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4-Hydroxyhexenal and 4-hydroxynonenal are mediators of the anti-cachectic effect of n-3 and n-6 polyunsaturated fatty acids on human lung cancer cells

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1598512> since 2019-04-29T15:58:08Z

Published version:

DOI:10.1016/j.freeradbiomed.2016.07.031

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1 4-hydroxyhexenal and 4-hydroxynonenal are mediators of the anti-cachectic effect of n-3 and n-6
2 polyunsaturated fatty acids on human lung cancer cells

3

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22

23 ABSTRACT

24 Cachexia, the most severe paraneoplastic syndrome, occurs in about 80% of patients with advanced
25 cancer; it cannot be reverted by conventional, enteral, or parenteral nutrition. For this reason,
26 nutritional interventions must be based on the use of substances possessing, alongside nutritional
27 and energetic properties, the ability to modulate production of the pro-inflammatory factors
28 responsible for the metabolic changes characterising cancer cachexia. In light of their nutritional
29 and anti-inflammatory properties, polyunsaturated fatty acids (PUFAs), and in particular n-3, have
30 been investigated for treating cachexia; however, the results have been contradictory.

31 Since both n-3 and n-6 PUFAs can affect cell functions in several ways, this research investigated
32 the possibility that the effects of both n-3 and n-6 PUFAs could be mediated by their major
33 aldehydic products of lipid peroxidation, 4-hydroxyhexenal (HHE) and 4-hydroxynonenal (HNE),
34 and by their anti-inflammatory properties. An “in vitro” cancer cachexia model, consisting of
35 human lung cancer cells (A427) and murine myoblasts (C2C12), was used.

36 The results showed that: 1) both n-3 and n-6 PUFAs reduced the growth of lung cancer cells
37 without causing cell death, increased lipid peroxidation and Peroxisome Proliferator-Activated
38 Receptor (PPAR) α , and decreased TNF α ; 2) culture medium conditioned by A427 cells grown in
39 the absence of PUFAs blocked myosin production and the differentiation of C2C12 muscle cells;
40 conversely, muscle cells grown in culture medium conditioned by the same cells in the presence of
41 PUFAs showed myosin expression and formed myotubes; 3) adding HHE or HNE directly to
42 C2C12 cells maintained in culture medium conditioned by A427 cells in the absence of PUFAs
43 stimulated myosin production and myotube formation; 4) putative consensus sequences for
44 (PPARs) have been found in genes encoding fast isoforms of myosin heavy chain, by a
45 bioinformatics approach.

46 The overall results show, first, the ability of both n-3 and n-6 PUFAs and their lipid peroxidation
47 products to prevent the blocking of myosin expression and myotube formation caused in C2C12
48 cells by medium conditioned by human lung tumor cells. The C2C12 cell differentiation can be due

49 to direct effect of lipid peroxidation products, as evidenced by treating C2C12 cells with HHE and
50 HNE, and to the decrease of pro-inflammatory TNF α in A427 cell culture medium. The presence of
51 consensus sequences for PPARs in genes encoding the fast isoforms of myosin heavy chain
52 suggests that the effects of PUFAs, HHE, and HNE are PPAR-mediated.

53

54 KEYWORDS: cachexia; lung cancer; muscle cells; n-3 PUFAs; n-6 PUFAs, lipid peroxidation;
55 HHE; HNE; myosin; PPARs; PPRE; TNF α .

56

57

58 INTRODUCTION

59

60 Cachexia is both the most common and the most severe paraneoplastic syndrome; it was recently
61 defined as a multifactorial condition. It is chiefly characterised by loss of skeletal muscle mass, and
62 negative protein and energy balance, driven by a variable combination of reduced food intake and
63 abnormal metabolism that is not fully reversed by conventional nutrition. It leads to a reduction in
64 the response and tolerance to therapy, and in the quality and duration of life [1]. Cachexia occurs in
65 about 80% of patients with advanced cancer; its incidence and prevalence differ in relation to the
66 type of cancer, the highest incidence being observed in pancreatic, gastric and lung cancer [2]. Lung
67 cancer induces sarcopenia associated to malnutrition, with a relatively high frequency (above 50%).
68 Depending on the cachexia classification, about 20% of patients with non small cell lung cancer
69 (NSCLC) are diagnosed as cachectic, and about 25% as pre-cachectic [3-5]. Malnutrition, due in
70 part to alterations in neurohormonal mechanisms controlling food intake, contributes to metabolic
71 alterations leading to a negative energy balance. This, in association with altered protein turnover
72 and inflammation, drives the muscle wasting characterizing cachexia to develop [6]. With regard to
73 the metabolic modifications occurring in cachexia, several cytokines produced by both tumour and
74 host (TNF- α , IL-6, IL-1, INF- γ) or by tumour alone (proteolysis-inducing factor, PIF, lipid
75 mobilizing factor, LMF) have been identified as molecular mediators of cachexia [7,8].
76 Due to its multifactorial pathogenesis, cachexia is unaffected by conventional dietary interventions,
77 and limited benefits have been demonstrated by enteral and parenteral nutrition [9,10]. For these
78 reasons, nutritional interventions focused on the use of substances with both nutritional and anti-
79 inflammatory properties, with the aim of improving energy balance and reducing inflammatory
80 status, have been proposed. In this perspective, and in light of their anti-inflammatory properties,
81 fish-oil and its primary components, n-3 polyunsaturated fatty acids (PUFAs), have been proposed
82 for treating cachexia. Moreover, in patients with cancer, a low concentration of n-3 PUFAs in the

83 plasma phospholipids has been shown at diagnosis, the value further declining during cancer
84 progression [11-13].

85 Among n-3 PUFAs, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid have been shown to
86 down-regulate production of pro-inflammatory cytokines and acute-phase proteins in cancer patient
87 blood [14-18], although a recent systematic review on the role of fish oil in treating cachexia
88 concluded that there is insufficient evidence of a net benefit of n-3 PUFAs in advanced cancer.
89 However, the same study reported that n-3 PUFA treatment can be beneficial for some selected
90 patient populations, acting as post-operative support able to improve wound healing and reduce
91 complications [19].

