University of Dundee

Lipid remodelling and an altered membrane proteome may drive the effects of EPA and DHA treatment on skeletal muscle glucose uptake and protein accretion

Jeromson, Stewart; Mackenzie, Ivor; Doherty, Mary K.; Whitfield, Phillip D.; Bell, Gordon; Dick, James; Shaw, Andy; Rao, Francesco; Ashcroft, Stephen; Philp, Andrew; Galloway, Stuart; Gallagher, Iain ; Hamilton, D. Lee

[Link to publication in Discovery Research Portal](#)

1 **Lipid remodelling and an altered membrane associated proteome may drive the**

2 **differential effects of EPA and DHA treatment on skeletal muscle glucose uptake and**

3 **protein accretion**

4 Stewart Jeromson[1], Ivor Mackenzie[2], Mary K. Doherty[2], Phillip D. Whitfield[2], Gordon Bell[3], James

5 Dick[3], Andy Shaw[1], Francesco Rao[4], Stephen P. Ashcroft[5], Andrew Philp[5], Stuart Galloway[1], Iain

6 Gallagher[1], D. Lee Hamilton[1]†

7 1. Health and Exercise Sciences Research Group, University of Stirling, Stirling, UK, FK9 4LA.

8 2. Department of Diabetes and Cardiovascular Science, University of Highlands and Islands,

9 Inverness, UK, IV2 3JH.

10 3. Institute of Aquaculture, University of Stirling, Stirling, UK, FK9 4LA.

11 4. Dundee Cell Products, Dundee Technopole, Dundee, UK, DD1 5JJ.

12 5. School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham,

13 UK, B15 2TT.

14

15 †Corresponding Author:

16 D. Lee Hamilton

17 Health and Exercise Sciences Research Group,

18 University of Stirling,

19 Stirling,

20 UK,

21 FK9 4LA

22 **Running Title:** Skeletal muscle lipid remodelling by EPA and DHA.

23

Abstract

In striated muscle, EPA and DHA have differential effects on the metabolism of glucose and differential effects on the metabolism of protein. We have shown that, despite similar incorporation, treatment of C_2C_{12} myotubes (CM) with EPA but not DHA improves glucose uptake and protein accretion. We hypothesized that these differential effects of EPA and DHA may be due to divergent shifts in lipidomic profiles leading to altered proteomic profiles. We therefore carried out an assessment on the impact of treating CM with EPA and DHA on lipidomic and proteomic profiles. FAME analysis revealed that both EPA and DHA led to similar but substantial changes in fatty acid profiles with the exception of arachidonic acid, which was decreased only by DHA, and DPA, which was increased only by EPA treatment. Global lipidomic analysis showed that EPA and DHA induced large alterations in the cellular lipid profiles and in particular, the phospholipid classes. Subsequent targeted analysis confirmed that the most differentially regulated species were phosphatidylcholines and phosphatidylethanolamines containing long chain fatty acids with 5 (EPA treatment) or 6 (DHA treatment) double bonds. As these are typically membrane associated lipid species we hypothesized that these treatments differentially altered the membrane-associated proteome. SILAC based proteomics of the membrane fraction revealed significant divergence in the effects of EPA and DHA on the membrane associated proteome. We conclude that the EPA specific increase in polyunsaturated long chain fatty acids in the phospholipid fraction is associated with an altered membrane associated proteome and these may be critical events in the metabolic remodelling induced by EPA treatment.

Introduction

Fatty acids play an important role in skeletal muscle metabolism, not only as substrates for oxidative phosphorylation or vital structural components of membranes but also as regulators of enzyme activities and signalling molecules (8). Furthermore, dysfunctions in the control of fatty acid metabolism can be an important factor in the aetiology of conditions such as insulin resistance and muscle atrophy (8). The lipid composition of skeletal muscle undergoes constant fluctuations and is reflective of dietary fat intake (6). There is strong evidence to suggest that the enrichment of skeletal muscle with omega-3 (n-3) fatty acids may have therapeutic benefits on muscle metabolism and function (14). Oral n-3 supplementation above the Reference Dietary Intake is known to result in significant incorporation of n-3 fatty acids into the skeletal muscle lipid pool (6, 13, 30).

Several n-3 supplementation studies in humans have observed beneficial effects ranging from an increased sensitivity to anabolic stimuli (43, 44) and muscle function (40, 45). Eight weeks n-3 supplementation improved the muscle protein synthetic response (MPS) to a hyperinsulinaemic amino acid infusion in both young (44) and elderly individuals (43). Furthermore, when taken alongside a resistance based exercise programme (12, 40) or in the absence of any strength training (45) n-3 fatty acids enhance strength and or physical function in the elderly. Concurrent with anabolic properties, n-3 FAs also display anti-catabolic effects. n-3 supplementation prevented muscle mass losses in burned guinea pigs (5) and protected against muscle mass loss during anti-neoplastic therapy in cancer patients (34). However, when combined in systematic reviews do not always show a beneficial effect on muscle mass during cancer treatment (39). Moreover n-3 FAs attenuated soleus atrophy in rodents that underwent 10 days of hind limb immobilization (55).

As well as effects on muscle protein metabolism there is building evidence to suggest that n-3 fatty acids may also modulate glucose metabolism. Current meta-analyses detect a neutral or small effect of n-3 FAs on measures of insulin sensitivity (4). However, there are several studies across a range of models utilizing higher doses of n-3s which support a role for n-3s in improving muscle mitochondrial function and glucose metabolism (23, 28, 35, 37, 47, 48). For instance, replacing 3.4% of the Kcals

71   with n-3 fatty acids on a high fat diet protects mice against declines in glucose tolerance during a 10-

72   week high fat diet despite similar increases in body weight compared to high fat alone (28). Whereas

73   supplementation studies in humans have not demonstrated a consensus (4), an interesting lipid

74   infusion trial has shown n-3 fatty acids to be bioactive in humans with respect to glucose metabolism

75   (46). More specifically, the addition of n-3 fatty acids to a lipid infusion of n-6 fatty acids attenuated

76   the decline in insulin stimulated glucose disposal caused by n-6 infusion alone suggesting that the n-3

77   fatty acids have a protective effect on glucose metabolism in the presence of an n-6 overload (46).

78   Fish oil and n-3 supplements are a heterogeneous mixture of fatty acids of which eicosapentaenoic

79   acid (EPA, 20:5) and docosahexaenoic acid (DHA 22:6) are thought to be the most biologically

80   active. Due to many studies using a combination of EPA and DHA and varying ratios of each fatty

81   acid it is difficult to assert whether EPA or DHA alone is causing the observed effects or if EPA and

82   DHA work synergistically or antagonistically for that matter. The molecular mechanisms of n-3

83   action are still poorly understood. Work from Olefsky's lab suggests that GPR120 acts as a general n-

84   3 receptor in macrophages and adipocytes that, when activated by n-3 fatty acids leads to increases in

85   whole body insulin sensitivity by reducing inflammation (37). EPA has also been shown to

86   antagonise the action of TNFα on C_2C_{12} myotube formation in a manner partially dependent on

87   PPARγ (29). Additionally, EPA reduces the activation of Nuclear Factor-κappa Beta (NF-Kb) leading

88   to a reduction in Muscle RING finger protein-1 (Murf-1) signalling, an important mediator of muscle

89   atrophy in cultured myotubes (22). Furthermore, a follow up study employing both EPA and DHA

90   demonstrated that DHA was more efficient in this mechanism than EPA (52). However, EPA has

91   been shown to improve metabolic flexibility in response to changing substrate availabilities (21). EPA

92   has also previously been shown to improve both basal and insulin stimulated glucose uptake in

93   cultured myotubes (1). However, it remains to be seen whether DHA similarly improves glucose

94   uptake in skeletal muscle. Despite the numerous similar intracellular effects and similar structure,

95   EPA and DHA may have divergent physiological effects in skeletal muscle. EPA improves skeletal

96   muscle protein metabolism while DHA has a non-significant effect (24). In other striated muscle

97   models such as cardiomyocytes, EPA but not DHA increases glucose and fatty acid uptake despite

98   similar effects on cell signalling (18). In plasma both EPA and DHA reduced triacylglycerol (TAG)

99   but only DHA modulates HDL (high density lipoprotein) and LDL (low density lipoprotein) particle

100   size (53). Collectively these data suggest that in certain contexts, EPA and DHA can have differential

101   biological effects.

