Reducing endothelial NOS activation and interstitial fluid pressure with \(n-3\) PUFA offset tumor chemoresistance

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The aim of this study was to determine how \(n-3\) polyunsaturated fatty acid (PUFAs) counteracted tumor chemoresistance by restoring a functional vascularization. Rats with chemically induced mammary tumors were divided into two nutritional groups: a control group and a group fed with an \(n-3\) PUFA-enriched diet. Both groups were treated with docetaxel. Functional vascular parameters (ultrasounds, interstitial fluid pressure) were determined for both nutritional groups before (W\(_0\)) and during docetaxel treatment [every 2 h up to 1 week (W\(_1\)) for interstitial fluid pressure, at W\(_4\) for Evans blue extravasation and at W\(_{2,4}\) and W\(_{4,6}\) for ultrasounds]. In vitro \(n-3\) PUFA-induced changes in endothelial cell migration, permeability and phosphorylation of endothelial nitric oxide synthase were evaluated using human umbilical vein endothelial cells. Whereas docetaxel stabilized tumor growth in the rat control group, it induced a 50% tumor regression in the \(n-3\) PUFA group. Ultrasound parameters were consistently lower in the \(n-3\) PUFA group at all time points measured, down to \(~50\%\) at W\(_{4,6}\). A single dose of docetaxel in the \(n-3\) PUFA group markedly reduced interstitial fluid pressure from 2 h after injection up to W\(_4\) when Evans blue extravasation was increased by 3-fold. A decreased activation of endothelial nitric oxide synthase in tumors of the \(n-3\) PUFA group, and in human umbilical vein endothelial cell cultured with \(n-3\) PUFA, points toward a PUFA-induced disruption of nitric oxide signaling pathway. This normalization of tumor vasculature functions under \(n-3\) PUFA diet indicates that such a supplementation, by improving drug delivery in mammary tumors, could be a complementary clinical strategy to decrease anticancer drug resistance.

Introduction

Among \(n-3\) polyunsaturated fatty acid (\(n-3\) PUFA), docosahexaenoic acid (DHA, \(22:6n-3\)) has generated increasing interest due to its ability to decrease resistance of experimental mammary tumors to anthracyclines or radiotherapy without additional side effects (reviewed in refs 1–3). In two Phase II studies, dietary supplementation with \(n-3\) PUFA has led to an increased survival and chemotherapy efficacy without affecting the toxicity profile of the conventional chemotheraphy (4,5). Different molecular mechanisms such as the amplification of the oxidative stress generated by anthracyclines or radiotherapy have been proposed to account for these effects (6,7).

Some studies have reported a reduced quantity of vessels in \(n-3\) PUFA-enriched tumors (8,9) but there is no data reported in the literature on a potential effect of \(n-3\) PUFA on the vessel quality. Up to now, the intriguing paradox of \(n-3\) PUFA diet, inducing both a decreased vascularization of tumor and an enhancement of tumor sensitivity to anticancer drugs, has not been addressed experimentally. However, it has already been observed that the efficacy of chemotherapy or radiations were improved when a conventional antiangiogenic agent was used concomitantly (10–12). Since the vascular quality in tumor is generally recognized to be poor, one would have anticipated that a further decreased vascularization would have led to a poor distribution of anticancer drug in tumors and to an increase in hypoxic areas known to be chemoresistant.

Recently, Jain et al. (13–15) developed and tested the idea that tumor vasculature was normalized during angiogenic therapy. Several experimental evidences indicated that antiangiogenic treatments, especially those directed against the vascular endothelial growth factor (VEGF) pathway, ‘normalized’ the abnormal structure and functions of tumor vasculature to make it more efficient for oxygen and drug delivery. This normalization was associated with a decrease in interstitial fluid pressure (IFP). In non-treated tumors, the induction of hyperpermeable vessels by VEGF and nitric oxide (NO) and a heterogenous blood flow led to elevation of IFP and hypoxia, two hallmarks of chemoresistance. The endothelial nitric oxide synthase (eNOS) is the NOS isofrom responsible for hyperpermeability and tumor angiogenesis (16). High IFP is an obstacle to anticancer drug diffusion and a decrease of IFP led to a deeper penetration of molecules (15,17–19). In agreement with the notion that IFP functions as a barrier to drug delivery, high tumor IFP has in some instance been shown to be associated with a poor response to treatment in patients. For example, in a study of patients with melanoma, all responders displayed a lower tumor IFP compared to non-responders (20). IFP has also been used as a prognostic factor and was found to be a best predictor (compared with oxygen pressure) of survival in patients with cancer of the uterine cervix (21).