92 Different mechanisms have been postulated to explain the effect of n-3 PUFA, including
93 modulation of the activity of some transcription factors, such as NFkB and PPARs, leading to a
94 decreased production of pro-inflammatory cytokines and acute-phase proteins. Moreover, EPA has
95 been reported to inhibit the activation of the ubiquitin-proteasome pathway induced by proteolysis-
96 inducing factor (PIF) and to decrease expression of the lipid mobilizing factor (LMF) [20,21]. More
97 recently, EPA and DHA have been reported to affect the balance between anabolism and catabolism
98 in muscles, through their effect on signalling transduction proteins and adipokines, in old and obese
99 persons, thus suggesting another possible mechanism underlying n-3 PUFA-mediated anti-wasting
100 effect [23,24].

101 The present research investigated the possibility that anti-cachectic properties of n-3 PUFAs could
102 be mediated by the aldehydic products derived from their lipid peroxidation. 4-hydroxyhexenal
103 (HHE) is the principal aldehyde generated by the non-enzymatic peroxidation of n-3 PUFAs. Due
104 to its electrophilicity, HHE interacts strongly with cellular nucleophilic molecules, thus positively
105 or negatively affecting several cell functions [22]. HHE-induced cytotoxicity has been reported in
106 different cell types, including rat neurons and muscle cells, and human lens and renal epithelial
107 cells. [25-29]. Moreover, the HHE concentration has been shown to be significantly higher in the
108 hippocampus/parahippocampal gyrus of patients with preclinical and late-stage Alzheimer's disease,

109 than in that of normal subjects [30]. Conversely, it has recently been shown that, in some cases,
110 lipoxidation of proteins can cause a gain of function/activity, suggesting that the formation of
111 covalent protein/ α,β -unsaturated aldehyde adducts, derived from lipid peroxidation, might be a step
112 in a redox signalling pathway of physiological significance [31]. The present study was also based
113 on a previous finding that, at a concentration found in many normal tissues and plasma, 4-
114 hydroxynonenal (HNE), the major lipid peroxidation product of n-6 PUFA, induced the
115 differentiation of murine erythroleukemia MEL cells and human promyelocytic HL-60 cells, by
116 modulating the expression of several genes involved in cell cycle control [32-34]. Moreover in
117 some tumours, arachidonic acid (AA), a fatty acid belonging to n-6 PUFA, was shown to be present
118 in a lower percentage in total fatty acids in comparison with corresponding normal tissues [35].
119 In this light, the research investigated the effect of both n-3 and n-6 PUFAs and their lipid
120 peroxidation products (HHE and HNE) on muscle cell differentiation, using an “in vitro” cancer
121 cachexia model, consisting of human lung cancer cells (A427) and murine myoblasts (C2C12).
122 Moreover, to evidence the mechanisms underlying the effect of PUFAs on C2C12 cell
123 differentiation, the expression of peroxisome proliferator activated receptor (PPAR) α and the
124 release of TNF α were also evaluated in A427 cells and in their culture medium, respectively.
125 Human lung cancer cells were chosen since, in lung cancer patients, cachexia is the main cause of
126 death despite the improvement in anticancer therapies. Moreover, little is known about the effect of
127 PUFAs on lung-cancer-induced cachexia.

128

129 MATERIALS AND METHODS

130 Treatment of A427 cells with PUFAs

131 Human lung adenocarcinoma cells A427 (ATCC, MD, USA) were seeded (20,000 cells/cm²) in
132 DMEM/F12 medium with 2 mM glutamine, 1% antibiotic/antimycotic solution and 10% FBS
133 (medium A). Twenty-four hours after seeding, medium A was replaced with medium B, containing
134 DMEM/F12 medium, 2 mM glutamine, 1% antibiotic/antimycotic solution, 2% horse serum (HS),

135 and n-3 (EPA plus DHA) or n-6 PUFAs (AA) prepared in HS. EPA and DHA were administered
136 simultaneously at a ratio of 1.5:1. For all treatments, the final concentrations were 10 or 50 μ M. In
137 control cells, a quantity of HS equivalent to the highest dose administered of PUFAs was added to
138 the culture medium, in addition to the 2% HS already present in medium B.
139 Twenty-four hours after addition of PUFAs or HS, culture media were collected and centrifuged at
140 2800 g for 10 min (centrifuge J6B Beckman, CA, USA) at room temperature. The collected media
141 were used to determine lactate dehydrogenase (LDH) release, and as conditioned medium to culture
142 C2C12 cells. After removing media, A427 cells were detached with trypsin/EDTA (0.25%/0.03
143 mM), centrifuged at 900 g for 10 min (centrifuge J6B Beckman, CA, USA) and used for the assays
144 listed below.

145

146 C2C12 cell culture conditions

147 Murine muscle C2C12 cells (ATCC, MD, USA) were seeded at 6,000 cells/cm² in medium A for 4
148 days. After this time, to induce differentiation into myotubes, medium A was replaced with medium
149 B, in which C2C12 cells were maintained for 3 further days. At the end of this period, medium B
150 was replaced with medium conditioned by A427 cells, grown for 24 hours in the presence or
151 absence of PUFAs. Cells were analyzed after a further 4 days. C2C12 cells maintained in medium B
152 for a further 4 days were labelled “Tdiff”, and were taken as positive control.

153

154 Treatment of C2C12 cells with HHE and HNE

155 In these experiments, after removal of medium B (after 3 days), C2C12 cells were cultured in
156 medium conditioned by A427 cells grown in the absence of PUFAs, and fortified with HNE or
157 HHE at a concentration of 1 μ M or 5 μ M. The addition of 1 μ M aldehyde was repeated every 45
158 minutes up to 10 treatments (total amount 10 μ M); in the case of 5 μ M aldehyde, it was added as a
159 single dose. C2C12 cells cultured in medium conditioned by A427 cells for 24 hours, in the absence
160 of PUFAs, were used as control cells. C2C12 cells were analyzed after a further 4 days.

161

162 Cell growth and viability

163 The numbers of A427 cells present in the monolayer and in the culture medium were evaluated by
164 counting cells in a Burkner chamber, and are expressed as cells/cm². Cell viability was also evaluated
165 in culture medium, as LDH release [36], and as DNA content after propidium iodide staining by
166 using flow cytometry [37]. The viability of C2C12 cells during the different treatments was
167 evaluated as LDH release.

168

169 Immunofluorescence staining

170 To evaluate the production of myosin and myotube formation, at the different experimental times
171 C2C12 cells were washed with PBS, fixed with acetone/methanol (1:1) at -20°C for 20 minutes, and
172 kept at 4°C until use. Cells were then treated and viewed as in [38].

173

174 Western blot analysis

175 A427 and C2C12 cells were analyzed for ALDH3A1 expression using Western blot analysis [38].
176 Polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or monoclonal anti-aldehyde
177 dehydrogenase (ALDH) 3A1 antibodies were used.

