ELSEVIER
Journal of Cystic Fibrosis 6 (2007) 320–326
Journal of Cystic Fibrosis
www.elsevier.com/locate/jcf

Fatty acids platelets and oxidative markers following intravenous n-3 fatty acids administration in cystic fibrosis: An open pilot observational study ☆

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Received 20 July 2006; received in revised form 14 December 2006; accepted 15 December 2006
Available online 25 January 2007

Abstract

Background: An imbalance in the ratio of arachidonic acid and docosahexaenoic acid (DHA) was found in cystic fibrosis (CF) affected tissues and was suggested to promote inflammation. Several studies have shown that the long chain n-3 fatty acids reduced inflammatory activity while others have highlighted prooxidant activity of DHA at high concentrations. The aim of our study was to evaluate the effects of an intravenous fish-oil emulsion enriched with n-3 FA in patients with CF on plasma and platelet FA composition and peroxidation markers.
Methods: 13 patients with CF received one IV emulsion per week of 2 mL/kg fish-oil n-3 emulsion for 12 weeks.
Results: There was a significant increase in 20:5 n-3 and 22:6 n-3 platelet FA composition, no variation in 20:4 n-6, a decrease in n-9. There was no variation in plasma FA composition. Specific urinary markers of lipid peroxidation derived from n-3 and n-6 showed a very high level before infusion compared with usual values in healthy subjects which was not affected by treatment. A significant weight loss and a decrease in reduced glutathione were observed in adult patients.
Conclusions: The intravenous administration of n-3 FA in CF patients induced a significant modification in platelet FA composition but no modification of oxidative markers. However, the weight loss and the decreased level in reduced glutathione observed in adult patients may suggest a potential deleterious activity for some patients. Further studies are necessary to determine the optimal dose and route for long chain FA administration required to reach a potential beneficial effect.

Keywords: Cystic fibrosis; n-3 fatty acids; Oxidative stress; Platelet; Intravenous fish-oil treatment

1. Introduction

Several studies have suggested a link between the genetic defect leading to Cystic Fibrosis (CF) and abnormal polyunsaturated fatty acid (FA) metabolism [1,2]. An intrinsic imbalance in the ratio of membrane fatty acids with an

☆ This work was supported by the Hospices Civils de Lyon and the AGIRàdom association.
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doi:10.1016/j.jcf.2006.12.005

excess of arachidonic acid (C20:4 n-6; AA) and a deficiency of docosahexaenoic acid (C22:6 n-3; DHA) was found in CFTR-expressing tissues [3] suggesting it may promote inflammation. A defective regulation of FA metabolism, with defective regulation and increased turnover of AA (a pro-inflammatory agent) and elevated production of eicosanoids [4], or an alteration of FA incorporation in membrane cells [5] have been suggested. The link between FA membrane profile and CF phenotype has been suggested by (i) the CF phenotype of EFA-deficient animals [6], (ii) the correction of some pathological manifestations in CF mice concomitantly with the correction of membrane lipid imbalance [3], and (iii) the link between CF genotype and essential FA plasma and mucosal profiles in CF patients [1,2].

Some authors have suggested a link between the FA profile and the high levels of inflammation and oxidative stress, particularly lipid peroxidation, observed in CF both at plasmatic and airway levels [7,8]. Previous studies have evaluated the effect of fatty acid supplementation in CF patients with different doses and ways of administration and various evaluated criteria [9–12], showing either little or no clinical beneficial effect but some biological anti inflammatory effects like decrease in LTB4/LTB5 ratio [13]. Eicosapentaenoic acid (C20:5 n-3 EPA) and DHA have demonstrated beneficial effects in some inflammatory and vascular diseases [14]. They belong, however, to the n-3 family of long chain polyunsaturated fatty acids, and DHA is particularly highly oxidizable owing to the presence of its six double bonds in the 22-carbon fatty acyl chain, suggesting that DHA may also increase the peroxidative process which is already present in cystic fibrosis. A previous study in healthy subjects showed that high-dose and long-time treatment with n-3 FA increased the susceptibility of red blood cell membranes to lipid peroxidation [15]. A recent study highlighted pro- or antioxidant activities of DHA at high and low concentrations respectively, on human blood platelets *in vitro* [16].

