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PRECLINICAL STUDY

Role of docosahexaenoic acid in enhancement of docetaxel action in patient-derived breast cancer xenografts

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Abstract

Purpose The objective of this study was to investigate if DHA dietary supplementation enhances the anticancer actions of docetaxel (TXT) in two different drug resistant triple negative breast cancer (TNBC) patient-derived xenografts (PDX)s.

Methods In two experiments, female NSG mice bearing TNBC PDXs were randomized to one of two nutritionally adequate diets (20% w/w): control (0% DHA), or DHA (3.9% w/w of total fat) and injected with 0 or 5 mg/kg TXT, twice weekly for 6 weeks ($n=8$ per group). Treatment response was determined by significant differences in tumor weight, and apoptotic, proliferation and cell cycle markers at endpoint.

Results Mice bearing MAXF574 xenografts fed DHA diet and treated with TXT had a 57% reduction in tumor weight compared to mice fed control diet ($P<0.004$), a 64% reduction compared to control + TXT ($P<0.01$) and a 34% reduction compared to DHA with no TXT ($P<0.04$). DHA + TXT reduced MAXF401 xenografts growth compared to control and control + TXT (by 43% and 34%, respectively, $P<0.05$). In both xenografts, DHA + TXT resulted in a higher expression of proapoptotic proteins Ripk1 and Bid, lower expression of proliferation marker Ki67 and anti-apoptotic proteins Bcl-2 and Parp, and a greater increase in cell cycle arrest as measured by decreased Survivin expression when compared to control + TXT mice ($P<0.05$).

Conclusions This work is the first to confirm that DHA supplementation during chemotherapy treatment improves TXT action in two PDX models of TNBC. The results suggest that decreases in tumor size occurred via changes in apoptosis, cell proliferation, and cell cycle pathways.

Keywords Fatty acids · Ki67 · Apoptosis · Cell cycle · Tumor growth

Abbreviations

ASK1	Apoptosis signal-regulating kinase 1
BAD	Bcl-2-associated death promoter
BAX	Bcl-2-associated X

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BCL-2	B cell CLL/lymphoma 2
BCLXL	B-cell lymphoma-extra large
BID	BH3 interacting domain death agonist
BSA	Bovine serum albumin
CD95	Cluster of differentiation 95 (Fas)
CDC2	Cyclin-dependent kinase 1 (CDK1)
CDC25C	Cell division cycle 25 homolog C
DR5	Death receptor 5
DHA	Docosahexaenoic acid
EGFR	Epidermal growth factor receptor
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
FADD	Fas-associated via death domain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX4	Glutathione peroxidase 4
HER2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
LCPUFA	Long chain polyunsaturated fatty acid
MAPK	Mitogen-activated protein kinase
MCM2	Minichromosome maintenance complex component 2
NSG	NOD.Cg-Prkdc^{scid}Il2rg
PAKT	Phospho-Akt
PARP	Poly (ADP-ribose) polymerase
PBCL-2	Phospho-Bcl-2
PCNA	Proliferating cell nuclear antigen
PDX	Patient-derived xenograft
PI3K	Phosphoinositide 3-kinase
PLK1	Polo-like kinase
PR	Progesterone receptor
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase
TBST	Tris-buffered saline and Tween20
TNBC	Triple negative breast cancer
TXT	Docetaxel
WEE1	WEE1 homolog

Background

Despite advances in prevention, screening, diagnoses, and treatment, breast cancer is expected to account for more than 627,000 deaths worldwide annually [1]. One of the difficulties that arises in successfully treating breast cancer is that it manifests as a heterogeneous group of diseases, rather than a single disease [2, 3]. Moreover, tumors employ multiple methods to survive and proliferate, creating a complex, continuously evolving environment which contributes to therapy resistance [4, 5]. This is particularly true for triple negative breast cancers (TNBC: estrogen, progesterone and human epidermal growth factor receptor negative, $ER^{-}PR^{-}HER2^{-}$). TNBC accounts for 15–20% of all breast cancer diagnoses in North America and is characterized by an aggressive trajectory that results in poorer patient prognoses [6–8]. Targeted drug therapy is not yet an option for most women diagnosed with TNBC. Rather, most patients are treated with adjuvant or neoadjuvant systemic chemotherapy such as docetaxel (TXT) [9]. TXT is known to be ineffective against certain breast cancers including metastatic cancers where the response rate is 30–50% [10]. However, it remains difficult to predict in the TNBC population who will respond favorably to docetaxel.