178

179 ELISA analysis

180 TNF- α content was evaluated in the culture medium of A427 cells by the Enzyme Linked Immuno
181 Sorbent Assay (Invitrogen Corporation, Frederick, MD, USA).

182

183 Real time PCR analysis

184 PPAR α mRNA content was examined in A427 cells treated (sample) or not (control) with PUFAs
185 for 24 hours.

186 Total RNA was extracted by using the TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). Real-
187 time PCR was performed with single-stranded cDNA prepared from total RNA (1 µg) using a High-
188 Capacity cDNA Archive kit (Applied Bio Systems, Foster City, CA).
189 The forward and reverse primers were designed using Beacon Designer® software (Bio-Rad,
190 Hercules, CA). Twenty-five microliters of a PCR mixture containing cDNA template equivalent 40
191 ng of total RNA, 5 pmoles each of forward and reverse primers, and 2× IQ SYBR Green SuperMix
192 (Bio-Rad, Hercules, CA) were amplified using an iCycler PCR instrument (Bio-Rad, Hercules, CA)
193 with an initial denaturation at 95°C for 3 min, followed by 35-40 cycles at 95°C for 30 s, annealing
194 at 52°C for 40 s, extension at 72°C for 40 s. Each sample was tested in duplicate, and threshold
195 cycle (Ct) values were averaged from each reaction. The change in expression was defined as that
196 detected in the A427 cells treated with PUFAs versus that detected in the control cells, calculated as
197 $2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_{t_{\text{sample}}} - C_{t_{\text{GAPDH}}}$ and $\Delta\Delta C_t = \Delta C_{t_{\text{sample}}} - \Delta C_{t_{\text{control}}}$.

198

199

200 Lipid peroxidation in A427 cells

201 Malondialdehyde was measured in HPLC according to the method of Nielsen et al [39], with slight
202 modifications. Briefly, aliquots of cell medium were mixed (volume/volume) with 0.6% (w/v)
203 aqueous solution of thiobarbituric acid (TBA). The mixture was acidified with 1/20 volume of
204 100% (w/v) trichloroacetic acid and heated at 100°C for 1 hour. The samples were then cooled in
205 ice and centrifuged at 13000 g for 5 minutes. Aliquots of 50 µl of the supernatant were injected into
206 the HPLC system.

207 The HPLC system was equipped with a Novapak C18 4µm 3.9x150 mm column. The elution was
208 isocratic. The mobile phase consisted of a mixture of a 10 mM potassium dihydrogen phosphate
209 solution adjusted at pH 6.8 with 1M KOH and methanol in ratio 60/40. The flow rate was 1 ml/min.

210 Detection was performed by a spectrofluorometer at 532 nm ex/553 nm em. The peak of the MDA-
211 TBA adduct was well resolved at baseline and its retention time in our conditions was 4.8 min.

212 Identification and quantification of MDA concentration in the samples was performed by
213 comparison to the chromatograms of various concentrations of standard MDA sodium salt,
214 synthesized by Dr Bruno Tasso, according to Nair et al [40], treated in the same way as the samples.
215 The lowest tested concentration of standard that was still quantifiable was 0.125 μ M.

216
217 Detection of putative peroxisome proliferator response element (PPRE)

218 The presence of the putative PPRE sequence in genes encoding fast isoforms of myosin heavy chain
219 (MyHC) was evaluated with the NHR SCAN software package.

220
221 Statistical analysis

222 All data are expressed as means $\pm$ S.D. Differences between means were assessed by analysis of
223 variance followed by the post-hoc Newman-Keuls test.

224
225 RESULTS

226 The effect of PUFAs on growth and viability of lung cancer cells is reported in Figure 1. Exposing
227 A427 cells to PUFAs caused a dose-dependent decrease in cell growth that was more marked for n-
228 3 PUFAs (panel A): 10 or 50 μ M EPA plus DHA reduced cell numbers by 22% and 38%,
229 respectively, whereas AA only affected A427 cell numbers significantly (-20%) at the highest
230 concentration used. Neither n-3 nor n-6 PUFAs significantly induced either necrosis (panel B) or
231 apoptosis (panel C).

232 Lipid peroxidation (Figure 2) was measured in the culture medium of A427 cells exposed to PUFAs
233 and expressed as the production of MDA. A similar and significant increase was observed in cells

234 exposed to EPA plus DHA or to AA (about +40% or +70%, in the presence of 10 or 50 μ M PUFAs,
235 respectively).

236 Figure 3 panel A shows that both concentrations of EPA+DHA decreased TNF α content in the
237 culture medium of A427 cells, whereas only the highest concentration of AA decreased this
238 cytokine. Figure 3 panel B reports that PPAR α mRNA was increased in A427 cells treated with
239 both 50 μ M PUFAs, being the highest increase in A427 cells treated with EPA+DHA.

240 Culture medium conditioned for 24 h by A427 cells, in the presence or absence of PUFAs, was used
241 to grow murine muscle C2C12 cells, with the aim of investigating its effect on muscle cell
242 differentiation. No significant change in C2C12 viability was evidenced by analyzing LDH activity
243 in the culture medium, at any of the experimental conditions investigated (data not shown).

244 Figure 4 shows that C2C12 cells grown in medium conditioned by A427 cells, in the absence of
245 PUFAs, showed no production of myosin and no myotube formation, unlike cells maintained in
246 differentiation medium (Tdiff). Conversely, in C2C12 cells grown in medium conditioned by A427
247 cells in the presence of both n-3 and n-6 PUFAs, myosin production and myotube formation both
248 occurred, the most significant effect being evident in cells grown in medium conditioned in the
249 presence of 50 μ M EPA plus DHA.

250 To investigate the role of lipid peroxidation products in reversing the anti-differentiation effect of
251 lung cancer cells on C2C12 cells, HHE and HNE were directly added to C2C12 cells grown in
252 medium conditioned by A427 cells, in the absence of n-3/n-6 PUFAs. Figure 5 shows the effect of
253 aldehyde administration on C2C12 cell viability: only HHE, at either of the concentrations used,
254 significantly increased LDH release.

255 Figure 6 shows that both myosin content and myotubes in C1C12 increased in a dose-dependent
256 manner, in C2C12 cells exposed for 4 days to aldehydes, the strongest effect being observed in cells
257 exposed to 5 μ M HNE.

