1 Docosahexaenoic acid enrichment in NAFLD is associated with improvements in hepatic
2 metabolism and hepatic insulin sensitivity: a pilot study
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Background/Objective: Treatment of subjects with non-alcoholic fatty liver disease (NAFLD) with omega-3 polyunsaturated fatty acids (PUFA) suggests high levels of docosahexaenoic acid (DHA) tissue enrichment decrease liver fat content. We assessed whether changes in erythrocyte DHA enrichment (as a surrogate marker of changes in tissue enrichment) were associated with alterations in hepatic *de novo* lipogenesis (DNL), postprandial fatty acid (FA) partitioning, and hepatic and peripheral insulin sensitivity in a sub-study of the WELCOME* trial.

Subjects/Methods: Sixteen participants were randomized to 4 g/day EPA+DHA (n=8) or placebo (n=8) for 15-18 months and underwent pre- and post-intervention measurements. Fasting and postprandial hepatic FA metabolism was assessed using metabolic substrates labelled with stable-isotope tracers ($^{2}H_2O$ and [$U^{13}C$]palmitate). Insulin sensitivity was measured by a stepped hyperinsulinaemic-euglycaemic clamp using deuterated glucose. Participants were stratified according to change in DHA erythrocyte enrichment (< or ≥2% post-intervention).

Results: Nine participants were stratified to DHA≥2% (8 randomised to EPA+DHA and 1 to placebo) and seven to the DHA<2% group (all placebo). Compared to individuals with erythrocyte <2% change in DHA abundance, those with ≥2% enrichment had significant improvements in hepatic insulin sensitivity, reduced fasting and postprandial plasma triglyceride concentrations, decreased fasting hepatic DNL, as well as greater appearance of ^{13}C from dietary fat into plasma 3-hydroxybutyrate (all $P<0.05$).

54 **Conclusions:** The findings from our pilot study indicate that individuals who achieved a
55 change in erythrocyte DHA enrichment ≥2% show favourable changes in hepatic FA
56 metabolism and insulin sensitivity, which may contribute to decreasing hepatic fat content.
57

58 *(**W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD (non-alcoholic
59 fatty liver disease) with **OM**acor th**E**rapy)
60

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver fat-related conditions that increase risk of chronic metabolic disease such as type 2 diabetes and cardiovascular disease, with obesity and insulin resistance (IR) being well-documented risk factors[1]. IR appears to be a key mediator in the initiation and progression of NAFLD, mainly through adverse changes in glucose, fatty acid (FA) and lipoprotein metabolism[2]; liver fat content appears to be the best independent predictor of peripheral and hepatic IR[3]. A net retention of triglyceride (TG) within the liver is a prerequisite for the development of NAFLD and increased FA flux to the liver, increased FA synthesis within the liver, and decreased hepatic FA oxidation have all been implicated in NAFLD development[1,4].

High-dose omega-3 FA (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are a licensed treatment to reduce plasma TG concentrations[5]. As NAFLD is associated with an overproduction of very low-density lipoprotein (VLDL)-TG[6], it is plausible that one contributor to the hypotriglyceridemic effect of omega-3 FA is through a lowering of liver fat content. We[7] and others[8,9] have reported that omega-3 FA have the potential to decrease liver fat. In addition to potentially lowering liver fat, short-term omega-3 FA treatment has been reported to improve whole-body insulin sensitivity[8,9]. However, the effects of omega-3 FA on insulin sensitivity in the context of NAFLD remains unclear, as some studies report neutral or negative results[10,11], despite a concomitant reduction in NAFLD severity[12].

The current study is a pre-specified sub-study of the WELCOME* randomized double-blind, placebo-controlled trial[7,13]. The aim of this pilot sub-study was to test if a pre-specified increase (≥2%) in erythrocyte enrichment of DHA[7,13] was associated with changes in hepatic FA synthesis, postprandial FA partitioning, and hepatic and peripheral insulin sensitivity.

Materials and Methods

Twenty-four individuals recruited from the main trial were randomly allocated to the sub-study (n=12 randomised to EPA+DHA, 4 g/d and n=12 randomised to placebo (olive oil, 4 g/d)) (see Figure 1)[7,13]. The duration of intervention was 15 to 18 months and inclusion and exclusion criteria were described previously[13]. For the main trial patients were randomised according to standardised procedures (computerised block randomisation in blocks of four, either to trial medication or placebo were used) by a research pharmacist at University Hospital Southampton NHS Foundation Trust[13]. This randomisation strategy was maintained for the sub-study. The study was approved by the Southampton and South West Hampshire Local Research Ethics Committee (REC 08/H0502/165). All subjects gave written informed consent for both the main trial and the sub-study.

