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► **To cite this version:**

Michel Lagarde, Miao Liu, Evelyne Véricel, Catherine Calzada, Ping Chen, et al.. Docosahexaenoic acid, protectin synthesis: relevance against atherothrombogenesis.: DHA

protectins against atherothrombosis. Proceedings of the Nutrition Society, Cambridge University Press (CUP), 2013, pp.1-4. <10.1017/S0029665113003704>. <inserm-00879583>

HAL Id: inserm-00879583

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Submitted on 4 Nov 2013

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1 **Docosahexaenoic acid, protectin synthesis: relevance against athero-thrombogenesis**

2

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11

12 **Running title:** DHA & protectins against atherothrombosis

13

14 **Abstract**

15 Docosahexaenoic acid (DHA) is an abundant nutrient from marine lipids: its specific
16 biological effects have been investigated in human volunteers, taking into consideration the
17 dose effects. We report herein that, at dosages below one g/day, DHA proved to be effective
18 in lowering blood platelet function and exhibited an “antioxidant” effect. However, this was
19 not anymore the case following 1.6g/day, showing then a U-shape response. The antioxidant
20 effect has been observed in platelets as well as low-density lipoproteins, of which the redox
21 status is assumed to be crucial in their relationship with atherosclerosis. Secondly, the
22 oxygenated products of DHA, especially protectins produced by lipoxygenases, have been
23 considered for their potential to affect blood platelets and leukocytes. It is concluded that
24 DHA is an interesting nutrient to reduce athero-thrombogenesis, possibly through
25 complementary mechanisms involving lipoxygenase products of DHA.

26

27 **Key-words**

28 Blood platelets, Leukocytes, Cyclooxygenases, Lipoxygenases

29

30 **Introduction**

31 Docosahexaenoic acid (DHA, 22:6n-3) is an abundant long-chain polyunsaturated fatty acid
32 (PUFA) of marine origin⁽¹⁾. In addition to be a major PUFA of the brain and retina in
33 animals⁽²⁾, DHA is well-known as a relevant nutrient which prevents adverse cardiovascular
34 events⁽³⁾. Together with its precursor eicosapentaenoic acid (EPA, 20:5n-3), another major
35 component of marine lipids⁽⁴⁾, DHA is one of the two main omega-3 long-chain PUFA
36 believed to be responsible for protection against cardiovascular events⁽⁵⁾. However, it is worth
37 mentioning the possible involvement in these biological events the third most abundant
38 component in marine lipids⁽⁶⁾, the intermediate between EPA and DHA, docosapentaenoic
39 acid (DPA, 22:5n-3), and that of the omega-3 family precursor alpha-linolenic acid (ALA,
40 18:3n-3)⁽⁷⁾.

41 During the last decade, a series of oxygenated derivatives of DHA have been described with
42 activities against inflammation, some speeding the resolution phase of inflammation, then
43 playing an interesting role in the prevention of atherogenesis. The oxygenated metabolites
44 have been named protectins and resolvins^(8,9). One protectin made by macrophages has been
45 named maresin⁽¹⁰⁾. Lipoxygenases (LOX) are key enzymes in the generation of these
46 derivatives, which makes the action of DHA quite specific and different from that of EPA that

47 is active through some of its cyclooxygenase (COX) products as well. In the eighties, DHA
48 had already been reported as a fairly good substrate of blood platelet 12-LOX⁽¹¹⁾. Also, its
49 mono-hydroxylated metabolites produced through LOX have been found to be inhibitors of
50 the thromboxane-induced platelet aggregation as well as blood vessel constriction⁽¹²⁾.

51 This short review will consider the *ex vivo* effect of dietary DHA used at low dosage, and the
52 *in vitro* biological effects of a double lipoxygenase product of DHA called protectin DX, in
53 the frame of the cardiovascular risk.

54

55 **DHA supplementation in humans**

56 A large number of trials have been conducted with long-chain n-3 PUFA with various
57 proportions of EPA and DHA, and also DPAn-3 when more or less crude fish oil or fish meat
58 were used for supplementing the diet. It is generally assumed that those supplementations
59 have preventive effects against cardiovascular events. However, depending on the status of
60 volunteers (age, dietary habits, possible usage of drugs, etc) the benefits of such
61 supplementations may be controversial. Indeed, a recent meta-analysis which did not take into
62 account the different situations of the people participating in the trials concluded that “omega-
63 3 PUFA supplementation was not associated with a lower risk of all-cause mortality”⁽¹³⁾. A
64 post-review further indicates that “Subgroup analyses suggested that this could be because of
65 a low absolute risk as a consequence of the state-of-the-art drug treatment”⁽¹⁴⁾.