The pleiotropic anti-cancer effects of the $n-3$ long chain polyunsaturated fatty acid (LCPUFA), docosahexaenoic acid (DHA), have been previously established in breast cancer cells in vitro and in vivo (reviewed by [11–13]). There is a growing evidence suggesting that DHA might also be beneficial when provided in conjunction with chemotherapy in a number of cancers in vitro [14–19] and in a few animal models of breast cancer [19–21]. While many of the multidimensional hallmarks of cancer [4, 5] appear to be targeted by LCPUFA (reviewed in [11–13]), much of this work has relied on the use of immortalized cell lines (in vitro and implanted in immunodeficient animals in vivo) that do not represent the intratumoral heterogeneity of patient tumors [2, 22].

A major challenge in translating laboratory findings to a clinical setting is the inability of cell line derived preclinical models to recapitulate genomic and microenvironmental heterogeneities [3, 23–26]. Patient-derived xenografts (PDXs) bridge this gap and more closely recapitulate the heterogenicity and gene expression of primary tumors [22, 27]. Herein, we employed two TNBC PDX models, one well differentiated and the other poorly differentiated, in order to determine if feeding DHA enhances the anticancer actions of docetaxel. We further sought to explore potential mechanisms involved in the anticancer effects of DHA.

Methods

Experimental diets

Nutritionally complete diets were composed of a basal mix diet from Teklad (TD.84172; Harlan Laboratories, Madison, WI), with macronutrient composition as previously described [28]) and contained 20% w/w fat. The fatty acid composition of the diets (Online Resource: Supplemental Table 1) was achieved by blending oils to obtain a DHA content in the DHA diets of 3.9% w/w of total fat (DHASCO™, DSM, Columbia, MD). The amount of DHA in the diet was selected to achieve a plasma phospholipid DHA concentration of > 5% w/w of total fatty acids. This concentration is believed to be clinically relevant as it has been associated with prolonged survival in women with metastatic breast cancer undergoing chemotherapy [29].

Because the animals in this study are immunocompromised, it is a requirement of our animal care facility that diets fed to these animals are irradiated to prevent potential exposure to foodborne pathogens [30]. Diets were irradiated for 108 h at 8 kGy and stored at − 20 °C until used. Fatty acid analysis by gas liquid chromatography pre- and post-irradiation confirmed that the fat composition was not altered by irradiation (data not shown). While DHA in combination with EPA has been shown to exert-anticancer effects [31], DHA alone has been shown to enhance the actions of chemotherapy (Supplemental Table 2) and therefore the diet was designed to contain only DHA.

Experimental animals

Animal experiments were reviewed and approved (AUP00000134) by the University of Alberta Animal Policy and Welfare Committee and were in accordance with the Canadian Council on Animal Care guidelines. Immune-deficient 6-week-old female NOD.Cg-Prkdc^{scid}Il2rg (NSG) mice were housed in bio-containment under aseptic conditions with autoclaved bedding and water. In separate experiments, PDX tumor sections approximately 30 mm^3, representative of basal-like triple negative breast cancers (MAXF574 and MAXF401, Charles River Oncotest™ PDX models) were implanted into the left flank of each NSG mouse. MAXF 574 is a poorly differentiated, well-vascularized PDX, whereas MAXF 401 is moderately differentiated and poorly vascularized (Online Resource: Supplemental Table 3 for description of PDXs). Once tumors were approximately 50 mm^3 (measured by calipers and the equation: volume (mm^3) = length (mm) × width^2 (mm^2) × 0.5), mice were randomized into diet groups: 0% DHA (control) or 3.9% w/w DHA (DHA) (Fig. 1a). Mice were fed ad libitum for one