We based the rationale of our study on the hypothesis that long chain polyunsaturated n-3 supplementation may have potential conflicting effects on oxidative status in CF patients; this justifies to assess in a preliminary pilot study their absence of biological criteria for potential deleterious effects. This caution seems necessary before further clinical trials to demonstrate potential clinical improvement. We chose the intravenous route because of potential lipids malabsorption in CF patients.

Thus, the aim of our preliminary study was (i) to evaluate the effects of an intravenous fish-oil emulsion enriched with n-3 FA on platelet and plasma composition in order to observe a potential modification at both plasmatic and membrane cell level, which is an indirect sign of n-3 membrane incorporation; (ii) to survey some peroxidation and antioxidant markers in order to check the biological safety of this administration in patients with CF. A significant membrane incorporation of n-3 FA and the absence of clinical and biological toxic effects are useful and essential criteria for further randomized clinical trials.

Table 1
Main clinical characteristics of patients before administration of treatment

Patient no	Age (year)	Height (cm)	Weight (kg)	BMI (kg/m2)	FEV1 (%)
1	7	126	24.5	15.54	94
2	7	120	22.2	15.3	88
3	8	132	27.3	15.67	64
4	8	128	27.2	16.7	95
5	10	140	28.5	14.4	77
6	15	157	52.2	21.2	71
Median	8	130	27.3	15.4	8382
(min–max)	(7–15)	(120–157)	(22.2–52.2)	(14.4–21.2)	(64–95)
7	32	173	62.7	21	44
8	23	159	45	17.8	45
9	26	164	48	17.8	76
10	37	167	56	20.1	49
11	35	150	45	20	41
12	19	166	50	19.4	69
13	25	167	53.5	17.9	33
Median	26	166	50	19.4	45
(min–max)	(19–37)	(150–173)	(45–62.7)	(17.8–21)	(33–76)

2. Patients and methods

2.1. Patients and study design

Thirteen patients with a well established diagnosis of CF attending the pediatric or the adult cystic fibrosis centers in Lyon, France, were included in the study. The study was approved by the ethics committee of the university hospital of Lyon. Informed consent was obtained from patients older than 18 years and from their parents when younger.

The inclusion criteria were: patients between 5 and 40 years old, with pancreatic insufficiency and stable pulmonary disease. Exclusion criteria were cirrhosis, transplanted patients or those waiting for transplantation, and patients treated with nocturnal enteral nutrition. All patients received pancreatic enzymes and adapted supplementation with retinol and tocopherol depending on annual plasmatic level dosing.

The clinical conditions are summarized in Table 1.

All subjects received a weekly intravenous administration of 2 mL per kg body weight of an enriched fish-oil emulsion (Omegavenous®. Fresenius Kabi) for 12 weeks. The Omegavenous® composition for 100 ml was 10 g of fish-oil, including 1.25 to 2.82 g of EPA, 1.44 to 3.09 g of DHA, 0.1 to 0.7 g of linoleic acid (C18:2 n-6), less than 0.2 g of alpha linolenic acid (C18:3 n-3), and 0.1 to 0.4 g of AA. Each patient received a two-week course of antibiotic treatment according to their sputum culture results and usual antibiotic regimen, before and at the end of the 12-week treatment. Each of the two evaluations was performed in fasted subjects, after the end of the antibiotic course in order to minimize the effects of bronchial infection on the evaluated parameters, particularly the inflammatory and oxidative markers. The

first evaluation was done after the first antibiotic course but before the first lipid infusion, the second evaluation was done one week after the last infusion at the end of the second antibiotic course. Each patient was his own control for each parameter measured before and after treatment, and, as the study was a preliminary observational one concerning only biological criteria, no control group was considered. We included an empirical number of twelve patients – six adults and six children– in order to have experimental biological analysis in both children and adult patients. One more adult patient was included after one of them retired.

The evaluated clinical parameters were: weight, height and body mass index (BMI: weight (kg)/height $(m)^2$), FEV1 and FVC expressed as a percentage of predicted value, sputum colonization. A sweat chloride concentration was also measured by the Gibson and Cook method before and after treatment.

