Interaction of ω-3 polyunsaturated fatty acids with radiation therapy in two different colorectal cancer cell lines

Cai, Fang; Sorg, Olivier; Granci, Virginie; Lecumberri Lima, Elena; Miralbell, Raymond; Dupertuis, Yves Marc; Pichard, Claude

How to cite

CAI, Fang et al. Interaction of ω-3 polyunsaturated fatty acids with radiation therapy in two different colorectal cancer cell lines. In: Clinical nutrition, 2014, vol. 33, n° 1, p. 164–170. doi: 10.1016/j.clnu.2013.04.005

This publication URL: https://archive-ouverte.unige.ch/unige:42510

Publication DOI: 10.1016/j.clnu.2013.04.005

Clinical Nutrition 33 (2014) 164–170

Contents lists available at SciVerse ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

Interaction of ω-3 polyunsaturated fatty acids with radiation therapy in two different colorectal cancer cell lines☆

Fang Cai [a], Olivier Sorg [b], Virginie Granci [a], Elena Lecumberri [a], Raymond Miralbell [c], Yves M. Dupertuis [a], Claude Pichard [a,*]

[a] Clinical Nutrition, Geneva University Hospital, Geneva 1211, Switzerland
[b] Swiss Centre for Applied Human Toxicology, University of Geneva, Switzerland
[c] Radio-Oncology, Geneva University Hospital, Switzerland

ARTICLE INFO

Article history:
Received 1 October 2012
Accepted 8 April 2013

Keywords:
Docosahexaenoic acid
Eicosapentaenoic acid
ω-3 polyunsaturated fatty acids
Human colorectal adenocarcinoma
Radiation therapy
Lipid peroxidation

SUMMARY

Background & aims: This study aims at evaluating if docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) increases the efficacy of radiation therapy (RT) on two human colorectal cancer cell lines with different radio-sensitivity.
Methods: LS174T and HT-29 cells were treated with 20 or 50 μmol/L EPA or DHA followed by single X-ray RT of 0, 2 or 4 Gy, to evaluate cell survival, apoptosis, peroxide and malondialdehyde productions. Inflammation- and apoptosis-related proteins were analyzed by Western Blot. ANOVAs were used for statistical analysis.
Results: LS174T was more sensitive to RT than HT-29. DHA and to a lesser extent EPA increased cell death, apoptosis and peroxide production after RT in LS174T and to a lesser extent in HT-29 ($p < 0.05$). This was associated with increased expression of heat shock protein 70, decreased expression of NF-kB p65, COX-2 and Bcl-2 proteins.
Conclusions: The effect of RT combination with DHA and to a lesser extent EPA was synergistic in the radio-sensitive LS174T cells, but additive in the radio-resistant HT-29 cells. This enhanced cytotoxicity was provoked at least partly by lipid peroxidation, which consequently modulated inflammatory response and induced apoptosis.

1. Introduction

Colorectal cancer (CRC) is currently one of the most prevalent causes of cancer death.[1] CRC treatment consists mainly in complete surgical resection with or without adjuvant radiation therapy (RT) and/or chemotherapy. Even though, CRC frequently remains refractory to these conventional treatments. More than 50% of patients develop local relapse or metastasis within two years after surgery.[2] New modalities to improve CRC treatment outcome are therefore of great interest.

ω-3 Polyunsaturated fatty acids (PUFAs) might be used as adjuvant for cancer therapy. They could inhibit tumor progression in breast, skin and prostate cancers.[3,4] The adjuvant use of ω-3 PUFAs increased chemo-[5] and radio-sensitivity in different cancers.[6,7] In our study, we investigated if ω-3 PUFAs could enhance radio-sensitivity in CRC cell lines.

Among the ω-3 PUFAs, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are the most studied. Both have been described to increase the therapeutic efficacy of RT in breast cancer cell lines,[8] rodent model[9] and also in patients.[10] In CRC, EPA or DHA, on its own, inhibits HT-29 cell growth, but DHA is more efficient than EPA due to its rapid cellular absorption and integration into cell membrane.[11] However, the combined effect of EPA/DHA and RT is rarely reported. We previously observed that EPA and DHA were able to increase radio-toxicity in different CRC cell lines, *i.e.* LS174T, Caco-2 and CO112. This synergic toxicity was negatively correlated with the differentiation degree of the cell lines.[6]

Oxidative stress might contribute to the cytotoxicity of PUFAs, because of an increase in oxidative stress markers, such as glutathione, disulfide glutathione and malondialdehyde (MDA) observed

Abbreviations: CRC, colorectal adenocarcinoma; DHA, docosahexaenoic acid; DNPH, 2,4-dinitrophenylhydrazine; EDTA, Ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PBS, phosphate-buffered saline; RT, radiation therapy; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ω-3 PUFAs, ω-3 polyunsaturated fatty acids.

☆ Conference presentation: 2012 Barcelona ESPEN poster presentation (Part of the results).

* Corresponding author. Tel.: +41 22 372 93 49; fax: +41 22 372 93 63.
E-mail address: Claude.Pichard@unige.ch (C. Pichard).

0261-5614/$ – see front matter © 2013 Elsevier Ltd and European Society for Clinical Nutrition and Metabolism. All rights reserved.
http://dx.doi.org/10.1016/j.clnu.2013.04.005

during DHA or EPA combination with chemotherapy in breast carcinoma cells.[5] When combined with RT, the mechanism of cytotoxicity of ω-3 PUFAs has not yet been evaluated in depth, especially in CRC. This study therefore aimed at evaluating the effect of RT combination with EPA or DHA on LS174T and HT29 CRC cell survival. These two cell lines have different proliferation rates and genetic backgrounds, especially in p53 and Bax protein expressions. The p53 and Bax positive HT29 cell line is more radioresistant than the p53 and Bax negative LS174T cell line. Lipid peroxidation and its downstream pathways such as inflammation and apoptosis, which cause cell survival inhibition, were also investigated.

2. Materials and methods

2.1. Reagents

EPA, DHA were obtained from Sigma–Aldrich (Buchs, Switzerland), diluted in ethanol and stored at 10 g/L at −20 °C under argon. All other chemicals were also purchased from Sigma–Aldrich (Buchs, Switzerland) unless otherwise stated.

