

1 Eicosapentaenoic Acid Decreases Post-prandial β -Hydroxybutyrate and Free Fatty Acid
2 Responses in Healthy Young and Elderly

3

4 RUNNING HEAD: EPA lowers plasma β -hydroxybutyrate response

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Abstract

41 **Abstract**
42 *Objectives:* We investigated whether a dietary supplement rich in eicosapentaenoic acid
43 (EPA) increases plasma fasting ketones or postprandial ketone responses in healthy
44 young and elderly subjects.

45 *Research Methods & Procedures:* 10 young (22 ± 2 y old) and 10 elderly subjects ($75 \pm$
46 4 y old) were recruited and participated in two identical study days, one before and one
47 6 weeks after providing an EPA-enriched supplement (1.4 g/d of EPA and 0.2 g/d of
48 docosahexaenoic acid). On the study days, blood samples were collected at fasting and
49 every hour for 6 h after giving a breakfast. Fasting and post-prandial plasma β -
50 hydroxybutyrate (β -OHB), free fatty acids (FFA), triglycerides, glucose and insulin
51 responses were measured. Fatty acid profiles were assessed in fasting plasma samples
52 before and after the EPA supplement.

53 *Results:* After the EPA supplement, postprandial plasma β -OHB responses dropped by
54 44% in the young and by 24% in the elderly, along with 20% and 34% lower FFA
55 responses in the young and elderly adults, respectively. β -OHB and FFA were positively
56 and significantly correlated in young but not in elderly subjects both before and after the
57 EPA supplement. In both groups, postprandial plasma triglycerides, glucose and insulin
58 were not significantly different after the intake of the EPA supplement. Before and after
59 the EPA supplement, fasting plasma EPA was 50% higher in the elderly but increased
60 by about 5 times in both groups following the intake of EPA supplement.

61 *Conclusions:* Contrary to our expectations, EPA supplementation lowered postprandial
62 β -OHB response and, in the elderly subjects, the concentration of postprandial β -OHB
63 was not lowered after the intake of EPA supplement.

64

65 Keywords: eicosapentaenoic acid; ketones; β -hydroxybutyrate; free fatty acids; elderly;

66 aging

67

68 **List of abbreviations**

69 β -OHB – beta-hydroxybutyrate

70 DHA – docosahexaenoic acid

71 EPA – eicosapentaenoic acid

72 FFA – free fatty acids

73 PPAR- α – peroxisome proliferator-activated receptor-alpha

74

75

76 Introduction

77 Glucose normally provides about 97% of cerebral energy requirements but when
78 glucose availability is limited, i.e. during fasting or starvation, ketones become important
79 brain energy substrates supplying up to 70% of brain energy requirements [1]. Brain
80 uptake of ketones is directly proportional to their circulating level [2-4]. During healthy
81 aging, brain glucose uptake decreases significantly in specific cortical regions [5], an
82 effect that is more pronounced in elderly with deteriorating cognitive function such as
83 Alzheimer's disease [6]. Therefore, the idea of safely inducing chronic, mild ketonemia
84 has been proposed as a strategy to counteract declining brain glucose uptake and
85 hence hopefully reduce the risk of deteriorating cognition in the elderly [7, 8].

86 Ketones refer to three molecules: acetoacetate, β -hydroxybutyrate (β -OHB) and
87 acetone. They are produced principally in liver mitochondria from successive
88 condensation of acetyl-CoAs derived from β -oxidation of free fatty acids (FFA). Despite
89 the possible need for a fuel to replace glucose in the aging brain, neither of the common
90 ways of increasing ketone production, i.e. fasting for several days [1] or a very high fat
91 ketogenic diet [9], seems realistic for the elderly. An alternative approach to safely
92 inducing mild ketonemia may be to increase FFA β -oxidation and up-regulate
93 transcription of enzymes involved in ketogenesis, particularly 3-hydroxy-3-methylglutaryl
94 coenzyme A synthase. Both β -oxidation and transcription of 3-hydroxy-3-methylglutaryl
95 coenzyme A synthase are regulated by the nuclear receptor - peroxisome proliferator-
96 activated receptor-alpha (PPAR- α), which is a ligand-activated transcription factor [10].