Three participants withdrew from the sub-study before completing all tests. Four patients with diabetes were not included in the analysis as their anti-diabetic regimens increased between baseline and end-of-study tests, which would have influenced change in insulin sensitivity measurements. Similarly, one participant who lost >10 kg in weight over the course of the trial was also excluded.

We compared participants who showed an increase in erythrocyte DHA enrichment of 2% or greater (DHA≥2%) between baseline and end of the study with participants showing little change in erythrocyte DHA enrichment (DHA<2%). In the DHA≥2% group eight participants had been randomised to EPA+DHA intervention and one participant had been randomised to placebo; the latter had a 4.2% increase in erythrocyte DHA between baseline and end of the study. All seven subjects in the DHA<2% group had been randomised to the placebo.

110

111 *Experimental procedures*

112 Blood samples were taken after an overnight fast (12 h) and serum separated within 1 hour to
113 undergo routine biochemical assay [7]. Blood pressure was measured using a Marquette Dash
114 3000 monitor (GE Healthcare, Bucks, UK), body composition by dual-energy X-ray
115 absorptiometry (DEXA) and liver fat content measured at baseline and end of the study by
116 MRS[13].

117

118 *Red blood cell FA composition*

119 To determine specific FA composition erythrocyte ghosts were prepared, membranes
120 isolated, total lipids isolated, fatty acid methyl esters (FAMEs) prepared and FA
121 compositions determined by gas chromatography (GC), as described elsewhere[14].

122

123 *Assessment of whole-body and hepatic insulin sensitivity*

124 Insulin sensitivity was measured using a two-step hyperinsulinaemic–euglycaemic clamp
125 with a deuterated glucose (6,6 $^{2}H_2$ glucose) infusion[15,16] to assess hepatic and peripheral
126 insulin sensitivity. Briefly, subjects arrived at the NIHR Wellcome Trust Clinical Research
127 Facility (CRF) after an overnight (12 h) fast and intravenous cannulae were placed in both
128 antecubital veins for sampling and infusion of labelled glucose and insulin during the two-
129 step hyperinsulinaemic-euglycaemic clamp[15,16].

130

131 Glucose isotopic enrichment was determined from deproteinised plasma using the
132 methoxime-trimethlysilyl ether derivative which was measured by gas chromatography mass
133 spectrometry (GC-MS) using selected ion monitoring[15]. A modified version of the equations
134 formulated by Steele *et al.*[17] for non-steady state was used to calculate total rate of

135 appearance (Ra) of glucose, endogenous glucose production (EGP) (at basal and low-dose
136 insulin stage) and rate of disappearance (Rd) as well as metabolic clearance rate (at the high-
137 dose insulin stage) of glucose adjusted to fat-free mass (FFM) $\mu mol.kg^{-1}.min^{-1}$. For the Steele
138 equations, 65% was used as the effective fraction and 0.22 l/kg as the distribution volume of
139 glucose to calculate Ra and Rd[17,18]. Plasma glucose and tracer to tracee ratio (TTR) time
140 courses were smoothed using optimal segment smoothing method[19]. We also measured a
141 validated index of hepatic insulin sensitivity by dividing the basal EGP rates by the fasting
142 insulin concentration[20].

143

144 *Fasting hepatic DNL and postprandial hepatic FA partitioning*

145 Approximately 2 weeks after the assessment of whole-body and hepatic insulin sensitivity,
146 subjects underwent a postprandial study day as previously described[21]. Briefly, to assess
147 fasting hepatic DNL, subjects consumed deuterated water (^2H_2O) (3 g/kg body water) the
148 evening prior to the study day to achieve a body water enrichment of 0.3%[21]. Subjects
149 arrived at the CRF after an overnight fast and were fed a standard test meal that contained
150 200 mg of [$U^{13}C$]palmitic acid (isotope purity 97%, CK Gas Products Ltd, Hook, UK) to
151 trace the fate of dietary FA[21]. Serial blood and breath samples were taken throughout the 5 h
152 postprandial period.

153

154 *Biochemical analysis*

155 Whole blood was collected into heparinized syringes (Starstedt, Leicester, UK) and plasma
156 was rapidly separated for the measurement of metabolite and insulin concentrations[21].
157 Separations of chylomicrons of Svedberg floatation rate (S_f) >400 and VLDL-rich fraction
158 (S_f 20–400) were made by sequential flotation using density gradient ultracentrifugation and
159 the S_f 20-400 fraction was then further separated by immunoaffinity chromatography[22,23].