2.2. Cell line

The human CRC cell line LS174T (ATCC no. CL-188™) and HT-29 (ATCC no. HTB-38™, USA) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen, Switzerland). The medium was changed twice a week to keep the cells in exponential growth phase at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were used no more than 10 passages in order to maintain their biological characteristics. According to ATCC product description, HT29 cells express p53 and Bax, while LS174T cells are Bax mutated and p53 mRNA positive but without p53 protein expression.

2.3. Cell treatment with EPA or DHA in combination with radiotherapy

Cells were treated with EPA, DHA at 20 or 50 μmol/L for 4 h before a single RT of 2 or 4 Gy, using a linear beam accelerator (X-rays, 6 MV). Non-treated cells were incubated with the same concentration of ethanol found in a treatment corresponding to 20 or 50 μmol/L EPA or DHA.

2.4. Cell survival

LS174T and HT29 cells were seeded in 6-well plates at a density of 250 cells/well. After the previously described treatment, cells were kept for 14 days at 37 °C, and then washed with phosphate-buffered saline (PBS) solution, fixed with methanol/acetic acid (3:1, v/v) for 20 min at 4 °C, and stained with 0.5% crystal violet in methanol/acetic acid (3:1, v/v). Colonies containing $\geq$ 50 cells were counted. The survival fraction was calculated as relative to untreated controls (S/So). The sensitizer enhancement ratio (SER) was defined as the ratio of required X-ray dose to reduce 80% of the survival fraction without ω-3 PUFAs, to the dose required to obtain the same survival fraction with PUFAs. The required dose was calculated based on the linear-quadratic regression model.[12]

2.5. Lipid peroxidation

Cells were seeded at a density of 600 000/well in 6-well plates 24 h before treatment of 50 μmol/L EPA or DHA followed by a 4-Gy RT. Four hours after irradiation, cells were collected and 50 μl cell lysate (in 50 mmol/L Tris–HCl pH 7.3, 150 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 1.0% Triton X-100) or standard (hydrogen peroxide 30%, Merck, Germany) was mixed with 50 μl xylenol orange reactive (50 mmol/L sulphuric acid, 500 μmol/L ammonium iron (II) sulfate, 200 μmol/L xylenol orange, 200 mmol/L D-sorbitol) and then incubated at room temperature (protected from light) for 1 h. The absorbance of the mixture was read using a colorimeter (Model 680, Bio-Rad Laboratories AG) at 595 nm.

MDA level was measured following a derivatization with 2,4-dinitrophenylhydrazine (DNPH) and HPLC analysis[13] with minor modifications. Fifty μl of reconstructed samples were injected into HPLC system (Agilent 1100 series) equipped with an autosampler, a quaternary pump, a UV diod-array detector, and a C18 column (Nucleodur® C18 Pyramid 3 μmol/L, Macherey–Nagel). The flow-rate of the mobile phase was 1 ml/min. The DNPH derivatization products of MDA (1-(2,4-dinitrophenyl) pyrazole) and methyl-MDA (4-methyl-1-(2,4-dinitrophenyl) pyrazole, internal standard, 100 μmol/L, synthesized by heating a mixture of 0.25 g 3-dimethylamino-2-methyl-2-propenal, 0.1 g sodium hydroxide and 350 μl HPLC grade water at 70 °C for 25 min) were analyzed at 306 nm and 322 nm, respectively. The calibration of the derivatization products was done using that of MDA and methyl-MDA standard solutions with molar extinction coefficients of 12'800 (M^{-1} cm^{-1}) at 249 nm for MDA in acidic medium and 22'800 (M^{-1} cm^{-1}) at 279 nm for methyl-MDA in alkaline medium.

2.6. Apoptosis quantification

Cells were seeded at a density of 50 000/well in 24-well plates for 24 h before treatment of 50 μmol/L EPA or DHA for 4 h followed by a 4-Gy RT. Apoptosis was quantified 2, 4 or 24 h after RT using a two-parameter fluorescence-activated cell sorting (FACS) analysis with annexin V/propidium iodide detection kit according to manufacturer's instructions (BD Biosciences, Switzerland). Briefly, cells were detached with trypsin, washed with 1×PBS and then resuspended in binding buffer (10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) at a concentration of 1×10^6 cells/ml. Samples were stained with 5 μL annexin V conjugated with fluorescein isothiocyanate (FITC) and 5 μL propidum iodide at room temperature for 15 min in the dark. They were then diluted in 400 μL of binding buffer and analyzed within 1 h using a flow cytometer (Accuri C6 personal flow cytometer, BD Biosciences; excitation 488 nm; emission 530 nm).

2.7. Western Blot

Two hours after a 4-Gy RT, cells were washed with PBS and collected with 100 μl of RIPA lysate buffer (NaCl 150 mmol/L, Tris–HCl pH8 50 mmol/L, NP40 1%, Triton X100 1%, SDS 0.1%)), and supplemented with proteinase inhibitors. The supernatant was then collected and denaturated at 95 °C for 5 min. 25 μg of protein from each sample were loaded on 9%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred, and immunoblotted onto PVDF membrane. Anti-NF-kB p65, anti-COX-2, anti-HSP-70, anti-Bcl-2, anti-Bax, anti-p53 antibodies (Santa-Cruz, USA) were used as primary antibodies. Anti-GAPDH antibody (Millipore, USA) was used as internal standard for protein.

2.8. Data analysis

For apoptosis analysis, data were acquired with CFlow® Plus software (BD biosciences, USA). All experiments were reproduced in triplicate and results were presented as means ± SD. Normality of data was tested using Shapiro–Wilk. Three-way ANOVA was used for clonogenic assay, apoptosis and xylenol orange, while two-way

ANOVA was performed for MDA, apoptosis for LS174T at 2 h, HT29 at 4 h, xylenol orange for LS174T at 4 h and HT29 at 24 h. All the ANOVA were followed by Tukey test using SPSS 11.5 (USA). Student's *t*-test was used to compare means between two specific conditions. *P*-value ≤ 0.05 was considered as statistically significant.