The effects of \(n-3\) PUFA during taxane therapy such as docetaxel, frequently used in cancer treatment, have not been explored so far. Using induced autochthonous mammary tumors, a rat model expressing the constituents of tumor stroma including vessels, we investigated how \(n-3\) PUFA can (i) restore a functional vascularization, (ii) potentiate the efficiency of docetaxel and (iii) modify the NO signaling pathway involved in angiogenesis and vessel permeability.

Materials and methods

Experimental design, carcinogenesis initiation and diets

All animal studies and experimental procedures were approved by the Animal Care Committee of Région Centre and Centre de Recherches Biologiques. Female Sprague–Dawley rats aged 6 weeks (Harlan, Gannat, France) received a single subcutaneous dose (25 mg/kg) of N-methylhydroxourea (Sigma, Saint Quentin-Fallavier, France) to initiate mammary carcinogenesis (22,23). Rats (\(n = 110\)) were then separated equally into a control group or an \(n-3\) PUFA group. The control group was fed with a diet containing peanut (12%) and rape (3%) oils (%/g/100 g of diet). The \(n-3\) PUFA group was fed with a diet containing peanut (8%), rape (2%) and fish (5%) oils (Phosphotech, Saint Herblain, France), which is composed of 50% DHA and 20% eicosapentaenoic acid. When tumors reached 1.5–2 cm\(^3\) (~8 weeks after carcinogenesis initiation), some rats (\(n = 24\)) were euthanized after the measurement of oxygen partial pressure. Tumors were cryopreserved for fatty acids and molecular analysis. Remaining rats (\(n = 66\)) were used to evaluate chemotheraphy efficacy and vascular quantity or to measure IFP and Evans blue extravasation. At the

Abbreviations: AUC, area under the curve; CAIX, carbonic anhydrase IX; CEUS, contrast-enhanced ultrasound; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; eNOS, endothelial nitric oxide synthase; FFC, fluorescence isothiocyanate; HUVEC, human umbilical vein endothelial cell; IFP, interstitial fluid pressure; mRNA, messenger RNA; NO, nitric oxide; \(n-3\) PUFA, \(n-3\) polyunsaturated fatty acids; PDI, power Doppler index; VEGF, vascular endothelial growth factor.

\(^1\)These two authors contributed equally to this work.

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end of chemotherapy protocol, tumors were also cryopreserved for fatty acids and molecular analysis (summary of experimental design in Figure 1).

Docetaxel treatment
When mammary tumors reached 2 cm², rats (n = 21 per nutritional group) were submitted to docetaxel treatment (Taxotere®, Sanofi Aventis, France; 6 mg/kg/week intraperitoneal) for 6 weeks. Rats were examined weekly and tumor area was calculated using the two largest diameters (Ø1 and Ø2) according to the ellipse formula: tumor area = π (Ø1 × Ø2)/4.

Functional vascular quantification
Tumor vascularization was investigated with both power Doppler ultrasound and contrast-enhanced ultrasound (CEUS) on rats (15 < n < 20). Ultrasound examinations were performed at three time points: prior to the first injection (W₀), after 2 weeks (W₂) and after 6 weeks of docetaxel injection (W₆).