97 *In vitro* studies show that the omega-3 fatty acid – eicosapentaenoic acid (EPA;
98 20:5 ω 3) – is a strong natural fatty acid ligand for PPAR- α [11]. Hence, our hypothesis

99 was that if PPAR- α is involved in activating the β -oxidation of FFA and up-regulating
100 enzymes of ketogenesis and since EPA is a good ligand of PPAR- α [11], ketogenesis
101 should increase after consuming an EPA supplement. Until now, the possible link
102 between activation of PPAR- α by fatty acids such as EPA and increased ketogenesis
103 had not been assessed in humans. The aims of this study were to determine, first,
104 whether supplementation with an EPA-enriched fish oil would increase ketone
105 concentration and, second, whether ketone concentration would differ in the elderly
106 compared to young adults after EPA supplementation.

107 Given the potentially important clinical application of ketones as alternative
108 cerebral energy substrates in the elderly, but the relative scarcity of information about
109 fasting and postprandial ketone production during healthy aging [12], we compared
110 these parameters in healthy elderly to young adults after giving an EPA supplement. Our
111 approach was to measure changes in plasma FFA and ketone concentration during two
112 identical metabolic study days, one before and one 6 wk after EPA supplementation
113 since a direct measure of ketogenesis enzymes and PPAR- α activation was not possible
114 in human liver.

115

116 **Subjects and Methods**

117 *Subjects:* Subjects were recruited in two age groups: 18-25 y old (young) and 70-85 y
118 old (elderly). All subjects were non-smokers and selected for relatively good health. As
119 part of the screening, blood chemistry was assessed after a 12 h overnight fast. Fasting
120 glucose and hemoglobin A_{1c} were used to rule out the presence of glucose intolerance
121 or overt diabetes. A complete blood cell count was used for blood disorders, electrolyte
122 profile, aspartate transaminase and alanine transaminase for liver function/nutritional
123 status, high and low density lipoprotein cholesterol, triglycerides and albumin for
124 nutritional status, C-reactive protein as a marker of inflammatory processes, and thyroid
125 stimulating hormone for thyroid function. Subjects with plasma omega-3 fatty acids
126 higher than 4% of total fatty acids were excluded. Except for glucose and cholesterol,
127 other parameters did not differ significantly between the two groups (**Table 1**).

128 Approval for the study was obtained from the Research Ethics Committee of the
129 Health and Social Services Center – Sherbrooke University Geriatrics Institute, which
130 oversees all human research done at the Research Center on Aging. All subjects gave
131 informed, written consent before participating. Thirty subjects underwent screening.
132 Complete metabolic data were obtained for 10 young and 10 elderly subjects (5 women
133 and 5 men in each group) who met our inclusion criteria. A group size of ten was
134 sufficient to meet the statistical power ($\beta = 0.80$) needed to achieve a significant
135 difference in doubling fasting plasma ketones after EPA supplementation [13].

136
137 *Protocol and sample collection:* During the study, each subject consumed 4 capsules /
138 day providing a total of 1480 mg/d of EPA and 250 mg/d of docosahexaenoic acid (DHA;

139 22:6 ω 3). The capsules used were a commercially-available omega-3 fatty acid
140 supplement (OM3, Isodis Natura, Brussels, Belgium), hereafter designated as the EPA
141 supplement. Subjects participated in two identical metabolic study days, one before and
142 one 6 wk after EPA supplementation. On each metabolic study day, subjects arrived at
143 7:30 a.m. after a 12 h fast. A forearm venous catheter was installed and was kept patent
144 by flushing hourly with non-heparinized saline after fasting blood draw (defined as time
145 0) and hourly blood draw for 6 h using a 5 ml latex-free syringe (Becton Dickinson,
146 Franklin Lakes, NJ). The subjects received a breakfast (between time 0 and time 1)
147 composed of eggs, bacon, cheese, one slice of tomato and toast with an average fat
148 content of 23 g or 43% of the breakfast calories and around 38 g of carbohydrates
149 accounting for around 34% of the breakfast calories. Blood samples were transferred
150 immediately to a 5 mL K₂-EDTA coated tube (Becton Dickinson) and kept on ice until the
151 end of the study day when they were all centrifuged at 2300 g for 18 min at 4°C. Plasma
152 was stored at -20°C until further analysis. During the study day, water was available *ad*
153 *libitum* and subjects were asked to remain in a resting position, with short walks allowed.
154
155 *Plasma fatty acid profile:* Plasma total lipids were extracted into 2:1 chloroform/methanol
156 solution, using heptadecanoate as an internal standard. The total lipids were then
157 saponified with 1 mol/L methanolic potassium hydroxide followed by transmethylation of
158 the FFA to fatty acid methyl esters using 14% methanolic boron trifluoride. Fatty acid
159 methyl esters were analyzed using a gas chromatograph (Agilent model 6890, Palo Alto,
160 CA) equipped with a 50 m BPX-70 fused capillary column (SGE, Melbourne, Australia,
161 0.25 mm i.d., 0.25 μ m film thickness). Splitless mode injection and flame ionization