3. Results

3.1. DHA and EPA decrease cell survival after RT

Clonogenic assays were performed to evaluate the cytotoxic effect of different treatments on LS174T (Fig. 1A) and HT29 cells (Fig. 1B). Cell survival depended on the addition of ω-3 PUFAs ($p < 0.001$), the dose of ω-3 PUFAs ($p < 0.001$) and RT ($p < 0.001$). DHA was more efficient than EPA ($p < 0.001$) to decrease cell survival in both LS174T (DHA, 39.7 ± 29.9%; EPA, 71.1 ± 10.4%) and HT29 cells (DHA, 45.9 ± 18.1%; EPA, 88.2 ± 7.1%). While HT29 cells (2 Gy, 75.5%, $p = 0.02$; 4 Gy, 66.7%, $p < 0.001$) were more resistant to RT than LS174T cells (2 Gy, 61.2%, $p < 0.001$; 4 Gy, 28.2%, $p < 0.001$), the effect of RT combination with different doses of ω-3 PUFAs was more evident in LS174T ($p = 0.052$) than in HT29 cells ($p = 0.1$). A tendency of synergy between ω-3 PUFAs and RT was observed in LS174T ($p = 0.09$) but not in HT29 cells ($p = 0.5$). SER induced by 50 μmol/L EPA was 1.4 and 1.5 in LS174T and HT29 cells, respectively, while SER induced by 50 μmol/L DHA was 1.6 and 1.7 in LS174T and HT29 cells, respectively. These results suggested that DHA is a better radio-sensitizer than EPA in both LS174T and HT29 cells.

3.2. DHA and EPA increase peroxide production after RT

In order to investigate the time-dependent production of peroxides after a 4-Gy RT, total peroxide concentration was measured 4, 24, and 48 h after a 4-h pre-treatment with 50 μmol/L ω-3 PUFAs in these two cell lines (Table 1). Total peroxide production in both cell lines depended not only on the addition of fatty acids ($p < 0.05$) but also on the exposure time ($p < 0.05$). Regardless of the combination with RT, DHA and to a lesser extent EPA increased peroxide production in LS174T ($p < 0.001$) and lesser extent in HT29 cells ($p < 0.001$). Without RT, the peroxide production reached a peak between 4 h and 24 h after incubation with EPA or DHA in both cell lines ($p < 0.001$), and then decreased at 48h in LS174T and remained stable in HT29 cells ($p = 0.22$). Whereas no significant effect could be observed within the first 24 h after RT alone, total peroxide production was increased significantly when RT was combined with ω-3 PUFA pre-treatment ($p < 0.05$). The peak production of peroxides was observed within the first 4 h after RT in LS174T cells and between 24 h and 48 h after RT in HT-29 cells, DHA being more efficient than EPA to increase total peroxide production (Table 1).

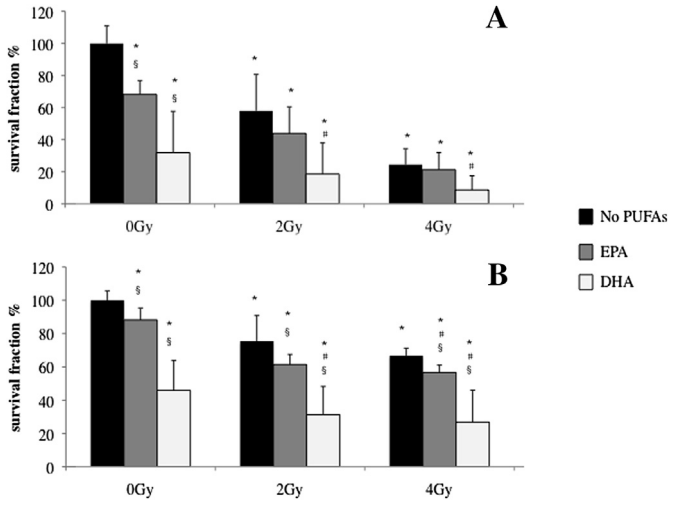

Fig. 1. Evaluation of cell survival. LS174T (A) and HT29 (B) cells were used for cell survival analysis after X-ray exposure to 0, 2 and 4 Gy combined or not with 50 μmol/L DHA or EPA. Values are expressed as cell survival (%) relative to untreated controls (S/So), and presented as means ± SD ($n > 3$). * $P < 0.05$, *vs.* control; ♯ $P < 0.05$ *vs.* RT; § $P < 0.05$ EPA *vs.* DHA (Student's-*t* test). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.

Table 1
Total peroxides production in LS174T or HT29 cells 4, 24, 48 h after an exposure of X-ray with a pre-incubation of EPA or DHA at 50 μmol/L for 4 h.

	4 h	24 h	48 h
LS174T			
Control	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.1
EPA	14.0 ± 13.9[a]	8.4 ± 4.6[a,b,c]	5.3 ± 1.6[a,b,c]
DHA	17.40 ± 13.9[a,b,]	20.7 ± 6.2[a,b,c]	12.8 ± 4.9[a,b,c,e]
4 Gy	1.9 ± 2.2	1.1 ± 0.5	1.3 ± 0.3
4 Gy + EPA	9.9 ± 8.4[a,b,c]	10.5 ± 3.8[a,b,c]	9.5 ± 2.6[a,b,c]
4 Gy + DHA	38.6 ± 15.5[a,b,c]	19.0 ± 6.3[a,b,c,d]	15.1 ± 1.7[a,b,c,d]
HT29			
Control	1.0 ± 0.1	1.0 ± 0.5	1.0 ± 0.1[,b]
EPA	0.9 ± 0.5[c]	5.6 ± 2.6[a,b,c,d]	8.1 ± 4.8[a,b,d]
DHA	3.2 ± 1.0[a,b,c]	11.8 ± 2.9[a,b,c,d]	12.0 ± 7.5[a,b]
4 Gy	0.6 ± 0.4	0.8 ± 0.4	1.3 ± 0.1[a]
4 Gy + EPA	0.7 ± 0.5	4.6 ± 2.2[a,b,c,d]	6.2 ± 3.7[a,b,c,d]
4 Gy + DHA	2.3 ± 1.2[b]	17.1 ± 8.7[a,b,c,d]	16.3 ± 2.7[a,b,c,d]

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
Values are calculated as fold to control and expressed as means ± SD ($n > 3$).
[a] $P < 0.05$ *vs.* control.
[b] $P < 0.05$ *vs.* RT.
[c] $P < 0.05$ EPA *vs.* DHA.
[d] $P < 0.05$ *vs.* 4 h.
[e] $P < 0.05$ *vs.* 24 h (Student's-*t* test).

