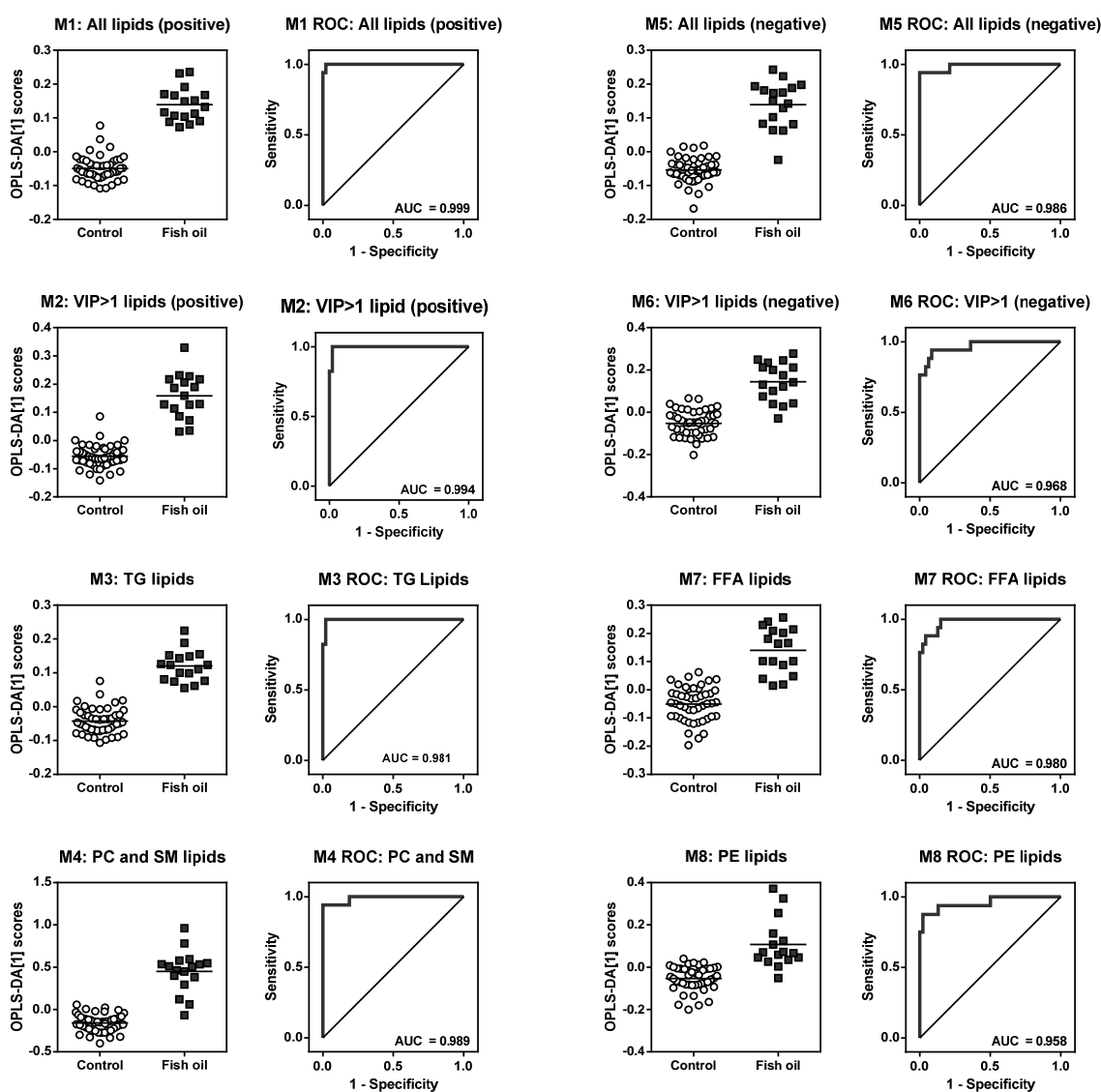
Figure 4: OPLS-DA scores maps and corresponding multivariate ROC curves for discriminating between 'control' and 'fish oil' supplementation derived from DI-MS lipid data models for M1: all positive mode lipids, M2: positive mode lipids with VIP >1, M3: TGs, M4: PCs and SMs, M5: all negative mode lipids, M6: all negative mode lipids with VIP >1, M7: NEFAs and M8: PEs. The accuracy, sensitivity and specificity for each model are given in Table 2.