2.2. Laboratory tests

2.2.1. Platelet lipid fatty acid composition

Platelet preparation: Blood samples were collected in tubes containing acid–citrate–dextrose (ACD; 0.8% citric acid, 2.2% sodium citrate, and 2.45% dextrose, 6:1 vol/vol). Platelet-rich plasma was prepared as previously described [17]. Briefly, platelet-rich plasma obtained by centrifugation of the blood at 100 *g* for 15 min was acidified to pH 6.4 with citric acid and centrifuged at 900 *g* for 20 min. The resulting platelet pellet was resuspended in a Tyrode-HEPES buffer solution (in mmol/L: 137 NaCl, 2.6 KCl, 11.9 $NaHCO_3$, 0.46 NaH_2 PO_4, 1 $MgCl_2$ and 5.5 dextrose, pH 7.35).

Total lipids were transmethylated for 90 min at 100 °C with 5% concentrated sulfuric acid in methanol [18]. The resulting fatty acid methyl esters and fatty aldehyde dimethyl acetals (DMA) were analyzed by gas-liquid chromatography (GC) using a Hewlett Packard (HP 6890 series) chromatograph equipped with an SP 2380 capillary column (30 m × 0.32 mm, Supelco, Bellefonte, PA, USA).

2.2.2. Platelet glutathione peroxidase activities

Measurement of cytosolic glutathione peroxidase (cGPx or GPx-1) activity was carried out according to the method described by Paglia and Valentine [19] as modified by Chaudière and Gérard [20].

Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx-4) activity was assayed by the procedure of Roveri et al. [21].

2.2.3. Plasma FA analysis in phospholipids and cholesterol ester fractions

Lipids were extracted by chloroform/methanol (1:1, v/v). The extract was washed by saline solution to remove proteins. The chloroform phase was concentrated under a stream of nitrogen and taken up in chloroform/methanol. The lipid classes were separated by thin-layer chromatography on silicagel plates (Merck 5721) using petroleum ether–ethylic ether–acetic acid as developing solvent. The plates were sprayed with bromophenol blue, and individual bands of phospholipids and cholesteryl esters were scraped off into separate tubes. The phospholipid fraction was saponified and transmethylated with sodium methylate, and the cholesteryl ester fraction was methylated with sulphuric acid and dehydrated methanol. The methyl esters of each fraction were removed by hexane and analyzed by gas liquid chromatography on a Fison GC-8000 gas chromatograph (Thermo Separation Products, Les Ulis, France) equipped with a CP-SIL fused silica capillary column (25 m × 0.25 mm internal diameter) coated with 100% cyanopropyl siloxane 88 phase 0.2 μm (Chrompack Les Ulis, France), helium as carrier gas, and a split ratio 1:20.

Initial temperature was 120 °C for 1 min then temperature programming was as follows: +20°C/min until 165 °C maintained for 20 min, +1 °C/min until 210 °C maintained for 1 min, +10 °C/min until 250 °C maintained for 5 min. Identification of the individual methyl ester components was made by frequent comparison with authentic standards and with their retention times. The results were expressed as a percentage of the total area of all fatty acid peaks in each lipid fraction, and quantified (mg/l) with the internal Standard added at the beginning of analysis.

2.2.4. Antioxidant and oxidative markers

Methods for measuring plasma vitamin A and E, carotenoids, true malonedialdehyde (MDA) and lipid hydroperoxides (LHP) have been described in detail in a previous article [22]. For information only: i) plasma concentrations of vitamin A, vitamin E, lutein, and beta-carotene were determined by high-performance liquid chromatography with a diode array detection [23]; ii) MDA was derivatized with a new highly specific reagent (diaminonaphtalene) and quantified by HPLC-UV chromatography [24]; iii) LHP were measured using an automatic method [25] derived from that originally published by Jiang and reevaluated by Nourooz-Zadeh [26]. Reduced glutathione (GSH) in whole blood was assessed by LC/MS, after thiol-derivatization with *N* ethyl maleimide as described recently [27].