3.3. DHA and EPA increase malondialdehyde production after RT

Cytoplasmic MDA level was measured by HPLC analysis 4 h and 24 h after RT in LS174T (Fig. 2A) and HT29 cells (Fig. 2B), respectively, to fit with the timing of peroxide production in these two cell lines. Again, the stronger effects of ω-3 PUFAs with or without RT were observed in LS174T compared to HT29 cells. DHA was more efficient than EPA to increase MDA production, regardless of RT combination ($p < 0.01$), whereas EPA showed a significant effect only when combined with RT in LS174T cells (Fig. 2C; $p = 0.01$), but not in HT29 cells (Fig. 2D; $p = 0.3$).

3.4. DHA and EPA accelerate early apoptosis after RT

FACS analysis with Annexin V/propidium iodide indicated that ω-3 PUFAs could increase early apoptosis in both LS174T ($p < 0.001$) and HT29 cells ($p = 0.01$). EPA induced apoptosis in a time-dependent manner in LS174T (Fig. 3A) and HT29 cells (Fig. 3B), while DHA caused a peak induction of 2.1 ± 0.5 fold ($p < 0.01$) and 1.8 ± 0.7 fold ($p = 0.07$) at 4 h in LS174T (Fig. 3C) and HT29 cells (Fig. 3D), respectively. Whereas RT alone stimulated apoptosis only in LS174T ($p = 0.01$), but not in HT29 cells ($p = 0.3$), RT combination with ω-3 PUFAs caused a time-dependent induction of apoptosis in both LS174T ($p = 0.04$) and HT29 cells ($p = 0.01$). In LS174T cells, a peak induction of 2.1 ± 0.3 fold ($p = 0.001$) and 2.5 ± 0.6 fold ($p < 0.05$) was observed at 2 h in the presence of RT combination with EPA (Fig. 3A, Table 2) and DHA (Fig. 3C, Table 2). In HT29 cells, a peak induction of 1.9 ± 0.6 ($p < 0.05$) and 2.4 ± 1.4 fold ($p < 0.05$) was rather observed at 4 h in the presence of RT combination with EPA (Fig. 3B) and DHA (Fig. 3D). ω-3 PUFAs synergized the pro-

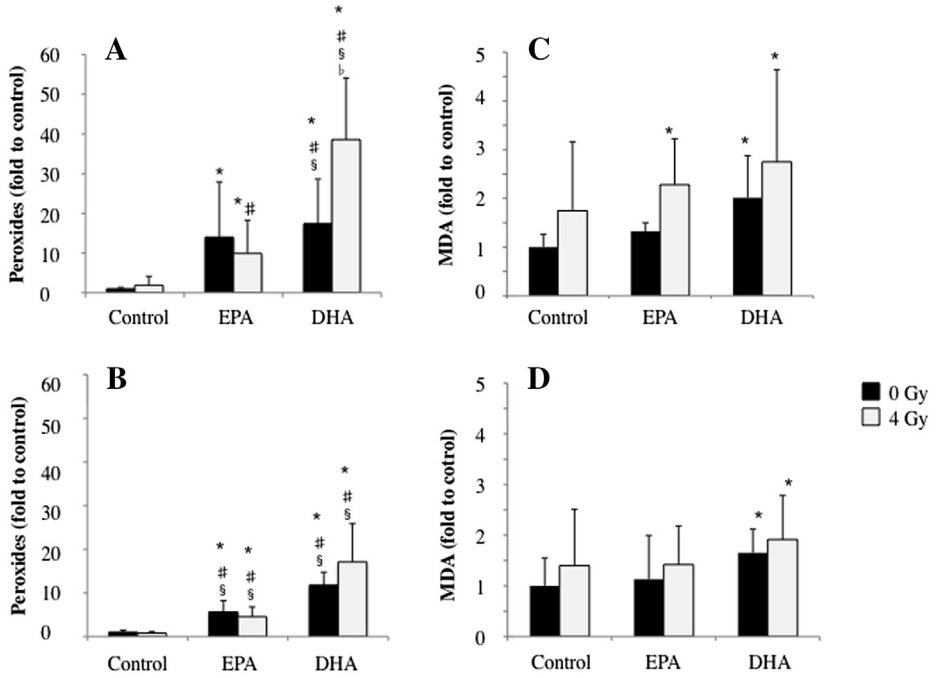

Fig. 2. Quantification of lipid peroxidation. Cells were treated with 50 μmol/L DHA or EPA for 4 h before X-ray exposure to 4 Gy. 4 h after irradiation, cells were collected for analysis. Peroxide production was measured by the xylenol orange method in LS174T (A) and HT29 (B) cells. MDA concentration was measured in LS174T (C) and HT29 (D) cells, using HPLC analysis. Values are normalized and expressed as fold to controls ($n > 3$). * $P < 0.05$, *vs.* control; ♯ $P < 0.05$ *vs.* RT; § $P < 0.05$ EPA *vs.* DHA; ♭ $P < 0.05$ EPA/DHA+4Gy *vs.* EPA/DHA (Student's-*t* test). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HPLC, high performance liquid chromatography; MDA, malondialdehyde.

apoptotic effect of RT only at 2 h after RT in LS174T cells ($p = 0.01$), while there was a tendency of synergy between ω-3 PUFAs and RT in HT29 cells 24 h after treatment ($p = 0.07$).

3.5. DHA and EPA affect protein expression after RT

As shown in Fig. 4, Heat shock protein 70 (HSP70), a biomarker of oxidative stress, increased with the addition of EPA, DHA and RT in LS174T and HT29 cells, while the combination of these two treatments decrease HSP70 expression compared to RT alone. The activated form of nuclear factor, NF-κB p65, decreased with DHA and to a lesser extent EPA, in both cells lines. RT also decreased NF-κB p65 expression. RT combination with ω-3 PUFAs, especially with DHA, considerably decreased NF-κB p65 expression in both LS174T and HT29 cells. A decrease in COX-2 with DHA with or without RT was observed in both cell lines. EPA, RT, but not DHA increased COX-2 expression in HT29 cells. Similar to DHA, EPA combination with RT decreased COX-2 expression in HT29 cells.