160

161 *FA analysis and isotopic enrichment*

162 To determine specific FA composition and isotopic enrichment, total lipids were extracted
163 from plasma and lipoproteins and FAMEs were prepared[22]. FA compositions in these
164 fractions were determined by GC, and palmitate concentrations were calculated as described
165 elsewhere[21].

166

167 $^{13}C/^{12}C$ ratios in plasma NEFA, S_f >400 (chylomicron-TG), S_f 20-400-TG (TRL) and VLDL-
168 TG FAME derivatives were determined using a 'Delta Plus XP' GC-combustion-isotope ratio
169 MS (GC-C-IRMS) (Thermo Electron Corporation, Bremen, Germany) and ^{13}C concentrations
170 calculated as previously described[23].

171

172 To assess ketone body production arising from the oxidation of dietary [U^{13}C]palmitate, we
173 measured isotopic enrichment of [^{13}C] in plasma 3OHB[21]. Fasting hepatic DNL was assessed
174 based on the incorporation of deuterium from $^{2}H_2O$ in plasma water (Finnigan GasBench-II,
175 ThermoFisher Scientific, UK) into VLDL-TG palmitate using GC-MS[21]. The concentration
176 of VLDL-TG derived from DNL was determined by multiplying %DNL and the
177 concentration of TG in VLDL[24].

178

179 *Sample size, calculations and statistical analysis*

180 The sample size for the main study[7] was powered to detect a change in liver fat content, as
181 described previously[13]; the sub-study reported here was run as a hypothesis-generating pilot
182 study. Areas under the curve (AUC) were calculated by the trapezoid method and were
183 divided by the relevant period to give a time-averaged value. Data were analysed using SPSS
184 for Windows v20 (SPSS, Chertsey, UK). Statistical significance was set at $P<0.05$. Data are

185 reported as mean (SEM) unless otherwise stated. All data sets were tested for normality
186 according to the Shapiro-Wilk test. Baseline results were compared with end of study results
187 using a paired t-test or the Wilcoxon signed rank test for non-parametric data. Comparisons
188 between the two groups were undertaken using independent t-test or Mann Whitney U test for
189 non-parametric data.

190 **Results**

191 *Subject Characteristics*

192 Baseline and end of study characteristics of participants with a change in erythrocyte DHA
193 enrichment of ≥2 % (n=9) or <2% (n=7) are shown in Table 1. One participant randomised to
194 the placebo group had a significant increase in erythrocyte EPA and DHA enrichment (0.8%
195 and 4.2%, respectively) between baseline and end of study measurements; the increase is
196 most likely because they started consuming more oily fish or over-the-counter fish oil
197 capsules during the course of the trial. There was no significant change in BMI or body fat
198 percentage between baseline and end of study in either group (Table 1). Baseline liver fat
199 content ranged from 5.3% to 85% and although liver fat decreased to a greater extent in the
200 DHA≥2% compared to the DHA<2% group, this difference did not reach statistical
201 significance.

202

203 We measured erythrocyte FA composition as a surrogate marker for tissue, specifically liver,
204 FA enrichment[25,26]. The DHA≥2% group had a significant ($P<0.001$) change in the
205 erythrocyte enrichment of EPA (by >300%) and DHA (by 92%) between baseline and end of
206 study measurements; there was no change in the DHA<2% group (Table 1).

207

208 There was no difference between baseline and end of study in the fasting plasma
209 concentrations of glucose, insulin, NEFA, total, LDL- or HDL-cholesterol (Table 2). Fasting
210 plasma TG and VLDL-TG concentrations were significantly ($P<0.001$) decreased by 0.6
211 mmol/L and 0.7 mmol/L, respectively, in the DHA≥2% group, whilst concentrations
212 remained unchanged in the DHA<2% group (Table 2).

213

214 We measured the incorporation of newly synthesised palmitate (DNL) into VLDL-TG in the
215 fasting state and found no difference in the relative contribution between baseline and end of
216 study in either group (Table 2). However, when expressed as the absolute concentration of
217 VLDL-TG derived from DNL there was a significant ($P<0.05$) decrease in the DHA≥2%
218 group between baseline and end of study measurements but there was no change in the
219 DHA<2% group (Table 2).

220

221 In line with the fasting data, there was a significant ($P<0.001$) decrease in postprandial
222 plasma TG and VLDL-TG concentrations between baseline and end of study measurements
223 in the DHA≥2% group but not the DHA<2% group (Table 2). There was a significant
224 ($P<0.05$) decrease in plasma 3OHB concentration over the postprandial period in the
225 DHA≥2% group (Table 2).