For power Doppler, the ultrasound machine (Esaote, Italy) was equipped with a 3.5–8 MHz linear probe (LA 523; Esaote). Gain and velocity scales remained stable over experiments. Tumor vascularization density was quantified with a 4–13 MHz linear probe (LA 532; Esaote). Microbubbles of SonoVue (Bracco Imaging SpA, Milano, Italy) were used as contrast agent (0.3 ml, 45 µg/ml, intravenous bolus injection) (25). Due to the important echogenicity and specific properties of the ultrasound contrast agent, CEUS detects only functional vessels (from large to small vessels) in which the contrast agent is able to flow. CEUS signal on video clip of manually drawn region of interest was analyzed with LICOX CMP software. The mean of oxygen pressure was obtained from the five stored images of the region of interest (ROI, defined as the tumor contours) expressed in % (24). The mean of PDI was obtained from the five stored images of the region of interest (ROI, defined as the tumor contours) expressed in % (24). The mean of PDI was obtained from the five stored images of the region of interest (ROI, defined as the tumor contours) expressed in % (24). The mean of PDI was obtained from the five stored images of the region of interest (ROI, defined as the tumor contours) expressed in % (24). The mean of PDI was obtained from the five stored images of the region of interest (ROI, defined as the tumor contours) expressed in % (24).

Oxygen partial pressure and carbonic anhydrase IX analysis in tumors
Tumor oxygen pressure (PO₂) was measured using a polarographic microprobe (LICOX® system). Docetaxel-treated (W₆) and untreated rats (W₀–10 tumors minimum per nutritional group) were evaluated. Data were captured by LICOX CMP software. The mean of oxygen pressure was obtained from measurements acquired in three different points of the tumor. To corroborate oxygen pressure measurements, we quantified carbonic anhydrase IX (CAIX) messenger RNA (mRNA) level, an hypoxia-inducible gene. Total RNAs from tumor samples were extracted with TRIzol reagent (Invitrogen, France) and reverse transcribed (‘Ready-to-go’; GE Healthcare). Quantitative real-time polymerase chain reaction was carried out with Myiq thermocycler (Bio-Rad, France) using Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen). The primer sequences of rat CAIX were 5'-CC'TT GGA AAT CCG AGA GG-3' (sense) and 5'- GCA ACA GTG CAG ATA CAT CCA-3' (antisense). HPRT and Rpl13 expression were used as reference (5'-CAG GCC AGA CTT TGT TGG AT-3' and 5'-TCC ACT TTC GAT GAC AC-3', 5'-CTG TGG CAG GGT CTA AAAT-3' and 5'-TGG ATG TGT TGG AT-3', respectively). Polymerase chain reaction protocol was 10 min start at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 57°C, and 1 min at 72°C. Using the comparative cycle threshold (CT) method (CT value), the amount of mRNAs was expressed as −ΔCT [CT CAIX-CT (mean HPRT-RPL13)].

Tumor IFP measurement and Evans blue extravasation
When the surface area of mammary tumors (3 < n < 11 per nutritional group) reached at least 2 cm², tumors were instrumented according to the technical method described by Schnell et al. (26). The radio telemetry system used in this study was composed of four basic components: an implantable transmitter (model: TL11M2-C50-PXT; Data Science) that continuously transmitted information, a receiver located under the cage, a matrix interface for coordination of signals, and an ART™ computerized acquisition system (Data Science) for collection, analysis and storage of data. Under anesthesia (2.5% isoflurane in O₂) and in aseptic conditions, the sterile transmitter was placed in the peritoneal cavity of the animal and the sensing pressure catheter was implanted at 0.5 cm of depth into the body of the tumor and secured at the site of entry with tissue adhesive (Histacycol®, B. Braun Medical, France). The animals were allowed to recover from the surgery for 24 h before starting data acquisition. IFP measures were stabilized for 6 days after instrumentation. Then, animals received a single dose of docetaxel (intraperitoneal, 6 mg/kg). Pressure data were recorded every 10 s at 500 Hz with the acquisition system, and for each rat, mean of IFP values were realized every 2 h for 1 week (W₁). At W₀, the dye Evans blue (27) was used to monitor molecules extravasation in tumors of each dietary group (n = 5 per group). Briefly, Evans blue solution (25 mg/kg in saline; Sigma) was injected in the tail vein of rat anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneal; CEVA, France) and rats were euthanized 30 min later. Evans blue fluorescence (Ex 620 nm, Em 680 nm) was used to quantify dye extravasation and evaluated on 20 µm tumor sections mounted onto glass side using an epi-fluorescence microscope equipped with a ×10 objective field (Nikon ‘Eclipse Ti-S’ microscope, Nikon ‘CFI')