Western blot analysis of ALDH3A1 protein content showed that neither A427 nor C2C12 cells express this enzyme, which is significant in metabolizing aldehydes derived from lipid peroxidation (Figure 7).

Table 1 reports the analysis of the putative PPRE motif in fast isoforms of myosin heavy chain (MyHC). The NHR SCAN software evidenced the putative presence of the PPRE consensus sequence in both exons /introns of IIa, IIb, IId/x genes, but not in the promoter regions.

DISCUSSION

In this research the anti-cachectic effect of both n-3 and n-6 PUFAs was investigated alongside with the possibility that this effect can be mediated by their major aldehydic products of lipid peroxidation, HHE and HNE, and by their anti-inflammatory properties. Aldehyde products derived from the lipid peroxidation of PUFAs have been shown to exert both regulatory and detrimental effects on several cell functions, mainly depending on their cellular concentration. When produced at physiological or low levels, lipidic aldehydes can act as signalling molecules targeting specific pathways, including tyrosine kinase signalling and the adaptive response mediated by Nrf2, AP-1, PPARs and NFkB [41]. Conversely, high intracellular levels of lipidic aldehydes are responsible for cytotoxic or cytostatic effects [42,43].

Among aldehydes derived from lipid peroxidation, α,β -unsaturated aldehydes are those that have been studied in most depth; they are the most important molecular mediators of the effects of both n-3 and n-6 PUFAs.

The “in vitro” model of cancer cachexia used in this research enabled both the effect of PUFA administration on pro-cachectic activity of human lung cancer cells, and the effects of culture medium conditioned by cancer cells in the presence or not of PUFAs on muscle cell differentiation, to be investigated.

The most important finding of the study is that both PUFA families, n-3 and n-6, decreased the proliferation of human lung cancer cells, and were able to prevent the inhibition of muscle cell

284 differentiation induced by lung cancer cells. The observation that AA decreased cancer cell
285 proliferation disagrees with the results of certain other studies [44-46], but confirms previous
286 studies by the present research group evidencing that this n-6 PUFA suppresses the growth of well
287 differentiated human lung tumour cells A549 and of rat hepatocarcinoma cell lines [47-50]. The
288 inhibitory effect of AA on cancer cell proliferation has been inversely related to the ability of cells
289 to metabolize aldehydic products of lipid peroxidation, in particular to the expression of aldehyde
290 dehydrogenase 3A1 (ALDH3A1) [49]. For this reason, in this research the protein content of
291 ALDH3A1 was evaluated in A427 and C2C12 cells, neither of which expresses this enzyme. The
292 lack of this enzyme in A427 cells could be important in maintaining the high level of lipid
293 peroxidation products observed after n-3 or n-6 PUFA supplementation, and, in consequence, might
294 be the explanation for their effect on both cell proliferation and pro-cachectic properties of A427
295 cells. At the same time, the poor ability of C2C12 cells to catabolise aldehydes might be crucial in
296 maintaining aldehyde levels that are able to induce muscle cell differentiation. In fact, unlike free
297 radicals, which have a very short half-life, HNE and other aldehydes deriving from lipid
298 peroxidation can diffuse from the site where they are produced and modulate signalling pathways in
299 other cells.

300 HNE has been reported to induce cell differentiation in human leukemic HL-60 cells and in murine
301 erythroleukemia MEL cells [32], the pathways proposed being different: translocation of PKC,
302 formation of adducts with Heat Shock 60 kDa Protein 1 [51], and inhibition of telomerase activity
303 [52]. In the light of these observations, the supposed involvement of lipid peroxidation products in
304 mediating the anti-cachectic properties of lung cancer cells was investigated. Exposing C2C12
305 muscle cells, maintained in medium conditioned by A427 cells in the absence of PUFAs, to HNE or
306 to HHE induced the expression of myosin and the formation of myotubes, indicating the ability of
307 both lipid peroxidation products to stimulate muscle cell differentiation, counteracting the inhibitory
308 effect of medium conditioned by lung cancer cells. These results show, firstly, that HHE also
309 possesses differentiation properties, and secondly that both the major lipid peroxidation products of

310 n-3 and n-6 PUFAs can block the anti-differentiation effect of lung cancer cells and induce
311 expression of myosin in murine muscle cells.

312 The lipid peroxidation products can induce muscle cell differentiation indirectly by blocking the
313 anti-differentiation effect of TNF α produced and released by cancer cells, and directly by affecting
314 myosin expression, as evidenced by treatment of C2C12 cells with HHE and HNE. The first
315 mechanism contributing to C2C12 cell differentiation, i.e. the decrease TNF α content in culture
316 medium of A427 cells, can be explained by the increased PPAR α expression, induced by PUFAs or
317 their lipid peroxidation products, including HHE and HNE. This statement is in agreement with our
318 previous researches, showing that PUFAs were able to induce PPARs, and, as consequence, to
319 decrease tumour cell proliferation and cytokine content in cell culture medium [49,53,54].

320 It has been shown that HNE acts as an intracellular agonist of peroxisome proliferator-activated
321 receptors (PPARs) and that it is thus involved in regulating several intracellular pathways [55,56].
322 PPARs are nuclear transcription factors that, after ligand binding, heterodimerize with RXR α
323 (PPAR α /RXR α) and bind to the consensus sequence named PPRE (Peroxisome proliferator
324 response element) in the target genes.

325

326 The PPRE motif consist of 2 half-sites (AGGTCA N AGGTCA) with an interspacing nucleotide
327 (DR-1, direct repeat 1) [57]. Based on these observations, the presence of putative PPRE sequences
328 in genes encoding fast isoforms of myosin heavy chain (MyHC) was investigated. The
329 bioinformatic approach used in this preliminary analysis evidenced the presence of putative PPRE
330 in both exons and intron of genes encoding fast myosin heavy chains, but not in the promoter
331 region. This suggests that the increased production of myosin observed in C2C12 cells, grown in
332 medium conditioned by A427 cells in the presence of PUFAs, and in the same cells directly
333 exposed to HHE or HNE, could be PPAR-dependent.