102   The molecular mechanisms underpinning the divergent physiological effects of EPA vs DHA are

103   currently under explored. However, differential remodelling of the lipid profile may partially explain

104   the divergent physiological response observed between EPA and DHA. Currently studies attempting

105   to address how n-3 fatty acids affect the lipidomic profile of skeletal muscle are limited. As expected

106   n-3 intake leads to incorporation into the lipid pool of multiple tissues i.e. plasma, muscle, adipose

107   tissue and liver with a significant proportion being directed towards phospholipid pools (7, 27, 30, 42-

108   44). This incorporation is not limited to the plasma membrane and is also incorporated into

109   subcellular organelles such as mitochondria (20). It is hypothesized that a primary driver of the effects

110   of EPA and DHA is the displacement of arachidonic acid (AA) from membranes with studies from a

111   range of models supporting this (27, 32, 42) while some *in vivo* human studies observe no change in

112   total AA in the skeletal muscle lipid pool (30). However, this does not discount the possibility that

113   AA might be displaced from specific lipid fractions. Multiple human studies have assessed the impact

114   of n-3 supplementation on skeletal muscle phospholipid pool however the use of a heterogeneous mix

115   of n-3 fatty acids precludes the ability to detect the isolated effects of EPA vs DHA on lipid profiles

116   (6, 13, 30). Furthermore, into which skeletal muscle phospholipid fractions EPA and DHA are

117   incorporated are poorly understood. In plasma phospholipids EPA and DHA induce a similar lipid

118   profile yet EPA increased docosapentanoic acid (DPA 22:5 n-3) and a differential but non-significant

119   increase in stearate acid (SA 18:0) (31). In smooth muscle cell phospholipids both EPA and DHA are

120   heavily incorporated into the phosphatidylcholine (PC) fraction but EPA is divergently incorporated

121   into the phosphatidylinositol (PI) and phosphatidylserine (PS) fraction while DHA is incorporated

122   into the phosphatidylethanolamine (PE) fraction (33).

123   One of the main cellular fates for fatty acids is incorporation into complex lipid species and so it

124   seems logical to hypothesize that the differential action of EPA and DHA may be due to differential

125   effects on the cellular lipidome. To date no study has characterised the impact of EPA and DHA

126   individually on lipidomic profiles in skeletal muscle. In this manuscript we demonstrate that the C_2C_{12}

127   cell line acts as a model in which EPA and DHA have differential effects on metabolism. We

128   followed these experiments by an extensive assessment of lipid changes hypothesizing that the

129   divergent effects of EPA and DHA are associated with differential regulation of the skeletal muscle

130   lipidome. The lipidomic profiling indicated that multiple membrane associated lipid species were

131   differentially altered by EPA and DHA treatments. We therefore hypothesized that the lipidomic

132   remodelling would be associated with remodelling of the membrane-associated proteome. SILAC

133   based proteomics of the membrane fraction indicated EPA and DHA differentially regulate the

134   membrane associated proteome. Therefore, the effects of EPA may be due to membrane associated

135   proteomic remodelling secondary to lipidomic remodelling of the membrane-associated lipids.

136

Materials and methods

Materials

All plasticware for tissue culture was purchased from fisher scientific (UK). Tissue culture media and sera were purchased from Invitrogen. Fatty acids EPA and DHA (>99%, liquid form) were purchased from Sigma Aldrich (Dorset, UK). $H^3$2-DG was purchased from Hartman Analytic. All solvents were LC-MS grade (Fisher Scientific, Loughborough, UK).

Cell culture

C_2C_{12} myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C and 5% C02. Myoblasts were maintained at ~60% confluence. Differentiation was induced once confluence reached 80-90% by changing the media to differentiation media (DMEM supplemented with 2% horse serum and 1% Penicillin/Streptomycin) for 72 hours. Following 72 hours differentiation cells were treated with 50 μM EPA or 50 μM DHA pre-bound to 2% fatty acid free BSA for 72 hours before collection. As a control cells were treated with 2% fatty acid free (FAF) BSA for 72 hours before collection. Fatty acids were conjugated to 2% fatty acid free BSA in DM by constant agitation for 1 hour at 37°C. Following treatment cell pellets were collected following 3 washes in 2% FAF BSA in PBS and centrifuged at 800 rpm for 4 minutes, excess liquid was removed and pellets were frozen in liquid nitrogen and stored at -80°C until further analysis.

FAME analysis

Total lipids were extracted by homogenising in 20 volumes of chloroform/methanol (2:1 v/v). Total lipids were prepared according to the method of Folch et al. (17) and non-lipid impurities were removed by washing with 0.88% (w/v) KCl. The weight of lipids was determined gravimetrically after evaporation of solvent and overnight desiccation under vacuum. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipids according to the method of Christie et al. (35). Extraction and purification of FAME was performed as described by Ghioni et al. (36). FAME were separated by gas-liquid chromatography using a ThermoFisher Trace GC 2000

163   (ThermoFisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60m

164   x 0.25 μm x 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using

165   on-column injection. The temperature gradient was form 50 to 150°C at 40°C/min and then to 195°C

166   at 1.5°C/min and finally to 220°C at 2°C/min. Individual methyl esters were identified by reference to

167   published data (Ackman, 1980). Data were collected and processed using the Chromcard for

168   Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy). All experiments

169   were carried in duplicate from 4 independent experiments. Data were represented as fold change from

170   the respective BSA control condition, logged to $\log_2$ and significance was determined by t-test and

171   corrected for false discovery rate.

172   **Global lipidomic analysis of C$_2$C$_{12}$ myotubes**

173   Lipid extraction was performed according to the method described above. The lipids were analysed by

174   liquid chromatography-mass spectrometry (LC-MS) using a Thermo Exactive Orbitrap mass

175   spectrometer (Thermo Scientific, Hemel Hempstead, UK), equipped with a heated electrospray

176   ionization probe and coupled to a Thermo Accela 1250 UHPLC system. All samples were analysed in

177   both positive and negative ion mode over the mass to charge (m/z) range 200-2000. The lipids were

178   separated on to a Thermo Hypersil Gold C18 column (1.9 μm, 2.1mm x 100 mm,). Mobile phase A

179   consisted of water containing 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B

180   consisted of 90:10 isopropanol/acetonitrile containing 10 mM ammonium formate and 0.1% (v/v)

181   formic acid. The initial conditions for analysis were 65%A/35%B. The percentage of mobile phase B

182   was increased to 100% over 10 min and held for 7 minutes before re-equilibration with the starting

183   conditions for 4 minutes. The raw LC-MS data were processed with Progenesis QI v2.0 software

184   (Non-linear Dynamics, Newcastle, UK) and searched against LIPID MAPS (www.lipidmaps.org) and

185   the Human Metabolome Database (http://www.hmdb.ca/) for identification. All experiments were

186   carried out in duplicate from three independent experiments.

187

188   **Phospholipid profiling of C$_2$C$_{12}$ myotubes**

189 In order to assess the incorporation of EPA and DHA into cellular phospholipids the lipid extracts

190 from C_2C_{12} myotubes were analysed by electrospray ionisation-tandem mass spectrometry (ESI-

191 MS/MS). All analyses were performed using a Thermo TSQ Quantum Ultra triple quadrupole mass

192 spectrometer equipped with a heated electrospray ionization probe. Samples were directly infused into

193 the ion source at a flow rate of 5 µL/min. Phosphatidylcholine, lysophosphatidylcholine and

194 sphingomyelin species were identified by precursor scanning for mass to charge ratio (m/z) 184 in

195 positive ion mode. Phosphatidylethanolamine and lysophosphatidylethanolamine species were

196 identified by neutral loss scanning for m/z 141 in positive ion mode. Phosphatidylserine species were

197 identified by neutral loss scanning for m/z 87 in negative ion mode. Phosphatidylinositol species were

198 identified by precursor scanning for m/z 241 in negative ion mode. The data were expressed as a

199 percentage composition of the relevant phospholipid fraction.

200

201 **Glucose uptake**

202 C2C12 myotubes, were exposed to 50µM EPA or 50µM DHA pre-bound to 2% fatty acid free BSA

203 or 2% fatty acid free BSA as a control for 48 h before a 2 h serum-starve. Following the 2 h serum

204 starve cells were exposed to insulin (100 nmol/l) or vehicle control for 30 mins. Myotubes were

205 incubated (12 min) with 10 µmol/l 2-deoxy-D-[3 H]glucose (2DG; 24.4 kBq/ml; Hartman Analytic) at

206 20°C. Non-specific uptake was determined using 10 µmol/l cytochalasin B (Sigma–Aldrich). After

207 lysis, cell-associated radioactivity was measured (Beckman, High Wycombe, UK; LS 6000IC

208 scintillation counter), and protein was quantified using the Bradford reagent. Data represented are the

209 average of 6 independent experiments carried out in duplicate.

210 **Mitochondrial function**

211 C2C12 myotubes, were exposed to 50µM EPA or 50µM DHA pre-bound to 2% fatty acid free BSA

212 or 2% fatty acid free BSA as a control for 48 h. Following 48 h in the respective treatments cells were

213 degassed and exposed to a mito-stress test in the Seahorse cellular respiration analyser as previously

214 described (16).