![Fig. 1. Summary of experimental design. Rats received a single dose of N-methylnitrosourea to initiate mammary carcinogenesis. Rats (n = 110) were separated equally into a control or an n-3 PUFA diet group. When tumors reached ~2 cm² (W₀), rats were euthanized after oxygen measure or submitted to docetaxel treatment with one injection/week (arrow). Functional vascular and molecular parameters were determined at time points indicated in the layout before (W₀) and after 1 (W₁), 2 (W₂) or 6 weeks (W₆) of docetaxel therapy.](https://academic.oup.com/carcin/article-abstract/33/2/260/2463709)
Fatty acids composition of tumor phospholipids (Table I) showed that the n–3 PUFA diet enhanced DHA and eicosapentaenoic acid level in tumor tissues compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in the n–3 PUFA group compared with control group. In the absence of docetaxel treatment, the n–3 PUFA diet did not modify tumor growth (Supplementary Figure 1, available at Carcinogenesis Online).

Ultrasound parameters have been used to quantify the tumor vascularization. Power Doppler ultrasound allowed quantifying the larger vessels, whereas CEUS allowed quantifying the total tumor vascularization including microvessels (Table II). At W0, before the first injection of docetaxel, peak intensity, AUC and blood flow (CEUS parameters) were, respectively, 26% (P < 0.05), 43% (P < 0.01) and 39% (P < 0.05) lower in the n–3 PUFA group than in the control group. PDI was slightly, but not significantly, lower at W0 in the n–3 PUFA group compared with control. Docetaxel appeared to have a sequential effect on the tumor vascularization according to the time of treatment. Docetaxel at W12 led to an increase in CEUS parameters in the control diet group (~40% for peak intensity and AUC, P < 0.05 and 65% for blood flow, P < 0.001). These vascular changes induced by docetaxel at W12 were not detected in the n–3 PUFA group. At W0, docetaxel displayed an antiangiogenic effect in both nutritional groups, with a more marked effect in the n–3 PUFA group. In control diet (W0), docetaxel led to a decrease of 37% for peak intensity (P < 0.05) and 52% for AUC (P < 0.001). In the n–3 PUFA group (W0), docetaxel led to a decrease of 50% in sonographic parameters (peak intensity, AUC) including PDI (P < 0.01 for all parameters). Whereas no significant difference in blood flow was observed in control diet at W0 (compared with W0), a decrease of 70% in blood flow was observed in the n–3 PUFA group (P < 0.05).

Table I. n–3 PUFA diet increases n–3 PUFA incorporation in phospholipids of tumor tissues

<table>
<thead>
<tr>
<th>Saturates</th>
<th>Control diet</th>
<th>n–3 PUFA diet</th>
<th>Statistics</th>
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<tbody>
<tr>
<td>16:0</td>
<td>22.27 ± 0.36</td>
<td>22.14 ± 0.53</td>
<td>ns</td>
</tr>
<tr>
<td>18:0</td>
<td>14.74 ± 0.42</td>
<td>14.67 ± 0.41</td>
<td>ns</td>
</tr>
<tr>
<td>Total</td>
<td>40.85 ± 0.23</td>
<td>40.80 ± 0.58</td>
<td>ns</td>
</tr>
<tr>
<td>Monounsaturates</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>16:1</td>
<td>0.78 ± 0.06</td>
<td>1.02 ± 0.10</td>
<td>*</td>
</tr>
<tr>
<td>18:1n–9c</td>
<td>13.88 ± 0.41</td>
<td>16.64 ± 0.60</td>
<td>**</td>
</tr>
<tr>
<td>Total</td>
<td>22.19 ± 0.52</td>
<td>25.07 ± 0.78</td>
<td>*</td>
</tr>
<tr>
<td>n–6 PUFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n–6c</td>
<td>3.28 ± 0.20</td>
<td>4.23 ± 0.72</td>
<td></td>
</tr>
<tr>
<td>20:3n–6</td>
<td>0.60 ± 0.04</td>
<td>1.00 ± 0.09</td>
<td>**</td>
</tr>
<tr>
<td>20:4n–6</td>
<td>17.11 ± 0.62</td>
<td>11.27 ± 0.81</td>
<td>***</td>
</tr>
<tr>
<td>Total</td>
<td>24.38 ± 0.75</td>
<td>18.18 ± 0.84</td>
<td>***</td>
</tr>
<tr>
<td>n–3 PUFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5n–3</td>
<td>0.03 ± 0.01</td>
<td>1.06 ± 0.15</td>
<td>***</td>
</tr>
<tr>
<td>22:6n–3</td>
<td>1.74 ± 0.05</td>
<td>2.94 ± 0.08</td>
<td>***</td>
</tr>
<tr>
<td>Total</td>
<td>1.99 ± 0.05</td>
<td>5.05 ± 0.27</td>
<td>***</td>
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</tbody>
</table>