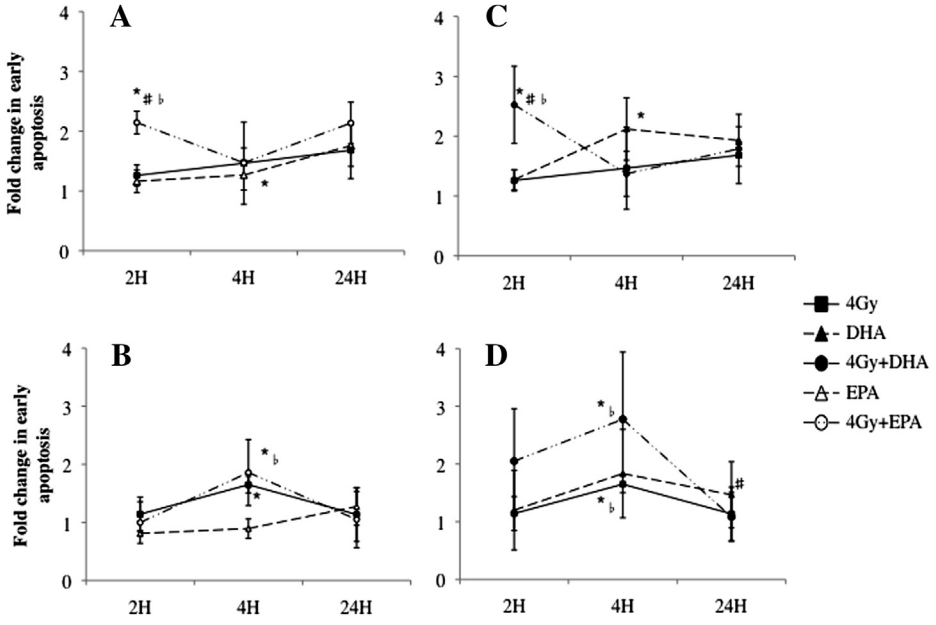

Fig. 3. Detection of early apoptosis. Early apoptosis were measured 2, 4, 24 h after X-ray exposure to 4 Gy in LS174T and HT29 cells with a pre-incubation of 50 μmol/L EPA (A. LS174T, B. HT29) and DHA (C. LS174T, D. HT29). Values are normalized as fold to control and represented as mean ± SD ($n > 3$). * $P < 0.05$, *vs.* control; ♯ $P < 0.05$ *vs.* RT; ♭ $P < 0.05$ EPA/DHA+4Gy *vs.* EPA/DHA (Student's-*t* test). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Table 2
Apoptosis analysis in LS174T and HT29 cells after pre-incubation of 4 h with EPA or DHA 50 μmol/L in combination with radiotherapy (4 Gy) by using fluorescence-activated cell sorter analysis with annexin V/fluorescein isothiocyanate labeling.

Early apoptosis (%)	LS174T	HT29
Control	7.5 ± 4.3	7.6 ± 4.3[b]
EPA	7.0 ± 4.5	7.6 ± 2.9[b,c]
DHA	7.5 ± 4.5	11.5 ± 2.6[a,c]
4 Gy	8.0 ± 5.7	12.5 ± 7.4[a]
4 Gy + EPA	13.0 ± 8.4[a,b,d]	12.3 ± 4.2[a,d]
4 Gy + DHA	14.2 ± 7.8[a,b,d]	14.6 ± 2.3[a]

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
Values are presented as means ± SD % ($n > 3$).
[a] $P < 0.05$ *vs.* control.
[b] $P < 0.05$ *vs.* RT.
[c] $P < 0.05$ EPA *vs.* DHA.
[d] $P <$ EPA/DHA *vs.* EPA/DHA+4Gy (Student's-*t* test).

RT and EPA or DHA decreased Bcl-2 expression, a member of apoptosis regulatory and pro-survival protein family, in both cell lines. The combination of EPA or DHA with RT had an additive effect especially when DHA was combined with 4 Gy. Increase in Bax expression, one of the pro-apoptotic proteins, was observed with the addition of EPA, DHA and RT in HT29 cells. Increase in p53 and also its small molecular isoforms were observed especially when RT was combined with DHA.

4. Discussion

DHA and to a lesser extent EPA had a strong dose-dependent additive effect with RT to suppress cell survival in the radio-sensitive LS174T cells. An additive effect was also observed in the radio-resistant HT29 cells. This suppressive effect could be explained by the induction of lipid peroxidation by fatty acids, which was enhanced by the addition of RT and consequently modified protein expression leading to apoptosis in these two cell lines.

ω-3 PUFAs have been shown to inhibit cell growth of several malignant cancer cells.[3,4] Habermann et al. found that DHA was the most cytotoxic among the fatty acids, including EPA.[11] This is in agreement with our findings that DHA was more efficient than EPA in suppressing cell survival in both LS174T and HT29 cells, possibly due to a better intracellular absorption rate of DHA than EPA in CRC cell lines.[11] DHA (22 carbons, 6-double-bonds) is more lipophilic and has thus more facility to be incorporated in cell membrane than EPA (20 carbons, 5-double-bonds). Therefore, DHA would be oxidized more quickly than EPA. Bougnoux et al. have revealed the exiting clinical benefit of DHA in breast cancer patients under chemotherapy. This tumor progression inhibition and survival prolongation is correlated with DHA plasma absorption level.[14] The biochemical characteristics of ω-3 PUFAs contributed to their different tumor-suppressive effects in breast cancer, which was proportional to their number of double bonds.[5]

ω-3 PUFAs increased the anti-tumor effect of RT in animal models of head and neck cancers[15] and mammary tumors.[16] RT is currently one of the main components of CRC treatment, especially for rectal cancer. A synergistic effect of PUFAs and RT in a radio-sensitive LS174T cell line and also an additive effect in a more radio-resistant HT29 cell line is therefore encouraging for a potential utilization in clinical practice.