Table 2: Summary of MVDA analysis comparing 'control' and 'fish oil' groups showing results for OPLS-DA (R^2 and Q^2) and ROC curve (AUC, accuracy, specificity and sensitivity) analyses.

Model	OPLS-DA analysis				ROC curve analysis			
	Lipid classes	K variables	R^2	Q^2	AUC ± SEM	Accuracy (%)	Specificity (%)	Sensitivity (%)
M1	All positive mode	137	0.81	0.60	0.999 ± 0.002	98 (86)	99 (96)	94 (59)
M2	Positive VIP >1	30	0.77	0.55	0.996 ± 0.005	93 (85)	94 (85)	91 (82)
M3	TG (positive)	102	0.77	0.56	0.996 ± 0.005	100 (78)	100 (88)	100 (53)
M4	PC+SM (positive)	35	0.76	0.59	0.989 ± 0.012	97 (83)	97 (83)	97 (82)
M5	All negative mode	183	0.77	0.56	0.988 ± 0.013	98 (79)	98 (74)	97 (94)
M6	Negative VIP >1	20	0.62	0.49	0.968 ± 0.023	91 (78)	91 (85)	91 (59)
M7	NEFA (negative)	38	0.62	0.47	0.980 ± 0.014	95 (78)	94 (76)	97 (82)
M8	PE (negative)	15	0.46	0.42	0.958 ± 0.032	89 (82)	88 (78)	91 (94)

For the ROC curve analysis the values in brackets are from the validation test set. All positive mode – all lipids detected using positive ion mode. Positive VIP >1 – all lipids detected in positive ion mode with a variable importance parameter (VIP) greater than 1. TG – triacylglycerols detected in positive ion mode. PC + SM phosphocholines and sphingomyelins detected in positive ion mode. All negative mode – all ion detected in negative ion mode. Negative VIP > 1 – all lipids detected in negative ion mode with a VIP greater than 1. NEFA – non-esterified fatty acids detected in negative ion mode. PE – phosphoethanolamines detected in negative ionisation mode.

Abbreviations: TG, triacylglycerols; PC, phosphocholines; SM, sphingomyelin; NEFA, non-esterified fatty acids; PE, phosphoethanolamines; VIP, variable influence on projection; AUC, area under curve.

The OPLS-DA S-plot p(corr) values for models of TG (M3), PC and SM (M4), NEFA (M7) and PE (M8) were used to identify the patterns of lipids associated with fish oil consumption. Thirteen TG

lipids were identified **(Figure 5)** with total chain lengths ≥48 carbons with twelve of these having a high degree of unsaturation (≥5 carbon double bonds). PC(38:5), PC(38:6) and PC(36:5) were identified reflecting incorporation of DHA and EPA into PC **(Table 3).** In negative mode, the NEFA forms of DHA and EPA and PE(38:6) and PE(36:5) were significantly higher in adipose tissue from subjects consuming fish oil **(Table 3)**.
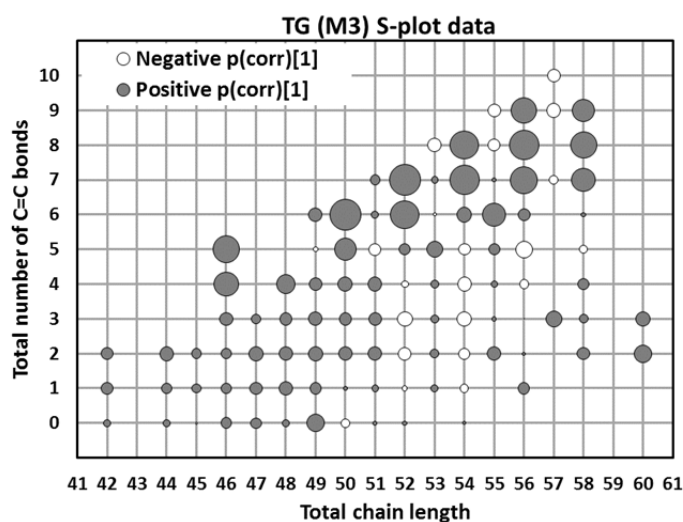