Muscle protein synthesis and muscle protein breakdown

Protein degradation was assessed by the quantification of the released L-[2,4,^{3}H] phenylalanine into the culture media. Following 4 days of differentiation myotubes were incubated with medium containing 2.5 µCi L-[2,4,^{3}H] phenylalanine/ml and the label was maintained for 24 hours in order to label long lived proteins. Following the pulse the cells were washed 2x in PBS and incubated in cold chase media (DMEM + 2mM L-phenylalanine) for 3 hours to allow for degradation of short lived proteins. Myotubes were then treated with either 50 µM EPA/DHA bound to 2% FAF-BSA or 2% FAF-BSA alone for 24 hrs. Following treatment an aliquot of the media was removed and radioactivity released was assessed by scintillation counting. The remaining myotubes were then thoroughly washed with ice cold saline (0.9%) and lysed with 50mM NaOH + 1% SDS for a minimum of 30 mins at room temperature. Residual radioactivity in cell lysates was then assessed by scintillation counting. Total radioactivity was calculated as the sum of the L-[2,4,^{3}H] phenylalanine released into the media and the residual cell retained L-[2,4,^{3}H] phenylalanine. Protein breakdown is presented as the fraction of the total incorporated L-[2,4,^{3}H] phenylalanine released into the media.

Basal protein synthesis was assessed by the incorporation of L-[2,4,^{3}H] phenyalalnine into peptide chains. Following differentiation, myotubes were treated with either 50 µM EPA/DHA bound to 2% FAF-BSA or 2% FAF-BSA alone for 24 hrs. At the end of the treatment period the media was removed and DMEM containing 1µCi L-[2,4^{3}H] phenylalanine (0.5 µCi/ml) was added for 180 mins. The reaction was stopped by 2x washes in ice cold saline (0.9%) before 3x washes with Tricholoroacetic acid (TCA) (10%) to remove any unincorporated tracer. Residual TCA was then removed by rinsing cells with methanol and plates left to dry. Myotubes were then lysed in 50mM NaOH + 1% SDS for a minimum of 30 minutes. An aliquot was collected for liquid scintillation counting to assess ^{3}H incorporation into proteins and the remaining lysate was used to determine protein content by the DC protein assay. Protein synthesis is presented as counts per minute/µg of protein.

Protein content

Protein content following 72 h treatment with 50μM EPA or 50μM DHA pre-bound to 2% fatty acid free BSA or 2% fatty acid free BSA as a control was determined by multiplying the concentration of the supernatant [as determined using the bicinchoninic acid protein assay according to the manufacturer's instructions (Sigma Aldrich, UK)] by the total volume of supernatant collected from a 6 well plate. Data are representative of 5 independent experiments carried out in triplicate.

Cell processing

Cell lysates were collected from 6 well plates by scraping on ice in RIPA buffer [50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] followed by snap freezing on liquid nitrogen. For preparation for western blotting samples were thawed and debris was removed by centrifugation at 4°C for 15 min at 13,000 g. The supernatant was then removed, and protein concentration was determined using the bicinchoninic acid protein assay according to the manufacturer's instructions (Sigma Aldrich, UK).

Western blotting

For WB, 100μg of supernatant was made up in Lamelli sample buffer, and 15 μg of total protein was loaded per well and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immunobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V overnight on ice. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature, followed by incubation in primary antibodies [PKBthr308 (#2965) or total PKB (#4691) GLUT 1(Santa cruz, sc-7903), GLUT4 (#2213S), Hexokinase 1 (#2204S), Hexokinase 2 (#2867S), Mito profile (abcam, #ab110413) (New England Biolabs unless stated)] at 4°C overnight. Membranes underwent three 5 min washes in TBST followed by incubation in the appropriate secondary antibodies [secondary horseradish peroxidase conjugated antibody was purchased from ABCAM (#6721)] for 1 h at room temperature. Membranes were again washed three

269 times for 5 min followed by incubation in enhanced chemiluninescence reagent (BioRad, Herts, UK).

270 A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. Phospho-

271 PKB was normalized to the corresponding total protein. Data are representative of 3 independent

272 experiments carried out in duplicate.

273 **Membrane proteome**

274 Proteins associated with membranes were assessed using the SILAC proteomic method (38). C_2C_{12}

275 myoblasts were grown in Dulbeccos' modified eagles medium (DMEM) supplemented with 20%

276 dialysed (10 kKDa) foetal bovine serum plus labelled amino acids lysine and arginine in a humidified

277 atmosphere of 37°C and 5% CO2. Cells intended to act as the control group were grown in with

278 unlabelled lysine and arginine (light), the EPA treatment group were grown in R6K4 media (l-

279 arginine-13C6 hydrochloride, l-lysine-4,4,5,5-d4 hydrochloride[medium]) while the DHA treatment

280 group were grown with R10K8 containing media (l-arginine-13C6, 15N4 hydrochloride, l-lysine-

281 13C6,15N2 hydrochloride [Heavy]). The use of combined labelled arginine and lysine ensures that

282 nearly all peptides will contain a label after tryptic digestion. Cells were allowed to grow for at least 6

283 population doublings to ensure full incorporation of labelled amino acids. We observed that use of

284 dialysed sera and labelled media did not affect doubling time, cell morphology or differentiation

285 capacity. Upon reaching 90-100% confluence the media was replaced with DMEM containing 2%

286 dialysed donor horse serum (10 kDa) to induce differentiation. After 3-4 days of differentiation,

287 myotubes were treated with either control, 50μM EPA or 50 μM DHA for 72 hours. For membrane

288 proteome analysis, membranes were isolated using the Thermo scientific Mem per plus protein

289 membrane extraction kit. The membrane proteome was assessed by LC-MS/MS.

290 **Mass spectrometry**

291 The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an

292 Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). In brief, peptides in 1% (vol/vol)

293 formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After

294 washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250

295 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150

296 min organic gradient, using 7 gradient segments (1-6% solvent B over 1min., 6-15% B over 58min.,

297 15-32%B over 58min., 32-40%B over 5min., 40-90%B over 1min., held at 90%B for 6min and then

298 reduced to 1%B over 1min.) with a flow rate of 300 nl min^{-1}. Solvent A was 0.1% formic acid and

299 Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-

300 electrospray ionization at 2.0 kV using a stainless steel emitter with an internal diameter of 30 μm

301 (Thermo Scientific) and a capillary temperature of 275°C.

302 All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur

303 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. FTMS1 spectra

304 were collected at a resolution of 120 000 over a scan range (m/z) of 350-1550, with an automatic gain

305 control (AGC) target of 300 000 and a max injection time of 100ms. Precursors were filtered using an

306 Intensity Range of 1E4 to 1E20 and according to charge state (to include charge states 2-6) and with

307 monoisotopic precursor selection. Previously interrogated precursors were excluded using a dynamic

308 window (40s +/-10ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a

309 width of 1.4m/z. ITMS2 spectra were collected with an AGC target of 20 000, max injection time of

310 40ms and CID collision energy of 35%.

311 **Quantification and bioinformatics analysis**

312 The raw mass spectrometric data files obtained for each experiment were collated into a single

313 quantitated data set using MaxQuant (version 1.2.2.5) (10) and the Andromeda search engine software

314 (11). Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline

315 residues and between aspartic acid and proline residues. Other parameters used were: (i) variable

316 modifications, methionine oxidation, protein N-acetylation, gln → pyro-glu, Phospho(STY); (ii) fixed

317 modifications, cysteine carbamidomethylation; (iii) database: target-decoy human MaxQuant

318 (ipi.HUMAN.v3.68); (iv) heavy labels: R6K4 and R10K8; (v) MS/MS tolerance: FTMS- 10ppm ,

319 ITMS- 0.6 Da; (vi) maximum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) maximum

320 of labeled amino acids, 3; and (ix) false discovery rate, 1%. Peptide ratios were calculated for each

321 arginine- and/or lysine-containing peptide as the peak area of labeled arginine/lysine divided by the

322 peak area of nonlabeled arginine/lysine for each single-scan mass spectrum. Peptide ratios for all

323   arginine- and lysine-containing peptides sequenced for each protein were averaged. Data is

324   normalised using 1/median ratio value for each identified protein group per labelled sample.

325   **Statistics**

326   Statistical analyses were carried out in Graphpad Prism with ANOVA followed by Tukey's HSD test.

327   For FAME analyses data were assessed in R statistical packages and tested by t-test and corrected by

328   false discovery rate. Statistical significance was determined with a $p < 0.05$. The global lipidomic data

329   sets were subjected to principal component analysis (PCA) and orthogonal projection latent structure-

330   discriminant analysis (OPLS-DA) with Pareto scaling using SIMCA-P v13.0 software (Umetrics,

331   Umea, Sweden). The OPLS-DA models were validated by using the internal cross-validation

332   function. Membrane protein abundance was considered altered if fold change was lower than 0.75 or

333   greater than 1.25. Enrichment of biological processes was determined using gene ontology, using the

334   whole genome of Mus musculus as a background reference list.

335

Results

EPA and DHA substantially increase the abundance of omega-3 species but have differential effects on individual omega-3 fatty acids.