162 detection were performed at 250°C. The oven temperature program was 50°C for 2 min,
163 increased to 170°C at a rate of 20°C/min and held there for 15 min, increased to 210°C
164 at a rate of 5°C/min and held there for 7 minutes. The inlet pressure of the carrier gas
165 (He) was 233 kPa at 50°C. The identity of individual fatty acids was determined by
166 comparing retention times with standard mixtures of fatty acids (NuChek 68A, NuChek
167 411, and NuChek 455; NuChek Prep, Inc., Elysian, MN) and a custom mixture of
168 saturated fatty acid standards.

169
170 *Other analyses:* Commercially available reagent kits were used for the analysis of β -
171 OHB (RX Daytona kit; Randox Laboratories Ltd., Antrim, UK), and FFA (Wako
172 Diagnostics, Richmond, VA) and triglycerides and glucose (Dade Behring Inc., Newark,
173 DE) using an automated clinical chemistry analyzer (Dimension XPand Plus, Dade
174 Behring Inc., Newark, DE). Insulin was analyzed by ELISA (Merckodia, Uppsala, Sweden)
175 using a microplate reader (model 3550, BioRad, Hercules, CA).

176
177 *Statistical analysis:* Results are given as mean \pm SEM in Figures and Tables.
178 Postprandial responses over the 6 h of the study day were defined by areas under the
179 curve calculated for plasma β -OHB, FFA, triglycerides, glucose and insulin (Prism
180 software version 4.0, GraphPad Prism, San Diego, CA) allowing comparison between
181 curves. Since data were not normally distributed and the sample size was small, we
182 used non-parametric tests to compare data. Hence, data for the two age groups were
183 compared by a Mann-Whitney test using SPSS software (version 12.0, SPSS Inc,
184 Chicago, IL). To determine statistical significance following the EPA supplementation,

185 we used Wilcoxon's signed rank test. To determine age-by-diet interactions, we
186 compared calculated variables (after – before EPA supplement) for fasting β -OHB, FFA,
187 triglycerides, glucose and insulin and for postprandial responses between age groups
188 using a Mann-Whitney test. Correlation between FFA and β -OHB was determined using
189 the Spearman correlation coefficient. Significance was set at $p < 0.05$.
190

191 **Results**

192 *Plasma β -OHB and FFA (Figure 1):* After the EPA supplement, fasting plasma β -OHB
193 and FFA were respectively 51% lower ($p = 0.007$) and 35% lower ($p = 0.022$) in young
194 subjects but unchanged in the elderly. Postprandial β -OHB response was significantly
195 lower in both groups after the EPA supplement, with a greater drop (44%) in the young
196 than in the elderly (24%). Similarly, after EPA, the postprandial FFA response was 20%
197 lower in both groups, but did not reach statistical significance in the young subjects ($p =$
198 0.059). Neither before nor after EPA was β -OHB response statistically different between
199 elderly and young subjects. In contrast, the postprandial FFA response was about 40%
200 higher in the elderly both before ($p = 0.014$) and after ($p = 0.013$) the EPA supplement.
201 Fasting plasma FFA and β -OHB were positively correlated in young ($p < 0.05$) both
202 before and after the intake of EPA supplement but not in elderly (Figure 2).