Lipid peroxidation products, including primary products, peroxides (—C—O—OH) and also one of its end products, MDA, might be the main cause of PUFA-induced DNA damages,[17] which may lead to apoptosis and then cell death. Lu et al. found an increase in plasma MDA concentration that might contribute to the inhibitory

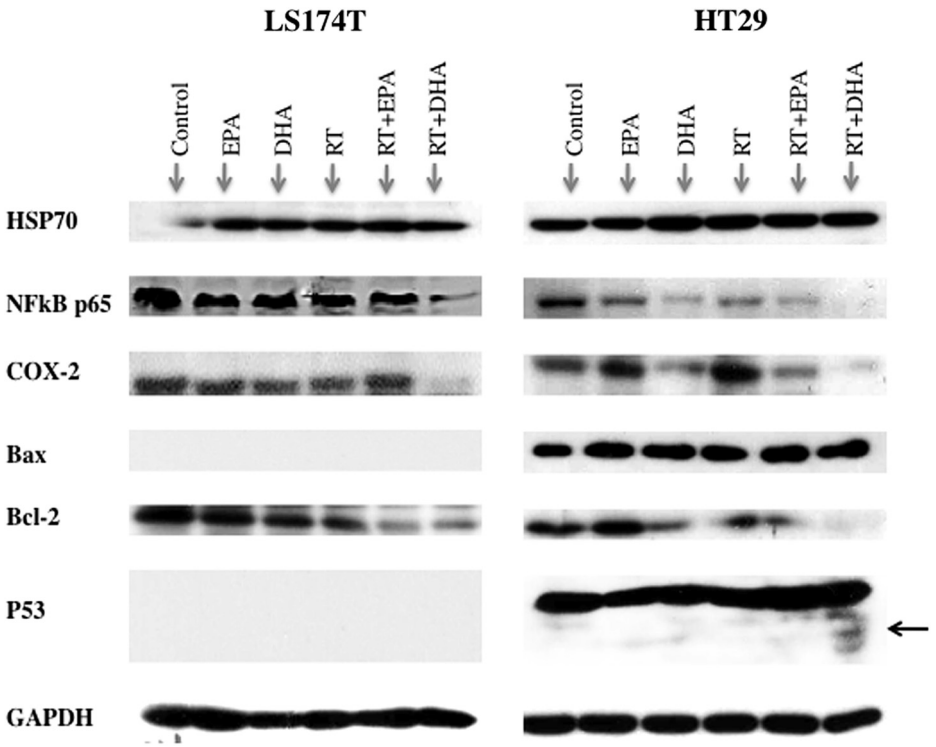

Fig. 4. Alteration of protein expression. Cells were pre-treated with DHA or EPA at 50 μmol/L and then exposed to X-rays of 4 Gy. 2 h after irradiation, cells were collected and lysed by RIPA solution (NaCl 150 mmol/L, Tris—HCl pH8 50 mmol/L, NP40 1%, Triton × 100 1%, SDS 0.1%). Indicator of reactive oxidation species, HSP70 (70 kDa); inflammation related protein, NFκB p65 (65 kDa) and COX-2 (72 kDa); apoptosis related protein, Bax (21 kDa), Bcl-2 (26 kDa) and p53 (53 kDa), were detected in using specific antibodies. GAPDH (36 kDa) was used as the internal standard for protein dosage. ←, small molecular weight isoforms of p53. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

effect of the PUFAs at a relatively high dose (100–300 μmol/L).[18] As shown in Fig. 2 and Table 1, addition of ω-3 PUFAs, especially DHA, increased production of peroxides and MDA in LS174T and to a lesser extent in HT29 cells. Moreover, a synergistic effect in peroxide production of PUFAs and RT existed in LS174T but not in HT29 cells, which corresponded to their strong additive effect in cell survival suppression in LS174T and to a lesser extent in HT29 cells.

The oxidative products would consequently modify several signaling pathways, including apoptosis. Apoptosis is a protective reaction for cells to fight against internal or external stimulation that will lead to aberrant cell transformation, which is one of the critical steps in cancer development and progression, and also one of the biological targets for cancer treatment. We found that DHA and EPA induced apoptosis in a time-dependent manner, which was consistent with peroxide production. DHA had more efficient pro-apoptotic effect than EPA in these two CRC cell lines (Fig. 3). DHA induced apoptosis in different cancers both *in vitro* and *in vivo*.[19,20] EPA supplementation reduced cell proliferation and increased apoptosis in the crypts of patients with CRC history by a selective increase in EPA uptake in the colonic mucosa.[21] When combined with RT, the pro-apoptotic effect of PUFAs was less studied. We found that PUFAs had a synergistic effect in apoptosis induction 2 h after exposure to RT in LS174T ($p = 0.01$), while in HT29 cells, a tendency of synergy ($p = 0.07$) was observed 24 h after exposure. Decrease in Bcl-2, an anti-apoptotic protein of the pro-survival protein family whose abnormal increase leads to malignant transformation,[22] together with an increase in pro-apoptotic Bax expression were observed in the presence of EPA or DHA using Western Blots. This effect was enhanced by the addition of RT. Therefore, DHA and to a lesser extent EPA enhanced the pro-apoptotic effect of RT in LS174T while in HT29 cells, the synergistic effect was not obvious. Similar results were found in cell survival and also in peroxide production as described before.