After exposure to 50uM EPA or DHA for 72 hrs C_2C_{12} myotubes were collected for FAME analysis to determine lipid profiles. EPA and DHA significantly increased total omega-3 fatty acid content from baseline values (EPA; 951 ± 81% p=0.0014, DHA; 750 ± 56% p=0.0009) with no significant difference detected between EPA and DHA treatment (p=0.115) (**Figure 1**). The changes in omega-3 abundance are a result of differential shifts in specific omega-3 fatty acids caused by EPA or DHA treatment. The increase in omega-3 content by DHA is a result of accumulation of mainly DHA [22:6 n-3, 3050% ± 310% (**Figure 5**)]. While, incubation of myotubes with EPA results in the accumulation of EPA [(20:5 n-3), 1630% ± 23.38] and DPA [(22:5 n-3), 1318% ± 199.8%] (**Figure 5**). These data suggest that EPA is elongated to DPA whilst DHA remains largely unmodified.

EPA and DHA have differential effects on skeletal muscle glucose uptake.

Insulin stimulated 2-deoxy-glucose (2DG) uptake was determined after 48 h in either EPA or DHA. EPA treatment significantly increased both basal and insulin stimulated 2DG uptake indicating that EPA treatment increases the capacity for glucose uptake (**Figure 2A**). DHA did not have any significant effects on 2DG uptake (**Figure 2A**). The observed changes in 2DG uptake did not appear to be related to any change in insulin stimulated PKB phosphorylation as phospho-blot analysis revealed that insulin stimulated PKB phosphorylation was the same between treatments and controls (**Figure 2B**). In addition EPA/DHA treatment did not appear to affect GLUT1 or GLUT4 expression (**Figure 1C**), nor did they appear to affect the expression of Hexokinase 1 or Hexokinase 2 (**Figure 1D**).

EPA and DHA treatment does not alter mitochondrial respiration.

As no changes in glucose transporters were detected we next assessed whether changes in mitochondrial oxygen consumption may explain the increase in glucose uptake following EPA treatment. C_2C_{12} myotubes were treated with 50uM EPA or DHA for 24 hours. Following treatment

362 multiple inhibitors/uncouplers (Oligomycin, FCCP, rotenone/anitmycin A) were used to probe various

363 parameters of mitochondrial function using the seahorse XF mito stress test. A two way ANOVA

364 found a significant interaction between oxygen consumption and inhibitor compound, indicating the

365 successful manipulation of mitochondrial function (**Figure 3A**). However, fatty acid treatment did not

366 lead to changes in cellular oxygen consumption on any parameter measured (**Figure 3A**). Consistent

367 with the lack of changes in mitochondrial function, there were no alterations in the abundance of ATP

368 synthase and UCQR2 (Complex V and Complex III respectively) (**Figure 3B/C).**

369 **EPA enhances protein accretion through a reduction in protein breakdown while DHA has a**
370 **neutral effect**

371 After a 72 hour incubation with EPA or DHA, myotubes were collected in order to determine total

372 protein content. When myotubes were incubated with EPA total protein content was enhanced while

373 incubation with DHA had a neutral effect (**Figure 4C**). Protein balance is determined by the balance

374 between synthesis and breakdown of proteins. Thus, the observed protein accretion may be reflected

375 in changes in either protein synthesis or breakdown. In order to understand the mechanisms

376 underlying the changes in protein content we directly assessed protein synthesis and breakdown and

377 related signalling processes. Neither fatty acid had any effect on basal protein synthesis after a 24-

378 hour incubation (**Figure 4A**). Anabolic signalling assessed by the phosphorylation status of mTOR,

379 P70S6K1 and 4E-BP1 were not different between groups, corresponding with lack of changes in

380 protein synthesis (**Figure 4E/F**). EPA reduced protein breakdown compared to both the vehicle and

381 DHA treatments (**Figure 4B**). No changes were detected in the level of ubiquitin tagged proteins

382 (**Figure 4D/F**).

383 **EPA treatment differentially regulates DPA levels whilst DHA treatment differentially regulates**

384 **arachidonic acid levels.** As previously mentioned, incubation of C2C12 with 50 µM EPA or 50 µM

385 DHA led to substantial cellular incorporation of total omega-3 fatty acids (**Figure 1A**). This was

386 associated with the above mentioned physiological changes. In order to determine the potential

387 mechanisms by which these effects occur we determined via FAME analysis the fatty acid changes

388 responsible for the increase in total n-3 levels in the cells treated with EPA/DHA. In order to clearly

389 distinguish differential fatty acid shifts we presented the complete fatty acid profiles as fold change

390 [(log2) **Figure 5**]. The most clearly differentiated fatty acid is DPA (22:5 n3) which demonstrates a

391 significant 1318 ± 200% increase with EPA treatment whilst DHA treatment induces a 17.83 ±

392 17.37% decrease in DPA content. Additionally there is a trend (p=0.06) for EPA treatment to increase

393 the DHA content of the cells (37.11 ± 12.62%) suggesting that only a small proportion of the EPA is

394 converted to DHA. Surprisingly arachidonic acid (20:4 n6) was only significantly decreased by DHA

395 treatment (-22.35 ± 3.174%) and remained unaffected by EPA treatment. Intriguingly we also

396 observed that both EPA and DHA increased the content of the saturated fatty acid palmitate (PA 16:0)

397 **(Figure 5**). In order to build a more complete picture of the impact of EPA and DHA on the lipidome

398 we proceeded with a global lipidomics assessment.

399 **Global lipidomics reveals that EPA and DHA treatments induce substantial divergence in the**

400 **lipidome.** Lipid extracts of cells treated with BSA (control), EPA or DHA were analysed by LC-MS

401 in positive and negative ion modes, processed and subjected to multivariate data analysis. PCA

402 highlights any natural clustering or separation within a data set and thereby enables similarities or

403 differences between study groups to be explored. The PCA scores plots of both the positive and

404 negative ion data sets revealed that EPA and DHA supplementation caused a substantial divergence in

405 the lipidome, effectively segregating control and treated cells (**Fig. 6A and 6B**). Having established

406 the existence of clustering behaviour between the sample cohorts more powerful multivariate methods

407 were used to characterise the specific lipid changes responsible for the observed shift in the lipidome

408 of the EPA and DHA treated myotubes. The OPLS-DA scores plots and associated 'S' plots of the

409 positive ion data are shown in **Fig. 7A and 7B**. The results indicated that many of the key

410 discriminating lipids associated with EPA and DHA treatment were phospholipids and in particular

411 molecular species of PC and PE.. In EPA treated cells elevations in PC and PE species containing

412 both 20:5 and 22:5 fatty acids (in agreement with FAME analysis) were observed, whereas there were

413 relative increases in the abundance of phospholipid species with a 22:6 fatty acid in DHA treated

414 cells. The analysis also revealed that both EPA and DHA treatments also resulted in an elevation of

415 PC 32:0, a saturated species. DHA was also found to be incorporated into a number of triglyceride

416   species (see supplemental). In order to fully understand the impact of EPA and DHA treatment we

417   followed up these experiments through the targeted analysis of the myotube phospholipids.

418   **Targeted phospholipid analysis reveals that EPA and DHA increase the fraction of lipid species**

419   **containing long chains and 5 or more double bonds at the expense of shorter chain, less**

420   **saturated species.** ESI-MS/MS was utilised to characterise the profiles of myocyte phospholipid

421   classes. Representative mass spectra of PC and PE are shown in **Figure 8A and 8B**. The general

422   trends (**Figure 9**) in both EPA and DHA were similar with the displacement of shorter chain in

423   apparent preference for longer chain highly unsaturated fatty acids. However, the magnitude of

424   change for these displacements was often higher with DHA. There was no evidence of alterations in

425   the profile of sphingomyelin species in response to fatty acid treatments. ESI-MS/MS analysis of PS

426   and PI was also performed however the low signal intensities of these lipids did not permit a robust

427   quantification. Interestingly EPA and DHA supplementation led to a higher abundance of

428   phospholipid species containing saturated fatty acids. For instance DHA induced an increase in PC

429   (32:0) and lysophosphatidylethanolamine (LPE) (16:0), whilst EPA induced an increase in PC (32:0,)

430   LPC (16:0), (18:0) and LPE (18:0). It is therefore evident that in spite of significant increases in

431   saturated fatty acids in these EPA and DHA treatment still improve or maintain glucose uptake

432   respectively.