2.2.5. Urinary markers of lipid peroxidation

Specific urinary markers of lipid peroxidation issued from n-3 and n-6 fatty acids recently characterized [28] were measured: 4-hydroxy-2E-nonenoic acid (4-HNA) representing all n-6 fatty acids, 4-hydroxy-2E-hexenoic acid (4-HHA) deriving from n-3 fatty acids and 4-hydroxy-2E-dodecadienoic acid (4-HDDA) as an index of 12-lipoxygenation of arachidonic acid. Urine was collected between 8 p.m. and 8 a.m. Samples were frozen and stored at −80 °C until analysis. Each analysis was repeated four times. Urinary samples (5 mL) were spiked with 20 ng of CD_3 internal standards (4-HHA-d_3, 4-HNA-d_3 and 4-HDDA-d_3), acidified to pH 3 using 3 M hydrochloric acid and extracted twice by 15 mL of ethyl acetate. The organic phase was removed

Table 2
Variations of weight before and after Omegavenous infusions

	Weight before	Weight after
Child 1	24.5	26
Child 2	22.2	23.5
Child 3	27.3	27.8
Child 4	27.2	27.6
Child 5	28.5	29.8
Child 6	52.2	50.8
Adult 1	62.7	60.2
Adult 2	45.0	45.6
Adult 3	48.0	48.2
Adult 4	56.0	55.2
Adult 5	45.0	45.0
Adult 6	50.0	48.9
Adult 7	53.5	52.6

under vacuum. The residue was dissolved in 500 μL of dichloromethane and applied to a Sep-Pak silica cartridge. Hydroxy acids were eluted with hexane/diethylether/acetic acid (50:50:1, v/v) and transferred into conical vials for their further derivatization.

2.2.5.1. Gas chromatography-mass spectrometry derivatization of 4-hydroxy-acids. The organic solvent was removed under nitrogen and the sample was dissolved into 60 μL of acetonitrile, after which 20 μL of 350 g/L α-bromo-2,3,4,5,6-pentafluorotoluene in acetonitrile and 20 μL of *N*-ethyldiisopropylamine were added, and the mixture was heated at 40 °C for 30 min. The pentafluorobenzyl (PFB) ester derivatives were then evaporated to dryness under nitrogen. The residues were dissolved with 500 μL of toluol and treated with 50 μL HFBI for 15 min at 60 °C. One mL of toluol was added, and samples were washed with water (2 mL of the organic layer was removed and evaporated under nitrogen). Residues were dissolved into hexane (100 μL). One microliter aliquot of pentafluorobenzyl heptafluorobutyryl ester derivatives was analyzed by GC-MS in the splitless mode. Signals at m/z 325, 367, 407, 328, 370 and 410 corresponding to 4-HHA, 4-HNA and 4-HDDA and their CD_3 analogues, respectively, were recorded.

2.2.6. Other laboratory tests

They included routine assays for total leucocytes and polymorphonuclear cells, C-reactive protein, liver function tests, creatininemia, and coagulation parameters.

3. Data analysis

The main outcomes were to evaluate the effect of n-3 FA administration firstly on platelet and plasma FA composition and secondly on oxidative status, each patient being its own control for each parameter measured before and after treatment.

Statistical analysis was realized with Stata8© (StataCorp, College Station, TX). Because of the small number of patients, data are presented as median (min–max) and a nonparametric test was used for analysis. Longitudinal data between the evaluations were compared using the Wilcoxon signed-rank test (when there were two sequential measurements), or one-way repeated measurement analysis of variance (when more than two sequential measurements) with Scheffe post hoc tests when appropriate. The level of significance was set at 0.05.

4. Results

4.1. Clinical evaluation

No significant variations in height and weight were observed in children. A significant decrease in weight was observed for the 7 adult patients: their median weight was 50 kg (45–62.7) before infusions and 48.2 kg (45–60.2) after infusions ($p=0.02$). A marked weight loss was individually noted for four patients (nos. 1, 4, 6 and 7, Table 2). It was not explained by bronchial exacerbation, digestive disease, reduced food intake or uncontrolled diabetes mellitus. Two of them (nos. 6 and 7) preferred to stop the study after the 4th and 5th infusions respectively because of the weight loss. Their second evaluation was underwent one week after their last infusion. They recovered their initial weight one month after the last infusion.

We observed no significant modification in pulmonary function tests (FEV1 and FVC).