Figure 5: Dot plots derived from the S-plot p(corr)[1] for the TG OPLS-DA model (M3) values showing the relationship between the degree of unsaturation (total number of carbon (C=C) double bonds) and total TG chain length with larger grey dots representing greater correlation with the fish oil intervention.

ROC curve and logistic regression analysis confirmed that phospholipid lipids provide the markers which discriminate best between no and high fish oil intake

The results of univariate statistical analysis, ROC curve analysis and logistic regression for the selected individual lipids are given in **Tables 3 and S2.** ROC curve analysis for a given lipid produced AUC values >0.7 **(Table 3; Figure S2)** but were consistently less than those calculated for the multivariate ROC curves derived from OPLS-DA models of the selected lipid classes. PC and PE, in particular PC(38:6), PC(36:5) and PE(36:5), had AUC values >0.9, comparable to those observed

with the multivariate ROC curves whilst also having the greatest fold changes (~5 compared to ~2 for TGs and NEFAs, **Table 3**). However, PE(36:5) is a less reliable indicator of fish oil consumption due to high degree of missing values (27%) in the analyses compared to the corresponding PC. This 'missingness' was particularly apparent for the control group where values fell below the DI-MS limit of detection as a result of the large dynamic range of NEFAs and PEs detected in negative mode.

Table 3: Summary of the univariate statistical and ROC curve analysis of individual lipids elevated in human adipose tissue as the result of n-3 PUFA supplementation equivalent to the habitual consumption of '4 portions' of oily fish per week.