66 Earlier, we have considered that the dosage in EPA-DHA supplementation could be an issue
67 as their high level of unsaturation makes them highly susceptible to peroxidation that could be
68 detrimental to their potential benefit. Relating to blood platelets that play a crucial role in the
69 initiation of atherothrombogenesis, we first considered that the increased oxidative stress
70 associated with aging⁽¹⁵⁾ could negatively affect the expected benefit of the intake of long-
71 chain n-3 PUFA. So, we conducted an assay with a small dosage of those (150mg DHA + 30
72 mg EPA esterified in triglycerides), each day for six weeks. The main results at the platelet
73 level were a significant accumulation of DHA in membrane ethanolamine phospholipids and
74 an increase in platelet vitamin E, the latter being of interest because platelet vitamin E is
75 lower in this population of elderly people compared to young adults, associated with a
76 decrease in malondialdehyde (MDA) concentration⁽¹⁶⁾. Also, a trend in decreased platelet
77 aggregation and basal thromboxane formation could be observed, but the most striking fact
78 was a significant lowering of the diastolic blood pressure. In contrast, no differences could be

79 observed in a placebo group receiving the same amount of sunflower oil, which of course
80 contained high proportion of linoleate and no n-3 PUFA⁽¹⁶⁾.
81 More recently we have conducted another investigation with increasing intakes of pure DHA
82 esterified in triglycerides (algal oil) in a population of middle-aged men (53-65 year-old) with
83 each of them following the supplementation program (two weeks of successively 200, 400,
84 800 and 1600 mg DHA per day), making each volunteer his own control. A significant dose-
85 dependent accumulation of DHA could be observed in platelet phospholipids, but platelet
86 aggregation was only significantly lowered after the intermediate dosages (400 and 800
87 mg/d). In terms of redox status, only the 200 mg/d dosage was able to increase platelet
88 vitamin E. Most interesting was to find that urinary isoprostanes, a recognized marker of
89 oxidative stress, were significantly decreased after 200mg/d and significantly increased after
90 1600 mg/d⁽¹⁷⁾. It must be noticed that the other markers of the oxidative stress, taken into
91 consideration in this study, were not significantly altered following the highest dosage.
92 Regarding the plasma low-density lipoproteins (LDL), their phospholipids and cholesteryl
93 esters dose-dependently accumulated DHA, but significant improvements of the redox status
94 were observed after 200, 400 and 800 mg/d DHA intake only. This concerned vitamin E with
95 the highest increase after 200 mg/d, and reciprocal U-shape curves for MDA, a global marker
96 of oxidative stress, and the oxidizability of LDL to copper ions, with a decreased MDA and
97 increased lag phase of oxidation in response to copper⁽¹⁸⁾. These results clearly indicate that
98 low daily intake of DHA (lower than 1 g/d) allows expression of an “antioxidant” profile
99 based on several blood markers. This beneficial profile was not any more observed following
100 the highest dosage, with even a global increased oxidative stress as stated above with urinary
101 isoprostanes.
102 Overall, and although the two latter intervention studies in healthy humans have not been
103 conducted on a long term basis, they clearly indicate that the amount of long-chain n-3 PUFA
104 intake is an issue that must be taken into consideration.

105

106 **Oxygenated metabolism of DHA and biological effects**

107 Contrary to EPA, that has the structural feature of arachidonic acid (ARA) with an additional
108 cis/Z double bond at carbon 17, DHA is not oxygenated into prostanoid-like products by
109 cyclooxygenases (COX) although it may inhibit the enzymes by competition, especially
110 against ARA⁽¹⁹⁾. DHA may however be hydroxylated into 13-hydroxylated derivative by
111 COX-2 or into 17(R)-hydroxylated derivative if COX-2 is treated by aspirin, the latter

112 derivative being a substrate of the neutrophil 5-LOX to produce resolvins D1 and D2⁽²⁰⁾. This
113 production is likely to be very low as the rate conversion of DHA into its 17(R)-hydroxylated
114 derivative has been found to be less than 1-5 % of the rate conversion of ARA into PGH₂⁽²¹⁾.
115 In contrast, DHA is a fairly good substrate of LOX to produce various hydroxylated end-
116 products after reduction of the hydroperoxide intermediates by glutathione peroxidase. They
117 are 4- and 7-OH through 5-LOX, 11- and 14-OH through 12-/n-9-LOX and 17-OH through
118 15-/n-6-LOX⁽²²⁾. The oxygenation of DHA through the latter LOX has been studied in details
119 for the production of protectin/neuroprotectin D1⁽⁸⁾. In this case the biosynthetic route seems
120 to mimic leukotriene B₄ production by 5-LOX from ARA, then leading to 10(R),17(S)diOH-
121 4Z,7Z,11E,13E,15Z,19Z-22:6^(23,24).