4.2. FA composition of platelets

Both EPA and DHA platelet molar percentage increased significantly: the median (min–max) value of molar percentage increased from 0.39 (0.22–0.49) before treatment to 0.54 (0.27–0.59) after treatment for EPA ($p=0.003$), and from 1.18 (0.76–1.71) to 1.49 (1.12–2.33) for DHA ($p=0.0044$) (Fig. 1). These increases were significant for both children ($p=0.02$ for EPA and DHA) and adults ($p=0.02$ for EPA and 0.04 for DHA).

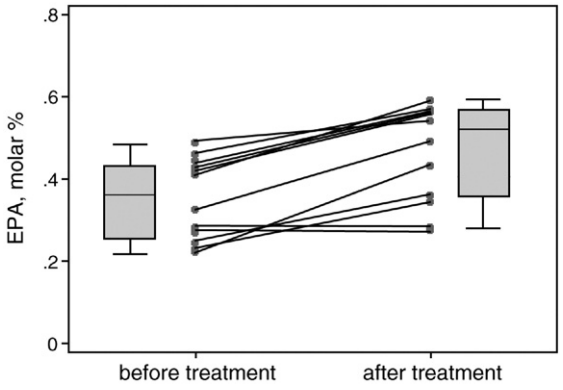

Fig. 1. Platelet fatty acid composition. Variation for EPA and DHA, expressed in molar percentage, before and after Omegavenous infusions (box-plots with median, and individual values).

Table 3
Median (min. and max.) values before and after the period of intravenous infusion for cGPx, PHGPx, total MDA and LHP, reduced glutathione

	Children		Adults	
	Before treatment	After treatment	Before treatment	After treatment
Platelet cGPx Nmol/min/mg of proteins	285.5 (258–334)	323 (263–434)	296 (191–412)	294 (170–407)
Platelet PHGPx Nmol/min/mg of proteins	4.55 (3.38–6.35)	5 (3.5–5.63)	6.3 (4.2–8.3)	6 (2.7–8)
MDA nmol/L	226 (138–431)	208 (174–476)	261 (160–301)	343 (274–541)
LHP μmol/L	12.4 (9.5–21)	14.5 (12.3–25)	12.2 (2.5–18)	9.5 (3.5–13)
GSH μmol/L	980 (894–1237)	1103 (804–1459)	1323 (967–1451)	1206 (1042–1588)

There was no variation for 20:4 n-6 percentage and a decrease in n-9 percentage ($p=0.05$).

4.3. Plasma FA composition

We observed no significant change in plasma cholesteryl ester (CE) and phospholipid (PL) FA profiles for n-3, n-6, n-7 and n-9, neither in children nor in adult patients. A slight but non significant increase in plasmatic PL EPA and DHA was observed: PL EPA 0.9% (0.6–1.1) before treatment and 1.1 (0.7–1.4) after treatment; PL DHA 2.2 (0.9–4.3) before and 2.5 (1.9–4.5) after.

4.4. Markers of oxidative stress

4.4.1. Platelet glutathione peroxidase activities

Cytosolic and phospholipid GPx (cGPx and PHGPx) activities were not modified by lipid infusions, either for adults or for children (Table 3).

4.4.2. Plasma LHP and MDA

Total MDA was measured before and after the twelve weeks of infusion and, in all patients, 4 h after the first infusion to avoid early oxidative reaction. No significant modification was observed after the first infusion and no modification after the end of treatment (Table 3).

The basal values for LHP were elevated compared with usual values measured in healthy controls (4.9±1.7 μmol/L) [25]. However we did not observe any significant variation after the period of treatment (Table 3).

4.4.3. Urinary markers of lipid peroxidation

Significantly elevated values of HHA, HNA and HDDA were observed for CF patients (mean±SD: 4-HHA 147±32, 4-HNA 72.4±20.7, 4-HDDA 14.2±3.2 pg/μmol creatinine – compared with values of controls previously studied – 75.4±20.4, 7.2±1.9, 1.4±0.4 respectively) [28]. These markers were not modified by Omegavenous® administration.