Lipid	*m/z*	Adduct	Mean ± SD 'Control' concentration (%TCS)	Mean ±SD 'Fish oil' concentration (%TCS)	Fold change with 95% confidence interval	Mann-Whitney U test P value	ROC curve AUC ± SEM	Concentration threshold[2] (%TCS)
TG(46:4)	788.673	$[M+NH_4]^{1+}$	0.015 ± 0.030	0.023 ± 0.040	1.5 (1.3 - 1.6)	0.0076**	0.717 ± 0.077	0.023
TG(46:5)	786.661	$[M+NH_4]^{1+}$	0.002 ± 0.000	0.004 ± 0.001	2.3 (1.8 - 2.6)	0.0006***	0.776 ± 0.073	0.002
TG(50:5)	842.723	$[M+NH_4]^{1+}$	0.078 ± 0.003	0.092 ± 0.004	1.2 (1.2 - 1.2)	0.0091**	0.712 ± 0.066	0.091
TG(50:6)	840.708	$[M+NH_4]^{1+}$	0.007 ± 0.001	0.017 ± 0.002	2.3 (2.3 - 2.4)	<0.0001****	0.878 ± 0.045	0.012
TG(52:6)	868.739	$[M+NH_4]^{1+}$	0.088 ± 0.003	0.119 ± 0.007	1.3 (1.3 - 1.4)	<0.0001****	0.816 ± 0.056	0.103
TG(52:7)	866.723	$[M+NH_4]^{1+}$	0.006 ± 0.001	0.017 ± 0.003	2.9 (2.7 – 3.0)	<0.0001****	0.852 ± 0.055	0.009
TG(54:7)	894.755	$[M+NH_4]^{1+}$	0.122 ± 0.006	0.180 ± 0.010	1.5 (1.4 - 1.5)	<0.0001****	0.858 ± 0.046	0.135
TG(54:8)	892.739	$[M+NH_4]^{1+}$	0.008 ± 0.001	0.018 ± 0.002	2.3 (2.2 - 2.5)	<0.0001****	0.824 ± 0.051	0.012
TG(55:6)	910.786	$[M+NH_4]^{1+}$	0.012 ± 0.001	0.016 ± 0.002	1.4 (1.3 - 1.5)	0.0183*	0.692 ± 0.074	0.015
TG(56:7)	922.786	$[M+NH_4]^{1+}$	0.283 ± 0.016	0.422 ± 0.034	1.5 (1.4 - 1.6)	<0.0001****	0.824 ± 0.051	0.312
TG(56:8)	920.770	$[M+NH_4]^{1+}$	0.087 ± 0.006	0.147 ± 0.012	1.7 (1.6 - 1.8)	<0.0001****	0.869 ± 0.044	0.102
TG(56:9)	918.755	$[M+NH_4]^{1+}$	0.008 ± 0.001	0.018 ± 0.002	2.1 (2.0 – 2.4)	<0.0001****	0.826 ± 0.054	0.013
TG(58:7)	950.817	$[M+NH_4]^{1+}$	0.068 ± 0.004	0.089 ± 0.007	1.3 (1.2 - 1.4)	0.0049**	0.728 ± 0.067	0.079
TG(58:8)	948.802	$[M+NH_4]^{1+}$	0.059 ± 0.004	0.091 ± 0.008	1.5 (1.5 - 1.6)	0.0002***	0.795 ± 0.055	0.06
TG(58:9)	946.786	$[M+NH_4]^{1+}$	0.022 ± 0.003	0.037 ± 0.003	1.7 (1.5 - 1.9)	0.0001***	0.802 ± 0.053	0.03
PC(36:5)	780.554	$[M+H]^{1+}$	0.004 ± 0.001	0.025 ± 0.003	5.7 (5.3 – 6.7)	<0.0001****	0.92 ± 0.048	0.016
PC(38:5)	808.585	$[M+H]^{1+}$	0.007 ± 0.001	0.020 ± 0.003	3.0 (2.8 - 3.2)	<0.0001****	0.835 ± 0.067	0.014
PC(38:6)	806.569	$[M+H]^{1+}$	0.008 ± 0.001	0.037 ± 0.004	4.5 (4.4 - 4.8)	<0.0001****	0.936 ± 0.038	0.023
FA(22:6)	327.233	$[M-H]^{1-}$	0.071 ± 0.006	0.156 ± 0.018	2.2 (2.0 - 2.4)	<0.0001****	0.867 ± 0.055	0.102
FA(20:5)	301.217	$[M-H]^{1-}$	0.159 ± 0.010	0.281 ± 0.023	1.8 (1.7 - 1.8)	<0.0001****	0.852 ± 0.057	0.206
PE(36:5)	736.492	$[M-H]^{1-}$	0.006 ± 0.001	0.029 ± 0.006	5.1 (5.0 – 5.2)	<0.0001****	0.892 ± 0.053	0.009
PE(38:6)	762.508	$[M-H]^{1-}$	0.040 ± 0.006	0.091 ± 0.021	2.3 (1.7 - 2.6)	0.0081**	0.716 ± 0.081	0.044
PE(40:6)	790.539	$[M-H]^{1-}$	0.020 ± 0.0120	0.035 ± 0.018	1.7 (1.5 - 1.9)	0.0901[NS]	0.651 ± 0.086	0.026

P values: NS non-significant, * <0.05, ** <0.01, *** <0.001, **** <0.0001

[2] Concentration threshold calculated from the ROC curve analysis d^2 value

Concentration thresholds (%TCS) were calculated from the ROC curve analysis with TGs and NEFAs having thresholds approximately 10-fold greater than those observed for PCs and PEs. Logistic regression analysis **(Table S2)** enabled the calculation of the probability of an adipose tissue sample originating from the 'fish oil' group, with the best models resulting from PCs, denoted by the lowest AIC values compared to the other lipid classes. Furthermore, multivariate logistic regression models **(Table S3; Figure S3)** for each lipid class confirmed that the PCs produced the most discriminative lipids both as individual markers and as part of a multivariate OPLS-DA or a logistic regression model. However, multivariate linear regression models showed that tissue concentrations of EPA+DHA (as measured previously by GC FAME analysis [34]) showed the best correlation with TGs ($R^2 = 0.61$) with weaker correlations for the other three lipid groups presumably reflecting the high TG content of adipose tissue **(Table S3; Figure S3)**.