334 Taken as a whole, these results demonstrate the ability of both n-3 and n-6 PUFAs, and of their
335 aldehydic lipid peroxidation products HHE and HNE, to decrease TNF α release and to prevent the

336 decrease of myosin expression and myotube formation that is determined by culturing C2C12
337 muscle cells in the presence of medium conditioned by lung tumour cells. The lower induction of
338 differentiation observed in C2C12 cells treated with HHE in comparison with C2C12 cells grown in
339 culture medium of A427 cells treated with EPA+DHA can be due to the fact that the treatment with
340 n-3 PUFAs caused, other than the production of lipid aldehydes, a decrease of TNF α . This decrease
341 was higher than in the case of A427 cells treated with AA, and this difference could explain the
342 higher differentiation effect induced by EPA+DHA respect to AA.
343 This finding could represent an important starting point to investigate in greater depth the
344 possibility of designing therapeutic protocols using natural dietary substances, such as n-3 and n-6
345 PUFAs, to both directly reduce the growth of cancer cells, and reduce their ability to decrease
346 myosin synthesis and differentiation in muscle cells, which occurs in cancer cachexia.

347

348 Acknowledgements.

349 This research was supported by University of Turin, Italy. The authors thank dr. Bruno Tasso
350 (DIFAR, University of Genoa, Italy) for the synthesis of MDA sodium salt.

351

352 Conflict of interest. No benefit of any kind will be received either directly or indirectly by the
353 authors.

354

355 REFERENCES

356

- 357 [1] K. Fearon, F. Strasser, S. D. Anker, I. Bosaeus, E. ruera, R.L. Fainsinger, A. Jatoi, C.
 358 Loprinzi, N. MacDonald, G.Mantovani, M. Davis, M. Muscaritoli, F. Ottery, L. Radbruch,
 359 P. Ravasco, D. Walsh, A. Wilcock, S. Kaasa, V.E. Baracos, Definition and classification of
 360 cancer cachexia: an international consensus, *Lancet Oncol.* 12 (2011) 489–495.
- 361 [2] S. von Haehling, S.D. Anker, Cachexia as a major underestimated and unmet medical need:
 362 facts and numbers, *J. Cachexia Sarcopenia Muscle* 1 (2010) 1–5.
- 363 [3] M. Kovarik, M. Hronek, Z. Zadak, Clinically relevant determinants of body composition,
 364 function and nutritional status as mortality predictors in lung cancer patients, *Lung Cancer*
 365 84 (2014) 1–6.
- 366 [4] C.M. Prado, J.R. Lieffers, L. Bowthorpe, V E. Baracos, M. Mourtzakis, L.J. McCargar,
 367 Sarcopenia and physical function in overweight patients with advanced cancer, *Can. J. Diet.*
 368 *Pract. Res.* 4 (2013) 69–74.
- 369 [5] B.S. van der Meij, C.P. Schoonbeek, E.F. Smit, M. Muscaritoli, P.A. van Leeuwen, J.A.
 370 Langius, Pre-cachexia and cachexia at diagnosis of stage III non-small-cell lung carcinoma:
 371 an exploratory study comparing two consensus-based frame-works, *Br. J. Nutr.* 109 (2013)
 372 2231–2239.
- 373 [6] J.M. Argilés, S. Busquets, B. Stemmler, F.J. López-Soriano, Cachexia and sarcopenia:
 374 mechanisms and potential targets for intervention, *Curr. Opin. Pharmacol.* 22 (2015) 100–
 375 106.
- 376 [7] J.K. Onesti, D.C. Guttridge, Inflammation based regulation of cancer cachexia, *Biomed.*
 377 *Res. Int.* 2014;2014:168407. doi: 10.1155/2014/168407.
- 378 [8] M. Ebadi, V. C. Mazurak, Potential Biomarkers of Fat Loss as a Feature of Cancer
 379 Cachexia, *Mediators Inflamm.* 2015;2015:820934. doi: 10.1155/2015/820934.

- 380 [9] L. Radbruch, F. Elsner, P. Trottenberg, F. Strasser, K. Fearon, Clinical practice guidelines
381 on cancer cachexia in advanced cancer patients with a focus on refractory cachexia,
382 European Clinical Guidelines. Aachen 2010.
- 383 [10] R.L. Koretz, Should patients with cancer be offered nutritional support: does the benefit
384 outweigh the burden? *Eur. J. Gastroenterol. Hepatol.* 19 (2007) 379–382.
- 385 [11] V.C. Pratt, S. Watanabe, E. Bruera, J. Mackey, M.T. Clandinin, V.E. Baracos, C.J. Field,
386 Plasma and neutrophil fatty acid composition in advanced cancer patients and response to
387 fish oil supplementation, *Br. J. Cancer* 87 (2002) 1370–1378.
- 388 [12] A. Chaudry, S. McClinton, L.E. Moffat, K.W. Wahle, Essential fatty acid distribution in the
389 plasma and tissue phospholipids of patients with benign and malignant prostatic disease, *Br.*
390 *J. Cancer* 64 (1991) 1157–1160.
- 391 [13] R.A. Murphy, M.S. Wilke, M. Perrine, M. Pawlowicz, M. J.R. Mourtzakis, Lieffers, M.
392 Maneshgar, E. Bruera, M.T. Clandinin, V.E. Baracos, V.C. Mazurak, Loss of adipose tissue
393 and plasma phospholipids: relationship to survival in advanced cancer patients, *Clin. Nutr.*
394 29 (2010) 482–487.
- 395 [14] R.A. Murphy, T.F. Bureyko, M. Mourtzakis, Q.S. Chu, M.T. Clandinin, T. Reiman, V.C.
396 Mazurak, Aberrations in plasma phospholipid fatty acids in lung cancer patients, *Lipids* 47
397 (2012) 363–369.
- 398 [15] C. García-Martínez, F.J. López-Soriano, J.M. Argilés, Interleukin-6 does not activate protein
399 breakdown in rat skeletal muscle, *Cancer Lett.* 76 (1994) 1–4.
- 400 [16] A. Enomoto, M.C. Rho, A. Fukami, O. Hiraku, K. Komiyama, M. Hayashi, Suppression of
401 cancer cachexia by 20S,21-epoxy-resibufogenin-3-acetate-a novel nonpeptide IL-6 receptor
402 antagonist, *Biochem. Biophys. Res. Commun.* 323 (2004) 1096–1102.
- 403 [17] D. Zhang, H. Zheng, Y. Zhou, X. Tang, B. Yu, J. Li, Association of IL-1beta gene
404 polymorphism with cachexia from locally advanced gastric cancer, *BMC Cancer* 7 (2007)
405 45. doi: 10.1186/1471-2407-7-45.