203
204 *Plasma triglycerides, glucose and insulin (Figure 1):* Before EPA supplementation,
205 fasting plasma triglycerides were similar in the young and the elderly. After EPA
206 supplementation, the elderly had 63% higher fasting triglycerides compared to the young
207 adults ($p = 0.041$) and postprandial plasma triglycerides levels were significantly higher
208 compared to young ($p = 0.041$). In both groups, fasting and postprandial plasma
209 triglycerides were not significantly lower after the EPA supplement. In the elderly, fasting
210 glucose was around 24% higher both before and after EPA supplementation compared
211 to young. In both groups, fasting glucose concentrations and postprandial glucose
212 responses were similar after the intake of EPA supplement. Fasting and postprandial

213 insulin of elderly was similar to young before and after the EPA supplementation. After
214 EPA supplementation, fasting plasma insulin was 42% and 39% higher in the elderly (p
215 = 0.017) and the young ($p = 0.114$), respectively whereas the postprandial insulin
216 response was not significantly different in both groups.

217
218 *Plasma fatty acid profile (Figure 4)*: Plasma fatty acid profiles were assessed to evaluate
219 the effectiveness of the EPA supplement in increasing fasting plasma EPA and DHA
220 levels. Before EPA supplementation and compared to the young adults, the elderly had
221 85% higher plasma EPA but DHA concentration was similar in both groups. After the
222 intake of EPA supplement, fasting plasma EPA concentration was 5.6 and 5.1 times
223 higher in the young and elderly, respectively, whereas fasting plasma DHA was 24%
224 higher only in the young subjects ($p = 0.037$). After the EPA supplement, fasting plasma
225 EPA (mg/L) remained 67% higher in elderly compared to young adults.

226

227 **Discussion**

228 This study aimed to evaluate the impact of EPA supplementation on fasting and
229 postprandial ketone concentration in both young and elderly adults. Our results suggest
230 that EPA supplementation *reduced* the postprandial β -OHB and FFA responses in both
231 groups. The concentration of ketones in plasma reflects the balance between
232 appearance in and removal from the plasma. Although β -oxidation of β -OHB is similar in
233 young and elderly adults [12], we have no indication whether after an EPA
234 supplementation the β -oxidation of β -OHB would be higher or its removal from plasma
235 would be altered. Therefore, the reason why EPA supplementation lowered rather than
236 raised β -OHB and FFA responses in this study is unclear.

237 The production of ketones requires increased mobilization of FFA from adipose
238 tissue to the liver by increasing lipolysis in adipose tissue and/or triglyceride-rich
239 lipoproteins [14, 15], coupled with enhancement of the liver's capacity to convert these
240 substrates into β -OHB and other ketones [16]. Since we observed a linear correlation
241 between fasting plasma FFA and β -OHB concentrations in young subjects (Figure 2)
242 [14] and because ketones are produced from FFA β -oxidation, the lower FFA response
243 may therefore have contributed significantly to lowered β -OHB response (Figure 1).

244 Indeed, we hypothesized that giving an EPA supplement would up-regulate
245 ketogenesis enzymes in the liver leading to increase ketone concentration. However,
246 EPA interacts with at least four families of transcription factors - PPAR- α , liver X
247 receptors, hepatic nuclear factor-4 α and sterol regulatory-element-binding protein - and

248 generates a large range of eicosanoids able to modulate transcription factor activity [17].
249 It is therefore possible that the EPA supplement may have simultaneously decreased
250 lipolysis through one of the other activated transcription factors since it is controlled by
251 gene transcription in the liver [18]. Two studies in humans [14, 19], reported that an EPA
252 supplement resulted in decreased peripheral lipolysis from adipose tissue thereby
253 reducing the availability of FFA. Moreover, EPA is suggested to lower liver TG synthesis
254 and increase adipose TG clearance thereby reducing the release of FFA by lipoprotein
255 lipase [17]. In this study, EPA supplementation did not significantly lower fasting or
256 postprandial plasma TG (Figure 3). This result may not be so unusual in normolipidic
257 humans since it occurs in about half of placebo-controlled trials [20] and is due to low
258 baseline TG concentration and EPA dose [17]. Hence, increasing plasma β -OHB
259 concentrations in humans appears complex and may require combined strategies for
260 increasing FFA lipolysis (substrate for ketonegenesis) from either adipose tissue and/or
261 triglyceride-rich lipoproteins while simultaneously increasing the liver's capacity to
262 produce ketones.