433   **Altered composition of phospholipids is associated with altered membrane associated proteomic**

434   **profiles.**

435   As the lipidomic remodelling indicated substantial changes induced by n-3 treatment in the membrane

436   associated lipid species we carried out SILAC based proteomics profiling of the membrane fraction to

437   assess if the lipid remodelling altered the proteins in the membrane fraction. Over 3000 proteins

438   associated with the membrane compartment were identified in the SILAC screen. These results were

439   filtered down to 625 proteins (see supplemental) with a coefficient of variance $\leq 5\%$ to describe

440   consistent changes in membrane abundance. Proteins were considered enriched or reduced in the

441   membrane with a fold change cut off of 0.25. Proteins similarly affected by EPA and DHA were

442   removed from analysis. Proteins with altered abundance were then subject to gene ontology analysis

443   for biological processes (see supplemental). Membrane proteins altered by EPA were associated with

444   protein folding (p = 4.76E-03). Additionally, these proteins were subjected to String analysis which

445   revealed that these proteins were highly likely to interact with eachother (Figure 10B). EPA also

446   increased Calumenin in the membrane fraction (1.34 fold). Interestingly, Calumenin plays a role in

447   calcium sensitive protein folding (49). DHA altered proteins associated with a number of processes,

448   primarily related to oxidative metabolism and ribosomal formation (see table 1). Further examination

449   of ribosomal proteins revealed that DHA induced the significant reduction in ribosomal proteins

450   associated with both small and large subunits at the membrane (Figure 10C). Conversely, EPA

451   induced a small increase in ribosomal proteins (**Figure 10C**).

452

Discussion

This study is the first to carry out a comprehensive analysis of the lipidomic profiles of a skeletal muscle cell line in response to two differentially bioactive n-3 fatty acids. Furthermore, it is the first study, to our knowledge, to combine this with a profile of the membrane associated proteome. We clearly demonstrate the differential metabolic activities of EPA vs DHA in the C_2C_{12} skeletal muscle cell line and provide data demonstrating the differential impact that EPA and DHA have on the skeletal muscle lipidome. Our data suggests that the bioactivity of EPA may be due to its preferential incorporation (and possibly elongation to DPA) into the phospholipid fraction where it substantially alters the long chain polyunsaturated fatty acid composition of major phospholipid classes. Likely secondary to the alterations in membrane associated phospholipids we see an altered membrane associated proteome. These changes in the membrane lipid-protein composition may be a key driver for the metabolic effects of n3 fatty acids.

Similar to previous cell culture based studies (1, 21), we show that EPA has a positive effect on glucose uptake. Both basal and insulin stimulated muscle glucose uptake were improved by EPA but not DHA. The increase in glucose uptake appears to be independent of changes in PKB signalling as measured by phosphorylation status which suggests that enhanced proximal insulin and possibly PKB signalling are not part of the mechanism of action. Furthermore, there was no significant change to the expression of the glucose transporters GLUT1/4, hexokinase1/2 or the mitochondrial enzymes UQCRC2 and ATP-synthase. Nor was there any significant change in mitochondrial function as assessed by the mitochondrial stress test. These data are difficult to consolidate, however, we hypothesize that the mechanism of action of EPA on glucose uptake may be dependent less upon changes in protein expression and more dependent upon protein localisation perhaps improving the functional coupling of glucose metabolism enzymes.

In addition to the EPA induced improvements in glucose uptake we also noted a significant improvement in protein accretion with EPA treatment, while DHA showed no significant effect. To determine the mechanism by which cells treated with EPA accumulate more protein we assessed

479 muscle protein synthesis and muscle protein breakdown. While Kamolrat et al (24) observed

480 enhanced leucine stimulated MPS following EPA treatment, we detected no significant changes in

481 basal muscle protein synthesis or the phosphorylation of anabolic signalling markers. Instead, we

482 determined that the effect of EPA on protein accretion was likely driven by an ~10% reduction in

483 muscle protein breakdown. Analysis of global ubiquitination via western blotting indicates that the

484 reduction in muscle protein breakdown may not be driven by a change in the activity of the ubiquitin

485 system. Rather, it may be driven by reduced lysosomal degradation (9). In saying that we must

486 concede however, that a 10% reduction in ubiquitin driven protein breakdown would be challenging

487 to detect via western blotting.

488 In an effort to determine the molecular mechanism of action of EPA on skeletal muscle glucose

489 uptake and protein accretion we tested the hypothesis that EPA and DHA treatments would induce

490 significant lipid remodelling leading to remodelling of the cellular proteome. We found that while the

491 total n-3 content was similar between treatments, EPA resulted in a larger variation in lipid species

492 accumulating mainly as EPA and DPA and to a lesser extent DHA while DHA treatment mainly

493 resulted in DHA accumulation with a decrease in DPA and a limited retro-conversion to EPA. The

494 main differentially regulated fatty acids were DPA, increased in EPA and decreased in DHA, while

495 only DHA decreased AA. Interestingly DPA accumulated to a similar extent as EPA [1630% (EPA)

496 vs 1318% (DPA)], findings consistent with previous literature in other tissues (2, 26). Given that DPA

497 increased to a similar extent to EPA we are unable to determine whether it is EPA or DPA that is the

498 main driver behind the metabolic effects observed. These data suggest that upon intake into the cell

499 EPA is elongated to DPA and to a lesser extent DHA. The elongation of EPA to DPA but not DHA

500 may be explained by the differential affinities of the desaturases and elongases involved in fatty acid

501 metabolism. In the n-3 pathway elovl2 catalyzes the conversion of EPA → DPA → 24:5 n-3, the

502 precursor to DHA. However, increasing EPA concentrations is known to lower the saturation point in

503 the conversion of DPA → 24:5 n-3 which may play a role the accumulation of DPA without being

504 further metabolised to DHA (19). Our data indicate that one of the primary fates of EPA and DHA

505 was incorporation into the phospholipid fraction. In the global lipidomics screen we found EPA or

506   DPA containing lipid species associated with the phospholipid pool whilst DHA containing species

507   were often associated with the TAG pool whilst EPA containing species were rarely associated with

508   the TAG pool (see supplemental spread sheet). Fatty acids in the TAG pool are stored in discrete lipid

509   droplets and therefore may be less metabolically active than the phospholipids associated with the

510   membranes. This differential incorporation into the various lipid pools may partially explain the

511   beneficial metabolic effects of EPA.

512   The potential relevance of DPA as a mediator of many of the physiological effects of n-3

513   supplementation is beginning to be further understood. DPA more potently inhibits platelet

514   aggregation than EPA or DHA (3) as well as more potently stimulating endothelial cell migration than

515   EPA or DHA (25). In macrophages EPA is a known inhibitor of the cyclooxygenase pathway and

516   elongation to DPA is an important factor in this inhibition (36). We would suggest that the elongation

517   of EPA to DPA seen in our study may also have important physiological roles in the increase in

518   skeletal muscle glucose uptake by EPA. As with the global lipidomic analysis we observed the

519   incorporation of long chain PUFAs into phospholipid species mainly at the expense of specific SFAs

520   and MUFAs, however some specific SFAs were increased by both EPA and DHA. We identified

521   multiple differentially regulated phospholipid species across PE, PC, LPE and LPC classes. In the

522   EPA treated group a number of species were enriched by long chain PUFAs with 5 or more double

523   bonds in addition to a number of EPA or DPA containing phospholipids in the PC and PE, fractions In

524   comparison, the DHA treated group increased the long chain PUFA containing phospholipids but did

525   not increase or increase as much as EPA the content of species containing 22:5 or 40:5. Interestingly,

526   with DHA treatment there was a trend for an increase in the palmitate containing lyso-PE content

527   above that of control and EPA. Additionally, while it appears that many phospholipids containing

528   saturated fatty acids are displaced in favour of polyunsaturated containing phospholipids we observe a

529   rise in PC (32:0) with both EPA and DHA, which may reflect the rise in palmitate observed with

530   FAME analysis. Incorporation of PUFAs into phospholipids is known to increase membrane fluidity

531   and we speculate that this increase may be a compensatory mechanism to maintain a base level of

532   membrane rigidity.

533 An attractive mechanism for the differential effects of EPA and DHA may lie in the reduced

534 production of different inflammatory eicosanoids. However, only DHA reduced the total amount of

535 AA. Additionally, the displacement of phospholipids alongside a lack of inflammatory stimulus

536 suggests it is unlikely that this would mediate the metabolic differences seen in this model. The G

537 protein coupled receptor GPR120 has previously been identified as a general n-3 sensor in a number

538 of tissues except skeletal muscle that elicits potent anti-inflammatory and consequently insulin

539 sensitizing effects (37). To our knowledge no such receptor exists in skeletal muscle that can

540 discriminate between EPA and DHA deeming it unlikely that the observed differential effects are

541 mediated by EPA or DHA through specific receptor activated signalling.