- 406 [18] P.C. Calder, Long-chain fatty acids and inflammation, *Proc. Nutr. Soc.* 71 (2012) 284–289.
- 407 [19] A. Ries, P. Trottenberg, F. Elsner, S. Stiel, D. Haugen, S. Kaasa, L.A. Radbruch, Systematic
408 review on the role of fish oil for the treatment of cachexia in advanced cancer: an EPCRC
409 cachexia guidelines project. *Palliat. Med.* 26 (2012) 294–304.
- 410 [20] A.S. Whitehouse, M.J. Tisdale, Increased expression of the ubiquitin-proteasome pathway in
411 murine myotubes by proteolysis-inducing factor (PIF) is associated with activation of the
412 transcription factor NF- κ B, *Br. J. Cancer* 89 (2003) 1116–1122.
- 413 [21] S.T. Russell, M.J. Tisdale, Effect of eicosapentaenoic acid (EPA) on expression of a lipid
414 mobilizing factor in adipose tissue in cancer cachexia, *Prostaglandins Leukot. Essent. Fatty*
415 *Acids* 72 (2005) 409–414.
- 416 [22] G.I. Smith, P. Atherton, D.N. Reeds, B.S. Mohammed, D. Rankin, M.J. Rennie, B.
417 Mittendorfer, Dietary omega-3 fatty acid supplementation increases the rate of muscle
418 protein synthesis in older adults: a randomized controlled trial, *Am. J. Clin. Nutr.* 93 (2011)
419 402–412.
- 420 [23] L.E. Robinson, A.C. Buchholz, V.C. Mazurak, Inflammation, obesity, and fatty acid
421 metabolism: influence of n-3 polyunsaturated fatty acids on factors contributing to
422 metabolic syndrome, *Appl. Physiol. Nutr. Metab.* 32 (2007) 1008–1024.
- 423 [24] E.K. Long, M.J. Picklo Sr, Trans-4-hydroxy-2-hexenal, a product of n-3 fatty acid
424 peroxidation: make some room HNE, *Free Radic. Biol. Med.* 49 (2010) 1–8.
- 425 [25] E.K. Long, T.C. Murphy, L.J. Leiphon, J. Watt, J.D. orrow, G.L. Milne, J.R. Howard, M.J.
426 Picklo Sr, Trans-4-hydroxy-2-hexenal is a neurotoxic product of docosahexaenoic (22:6; n-
427 3) acid oxidation, *J. Neurochem.* 105 (2008) 714–724.
- 428 [26] J.Y. Lee, J.H. Je, D.H. Kim, S.W. Chung, Y. Zou, N.D. Kim, M. Ae Yoo, H. Suck Baik,
429 B.P. Yu, H.Y. Chung, Induction of endothelial apoptosis by 4-hydroxyhexenal, *Eur. J.*
430 *Biochem.* 271 (2004) 1339–1347.

- 431 [27] S. Choudhary, T. Xiao, S. Srivastava, W. Zhang, L.L. Chan, L.A. Vergara, F.J. Van Kuijk,
432 N.H. Ansari, Toxicity and detoxification of lipid-derived aldehydes in cultured retinal
433 pigmented epithelial cells, *Toxicol. Appl. Pharmacol.* 204 (2005) 122–134.
- 434 [28] E.H. Bae, S. Cho, S.Y. Joo, S.K. Ma, S.H. Kim, J. Lee, S.W. Kim, 4-Hydroxy-2-hexenal-
435 induced apoptosis in human renal proximal tubular epithelial cells, *Nephrol. Dial.*
436 *Transplant.* 26 (2011) 3866–3873.
- 437 [29] N.J. Pillon, L. Soullère, R.E. Vella, M. Croze, B.R. Caré, H.A. Soula, A. Doutheau, M.
438 Lagarde, C.O. Soulage, Quantitative structure-activity relationship for 4-hydroxy-2-alkenal
439 induced cytotoxicity in L6 muscle cells, *Chem. Biol. Interact.* 188 (2010) 171–180.
- 440 [30] M.A. Bradley, S. Xiong-Fister, W.R. Markesbery, M.A. Lovell, Elevated 4-hydroxyhexenal
441 in Alzheimer's disease (AD) progression, *Neurobiol. Aging* 33 (2012) 1034–1044.
- 442 [31] R.M. Domingues, P. Domingues, T. Melo, D. Pérez-Sala, A. Reis, C.M. Spickett,
443 Lipoxidation adducts with peptides and proteins: deleterious modifications or signaling
444 mechanisms? *J. Proteomics* 92 (2013) 110–131.
- 445 [32] M. Rinaldi, G. Barrera, A. Aquino, P. Spinsanti, S. Pizzimenti, M.G. Farace, M.U. Dianzani,
446 V.M. Fazio, 4-Hydroxynonenal-induced MEL cell differentiation involves PKC activity
447 translocation, *Biochem. Biophys. Res. Commun.* 272 (2000) 75–80.
- 448 [33] G. Barrera, S. Pizzimenti, S. Laurora, E. Moroni, B. Giglioni, M.U. Dianzani, 4-
449 Hydroxynonenal affects pRb/E2F pathway in HL-60 human leukemic cells, *Biochem.*
450 *Biophys. Res. Commun.* 295 (2002) 267–275.
- 451 [34] S. Pizzimenti, M. Ferracin, S. Sabbioni, C. Toaldo, P. Pettazzoni, M.U. Dianzani, M.
452 Negrini, G. Barrera, MicroRNA expression changes during human leukemic HL-60 cell
453 differentiation induced by 4-hydroxynonenal, a product of lipid peroxidation, *Free Radic.*
454 *Biol. Med.* 46 (2009) 282–288.