122 A double 15-/n-6-LOX end-product can also be produced, which is a geometric and
123 stereoisomer of PD1. It is 10(S),17(S)diOH-4Z,7Z,11E,13Z,15E,19Z-22:6 and has been
124 named protectin DX (PDX)⁽²⁵⁾. As mono-hydroxylated derivatives have been shown to inhibit
125 the thromboxane-induced aggregation of human blood platelets⁽¹²⁾, we have investigated the
126 inhibition of that function by PDX and some isomers. In summary, PDX inhibits dose-
127 dependently platelet aggregation induced by collagen, ARA and the stable thromboxane A₂
128 mimetic U-46619 (a stable analog of prostaglandin H₂). The inhibition power of PDX towards
129 the aggregation induced by collagen or ARA was the same, but the inhibition of the U-46619-
130 induced aggregation was around half, suggesting that PDX did not affect the release of ARA
131 from phospholipids in response to collagen but equally inhibits platelet COX-1 and
132 thromboxane A₂ response⁽²⁶⁾. The inhibition of COX-1 was confirmed by studying the
133 oxygenation of radiolabelled ARA, which proved the specific inhibition of COX-1 as the 12-
134 /n-9-LOX activity was not affected. Interestingly, a stereoisomer of PDX (10(R) instead of
135 10(S)), other double 15-/n-6-LOX end-products from ARA and 22:3n-6 were as inhibitory as
136 PDX, providing they have the *E,Z,E* conjugated triene geometry. In contrast, isomers having
137 an *E,E,Z* or *E,E,E* (all-trans) conjugated triene geometry were inactive. The *E,Z,E* conjugated
138 trienes oxygenated PUFA have been collectively named "poxytrins"⁽²⁶⁾. It is worth noting that
139 PD1, which has an *E,E,Z* conjugated triene motif, is described as an anti-inflammatory
140 molecule but as a weak inhibitor of ADP-induced aggregation, without being further
141 potentiated by aspirin treatment⁽²⁷⁾.

142 More recently, we have extended our investigations regarding PDX activities. First we found
143 that it inhibits purified COX-1 and COX-2 with a slightly stronger inhibition of COX-2
144 (submitted). Second, we addressed the possibility of inhibiting the reactive oxygen species

145 (ROS) generation in human neutrophils, as a previous work has shown that punicic acid,
146 which exhibits a Z,E,Z conjugated triene motif, inhibits NADPH oxidase-induced ROS
147 production⁽²⁸⁾. Indeed, we found such an inhibition with a dose-dependent effect^(29 & submitted).
148 However, whereas PDX is active against platelet aggregation and COX activities in the sub-
149 micromolar range, the inhibition of ROS generation requires micromolar concentrations.
150 Leukotriene production from ARA being an important feature in neutrophils, we also tested
151 the effect of PDX upon the formation of 5-LOX products in these cells. No inhibition can be
152 found while the endogenous COX-2 activity was inhibited at similar range concentrations as
153 those inhibiting ROS production (submitted). This indicates that in addition to its potential for
154 inhibiting platelet function, PDX may also exhibit some anti-inflammatory activity, likely
155 through the inhibition of COX-2.

156

157 **Conclusion**

158 DHA is a nutrient with several beneficial effects in preventing athero-thrombogenesis if it is
159 consumed in moderate amounts. In this respect, several international recommendations
160 agreeing with half-a-gram per day seem reasonable to avoid some possible side-effects in
161 terms of oxidative stress. Several mechanisms could contribute to the beneficial effects.
162 Among them, the inhibition of COX activities and the DHA oxygenated products, mainly
163 through LOX activities, may act by complementary effects such as inhibition of platelet
164 aggregation and immune-competent cell function (Figure 1). Altogether the athero-
165 thrombogenesis could be reduced. However, it remains to prove that enough oxygenated
166 products are generated *in situ* to account for the effects observed *in vitro*.

167

168 **Acknowledgements**

169 The authors thank the support of Inserm, the Ministry of Education and Research, ANR, and
170 the Carnot LISA institute.

171 The authors declare no conflicts of interest.

172 M. Lagarde wrote the review and supervised the experimental work

173 M. Liu, P. Chen & F. Driss conducted experiments

174 E. Véricel, C. Calzada & M. Guichardant conducted and supervised the experimental work

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176

177 **Figure: Summary of the DHA effect on blood platelets.**

178 DHA from the blood flow is partly taken up by platelets and mainly esterified into
179 ethanolamine plasmalogens. This may be accompanied with an "antioxidant" effect as shown
180 by *in vitro* enrichment⁽³⁰⁾. DHA may be released by calcium-independent phospholipase A₂
181 (iPLA₂)⁽³¹⁾, and be converted into 14-HDoHE by 12-/n-9-LOX to inhibit thromboxane action.
182 Some non-esterified DHA entering platelets may directly be converted into 14-HDoHE. Non-
183 esterified DHA may also inhibit thromboxane A₂ (TxA₂) formation, in cPLA₂-dependent
184 activated platelets, through inhibition of COX-1. Besides platelets, DHA may be converted
185 into PD1 and PDX by other cells doted of 15-/n-6-LOX such as endothelial cells and
186 leukocytes, and PDX may inhibit platelet aggregation.

187 PL-ARA & PL-DHA: ARA & DHA-containing phospholipids, respectively. TxS:
188 thromboxane synthase.

189

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Figure 1