226

227 We included [$U^{13}C$]palmitate as part of the mixed test meal, to trace the fate of dietary FA
228 over the 5 h postprandial period. We observed a significant ($P<0.05$) decrease in the
229 appearance of [$U^{13}C$]palmitate in the plasma NEFA pool in the DHA<2% group, but not in
230 the DHA≥2% group (Table 2). There was a significant ($P<0.01$) decrease in the appearance
231 of [$U^{13}C$]palmitate in VLDL-TG and a significant ($P<0.01$) increase in the incorporation of
232 ^{13}C into plasma 3OHB in the DHA≥2% group whilst the DHA<2% group remained
233 unchanged (Table 2).

234

235 Whole-body insulin sensitivity (M-value) and peripheral glucose disposal (Rd) during the
236 high-dose insulin stage did not change in either the DHA≥2% or DHA<2% group between
237 baseline and end of study (Table 3). Hepatic insulin sensitivity significantly ($P<0.01$)
238 increased in the DHA≥2% group over the course of the study, with no change being observed

239 in the DHA<2% group (Table 3). We investigated whether a change in liver fat percentage
240 was associated with changes in insulin sensitivity. In exploratory analyses, we stratified the
241 cohort into two equal groups by the median change in liver fat percentage during the trial.
242 Group 1 represented a “high” reduction in liver fat (range -3% to -53%), whilst Group 2
243 represented minimal change or increase in liver fat (range -1.3% to +33%). When we
244 analysed the difference in percentage suppression of EGP at the low-dose insulin step
245 between the groups (as another measure of hepatic insulin sensitivity), percentage
246 suppression was significantly better in Group 1 vs Group 2 (13.7% vs -3.8% (95% C.I. 1.4,
247 33.5, $P<0.05$)). However, the difference in percentage increase of glucose disposal (measure
248 of whole-body insulin sensitivity) was not significantly different (Group 1, 26.7% vs Group
249 2, 8.7% (95% C.I. -56.2, 92.3, $P=0.61$)).
250

251 We found an inverse association between change in erythrocyte DHA enrichment and change
252 in fasting plasma VLDL-TG concentrations ($r_s=-0.59$, $P<0.05$) (Figure 2A). We also
253 assessed the association between change in hepatic DNL and change in erythrocyte DHA
254 enrichment and found an inverse association ($r_s=-0.58$, $P<0.05$) (Figure 2B). Lastly, we
255 assessed the association between change in erythrocyte DHA status and the change in the
256 incorporation of ^{13}C from dietary fat into plasma 3OHB and found a significant positive
257 correlation ($r_s=0.74$, $P<0.01$) (Figure 2C).
258

259 **Discussion**

260 We report here data demonstrating that individuals with NAFLD, who have an increase in
261 erythrocyte DHA enrichment of ≥2% (as a marker of tissue enrichment[25,26]) through
262 treatment with omega-3 FA, show favourable changes in both hepatic insulin sensitivity and
263 hepatic FA metabolism. Although erythrocyte DHA enrichment ≥2% was associated with a
264 non-significant (26%) decrease in liver fat content, hepatic DNL significantly decreased
265 whilst hepatic FA oxidation and hepatic insulin sensitivity significantly increased. Further
266 analysis of our data revealed a reduction in liver fat was also significantly associated with
267 improved hepatic, but not peripheral, insulin sensitivity. Given that increased liver fat is
268 associated with defects in insulin-mediated suppression of glucose production[27], our study
269 extends this observation by showing a significant improvement in hepatic insulin sensitivity
270 over 18 months in association with a reduction in liver fat.

271

272 Liver fat content is associated with an overproduction of VLDL, which may result in
273 dyslipidemia[6] and a decrease in liver fat content may contribute to a decrease in VLDL-TG
274 production. In line with previous work,[28,29] we found a significant decrease in fasting and
275 postprandial VLDL-TG concentrations in the DHA≥2% group only. Evidence from kinetic
276 studies has demonstrated that omega-3 FA decrease TRL particle concentrations by
277 decreasing hepatic TRL apoB-100 synthesis and secretion rates and increasing TRL-to-low
278 density lipoprotein (LDL) conversion[28,29]. We did not measure TRL production, secretion or
279 conversion rates in the current study.