Values are mean percent area ± SEM, sampled from eight randomly selected rats per dietary groups.

P < 0.05, **P < 0.01, ***P < 0.001, Mann–Whitney test.

Results

n–3 PUFA diet enhanced DHA and eicosapentaenoic acid level in tumor tissues

Fatty acids composition of tumor phospholipids (Table I) showed a 2.5-fold increase in total n–3 PUFA (P < 0.001) in the n–3 PUFA group compared with control group, with a 1.7-fold enrichment in DHA (22:6n–3, P < 0.001). Although eicosapentaenoic acid (20:5n–3) was undetectable in control rats, its level reached 1.1 ± 0.15% in the n–3 PUFA group (P < 0.001). The n–3 PUFA group showed a decrease by 25% of n–6 PUFA amount (P < 0.001), especially for arachidonic acid (20:4n–6), which was reduced by 1.5-fold. A weak increase in monounsaturated (13%, P < 0.05) was observed in the n–3 PUFA group. No modifications were detected in saturated fatty acids.

n–3 PUFA diet increased tumor response to docetaxel treatment and decreased tumor vascularization

The effect of the n–3 PUFA diet, compared with the control diet, on the tumor size during docetaxel therapy is presented in Figure 2. With the control diet (left), tumor growth under docetaxel was stopped and stabilized at 2.1 cm². With the n–3 PUFA diet (right), the chemotherapy led to a tumor size regression significantly different from 2 to 6 weeks compared with control group. The tumor regression reached 50% at the end of chemotherapy (P < 0.01) without additional adverse effects (no significant difference in blood formula and animal weight was observed between the two nutritional groups). In the absence of docetaxel treatment, the n–3 PUFA diet did not modify tumor growth (Supplementary Figure 1, available at Carcinogenesis Online).

Statistical analysis

Statistical analyses were carried out using an analysis of variance with repeated measurements followed by post hoc Fisher least significant difference test for monitoring of IFP and tumor size. Spearman test was used for correlation. Analysis of variance/Newman–Keuls and Mann–Whitney tests were used, respectively, for in vitro assays and molecular analysis (GraphPad Prism; GraphPad Software, La Jolla, CA); P < 0.05 was considered to be significant.

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n–3 PUFA potentiate docetaxel treatment by decreasing IFP

**Fig. 2.** n–3 PUFA diet increased the tumor sensitivity to docetaxel (control group, left and n–3 PUFA group, right). When the tumor reached 2 cm², docetaxel was injected at 6 mg/kg/week (arrows, injections) and tumor size was measured once a week for 6 weeks. Points, mean of tumor size in each group (n = 21), bars and standard error of the mean. Difference between control and n–3 PUFA diet: analysis of variance with repeated measurements followed by Fisher least significant difference test, *P < 0.05, **P < 0.01.

**Table II.** n–3 PUFA diet decreases tumor vascularization before (W₀) and during docetaxel chemotherapy (W₀ ± 6 weeks).