542 Phospholipid species are not merely inert structural components of cellular membranes and their

543 various roles in intracellular processes are beginning to be further understood. PS and PE are related

544 phospholipid species found predominantly in the inner membrane and contribute to the membrane

545 targeting and activation and modification of protein kinases [as reviewed (51)]. It has also previously

546 been seen that the lyso-PC, a hyrdrolyzed form of phospholipid, stimulated adipocyte glucose uptake

547 in a manner dependent upon chain length and saturation of the acyl group (54). We observed an

548 incorporation of long chain PUFAs (possibly EPA and DPA) into the PC, PS, PE and certain lyso-PL

549 species and therefore cannot discount that the change in acyl chain length and unsaturation level alters

550 the function of these PL species and leads to an increase in glucose uptake through a currently

551 unknown mechanism. Because, the lipid composition of the membrane can alter the targeting of

552 various proteins to the membrane (50, 51) we speculated that part of the mechanism of action of EPA

553 might be via a change in the composition of the membrane associated proteome. To test this theory

554 we carried out a 3 way SILAC experiment on the membrane fractions of cells treated with vehicle,

555 EPA or DHA.

556 Our SILAC experiment illustrated the proof of concept that the incorporation of EPA and DHA into

557 phospholipid species was associated with the alteration of proteins interacting with the membrane

558 compartment. There are a number of mechanisms by which proteins can bind to the membrane which

559 are influenced by the fatty acid composition of the lipid bilayers (50, 51). Gene ontology analysis

560   indicated that proteins associated with protein folding at the membrane were overrepresented

561   following EPA incorporation. There is experimental evidence that a number of the proteins identified

562   with this process interact, as probed by the STRING database. Furthermore, analysis of ribosomal

563   proteins indicated a small but significant shift of ribosomal proteins towards the membrane fraction in

564   EPA treated cells. Since the endoplasmic reticulum is a membranous structure these data suggest that

565   EPA is increasing the content of ribosomes at the ER. This shift in ribosomes towards the ER could

566   lead to improved fidelity of protein production as the ER is key to protein quality control (15). We

567   propose a mechanism in which protein folding is enhanced, thereby enhancing the fidelity with which

568   proteins are synthesised thereby reducing protein breakdown leading to increased protein accretion.

569   Further experimental work is needed to confirm this hypothesis since mis-folded proteins tend to be

570   degraded by the ubiquitin-proteasome system (9) and our data indicated that global ubiquitination was

571   not reduced by EPA. By comparison, DHA caused a striking reduction in the abundance of a number

572   of ribosomal proteins with the membrane fraction. If future work confirms that total ribosomal content

573   is unchanged with these conditions then these data would indicate an increase in cytosolic ribosomes.

574   This differential shift in ribosomal compartmentalisation may indicate a shift in protein expression

575   profiles. Proteins that enter the secretory pathways or integral membrane proteins are synthesised in

576   the ER while other proteins are translated in the cytosolic ribosome pool (41).

577   DHA also altered the abundance of proteins involved in ATP coupled proton transport and acetyl-

578   CoA metabolism. The proteins identified with ATP synthesis coupled transport were mainly

579   downregulated proteins in the ATP synthase complex. This would be expected to manifest as a

580   reduced ability to generate ATP yet no changes were observed in ATP synthase dependent oxygen

581   consumption. The effects of DHA may not have been severe enough to observe at basal levels.

582   Although maximal respiration is measured during the mito stress test, it is induced by uncoupling so

583   may not be indicative of changes in ATP synthesis. Previous studies have observed that omega-3 fatty

584   acids can alter mitochondrial function, altering ADP kinetics without altering maximal respiration

585   (20). It remains to be seen if this reduction in ATP synthase proteins would alter the mitochondrial

586   response to cellular stress or changing substrate availability.

587 In summary, we demonstrate that EPA and DHA display divergent metabolic activities in a skeletal

588 muscle cell line, which may be partially mediated by differential remodelling of the lipidome. We

589 speculate that the remodelling of the membrane-associated proteome is secondary to the changes

590 observed in the saturation profile of the membrane-associated phospholipid species. Whilst the

591 proteomic data did not reveal a mechanism for the effects of EPA on glucose uptake, our data support

592 the proof of concept that a redistribution of the proteome may be responsible. Gene ontology analysis

593 of the proteomic data indicate that the mechanism of action of EPA on protein metabolism may be

594 driven by an improved fidelity with which proteins are synthesised. Based on the shifts in ribosomal

595 proteins found in the membrane fractions future work should determine if EPA and DHA alter the

596 transcript profiles in various ribosomal fractions.

597

598   References

599   1.   **Aas V, Rokling-Andersen MH, Kase ET, Thoresen GH, and Rustan AC**. Eicosapentaenoic acid
600   (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. *J Lipid Res*
601   47: 366-374, 2006.
602   2.   **Achard F, Benistant C, and Lagarde M**. Interconversions and distinct metabolic fate of
603   eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in bovine aortic endothelial cells.
604   *Biochim Biophys Acta* 1255: 260-266, 1995.
605   3.   **Akiba S, Murata T, Kitatani K, and Sato T**. Involvement of lipoxygenase pathway in
606   docosapentaenoic acid-induced inhibition of platelet aggregation. *Biol Pharm Bull* 23: 1293-1297,
607   2000.
608   4.   **Akinkuolie AO, Ngwa JS, Meigs JB, and Djousse L**. Omega-3 polyunsaturated fatty acid and
609   insulin sensitivity: a meta-analysis of randomized controlled trials. *Clin Nutr* 30: 702-707, 2011.
610   5.   **Alexander JW, Saito H, Trocki O, and Ogle CK**. The importance of lipid type in the diet after
611   burn injury. *Ann Surg* 204: 1-8, 1986.
612   6.   **Andersson A, Nalsen C, Tengblad S, and Vessby B**. Fatty acid composition of skeletal muscle
613   reflects dietary fat composition in humans. *Am J Clin Nutr* 76: 1222-1229, 2002.
614   7.   **Browning LM, Walker CG, Mander AP, West AL, Gambell J, Madden J, Calder PC, and Jebb
615   SA**. Compared with daily, weekly n-3 PUFA intake affects the incorporation of eicosapentaenoic acid
616   and docosahexaenoic acid into platelets and mononuclear cells in humans. *J Nutr* 144: 667-672,
617   2014.
618   8.   **Burdge GC, and Calder PC**. Introduction to fatty acids and lipids. *World Rev Nutr Diet* 112: 1-
619   16, 2015.
620   9.   **Ciechanover A**. Intracellular protein degradation: from a vague idea thru the lysosome and
621   the ubiquitin-proteasome system and onto human diseases and drug targeting. *Cell Death Differ* 12:
622   1178-1190, 2005.
623   10.   **Cox J, and Mann M**. MaxQuant enables high peptide identification rates, individualized
624   p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26: 1367-
625   1372, 2008.
626   11.   **Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, and Mann M**. Andromeda: a
627   peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 10: 1794-1805,
628   2011.
629   12.   **Da Boit M, Sibson R, Sivasubramaniam S, Meakin JR, Greig CA, Aspden RM, Thies F,
630   Jeromson S, Hamilton DL, Speakman JR, Hambly C, Mangoni AA, Preston T, and Gray SR**. Sex
631   differences in the effect of fish oil supplementation on the adaptive response to resistance exercise
632   training in older people: a randomized control trial. *Am J Clin Nutr* 2016.
633   13.   **Dangardt F, Chen Y, Gronowitz E, Dahlgren J, Friberg P, and Strandvik B**. High physiological
634   omega-3 Fatty Acid supplementation affects muscle Fatty Acid composition and glucose and insulin
635   homeostasis in obese adolescents. *J Nutr Metab* 2012: 395757, 2012.
636   14.   **Di Girolamo FG, Situlin R, Mazzucco S, Valentini R, Toigo G, and Biolo G**. Omega-3 fatty
637   acids and protein metabolism: enhancement of anabolic interventions for sarcopenia. *Curr Opin Clin
638   Nutr Metab Care* 17: 145-150, 2014.
639   15.   **Ellgaard L, and Helenius A**. Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell
640   Biol* 4: 181-191, 2003.
641   16.   **Findlay JA, Hamilton DL, and Ashford ML**. BACE1 activity impairs neuronal glucose
642   oxidation: rescue by beta-hydroxybutyrate and lipoic acid. *Front Cell Neurosci* 9: 382, 2015.
643   17.   **Folch J, Lees M, and Sloane Stanley GH**. A simple method for the isolation and purification
644   of total lipides from animal tissues. *J Biol Chem* 226: 497-509, 1957.
645   18.   **Franekova V, Angin Y, Hoebers NT, Coumans WA, Simons PJ, Glatz JF, Luiken JJ, and Larsen
646   TS**. Marine omega-3 fatty acids prevent myocardial insulin resistance and metabolic remodeling as
647   induced experimentally by high insulin exposure. *Am J Physiol Cell Physiol* 308: C297-307, 2015.