Addition of EPA or DHA increased intracellular reactive oxidation species (ROS) as indicated by an augmentation of HSP70 (Fig. 4). HSP70, a member of heat shock protein family, is usually induced by oxidative stress to protect cells from this challenge. In our study, we found an increase in HSP70 expression with ω3-PUFAs or RT alone. Because our dose of ω3-PUFAs or RT are not critical as we aimed at evaluating a potential synergy between these treatments. Decrease in HSP70 by the combination of these two treatments compared to RT alone suggests a radiosensitizer effect of DHA, because high expression of HSP70 can lead to radioresistance.[23]

ROS consequently mediate activation of NF-κB,[24] whose high constitutive expression is important to maintain cell viability in cancer cells.[25] NF-κB is also the most important mediator in radioresistance.[26] Sandur et al. concluded that NF-κB dependent cell survival pathway was crucial for radio-resistance in CRC cell lines and that suppression of inducible NF-κB led to radio-sensitivity.[27] We found a decrease in NF-κB p65 expression after single DHA or EPA treatment. With the combination of PUFAs and RT, the decrease was more evident in both cell lines, especially when DHA was combined with RT. Zand et al. also found a synergistic apoptosis-inductive effect of DHA with RT via suppression of NF-κB pathway in a high radio-resistant cancer cell line.[28] Therefore, NF-κB probably has a primordial effect in maintaining cell apoptosis induced by inflammatory challenge and at the same time modulating release of factors that are involved in cell survival.[24]

Modulation of NF-kB consequently led to change in expression of COX-2, a down-stream inflammatory protein that plays an essential role in CRC carcinogenesis and treatment.[29] ω-3 PUFAs could inhibit CRC cell proliferation with a decrease in pro-inflammatory proteins, including iNOS and COX-2.[30] Furthermore, long-term intake of COX-2 inhibitor was effective in decreasing colorectal cancer risk.[31] These observations indicate that COX-2 does not only play an important role in promoting tumor cell growth but also is a potential target for anti-cancer treatment. In our study, decreased expression of COX-2 was observed in the presence of ω-3 PUFAs, especially when DHA was combined with RT. This is consistent with the cell survival inhibition and NF-κB p65 modulation as described before.

Tumor suppressor protein p53 is involved in conserving genome stability. Isoforms of p53 have recently been identified and their function is widely debated. Avery-Kiejda et al. found an aberrant increase in Δ40p53, a small molecular weight isoform of p53, in different cancer cells, which was related to chemotherapeutic treatment in a time-dependent manner.[32] We also found an increase in this p53 isoform of about 40 kDa after the treatment combination of DHA and with RT (Fig. 4), which might possibly contribute to the radio-sensitive effect of the combined treatment.

Our study was carried out using two CRC cell lines with different genetic backgrounds, which affected their proliferation rate and also their different response to RT. Although the peroxide level of ω-3 PUFAs solution was not measured before use, both stock solutions were kept at −20 °C under argon to prevent oxidative degradation, and all the treatments were performed cautiously in parallel with EPA and DHA in order to compare their effect. The intracellular incorporation of ω-3 PUFAs was not quantified in our study. As reported by Habermann et al., intake of fatty acids into colon cells occurs continuously from 1 h to 72 h after incubation. This intake depends also on the cell type.[11] Therefore, quantification of ω-3 PUFAs is of less important in our study, since the subsequent cascade for this incorporation, which are peroxyl production, peroxidation and apoptosis have been tested thereafter. The results suggest that ω-3 PUFAs strongly enhance the radio-cytotoxicity in radio-sensitive LS174T cells while an additive effect was observed in radio-resistant HT29 cells. Nevertheless animal experiment is needed to confirm these observations before its potential clinical application.

5. Conclusion

DHA and to a lesser extent EPA strongly enhance the cytotoxic effect of RT in radio-sensitive LS174T cells, while an additive effect of ω-3 PUFAs with RT was observed in the radio-resistant HT-29 cells. ω-3 PUFAs enhanced RT efficacy by inducing lipid peroxidation, with subsequent modulation of inflammatory response and finally led to cell apoptosis. Our observations suggest that ω-3 PUFAs might be used as radio-sensitizer for CRC treatment. Further investigations, especially experiments on animals, are needed to confirm these observations for potential clinical application.

Authors' contributions

FC: data acquisition and analysis and manuscript drafting; OS: lipid peroxidation analysis processing and data interpretation; VG and EL: data acquisition; RM: irradiation protocol development; YMD: study conception and design, data interpretation, manuscript revision; CP: study conception and design, data interpretation, manuscript revision and funding. This work was supported by research grants from the Swiss National Science Foundation n° 310000-116738/1 and the Foundation Nutrition 2000Plus.

Conflict of interest statement

None of the authors had any personal or financial conflict of interest.