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>n–3 PUFA diet</th>
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<tr>
<td></td>
<td>W₀</td>
<td>W₂ ± 6</td>
</tr>
<tr>
<td>CEUS</td>
<td></td>
<td></td>
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<tr>
<td>Peak intensity (db)</td>
<td>4.6 ± 0.4</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>AUC (db/s)</td>
<td>120 ± 14</td>
<td>168 ± 29*</td>
</tr>
<tr>
<td>Blood flow (db/s)</td>
<td>3.8 ± 0.7</td>
<td>6.3 ± 1°</td>
</tr>
<tr>
<td>Power Doppler ultrasound PDI</td>
<td>9.5 ± 1.1</td>
<td>11.7 ± 1.5</td>
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</table>

Vascular parameters were obtained by CEUS and power Doppler ultrasound. For CEUS, microbubbles of SonoVue were used as contrast agent. Vascular parameters such as AUC, peak intensity (representative of vascular volume) and blood flow were calculated (mean ± SEM, 15 < n < 18 per group). For power Doppler ultrasound, PDI was calculated using the mean value of five stored images for each tumor (PDI mean values ± SEM, 17 < n < 20 per group). *P < 0.05, **P < 0.01, ***P < 0.001 (compared with W₀ in the same dietary group, Mann–Whitney test); *P < 0.05, ##P < 0.01 (compared with control group at the corresponding time points, Mann–Whitney test).

15.2 ± 1.3 mmHg for control and 15.0 ± 1.9 mmHg for n–3 PUFA group. After docetaxel injection, in control group, IFP slightly decreased to 13.5 ± 1.1 mmHg (−11%) 24 h post-injection and to 12.9 ± 1.2 (−15%) at 48 h (compared with W₀). In n–3 PUFA group, IFP decreased rapidly and markedly 2 h (−14%) or 24 h (−26%) after docetaxel injection. IFP was ∼ 9.4 mmHg (−37%) in the PUFA group at the end of Day 2 (48 h) corresponding to a reduction of 27% compared with the control group (P < 0.01). At Day 7, the IFP values were stabilized without any further IFP difference between the both diet groups.

To determine whether IFP may be an early biomarker for docetaxel efficacity, correlation between the percent of decrease in IFP at 48 h and the percent of tumor regression has been analyzed (Figure 3A). The two variables were moderately correlated (Spearman ρ² = 0.37) and close to statistical significance (P = 0.057). The extravasation of Evans blue was assessed in each dietary group at W₆ (Figure 3B). The fluorescence intensity of Evans blue was increased by 3-fold in tumors of the n–3 PUFA group compared with the control group (P < 0.05). These data were confirmed by the spectrofluorometric quantification of Evans blue extracted from tumors (Supplementary Figure 2, available at Carcinogenesis Online).

The number of hypoxic tumors was evaluated with a polargraphic probe. Before docetaxel treatment (W₀), the hypoxic tumor percentage (<10% mmHg) was the same in both nutritional groups (28 and 25% in control and n–3 PUFA groups, respectively). After docetaxel treatment (W₀ ± 6 weeks), none of the tumors of the two nutritional groups were hypoxic (data not shown). These results have been completed by an analysis of CAIX mRNA expression, an enzyme induced by hypoxia (Figure 3C). CAIX mRNA expression was significantly decreased by docetaxel treatment (P < 0.001) in the diet control group, indicating a decrease in hypoxia. At W₀ ± 6 weeks, CAIX mRNA expression further decreased in the n–3 PUFA group (P < 0.05), suggesting an improvement of tumor oxygenation in tumors of the n–3 PUFA group under docetaxel treatment.