648 19.   **Gregory MK, Gibson RA, Cook-Johnson RJ, Cleland LG, and James MJ**. Elongase reactions as
649 control points in long-chain polyunsaturated fatty acid synthesis. *PLoS One* 6: e29662, 2011.
650 20.   **Herbst EA, Paglialunga S, Gerling C, Whitfield J, Mukai K, Chabowski A, Heigenhauser GJ,**
651 **Spriet LL, and Holloway GP**. Omega-3 supplementation alters mitochondrial membrane composition
652 and respiration kinetics in human skeletal muscle. *J Physiol* 592: 1341-1352, 2014.
653 21.   **Hessvik NP, Bakke SS, Fredriksson K, Boekschoten MV, Fjorkenstad A, Koster G, Hesselink**
654 **MK, Kersten S, Kase ET, Rustan AC, and Thoresen GH**. Metabolic switching of human myotubes is
655 improved by n-3 fatty acids. *J Lipid Res* 51: 2090-2104, 2010.
656 22.   **Huang F, Wei H, Luo H, Jiang S, and Peng J**. EPA inhibits the inhibitor of kappaBalpha
657 (IkappaBalpha)/NF-kappaB/muscle RING finger 1 pathway in C2C12 myotubes in a PPARgamma-
658 dependent manner. *Br J Nutr* 105: 348-356, 2011.
659 23.   **Jucker BM, Cline GW, Barucci N, and Shulman GI**. Differential effects of safflower oil versus
660 fish oil feeding on insulin-stimulated glycogen synthesis, glycolysis, and pyruvate dehydrogenase flux
661 in skeletal muscle: a 13C nuclear magnetic resonance study. *Diabetes* 48: 134-140, 1999.
662 24.   **Kamolrat T, and Gray SR**. The effect of eicosapentaenoic and docosahexaenoic acid on
663 protein synthesis and breakdown in murine C2C12 myotubes. *Biochem Biophys Res Commun* 432:
664 593-598, 2013.
665 25.   **Kanayasu-Toyoda T, Morita I, and Murota S**. Docosapentaenoic acid (22:5, n-3), an
666 elongation metabolite of eicosapentaenoic acid (20:5, n-3), is a potent stimulator of endothelial cell
667 migration on pretreatment in vitro. *Prostaglandins Leukot Essent Fatty Acids* 54: 319-325, 1996.
668 26.   **Kaur G, Sinclair AJ, Cameron-Smith D, Barr DP, Molero-Navajas JC, and Konstantopoulos N**.
669 Docosapentaenoic acid (22:5n-3) down-regulates the expression of genes involved in fat synthesis in
670 liver cells. *Prostaglandins Leukot Essent Fatty Acids* 85: 155-161, 2011.
671 27.   **Lamaziere A, Wolf C, Barbe U, Bausero P, and Visioli F**. Lipidomics of hepatic lipogenesis
672 inhibition by omega 3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 88: 149-154, 2013.
673 28.   **Lanza IR, Blachnio-Zabielska A, Johnson ML, Schimke JM, Jakaitis DR, Lebrasseur NK,**
674 **Jensen MD, Sreekumaran Nair K, and Zabielski P**. Influence of fish oil on skeletal muscle
675 mitochondrial energetics and lipid metabolites during high-fat diet. *Am J Physiol Endocrinol Metab*
676 304: E1391-1403, 2013.
677 29.   **Magee P, Pearson S, Whittingham-Dowd J, and Allen J**. PPARgamma as a molecular target
678 of EPA anti-inflammatory activity during TNF-alpha-impaired skeletal muscle cell differentiation. *J*
679 *Nutr Biochem* 23: 1440-1448, 2012.
680 30.   **McGlory C, Galloway SD, Hamilton DL, McClintock C, Breen L, Dick JR, Bell JG, and Tipton**
681 **KD**. Temporal changes in human skeletal muscle and blood lipid composition with fish oil
682 supplementation. *Prostaglandins Leukot Essent Fatty Acids* 90: 199-206, 2014.
683 31.   **Mori TA, Burke V, Puddey IB, Watts GF, O'Neal DN, Best JD, and Beilin LJ**. Purified
684 eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and
685 lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am J Clin Nutr* 71:
686 1085-1094, 2000.
687 32.   **Mori TA, Codde JP, Vandongen R, and Beilin LJ**. New findings in the fatty acid composition
688 of individual platelet phospholipids in man after dietary fish oil supplementation. *Lipids* 22: 744-750,
689 1987.
690 33.   **Morisaki N, Kanzaki T, Fujiyama Y, Osawa I, Shirai K, Matsuoka N, Saito Y, and Yoshida S**.
691 Metabolism of n-3 polyunsaturated fatty acids and modification of phospholipids in cultured rabbit
692 aortic smooth muscle cells. *J Lipid Res* 26: 930-939, 1985.
693 34.   **Murphy RA, Mourtzakis M, and Mazurak VC**. n-3 polyunsaturated fatty acids: the potential
694 role for supplementation in cancer. *Curr Opin Clin Nutr Metab Care* 15: 246-251, 2012.
695 35.   **Neschen S, Morino K, Dong J, Wang-Fischer Y, Cline GW, Romanelli AJ, Rossbacher JC,**
696 **Moore IK, Regittnig W, Munoz DS, Kim JH, and Shulman GI**. n-3 Fatty acids preserve insulin
697 sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner.
698 *Diabetes* 56: 1034-1041, 2007.

699   36.   **Norris PC, and Dennis EA**. Omega-3 fatty acids cause dramatic changes in TLR4 and
700   purinergic eicosanoid signaling. *Proc Natl Acad Sci U S A* 109: 8517-8522, 2012.
701   37.   **Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, and
702   Olefsky JM**. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and
703   insulin-sensitizing effects. *Cell* 142: 687-698, 2010.
704   38.   **Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, and Mann M**. Stable
705   isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to
706   expression proteomics. *Mol Cell Proteomics* 1: 376-386, 2002.
707   39.   **Ries A, Trottenberg P, Elsner F, Stiel S, Haugen D, Kaasa S, and Radbruch L**. A systematic
708   review on the role of fish oil for the treatment of cachexia in advanced cancer: an EPCRC cachexia
709   guidelines project. *Palliative medicine* 26: 294-304, 2012.
710   40.   **Rodacki CL, Rodacki AL, Pereira G, Naliwaiko K, Coelho I, Pequito D, and Fernandes LC**.
711   Fish-oil supplementation enhances the effects of strength training in elderly women. *Am J Clin Nutr*
712   95: 428-436, 2012.
713   41.   **Ron D**. Translational control in the endoplasmic reticulum stress response. *The Journal of
714   Clinical Investigation* 110: 1383-1388, 2002.
715   42.   **Sjovall P, Rossmeisl M, Hanrieder J, Kuda O, Kopecky J, and Bryhn M**. Dietary uptake of
716   omega-3 fatty acids in mouse tissue studied by time-of-flight secondary ion mass spectrometry (TOF-
717   SIMS). *Anal Bioanal Chem* 407: 5101-5111, 2015.
718   43.   **Smith GI, Atherton P, Reeds DN, Mohammed BS, Rankin D, Rennie MJ, and Mittendorfer B**.
719   Dietary omega-3 fatty acid supplementation increases the rate of muscle protein synthesis in older
720   adults: a randomized controlled trial. *Am J Clin Nutr* 93: 402-412, 2011.
721   44.   **Smith GI, Atherton P, Reeds DN, Mohammed BS, Rankin D, Rennie MJ, and Mittendorfer B**.
722   Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to
723   hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged men and women. *Clin
724   Sci (Lond)* 121: 267-278, 2011.
725   45.   **Smith GI, Julliand S, Reeds DN, Sinacore DR, Klein S, and Mittendorfer B**. Fish oil-derived n-
726   3 PUFA therapy increases muscle mass and function in healthy older adults. *Am J Clin Nutr* 102: 115-
727   122, 2015.
728   46.   **Stephens FB, Mendis B, Shannon CE, Cooper S, Ortori CA, Barrett DA, Mansell P, and
729   Tsintzas K**. Fish oil omega-3 fatty acids partially prevent lipid-induced insulin resistance in human
730   skeletal muscle without limiting acylcarnitine accumulation. *Clin Sci (Lond)* 127: 315-322, 2014.
731   47.   **Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, and Kraegen EW**. Influence of
732   dietary fat composition on development of insulin resistance in rats. Relationship to muscle
733   triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes* 40: 280-289, 1991.
734   48.   **Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG, and Pascoe WS**. Fish oil prevents
735   insulin resistance induced by high-fat feeding in rats. *Science* 237: 885-888, 1987.
736   49.   **Tripathi R, Benz N, Culleton B, Trouve P, and Ferec C**. Biophysical characterisation of
737   calumenin as a charged F508del-CFTR folding modulator. *PLoS One* 9: e104970, 2014.
738   50.   **Vance JE**. Phospholipid synthesis and transport in mammalian cells. *Traffic* 16: 1-18, 2015.
739   51.   **Vance JE, and Tasseva G**. Formation and function of phosphatidylserine and
740   phosphatidylethanolamine in mammalian cells. *Biochim Biophys Acta* 1831: 543-554, 2013.
741   52.   **Wang Y, Lin QW, Zheng PP, Zhang JS, and Huang FR**. DHA inhibits protein degradation more
742   efficiently than EPA by regulating the PPARgamma/NFkappaB pathway in C2C12 myotubes. *Biomed
743   Res Int* 2013: 318981, 2013.
744   53.   **Woodman RJ, Mori TA, Burke V, Puddey IB, Watts GF, Best JD, and Beilin LJ**.
745   Docosahexaenoic acid but not eicosapentaenoic acid increases LDL particle size in treated
746   hypertensive type 2 diabetic patients. *Diabetes Care* 26: 253, 2003.
747   54.   **Yea K, Kim J, Yoon JH, Kwon T, Kim JH, Lee BD, Lee HJ, Lee SJ, Kim JI, Lee TG, Baek MC, Park
748   HS, Park KS, Ohba M, Suh PG, and Ryu SH**. Lysophosphatidylcholine activates adipocyte glucose

749 uptake and lowers blood glucose levels in murine models of diabetes. *J Biol Chem* 284: 33833-33840,
750 2009.
751 55.   **You JS, Park MN, Song W, and Lee YS**. Dietary fish oil alleviates soleus atrophy during
752 immobilization in association with Akt signaling to p70s6k and E3 ubiquitin ligases in rats. *Appl*
753 *Physiol Nutr Metab* 35: 310-318, 2010.