Acknowledgments

The authors gratefully acknowledge the assistance and availability of Aurélie Clerc for the statistical analyses and the Radio-Oncology team, especially Michel Rouzaud, Corinne Sedmak Hotier, Marin Barada, Sabrina Decaillet, Gloria Lee for their assistance in the radiation procedure.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010 Sep–Oct;**60**(5):277–300.
2. Andre N, Schmiegel W. Chemoradiotherapy for colorectal cancer. *Gut* 2005 Aug;**54**(8):1194–202.
3. Rose DP, Connolly JM. Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol Ther* 1999 Sep;**83**(3):217–44.
4. Rao CV, Hirose Y, Indranie C, Reddy BS. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. *Cancer Res* 2001 Mar 1;**61**(5):1927–33.
5. Germain E, Chajes V, Cognault S, Lhuillery C, Bougnoux P. Enhancement of doxorubicin cytotoxicity by polyunsaturated fatty acids in the human breast tumor cell line MDA-MB-231: relationship to lipid peroxidation. *Int J Cancer* 1998 Feb 9;**75**(4):578–83.
6. Benais-Pont G, Dupertuis YM, Kossovsky MP, Nouet P, Allal AS, Buchegger F, et al. omega-3 polyunsaturated fatty acids and ionizing radiation: combined cytotoxicity on human colorectal adenocarcinoma cells. *Nutrition* 2006 Sep;**22**(9):931–9.
7. Bougnoux P, Hajjaji N, Maheo K, Couet C, Chevalier S. Fatty acids and breast cancer: sensitization to treatments and prevention of metastatic re-growth. *Prog Lipid Res* 2010 Jan;**49**(1):76–86.
8. Calviello G, Serini S, Piccioni E, Pessina G. Antineoplastic effects of n-3 polyunsaturated fatty acids in combination with drugs and radiotherapy: preventive and therapeutic strategies. *Nutr Cancer* 2009;**61**(3):287–301.
9. Colas S, Maheo K, Denis F, Goupille C, Hoinard C, Champeroux P, et al. Sensitization by dietary docosahexaenoic acid of rat mammary carcinoma to anthracycline: a role for tumor vascularization. *Clin Cancer Res* 2006 Oct 1;**12**(19):5879–86.
10. Bougnoux P, Germain E, Chajes V, Hubert B, Lhuillery C, Le Floch O, et al. Cytotoxic drugs efficacy correlates with adipose tissue docosahexaenoic acid level in locally advanced breast carcinoma. *Br J Cancer* 1999 Apr;**79**(11–12):1765–9.
11. Habermann N, Christian B, Luckas B, Pool-Zobel BL, Lund EK, Glei M. Effects of fatty acids on metabolism and cell growth of human colon cell lines of different transformation state. *Biofactors* 2009 Sep–Oct;**35**(5):460–7.
12. Kim S, Wu HG, Shin JH, Park HJ, Kim IA, Kim IH. Enhancement of radiation effects by flavopiridol in uterine cervix cancer cells. *Cancer Res Treat* 2005 Jun;**37**(3):191–5.
13. Sim AS, Salonikas C, Naidoo D, Wilcken DE. Improved method for plasma malondialdehyde measurement by high-performance liquid chromatography using methyl malondialdehyde as an internal standard. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003 Mar 5;**785**(2):337–44.
14. Bougnoux P, Hajjaji N, Ferrasson MN, Giraudeau B, Couet C, Le Floch O. Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: a phase II trial. *Br J Cancer* 2009 Dec 15;**101**(12):1978–85.
15. Wen B, Deutsch E, Opolon P, Auperin A, Frascogna V, Connault E, et al. n-3 polyunsaturated fatty acids decrease mucosal/epidermal reactions and enhance antitumour effect of ionising radiation with inhibition of tumour angiogenesis. *Br J Cancer* 2003 Sep 15;**89**(6):1102–7.
16. Colas S, Paon L, Denis F, Prat M, Louisot P, Hoinard C, et al. Enhanced radiosensitivity of rat autochthonous mammary tumors by dietary docosahexaenoic acid. *Int J Cancer* 2004 Apr 10;**109**(3):449–54.
17. Cai F, Dupertuis YM, Pichard C. Role of polyunsaturated fatty acids and lipid peroxidation on colorectal cancer risk and treatments. *Curr Opin Clin Nutr Metab Care* 2012 Mar;**15**(2):99–106.
18. Lu X, Yu H, Ma Q, Shen S, Das UN. Linoleic acid suppresses colorectal cancer cell growth by inducing oxidant stress and mitochondrial dysfunction. *Lipids Health Dis* 2010;**9**:106.
19. Song KS, Jing K, Kim JS, Yun EJ, Shin S, Seo KS, et al. Omega-3-polyunsaturated fatty acids suppress pancreatic cancer cell growth in vitro and in vivo via down-regulation of Wnt/Beta-catenin signaling. *Pancreatology* 2011;**11**(6):574–84.
20. Serini S, Fasano E, Piccioni E, Monego G, Cittadini AR, Celleno L, et al. DHA induces apoptosis and differentiation in human melanoma cells in vitro: involvement of HuR-mediated COX-2 mRNA stabilization and beta-catenin nuclear translocation. *Carcinogenesis* 2012 Jan;**33**(1):164–73.
21. Courtney ED, Matthews S, Finlayson C, Di Pierro D, Belluzzi A, Roda E, et al. Eicosapentaenoic acid (EPA) reduces crypt cell proliferation and increases apoptosis in normal colonic mucosa in subjects with a history of colorectal adenomas. *Int J Colorectal Dis* 2007 Jul;**22**(7):765–76.
22. Kelly PN, Strasser A. The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. *Cell Death Differ* 2011 Sep;**18**(9):1414–24.
23. Gehrmann M, Radons J, Molls M, Multhoff G. The therapeutic implications of clinically applied modifiers of heat shock protein 70 (Hsp70) expression by tumor cells. *Cell Stress & Chaperones* 2008 Spring;**13**(1):1–10.
24. Magne N, Toillon RA, Bottero V, Didelot C, Houtte PV, Gerard JP, et al. NF-kappaB modulation and ionizing radiation: mechanisms and future directions for cancer treatment. *Cancer Lett* 2006 Jan 18;**231**(2):158–68.
25. Smirnov AS, Ruzov AS, Budanov AV, Prokhortchouk AV, Ivanov AV, Prokhortchouk EB. High constitutive level of NF-kappaB is crucial for viability of adenocarcinoma cells. *Cell Death Differ* 2001 Jun;**8**(6):621–30.
26. Eschrich S, Zhang H, Zhao H, Boulware D, Lee JH, Bloom G, et al. Systems biology modeling of the radiation sensitivity network: a biomarker discovery platform. *Int J Radiat Oncol Biol Phys* 2009 Oct 1;**75**(2):497–505.
27. Sandur SK, Deorukhkar A, Pandey MK, Pabon AM, Shentu S, Guha S, et al. Curcumin modulates the radiosensitivity of colorectal cancer cells by suppressing constitutive and inducible NF-kappaB activity. *Int J Radiat Oncol Biol Phys* 2009 Oct 1;**75**(2):534–42.
28. Zand H, Rahimipour A, Salimi S, Shafiee SM. Docosahexaenoic acid sensitizes Ramos cells to Gamma-irradiation-induced apoptosis through involvement of PPAR-gamma activation and NF-kappaB suppression. *Mol Cell Biochem* 2008 Oct;**317**(1–2):113–20.
29. Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, et al. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009 Mar;**30**(3):377–86.
30. Ritchie SA, Jayasinghe D, Davies GF, Ahiahonu P, Ma H, Goodenowe DB. Human serum-derived hydroxy long-chain fatty acids exhibit anti-inflammatory and anti-proliferative activity. *J Exp Clin Cancer Res* 2011;**30**:59.
31. Vinogradova Y, Coupland C, Hippisley-Cox J. Exposure to cyclooxygenase-2 inhibitors and risk of cancer: nested case-control studies. *Br J Cancer* 2011 Jul 26;**105**(3):452–9.
32. Avery-Kiejda KA, Zhang XD, Adams LJ, Scott RJ, Vojtesek B, Lane DP, et al. Small molecular weight variants of p53 are expressed in human melanoma cells and are induced by the DNA-damaging agent cisplatin. *Clin Cancer Res* 2008 Mar 15;**14**(6):1659–68.