280

281 FA availability is a determinant of VLDL-TG production. We found the absolute contribution
282 of FA derived from hepatic DNL to VLDL-TG in the fasting state was significantly
283 decreased in the DHA≥2% group. A plausible explanation for the decrease in DNL is that

284 omega-3 FA inhibit sterol regulatory element-binding protein 1c (SREBP1c), which leads to
285 a downregulation in the expression of several key lipogenic genes involved in FA synthesis[30].
286 We observed a significant decrease in the appearance of [^{13}C]palmitate in VLDL-TG over the
287 postprandial period in the DHA≥2% group, with no change in the DHA<2% group,
288 suggesting a change in the intrahepatic partitioning of dietary FA. In support of this
289 observation, we found a notable increase in the appearance of ^{13}C (from dietary FA) into
290 plasma 3OHB, suggesting intrahepatic metabolism was being moved away from
291 esterification toward ketogenic pathways over the postprandial period. These observations are
292 also in line with the liver becoming more insulin sensitive in the DHA≥2% group.
293
294 Supplementation with EPA+DHA has been reported to decrease liver fat in some[7,8] but not
295 all[31] studies. In the present study individuals who increased their erythrocyte DHA
296 enrichment by ≥2% had, on average a non-significant 26% decrease in liver fat content. In
297 the WELCOME Study results[7,32] we noted some individuals benefitted markedly from
298 treatment with 4 g/day DHA+EPA whilst others derived no benefit; a result which cannot be
299 explained through lack of adherence to DHA+EPA treatment.
300
301 It is important to consider the strengths and limitations of our study. Although the sample size
302 is small in this proof of concept pilot study, we have undertaken a randomised double-blind,
303 placebo-controlled trial testing the effects of the high-dose omega-3 FA intervention over a
304 minimum of 15 months. Subjects consumed either a combination of EPA+DHA or placebo
305 (olive oil). Recent work by Allaire et al[33] reported, in a head-to-head comparison of EPA and
306 DHA, that the latter was more effective in modulating markers of inflammation and blood
307 lipids. It would be of interest to study the effect of a DHA treatment only on hepatic
308 metabolism and insulin sensitivity given improvements in hepatic insulin sensitivity, and

309 hepatic fatty acid partitioning were noted in individuals with a ≥2% change in erythrocyte
310 DHA abundance. Although *in vitro* cellular work has suggested oleic acid, when compared
311 to palmitic acid has a protective role against insulin resistance[34] it is likely subjects in the
312 placebo group of the current study were not supplemented with high enough doses of oleic
313 acid to have an effect. Subjects in the placebo group consumed 4 g olive oil per day, which
314 provided 2.4 g oleic acid per day in addition to their habitual diet; oleic acid is very prevalent
315 in many foods of both plant and animal origin. Subjects underwent very extensive
316 phenotyping to characterise key aspects of liver metabolism and gold-standard techniques
317 were applied to assess liver fat, insulin sensitivity and hepatic FA partitioning. As our
318 postprandial period was only of 5 h duration, it is plausible that we have underestimated the
319 effects, and had we traced the meal for longer, or investigated hepatic FA partitioning after
320 multiple meals, we may have found greater divergence between the groups. We also only
321 measured hepatic DNL in the fasting state and it would be of interest to measure hepatic DNL
322 in the postprandial state. Additionally, we cannot determine how early or in what order
323 changes in liver fat, hepatic FA synthesis and partitioning, and hepatic insulin sensitivity
324 occurred.
325
326 In conclusion, individuals with NAFLD who increase erythrocyte DHA enrichment (≥2%)
327 (as a marker for tissue enrichment) show notable changes in hepatic insulin sensitivity and
328 hepatic FA metabolism that favour decreased hepatic DNL and increased hepatic FA
329 oxidation which would be expected to decrease hepatic TG synthesis and storage. Variable
330 tissue enrichment with DHA may in part, explain the differential effects of omega-3 fatty
331 acid treatment on hepatic fat quantity via the different pathways influenced by omega-3 fatty
332 acid treatment. Subjects achieving higher DHA enrichment ≥2% tended to have higher

333 hepatic insulin sensitivity, lower fasting hepatic DNL and higher levels of hepatic fat

334 oxidation.

335

336

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347

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349

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456 **Figure legend**

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458 **Figure 1.** Consort diagram showing recruitment for the WELCOME sub-study and the
459 numbers of subjects available for hepatic fatty acid metabolism and insulin sensitivity
460 studies. See text for the reasons for withdrawal from the study.

461

462 **Figure 2.** Correlations between change in erythrocyte DHA (%) and change in fasting plasma
463 VLDL-triacylglycerol (TG) concentrations (µmol/L) (**2A**); change in erythrocyte DHA (%)
464 and change in the fasting contribution (µmol/L) of DNL fatty acids to VLDL-TG (**2B**);
465 change in erythrocyte DHA (%) and change in postprandial plasma ^{13}C-3OHB concentrations
466 (µmol/L) (**2C**).

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