263 Since cognitive decline affects the elderly and because ketones are the major
264 alternative brain fuel to glucose, we investigated whether fasting and postprandial
265 ketone concentrations would be *increased* similarly in young and elderly after the EPA
266 supplementation. In our elderly group, neither fasting nor postprandial plasma β -OHB
267 was statistically different from that seen in our young adults but the elderly had a higher
268 postprandial FFA response compared to our young subjects. Moreover, our data do not
269 support a significant correlation of fasting plasma FFA with β -OHB in the elderly either
270 before or after EPA supplementation (Figure 2). Therefore, lipid metabolism, specifically

271 regarding FFA release and/or β -oxidation, is possibly altered during aging [22].
272 However, the ratio of fasting plasma β -OHB/FFA, which is suggested to be a marker for
273 fatty acid β -oxidation capacity and/or ketogenesis in the liver [14], was not statistically
274 different between young and elderly (data not shown).

275 Thus, the higher postprandial response of FFA in the elderly (Figure 1) [22] may
276 be a result of lower β -oxidation in muscle possibly resulting from lower muscle mass in
277 elderly or lower muscle capacity for β -oxidation [22]. Despite possibly altered lipid
278 metabolism in the elderly, our results support a similar concentration of both fasting and
279 postprandial β -OHB response in elderly compared to young adults.

280 EPA incorporation into plasma lipids following an EPA supplementation may differ
281 during aging [23, 24]. In this study, fasting plasma EPA concentration before EPA
282 supplementation was 85% higher in our elderly compared to young adults, which agrees
283 with previous work [23, 24]. The reasons why plasma EPA differs in the young and
284 elderly is unknown [23] but may result from lower β -oxidation of dietary EPA, thus
285 leaving a greater proportion for incorporation into plasma lipids in the elderly [24]. The
286 impact of higher incorporation of EPA into plasma lipids during aging has not yet been
287 fully investigated but should be considered as a possible confounder in results of studies
288 using fish oil supplementation with elderly. Our EPA supplement also provided 200 mg
289 of DHA/d, but this did not significantly raise fasting plasma DHA in our elderly group.
290 This indirectly suggests that 200 mg of DHA/d for 6 wk may not be sufficient to raise
291 fasting plasma DHA in the elderly.

292 We conclude that short term EPA supplementation lowers β -OHB and FFA
293 responses, an effect apparently not influenced by healthy aging.

294

295

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360

361 Figure Legends

362 Figure 1: Plasma β -hydroxybutyrate (β -OHB, upper, mean \pm SEM) and free fatty acid
363 (FFA, lower) postprandial responses of young and elderly before and 6 wk after the
364 intake of eicosapentaenoic acid (EPA) supplement. Subjects had breakfast between
365 time 0 and time 1 (\uparrow).

366 *Statistically different for fasting plasma measures, $p < 0.05$.

367 † Area under the curves significantly decreased after EPA supplement, $p < 0.05$

368

369 Figure 2: Correlation between fasting plasma β -hydroxybutyrate (β -OHB) and free fatty
370 acids (FFA) in young and elderly before and 6 weeks after the intake of
371 eicosapentaenoic acid (EPA) supplement. Both correlations in young were statistically
372 significant ($p < 0.05$) while both correlations in elderly were not statistically significant.

373

374 Figure 3: Concentration (mg/L) of fasting plasma eicosapentaenoic acid (EPA), and
375 docosahexaenoic acid (DHA) in young and elderly before and 6 weeks after the intake of
376 EPA supplement.

377 *Statistically significant between young and elderly on same EPA treatment, $p < 0.05$

378 † Significantly increased after EPA supplement, $p < 0.05$

379

380 Figure 1: Plasma triglycerides (upper, mean \pm SEM), glucose (middle) and insulin
381 (lower) postprandial responses of young and elderly before and 6 wk after the intake of

382 eicosapentaenoic acid (EPA) supplement. Subjects had breakfast between time 0 and
383 time 1 (↑).

384 *Statistically different for fasting plasma measures, $p < 0.05$.

385