**n–3 PUFA diet prevented endothelial NO synthase phosphorylation induced by docetaxel treatment**

Since angiogenesis and vasculature permeability are controlled in part by production and release of free radical gas NO, we hypothesized that n–3 PUFA-enriched diet might reduce eNOS activation. Western blot analyses of Ser1177P-eNOS and pan-eNOS were performed from tumor tissues in control and n–3 PUFA groups before (no docetaxel—W₀) and after docetaxel treatment (docetaxel—W₀ ± 6). Densitometry analyses (Ser1177P-eNOS/pan eNOS) showed no change in phosphorylation of Ser1177P-eNOS in the two nutritional groups before docetaxel treatment (Figure 3D). The phosphorylation of Ser1177P-eNOS was increased by ∼6-fold in the control nutritional group under docetaxel treatment (P < 0.01). Interestingly, this docetaxel-induced eNOS phosphorylation was repressed in the n–3 PUFA group since the median values were only increased by 2-fold in this group before (median = 0.45) and after docetaxel treatment (median = 1.02) (not statistically different).

**DHA blocked the activation of endothelial cells by VEGF**

Since VEGF regulates tumor angiogenesis and vessel permeability, we wondered whether n–3 PUFA, such as DHA, could regulate VEGF signaling pathway in endothelial cells. We examined the effect of DHA on the permeability of HUVEC monolayer to FITC-labeled dextran (Figure 4A). Although VEGF increased the permeability of the monolayer by ∼3-fold in control cells (P < 0.001), this effect was not observed in DHA-supplemented HUVEC (P < 0.001). As expected (30), VEGF increased HUVEC migration by 46% (P < 0.001) (Figure 4B). In DHA-supplemented HUVEC, the stimulation of migration by VEGF was markedly lower compared with the migration induced by VEGF in control cells (P < 0.001). This inhibition was abolished by the addition of exogenous NO (Delta-NO), suggesting an interaction of DHA with NO signaling pathway. Since the effects of VEGF are largely dependent on NO release, we have studied the
As anticipated, VEGF induced the phosphorylation of eNOS by 1.6-fold ($P < 0.05$) (Figure 4C). In DHA-supplemented cells, this phosphorylation of eNOS by VEGF was prevented ($P < 0.05$).

**Discussion**

Many solid tumors treated with chemotherapy present a high IFP that is associated to chemoresistance. Our study reports for the first time that PUFA, used as an adjuvant to standard chemotherapy, markedly improved the efficacy of taxanes. This chemosensitization of mammary tumors to taxanes was associated to the decrease of IFP, which is a major obstacle to effective delivery of anticancer agent within the tumor. These vascular effects of $n$–3 PUFA were further confirmed by a decreased activation of the eNOS in mammary tumors and in endothelial cells.

It has been previously reported by us and others that $n$–3 PUFA display an antiangiogenic effect in rats (8, 9) or in cell culture (31, 32). The reduction in tumor vascularization was accompanied by an enhancement of tumor sensitivity to anthracyclines (9). In the present study, we showed that the therapeutic efficacy of taxanes was also markedly improved by $n$–3 PUFA supplementation. An important finding was that the tumor IFP was rapidly and steadily reduced in $n$–3 PUFA group after a single taxane injection. Tumor IFP is traditionally measured using the wick-in needle technique that can only be performed on anaesthetized animals, hence with limited time point. In the present study, a miniaturized radio-telemetry system has been used for a continuous recording of IFP as described by Schnell et al. (26).

IFP values measured within untreated tumors during this study were within the same order as those measured by wick-in needle in a similar rat mammary tumor model (33). We showed that the decrease in tumor IFP in the $n$–3 group under docetaxel treatment preceded the tumor response to the anticancer drug. This supports the idea that IFP plays a major role in anticancer agent delivery and, as previously reported, it may be an early biomarker for drug efficacy (34, 35). The movement of molecules from blood vessels to the interstitial space is governed by pressure gradients. Indeed, elevated IFP in tumors is considered to be a major barrier to the delivery of
chemotherapeutics (36). Several studies showed that the uptake of anticancer agents (5-fluorouracil, taxol, CPT11 and topotecan) in rodent tumors was increased when the IFP was pharmacologically lowered (18,19,37,38). For example, a 40% reduction in IFP 1 h after platelet-derived growth factor receptor antagonist administration was associated with an increase in taxol uptake by 4-fold at 24 h (19). In our study, the more marked decrease of IFP in n–3 PUFA tumors (−25% compared to control) was associated with an increase by 3-fold of Evans blue extravasation. These results are consistent with those of Tong et al. (39) who showed that a decrease in IFP by anti-VEGFR2 monoclonal antibody was associated with a significantly deeper penetration of molecules into the rodent tumors. Thus, an increase of docetaxel delivery in mammary tumors subsequent to a lower IFP might account for the greater efficacy of taxane in n–3 PUFA nutritional group.