DISCUSSION

In this study, where healthy individuals received EPA and DHA as dietary supplements, the initial analyses showed that the greatest source of variation in the lipid data from adipose tissue was related to inter-individual variation especially in TGs containing the highest concentration of fatty acids C16:0 (20% total fatty acid (TFA) content as measured by GC), C16:1n-7c (5% TFA), C18:0 (3% TFA), C18:1n-9c (50% TFA) and C18:2n-6c (15% TFA) (as determined by GC FAME analysis [34]). The two distinct groups detected by the PCA and HCA models reflected the balance between TGs containing predominantly fatty acids derived from *de novo* lipogenesis such as palmitic acid (C16:0) and stearic acid (C18:0) and those derived from dietary essential fatty acids such as linoleic acid (C18:2n-6c) and   -linolenic acid (C18:3n-3c). Expression of the desaturase enzymes, including Δ^9 desaturase (EC 1.14.19.1) responsible for conversion of SFA to MUFA and the Δ^6 and Δ^5 desaturases involved in the synthesis of highly unsaturated PUFAs including EPA, DHA and AA along with elongase enzymes (EC 6.2.1.3), are also implicated in the variability in levels of these fatty acids in TGs. In two studies based on the KORA and Twins UK cohorts, Illig and colleagues have examined how common SNPs influence the human metabolome [41, 42], identifying a range of common SNPs associated with genes involved in lipid metabolism in the two cohorts including acyl-CoA dehydrogenase, long chain (ACADL), acyl-CoA dehydrogenase, medium chain (ACADM), acyl-CoA dehydrogenase, short chain (ACADS), electron-transferring-flavoprotein dehydrogenase (ETFDH), stearoyl-CoA desaturase (SCD), fatty acid desaturase 1 (FADS1), elongation of very long chain fatty acids-like 2 (ELOVL2) and serine palmitoyltransferase, long chain base subunit 3 (SPTLC3). While it is unlikely that variations in any of these SNPs could explain the two distinct clusters detected in the HCA dendrograms as the distribution of different polymorphs does not approach 50% for a given gene, common variants may explain the inter-individual variability detected in the initial multivariate analyses performed on the lipidomic dataset.

Independent of the dietary changes induced by the n-3 fatty acid supplementation, this study showed that TGs incorporating the highly unsaturated C20:4 were correlated with the degree of obesity of the participant. Although our DI-MS method could not determine where the double bonds were in the

C20:4 detected in lipid fragmentation patterns, they presumably largely relate to arachidonic acid (C20:4n-6) given its relative proportion in the diet. Both age and sex have been shown previously to affect levels of n-3 PUFAs in adipose tissue with docosapentaenoic acid (DPA) and DHA increasing with age and these fatty acids being higher in females [18]. In the original study, analysis by GC also showed EPA and DHA in adipose tissue to be higher with increasing age, and plasma TG EPA to be significantly higher in females [43]. Age was also associated with increased adiposity (defined by percentage body fat) for this sub-cohort of the original study, with females generally having a higher percentage body fat and, therefore, this contributed to the inter-individual variability of the data-set. In addition to these changes associated with age and sex, supplementation with n-3 fatty acids also led to increases in arachidonic acid, EPA and DHA in adipose tissue. The incorporation of highly unsaturated C20:4 into TGs, which presumably reflects a higher proportion of arachidonic acid, in adipose tissue may reflect the beneficial effects of fish oil by decreasing the n-6/n-3 ratio in blood plasma in favour of a more anti-inflammatory profile [44], which could potentially have a protective role in terms of systemic metabolism.