- 455 [35] M. Oraldi, A. Trombetta, F. Biasi, R.A. Canuto, M. Maggiora, G. Muzio, Decreased
456 polyunsaturated Fatty Acid content contributes to increased survival in human colon cancer,
457 J. Oncol. 2009:867915; 2009. doi: 10.1155/2009/867915.
- 458 [36] A. Kornberg, Lactic dehydrogenase of muscle, in S.P. Colowick, N. D. Kaplan (Eds),
459 Methods of Enzymology, Academic Press, New York, 1955, vol.1, pp. 441–443.
- 460 [37] G. Barbiero, F. Duranti, G. Bonelli, J.S. Amenta, F.M. Baccino, Intracellular ionic
461 variations in the apoptotic death of L cells by inhibitors of cell cycle progression, Exp. Cell
462 Res. 217 (1995) 410-418.
- 463 [38] M. Oraldi, S. Saracino, M. Maggiora, A. Chiaravalloti, C. Buemi, G. Martinasso, E. Paiuzzi,
464 D. Thompson, V. Vasiliou, R.A. Canuto, Importance of inverse correlation between
465 ALDH3A1 and PPAR γ in tumour cells and tissue regeneration, Chem. Biol. Interact. 191
466 (2011) 171–176.
- 467 [39] F. Nielsen, B.B. Mikkelsen, J.B. Nielsen, H.R. Andersen, P. Grandjean, Plasma
468 malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-
469 style factors, Clin. Chem. 43 (1997) 1209–1214.
- 470 [40] V. Nair, D.E. Vietti, C.S. Cooper, Degenerative Chemistry of Malondialdehyde. Structure,
471 Stereochemistry, and Kinetics of Formation of Enaminals from Reaction with Amino Acids,
472 J. Am. Chem. Soc. 103 (1981) 3030–3036.
- 473 [41] S. J. Chapple, X. Cheng, G.E. Mann, Effects of 4-hydroxynonenal on vascular endothelial
474 and smooth muscle cell redox signaling and function in health and disease, Redox Biol. 1
475 (2013) 319–331.
- 476 [42] H. Esterbauer, Cytotoxicity and genotoxicity of lipid-oxidation products, Am. J. Clin. Nutr.
477 57(5 Suppl) (1993) 779S–785S; discussion 785S–786S.
- 478 [43] E. Niki, Lipid peroxidation: physiological levels and dual biological effects, Free Radic.
479 Biol. Med. 47 (2009) 469–484.

- 480 [44] M. Hughes-Fulford, Y. Chen, R.R. Tjandrawinata, Fatty acid regulates gene expression and
481 growth of human prostate cancer PC-3 cells, *Carcinogenesis* 22 (2001) 701–707.
- 482 [45] B. Chénais, V. Blanckaert, The janus face of lipids in human breast cancer: how
483 polyunsaturated Fatty acids affect tumor cell hallmarks, *Int. J. Breast Cancer*
484 2012;2012:712536. doi: 10.1155/2012/712536.
- 485 [46] N.W. Chang, C.T. Wu, D.R. Chen, C.Y. Yeh, C. Lin, High levels of arachidonic acid and
486 peroxisome proliferator-activated receptor- α in breast cancer tissues are associated with
487 promoting cancer cell proliferation, *J. Nutr. Biochem.* 24 (2013) 274–281.
- 488 [47] R.A. Canuto, G. Muzio, A.M. Bassi, M. Maggiora, G. Leonarduzzi, R. Lindahl, M.U.
489 Dianzani, M. Ferro, Enrichment with arachidonic acid increases the sensitivity of hepatoma
490 cells to the cytotoxic effects of oxidative stress, *Free Radic. Biol. Med.* 18 (1995) 287–293.
- 491 [48] R.A. Canuto, M. Ferro, R.A. Salvo, A.M. Bassi, A. Trombetta, M. Maggiora, G. Martinasso,
492 R. Lindahl, G. Muzio, Increase in class 2 aldehyde dehydrogenase expression by
493 arachidonic acid in rat hepatoma cells. *Biochem. J.* 357 (2001) 811–818.
- 494 [49] G. Muzio, A. Trombetta, M. Maggiora, G. Martinasso, V. Vasiliou, N. Lassen, R.A. Canuto,
495 Arachidonic acid suppresses growth of human lung tumor A549 cells through down-
496 regulation of ALDH3A1 expression. *Free Radic. Biol. Med.* 40 (2006) 1929–1938.
- 497 [50] R.A. Canuto, M. Ferro, M. Maggiora, R. Federa, O. Brossa, A.M. Bassi, R. Lindahl, G.
498 Muzio, In hepatoma cell lines restored lipid peroxidation affects cell viability inversely to
499 aldehyde metabolizing enzyme activity. *Adv. Exp. Med. Biol.* 414 (1997) 113–122.
- 500 [51] A. Arcaro, M. Daga, G.P. Cetrangolo, E.S. Ciamporcerio, A. Lepore, S. Pizzimenti, C.
501 Putrella, M. Graf, K. Uccida, G. Mamone, P. Ferranti, P.R. Ames, G. Palombo, G. Barrera,
502 F. Gentile, Generation of Adducts of 4-Hydroxy-2-nonenal with Heat Shock 60 kDa Protein
503 1 in Human Promyelocytic HL-60 and Monocytic THP-1 Cell Lines, *Oxid. Med. Cell*
504 *Longev.* 2015;2015:296146. doi: 10.1155/2015/296146.

- 505 [52] S. Pizzimenti, E. Menegatti, D. Berardi, C. Toaldo, P. Pettazzoni, R. Minelli, B. Giglioni, A.
506 Cerbone, M.U. Dianzani, C. Ferretti, G. Barrera, 4-hydroxynonenal, a lipid peroxidation
507 product of dietary polyunsaturated fatty acids, has anticarcinogenic properties in colon
508 carcinoma cell lines through the inhibition of telomerase activity J. Nutr. Biochem. 21
509 (2010) 818–826.
- 510 [53] M. Oraldi, M. Maggiora, E. Paiuzzi, R.A. Canuto, G. Muzio, CLA reduces inflammatory
511 mediators from A427 human lung cancer cells and A427 conditioned medium promotes
512 differentiation of C2C12 murine muscle cells, Lipids 48 (2013) 29–38.
- 513 [54] A. Trombetta, M. Maggiora, G. Martinasso, P. Cotogni, R.A. Canuto, G. Muzio,
514 Arachidonic and docosahexaenoic acids reduce the growth of A549 human lung-tumor cells
515 increasing lipid peroxidation and PPARs. Chem. Biol. Interact. 165 (2007) 239–250.
- 516 [55] A. Cerbone, C. Toaldo, S. Laurora, F. Briatore, S. Pizzimenti, M.U. Dianzani, C. Ferretti, G.
517 Barrera, 4-Hydroxynonenal and PPARgamma ligands affect proliferation, differentiation,
518 and apoptosis in colon cancer cells, Free Radic. Biol. Med. 42 (2007) 1661–1670.
- 519 [56] A. Manea, S.A Manea, A. Todirita, I.C. Albulescu, M. Raicu, S. Sasson, M. Simionescu,
520 High-glucose-increased expression and activation of NADPH oxidase in human vascular
521 smooth muscle cells is mediated by 4-hydroxynonenal-activated PPAR α and PPAR β/δ , Cell
522 Tissue Res. 361 (2015) 593–604.
- 523 [57] L. Michalik, J. Auwerx, J.P. Berger, V.K. Chatterjee, C.K. Glass, F.J. Gonzalez, P.A.
524 Grimaldi, T. Kadowaki, M.A. Lazar, S. O'Rahilly, C.N. Palmer, J. Plutzky, J.K. Reddy,
525 B.M. Spiegelman, B. Staels, W. Wahli, International Union of Pharmacology. LXI.
526 Peroxisome proliferator-activated receptors, Pharmacol. Rev. 58 (2006) 726–741.
527

528 FIGURE CAPTIONS

529

530 Figure 1. Effect of n-3 or n-6 PUFAs on growth and viability of human lung cancer cells A427.

531 Data are means $\pm$ S.D. from 4 experiments. For each panel, means with different letters are
532 significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by
533 post-hoc Newman-Keuls analysis.