In the control nutritional group during docetaxel treatment, we noticed an increase in tumor vascularization (AUC, peak intensity and blood flow at $W_{1/2}$) that can be attributed to vessel enlargement and/or vessel recruitment. A vessel enlargement appeared to be more relevant since a vasodilator effect of docetaxel on tumor vessels has been previously mentioned (40). In addition, we determined that docetaxel increased Ser1177-eNOS phosphorylation in mammary tumors, suggesting a disruption of NO signaling. Results obtained on endothelial cells were in line with the in vivo data since a lack of eNOS phosphorylation on Ser 1177 was observed in DHA-supplemented cells. NO promotes tumor angiogenesis and induces vessel hyperpermeability which is a cause of elevated IFP (45,46). Inhibition of NO synthesis in eNOS-deficient mice or in wild type treated with NO inhibitors abrogates tumor angiogenesis and vascular permeability (16,47). As reported by Gratton and Dimmeler using NO inhibitors, our data showed that DHA was also able to block permeability of endothelial cell monolayer and endothelial cell migration (30,48). The disruption of NO signaling is likely to be a key mechanism of n–3 PUFA leading to antiangiogenic effect and decrease of IFP in tumors. In addition, since tumor IFP is influenced to systemic blood pressure as reported previously (49), a potential variation of systemic blood pressure following n–3 PUFA diet might affect the magnitude of the IFP decrease. In this study, the cardiac frequency has been measured and no difference was found regardless of diet (data not shown). These results were consistent with a previous study where no hemodynamic cardiac alterations were observed in rats receiving anthracyclines under n–3 PUFA diet (50).

The consequence of eNOS inhibition on decreased permeability and increased molecule extravasation highlighted a paradox already described by others (51,52). Tong et al. (39) reported that a drop in vascular permeability induces transvascular pressure gradients implied in convection. Convection is governed by pressure gradients, made up of three components: microvascular pressure, IFP and osmotic pressure. Convection with diffusion governed the movement of anticancer drugs across vessel walls. In the context of permeable vessels in tumor, osmotic pressure gradient is weak and has minimal effects on transvascular flow (39). We propose a model to explain the effects of n–3 PUFA on transvascular convection leading to the increased extravasation of molecules (Figure 5). In untreated tumors,
n-3 PUFA (15 mmHg) could equalize or exceed the microvascular pressure and, thus, cause a null or negative net outward filtration pressure (14). In tumors treated by docetaxel alone, eNOS activation could explain the vasodilatation and decrease in microvascular pressure as already reported (40). The resultant filtration pressure would be neutralized since we measured that IFP was also slightly decreased (12 mmHg) under docetaxel. In these conditions, extravasation of anticancer drugs would be mostly governed by diffusion. In n-3 PUFA nutritional group treated by docetaxel, eNOS remained inactivated, resulting in a decrease of permeability and a subsequent IFP reduction (9 mmHg). Therefore, n-3 PUFA would promote the establishment of a net outward positive pressure in the same direction as diffusion and subsequently improved anticancer drug extravasation and therapeutic effects.

Inadequate drug delivery is a major cause of drug resistance and methods of enhancing chemotherapy delivery to solid tumors remain a major interest in medicine. This study reports that n-3 PUFA display antiangiogenic properties and normalize function of tumor vasculature to make it more efficient for molecule delivery. Finally, the preclinical data reported here strengthen the findings of two published Phase II trial using PUFA for patients treated for breast and lung cancer (3,5) and reinforce the rationale for a Phase III clinical studies testing n-3 PUFA supplementation during conventional cancer treatment.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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n-3 PUFA potentiate docetaxel treatment by decreasing IFP