754

ACKNOWLEDGEMENTS

761

Figure Legends

Figure 1. EPA and DHA enhance n-3 content to a similar degree. C_2C_{12} myotubes were incubated

in either fatty acid free 2% BSA or fatty acid free 2% BSA pre-conjugated to 50 µM EPA or 50 µM

DHA for 72hrs. Fatty acid analysis was carried out by FAME analysis (n=4 in duplicate) and data are

presented as % change from BSA control grouped by fatty acid species; SFA – saturated fatty acids,

MUFA – monounsaturated fatty acids, n-9 PUFA – omega-9 polyunsaturated fatty acids, n-6 PUFA –

omega-6 polyunsaturated fatty acids, n-3 PUFA – omega-3 polyunsaturated fatty acids.

Figure 2. EPA and DHA have differential effects on C_2C_{12} glucose uptake independently of

changes in PKB Thr308 phosphorylation or GLUT1/4 and Hexokinase1/2 expression. Glucose

uptake was determined using a radiolabelled 2 Deoxy-glucose uptake assay (n=6 in duplicate) (A) and

PKB Thr308 phosphorylation (n=4 in duplicate) (B) was determined using SDS-PAGE and phospho-

specific antibodies, the signal for which was normalised to t-PKB expression (representative blots are

inset). GLUT1/4 expression (C) were normalised to t-eEF2 whilst Hexokinase1/2 expression (D) were

normalised to α-tubulin. Bars not connected by the same letter are significantly different from each

other (p<0.05).

Figure 3. EPA and DHA treatment do not modulate mitochondrial function or mitochondrial

protein expression. C_2C_{12} myotubes were incubated in either fatty acid free 2% BSA or fatty acid

free 2% BSA pre-conjugated to 50 µM EPA or 50 µM DHA for 48hrs. One set of myotubes was

exposed to a mitochondrial stress test in the SeaHorse XFA and oxygen consumption was assessed

(A). A parallel set of myotubes were collected for western blot analysis of mitochondrial protein

expression with the mito-blot panel (B). Representative blots are shown in (C).

Figure 4. EPA enhances protein accretion through a reduction in protein breakdown. A) Protein

synthesis was measured by a L-[2,4,^{3}H] phenylalanine incorporation assay following a 24 hr

incubation with n-3 fatty acids (n=5 in duplicate) B) protein breakdown was assessed by the release of

L-[2,4,^{3}H] phenylalanine into culture media after 24hr treatment with n-3 fatty acids (n=10, in

duplicate) C) total protein content was assessed after a 72hr incubation with EPA or DHA (n=5, in

788 duplicate) D) Phosphorylation of proteins within the mTOR signalling pathway were assessed by

789 western blotting after a 24 hr incubation with n-3 fatty acids (n=3 in duplicate) E) ubiquitination of

790 proteins were assessed by western blotting after a 24hr incubation with fatty acids (n=3 in duplicate).

791 # indicates significantly different from Veh and DHA, * indicates significantly different from

792 corresponding control condition (p<0.05).

793 **Figure 5. Complete fatty acid profiles reveal a number of fatty acids differentially regulated by**

794 **EPA and DHA.** C_2C_{12} myotubes were incubated in either fatty acid free 2% BSA or fatty acid free

795 2% BSA pre-conjugated to 50 µM EPA or 50 µM DHA for 72hrs (n=4 in duplicate). Cells were

796 pelleted and washed 3x using PBS with 2% fatty acid free BSA. Fatty acid analysis was carried out by

797 FAME analysis. The fold change was determined from the BSA control condition and logged (log2).

798 ND indicates non detectable, * indicates significant difference between EPA vs DHA (p<0.05).

799 **Figure 6. Global lipidomic analysis characterises shifts in lipid composition of C_2C_{12} myotubes.**

800 Principal component analysis (PCA) scores plots of lipid profiles generated by LC-MS in (A) positive

801 ion and (B) negative ion modes. Cultured cells were incubated with either fatty acid free 2% BSA

802 (green circles), fatty acid free 2% BSA pre-conjugated to 50 µM EPA (red triangles) or 50 µM DHA

803 (blue squares) for 72hrs. Global lipidomic analysis was performed on the cells and the data sets were

804 subjected to PCA with Pareto scaling. Each point represents a single cell sample (n=6). The PCA

805 revealed that the control, EPA and DHA groups could be discriminated on the basis of their lipid

806 profiles. The control cells were found to cluster in one area of the scores plot whilst cells treated with

807 EPA or DHA appeared in regions away from the controls indicating that were alterations in their lipid

808 composition as a result of the fatty acid treatments.

809 **Figure 7. Lipidomic profiling reveals cellular lipid species containing EPA, DPA and DHA.**

810 Positive ion orthogonal partial least-squares discriminant analysis (OPLS-DA) for (A) BSA vs DHA

811 and (B) BSA vs EPA (n=6). Green circles indicate BSA treated C_2C_{12} myotubes; blue squares indicate

812 DHA treated C_2C_{12} myotubes and red triangles indicate EPA treated C_2C_{12} myotubes. The OPLS-DA

813 scores plots indicated that EPA and DHA groups had distinct lipid profiles compared to the controls.

814 In order to determine the lipids responsible for the inter-class differences associated S-plots of

815   covariance versus the correlation were generated. Each point in the S-plots represents a lipid detected

816   in the LC-MS analysis with the lipids at the top and bottom of the plots showing the greatest changes.

817   The analysis revealed that there was a relative increase in the abundance of lipid species containing

818   20:5, 22:5 or 22:6 fatty acids in EPA and DHA treated cells respectively.

819

820   **Figure 8. Targeted phospholipid analysis indicates preferential incorporation of long chain**

821   **polyunsaturated fatty acids into specific phospholipid classes.** C_2C_{12} myotubes were solvent

822   extracted and molecular species of (A) phosphatidylcholine and (B) phosphatidylethanolamines were

823   detected by ESI-MS/MS in positive-ion mode by means of a precursor ion scan for m/z 184 and a

824   neutral loss scan of m/z 141 respectively.

825   **Figure 9. Targeted phospholipid analysis presented as % abundance for PC, PE, LPC and LPE**

826   **species.** C_2C_{12} myotubes were solvent extracted and molecular species of (A) lyso-

827   phosphatidylcholine, (B) phosphatidylcholine, (C) lyso-phosphatidylethanolamine and (D)

828   phosphatidylethanolamine were detected by ESI-MS/MS in positive-ion mode by means of a

829   precursor ion scan for m/z 184 and a neutral loss scan of m/z 141 respectively. Data are presented as

830   % abundance ± SEM. Bars not connected by the same letter are significantly different from one

831   another ($p < 0.05$).

832   **Figure 10. Membrane associated proteomics reveals protein folding machinery and ribosomal**

833   **proteins differentially shift to the membrane in response to EPA treatment.** C_2C_{12} myotubes were

834   incubated in SILAC media for 7 doublings followed by plating and differentiation for 72hrs in SILAC

835   differentiation media. Following differentiation myotubes were incubated in either fatty acid free 2%

836   BSA or fatty acid free 2% BSA pre-conjugated to 50 μM EPA or 50 μM DHA for 72hrs (n=3 in

837   duplicate). Cells were collected and fractionated, the membrane fraction was then submitted to

838   proteomics analysis. (A) Venne diagram illustrating the number of proteins found to be changed in the

839   membrane fraction in response to EPA/DHA. (B) String diagram illustrating the interactions between

840   the proteins identified from the GO analysis as being involved in 'protein folding.' (C) Fold change in

841   ribosomal proteins in the membrane fraction in response to EPA/DHA. * indicates a significant fold

842   change from control (p<0.05). All differences between EPA and DHA were significant.

843

844

845

846

847