In general, multivariate OPLS-DA and logistic models provided a greater degree of accuracy, sensitivity and specificity in predicting the dietary origin of a sample than analyses based on any one individual lipid class. Detection of TGs which had directly incorporated EPA and DHA provided a reasonable indicator of EPA and DHA consumption especially when considering the OPLS-DA models and multivariate ROC curve analysis. However, although the multivariate TG ROC curve analysis provided accurate prediction of group membership ('control' or 'fish oil') it lacked sensitivity (true positive rate) as well as having the highest AIC values for the logistic regression analysis. This lack of sensitivity has been attributed to the confounding effects of arachidonic acid contributing to models of obesity, sex and diet. Non-esterified EPA and DHA also showed high logistic regression AIC values and lower ROC curve AUC values especially in comparison with the phospholipids.

NEFA concentrations in adipose tissue were highly variable and dominated by the saturated palmitic and stearic acids. These two fatty acids are particularly affected by the rate of *de novo* lipogenesis and plasma levels of SFAs, MUFAs, n-3 PUFAs and n-6 PUFAs have been shown previously to be poor

predictors of adipose tissue concentrations [45]. Furthermore, the concentrations of NEFAs in adipose tissue have been shown to exhibit sex-specific effects on body fat distribution [46], contributing to the high variability of these lipids in the present study. One thing that should be noted is that samples were taken in the fasted state where TGs will be undergoing hydrolysis, releasing NEFAs into the bloodstream. It is interesting to note that the NEFAs detected do not reflect the n-3 PUFAs taken as part of the fish oil supplement, but instead are dominated by palmitic, oleic and stearic acids which are the main stored fatty acids found in adipose tissue, which in turn reflect the dominant fatty acids found in TGs in adipose tissue. The phospholipids, especially those containing palmitic acid with a PUFA, including PC(36:5) and PC(38:6) and PE(36:5) produce both accurate and sensitive OPLS-DA models and logistic regression models with the lowest AIC values. This reflects the previous GC FAME findings that the EPA and DHA levels in blood plasma phospholipids were found to be the most reliable marker of short-term n-3 PUFA consumption [34]. Adipose tissue PCs were generally more reliable than PEs for the prediction of n-3 PUFA consumption, in part reflecting the better detection of PCs in positive ion mode compared with PEs in negative ion mode resulting in a number of missing values which affected the quantification of these PEs in general. Phospholipids form the bilayer of the cell membrane, and increasing supply of EPA and DHA will increase their incorporation into the phospholipid bilayer, producing potentially a less inflammatory environment. In addition, incorporation of PUFAs has been suggested as a mechanism to maintain membrane fluidity [47], and these membrane associated lipids may reflect a longer term store of lipids than TGs which are re-modelled during the switch between fed and fasted states on a daily basis.

While no single lipid species was identified that acted as a marker of n-3 PUFA consumption, it is worth commenting that by using mass spectrometry a panel of lipids can be quantified in a single analysis. Approaches based on so-called 'open profiling methods', as used in this study, aim to detect all the high concentration metabolites/lipids in a sample. In addition, when using targeted analyses based on triple quadrupoles, it is routine to quantify individual lipid species according to their fragmentation patterns either using direct infusion or short liquid chromatography gradients. Our panel of lipids could be routinely incorporated into a targeted lipid profile to provide objective

markers of n-3 PUFA consumption. In addition, it has been recently suggested that TGs containing shorter, more saturated fatty acids act as markers for de novo lipogenesis arising from dietary sugar intake [36, 48]. Thus, lipid profiles may provide fast, convenient, objective measures of dietary intake for the use in population studies.