534 C, control cells; E + D 10, cells treated with 10 μ M EPA + DHA (1.5:1 ratio); E + D 50, cells
535 treated with 50 μ M EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10 μ M AA; AA 50; cells
536 treated with 50 μ M AA.

537

538 Figure 2. Lipid peroxidation in culture medium of human lung cancer cells A427 treated or not with
539 n-3 or n-6 PUFAs for 24 hours.

540 Data are means $\pm$ S.D. from 4 experiments and are expressed as percentage of control value taken
541 as 100. Means with different letters are significantly different from one another ($p < 0.05$) as
542 determined by analysis of variance followed by post-hoc Newman-Keuls analysis.

543 MDA, malondialdehyde; C, control cells; E + D 10, cells treated with 10 μ M EPA + DHA (1.5:1
544 ratio); E + D 50, cells treated with 50 μ M EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10
545 μ M AA; AA 50; cells treated with 50 μ M AA.

546

547 Figure 3. TNF α (panel A) and PPAR α (panel B) in culture medium of A427 cells treated or not with
548 n-3 or n-6 PUFAs for 24 hours and in A427 cells, respectively.

549 Data are means $\pm$ S.D. from 4 experiments and are expressed as percentage of control value taken
550 as 100. For each panel, means with different letters are significantly different from one another
551 ($p < 0.05$) as determined by analysis of variance followed by post-hoc Newman-Keuls analysis.

552 C, control cells; E + D 10, cells treated with 10 μ M EPA + DHA (1.5:1 ratio); E + D 50, cells
553 treated with 50 μ M EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10 μ M AA; AA 50; cells
554 treated with 50 μ M AA.

555

556 Figure 4. Myosin content evidenced by immunofluorescence in C2C12 murine myoblasts grown in
557 culture medium conditioned by human lung cancer cells A427, in the presence or absence of
558 PUFAs.

559 Tdiff, cells maintained in medium B; none, cells grown in culture medium conditioned by A427
560 cells in the absence of PUFAs; E + D 10, cells treated with 10 μ M EPA + DHA (1.5:1 ratio); E + D
561 50, cells treated with 50 μ M EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10 μ M AA; AA
562 50; cells treated with 50 μ M AA.

563

564 Figure 5. Effect of HHE and HNE on viability of C2C12 murine myoblasts grown in culture
565 medium conditioned by human lung cancer cells A427 in the absence of PUFAs.

566 Data are means $\pm$ S.D. from 4 experiments. Means with different letters are significantly different
567 from one another ($p < 0.05$) as determined by analysis of variance followed by post-hoc Newman-
568 Keuls analysis.

569 Tdiff, cells maintained in medium B; none, cells grown in culture medium conditioned by A427
570 cells in the absence of PUFAs; HHE 1, cells grown in culture medium conditioned by A427 cells in
571 the absence of PUFAs and treated with 1 μ M HHE; HHE 5, cells grown in culture medium
572 conditioned by A427 cells in the absence of PUFAs and treated with 5 μ M HHE; HNE 1, cells
573 grown in culture medium conditioned by A427 cells in the absence of PUFAs and treated with 1
574 μ M HNE; HNE 5, cells grown in culture medium conditioned by A427 cells in the absence of
575 PUFAs and treated with 5 μ M HNE (for treatment details see Materials and Methods).

576

577 Figure 6. Myosin content evidenced by immunofluorescence in C2C12 murine myoblasts grown in
578 culture medium conditioned by human lung cancer cells A427 in the absence of PUFAs and treated
579 with HHE and HNE.

580 Tdiff, cells maintained in medium B; none, cells grown in culture medium conditioned by A427
581 cells in the absence of PUFAs; HHE 1, cells grown in culture medium conditioned by A427 cells in
582 the absence of PUFAs and treated with 1 μ M HHE; HHE 5, cells grown in culture medium
583 conditioned by A427 cells in the absence of PUFAs and treated with 5 μ M HHE; HNE 1, cells
584 grown in culture medium conditioned by A427 cells in the absence of PUFAs and treated with 1
585 μ M HNE; HNE 5, cells grown in culture medium conditioned by A427 cells in the absence of
586 PUFAs and treated with 5 μ M HNE (for treatment details see see Materials and Methods).

587

588 Figure 7. Aldehyde dehydrogenase 3A1 protein content in human lung cancer cells A427 and in
589 C2C12 murine myoblasts.

590 Lane 1, A427 cells; lane 2, C2C12 cells; C+, positive control.

TABLE 1 –PUTATIVE PPRE MOTIF IN GENES ENCODING FAST ISOFORMS OF MYOSIN HEAVY CHAIN

MyHC	Gene I.D.	PUTATIVE PPRE MOTIF	
		Promoter region	Exons / Introns
			bp
IIa	17882	Not present	1 DR1 (intron 3) 3543 -3555
			1 DR1 (exon 4) 4032 - 4044
			1 DR1 (intron 16) 11674 - 11686
			1 DR1 (exon 19) 14111 - 14123
IIb	17884	Not present	1 DR1 (intron 1) 229 - 241
			1 DR1 (intron 4) 3890 - 3902
			1 DR1 (intron 15) 10814 - 10826
			1 DR1 (exon 27) 16059 - 16071
IId/x	17879	Not present	1 DR1 (intron 5) 2502 - 2514
			1 DR1 (intron 9) 5309 - 5321
			2 DR1 (intron 15) 7975 - 7987
			8606 - 8618

MyHC, myosin heavy chain

Figure 1

Figure 2

Figure 3

TNF α

A

PPAR α

B

Figure 4

DAY 4

T DIFF

NONE

E + D 10

E + D 50

AA 10

AA 50

Figure 5

Figure 6

DAY 4

T DIFF

NONE

HHE 1

HHE 5

HNE 1

HNE 5

Figure 7