Table 4
Variations of reduced glutathione (μmol/L) before and after Omegavenous infusions in the four patients undergoing weight loss

	Weight before	Weight after	rGSH before	rGSH after
Adult 1	62,7	60,2	1224	1042
Adult 4	56.0	55.2	1432	1206
Adult 6	50.0	48.9	1451	1123
Adult 7	53.5	52.6	1323	1136

4.4.4. Vitamin A and E, β-carotene, glutathione

We observed no significant variation in vitamin A and E concentrations, and no significant variation in reduced GSH (Table 3). However a marked decrease in GSH level was noted in the four adult patients who developed unexplained weight loss (nos. 1, 4, 6, and 7, Table 4).

4.5. Miscellaneous

There was no modification in sweat chloride concentrations (data not shown).

There were no significant variations for the other biological parameters including total leucocytes, C-reactive protein, liver function tests, creatininemia and coagulation test (data not shown).

5. Discussion

This preliminary study shows that 3 months of weekly intravenous administration of n-3 enriched FA emulsion led to a significant incorporation of EPA and DHA in platelet FA as previously demonstrated with long-term oral supplementation on erythrocyte FA composition [9,11]. It would be interesting to check the modifications observed in FA membrane.

We observed slight but no significant variations in plasma FA concentrations, in contrast to another study which described an increase in n-3 plasma FA concentrations [10]. This study concerned daily intravenous administration during one month. In our study, a weekly administration may suppose a rapid incorporation of circulating n-3 in cell membrane phospholipids, particularly for EPA and DHA which represent a very low percentage of total FA composition; the daily monitoring of plasma composition after infusion may allow to observe the kinetic of plasma changes.

Some major circulating markers of oxidative stress, such as MDA, LHP and specific urinary markers of lipid hydroperoxides, have been measured either normal or elevated in previous studies in cystic fibrosis patients [22,28]; n-3 long chain FA administration in the present report did not induce either increase or decrease of these markers. We

observed, however, a decrease in the blood level of one major antioxidant parameter, reduced glutathione, for four adult patients who also presented a significant weight loss, suggesting potential oxidative stress, while vitamins such as vitamin A and E, for which patients have regular supplementation, were not modified. It is known that GSH scavenges free radicals and lipoperoxidation by-products directly and indirectly through enzymatic reactions during which glutathione is oxidized to glutathione disulfide (GSSG). GSSG is quickly reduced to glutathione by NADPH-dependant glutathione reductase in order to maintain a normal reduced-to-oxidized glutathione ratio and a very low level of circulating GSSG [29]. The decrease in total and reduced glutathione could be interpreted as a sign of insufficient induction of GSH synthesis normally observed during oxidative stress.

No clinical benefit concerning respiratory function was observed. This uncontrolled study with a small number of patients was not planned in order to document a clinical effect, which needs a randomized control trial.

The reversible weight loss observed in some adult patients is difficult to understand. We noted no complication related to CF disease (bronchial exacerbations, uncontrolled diabetes mellitus) or any reduction in food intake related to weekly FA administration. The weight loss and the decrease in reduced GSH concerned the same patients, but our data do not support a direct link between weight loss and oxidative stress.

It has been suggested that DHA can have different effects on cell function and susceptibility to oxidative damage depending on its phospholipid accumulation in phosphatidylcholine or phosphatidylethanolamine plasmalogens which differs according to DHA concentration [16]. This results in a biphasic effect of DHA with antioxidant and prooxidant effects at low and high concentrations [16,30,31]. We observed no direct signs of oxidative stress or lipid peroxidation induced by treatment either in plasma or in platelets. However some clinical and biological results observed in adult patients suggest that specific fatty acid treatment should be approached with great caution in cystic fibrosis [32]; careful attention should be claimed to determine the dose effect of DHA on redox status in the specific condition of CF patients.

The beneficial effect of DHA administration in CF patients remains to be demonstrated. A better understanding of FA metabolism and the dose effect of DHA on redox status in CF patients is necessary before clinical trials can be undertaken.

Acknowledgement

This work was supported by the Hospices Civils de Lyon and the AGIRàdom association.

We thank M. Carreras, P. Molière, I. Duperray and G. Tourette for their technical assistance. Many thanks also to Sarah Somerville (IARC) for careful English editing.

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