In terms of limitations of the study it should be noted that the 'fish oil' group had fewer subjects than the 'control' group and in addition the 'fish oil' group had a significantly lower average BMI compared with the 'control' group despite a similar sex distribution, comparable ages and similar body fat percentage. This arose in part because we made use of samples that remained from the analyses performed on the original study [34]. However, one of the advantages of the multivariate approach employed in this study is that it considers each subject individually when forming clusters. Furthermore, the multivariate analyses highlighted a number of lipid species that were associated with n-3 PUFA consumption and which were not associated with variations in BMI.

Concluding remarks

Overall, we have detected a pattern of intact lipids across a range of lipid classes in human adipose tissue that predict with accuracy dietary exposure to n-3 PUFAs whilst also considering confounding factors such as the degree of obesity/adiposity of the subject. The advantage of the direct infusion mass spectrometry method is that it provides rapid sample analysis (< 5 minutes per sample) with a minimal amount of sample preparation whilst covering a wide range of lipids in three main lipid classes found in adipose tissue. When combined with multivariate modelling, ROC curve analysis and logistic regression of the resulting data sets provide a powerful tool for the detection and characterisation of lipid patterns associated with dietary intervention. This may be useful when assessing the impact of diet on lipidomic profiles and deconvolving dietary effects from disease or pathophysiological states, and could be readily used in other sample formats (e.g. blood, plasma) and in population studies to provide metabolic markers of dietary intake.

Supporting information:

Figure S1: LCMS/MS data showing ms^2 spectra (m/z = 500-1000) of selected triglyceride 56- and 58-chain lipids with assignment of the loss from parent ion m/z for the highly unsaturated PUFAs: EPA (-302.24), DHA (-328.25), DPA (-330.27) and AA (-304.26).

Figure S2: Plots showing the descriptive statistics (minimum-maximum box-whisker plots), ROC curve analysis, frequency distributions and the logistic regression probabilities for selected intact lipids shown to be increased after fish oil intervention.

Figure S3: Results of multivariate logistic and linear regression modelling showing the probability of detecting a fish oil sample and the correlation between observed and predicted EPA+DHA values respectively for the different lipid classes (TG, PC and SM, NEFA and PE) affected by dietary fish oil intervention.

Table S1: Volunteer characteristics for the sub-cohort of the original study[34] used in the manuscript.

Table S2: Summary of logistic regression parameters for lipids elevated in human adipose tissue as the result of n-3 PUFA supplementation equivalent to the habitual consumption of '4 portions' of oily fish per week.

Table S3: Summary of multivariate logistic and linear regression parameters for the four classes of intact lipids detected in human adipose tissue affected by n-3 PUFA supplementation equivalent to the habitual consumption of '4 portions' of oily fish per week.

AUTHOR CONTRIBUTIONS

The contributions to this manuscript are as follows: EGS (lipidomic analysis, lipidomic data processing and manuscript preparation); BJJ (LCMS/MS analysis); AK (lipidomic and LCMS/MS analysis); CGW (provision of adipose tissue, FAME data and metadata); ALW and LB (conduct of

the original study); SAJ (design of original study, provision of adipose tissue, review of manuscript); PCC (design of original study, provision of adipose tissue, review of manuscript); JLG (design of the current study, scientific discussion and preparation of manuscript).

CONFLICTS OF INTEREST

Abbreviations: AA: Arachidonic acid, ALA: α-linolenic acid, DHA: Docosahexaenoic acid, DIMS: Direct Infusion-Mass Spectrometry, EPA: Eicosapentaenoic acid, FA: fatty acid, HCA: Hierarchical Clustering Analysis, LA: Linoleic acid, LC-MS Liquid Chromatography Mass Spectrometry, MLR: Multivariate Logistic Regression, NEFA: non-esterified fatty acids OPLS(-DA): Orthogonal Partial Least Squares Projection to Latent Structures (-Discriminant Analysis), PC: Phosphocholine, PCA: Principal Components Analysis, PE: Phosphoethanolamine, PUFA: Polyunsaturated fatty acid, ROC: Receiver Operator Characteristic, TG: Triacylglycerol.

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Table of Contents Graphical Abstract

Diet predictions

M1: All lipids (positive)

M1 ROC: All lipids (positive)