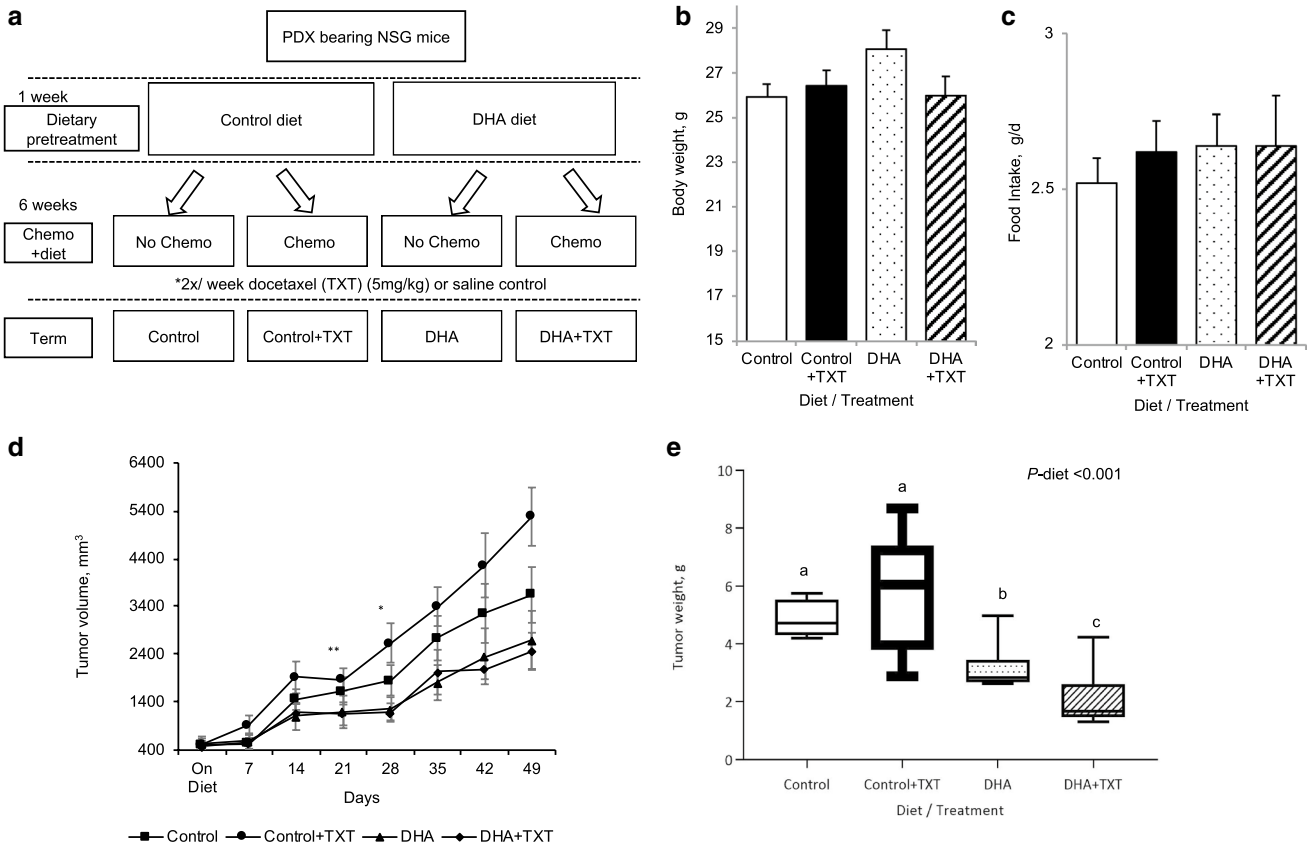

Fig. 1 Effect of dietary DHA with or without TXT on MAXF574 TNBC PDX growth in NSG mice. **a** Experimental design; mice were implanted with TNBC MAXF574 tumor sections approximately 30 mm^3. One week prior to commencing chemotherapy the mice were randomized into control or DHA diet groups and subsequently into chemotherapy (twice weekly) or control groups for an additional 6 weeks. Experimental groups are defined as: control, control + TXT, DHA, and DHA + TXT. Values represent the mean ± SEM ($n = 8$ mice per group). **b** Average daily food intake of MAXF574 tumor bearing mice. **c** Final body weight of MAXF574 tumor bearing mice. **d** Average tumor volume of MAXF574 tumor bearing mice, *statistical difference from day 28 ($P < 0.05$) of DHA compared to Control + TXT, **statistical difference from day 21 ($P < 0.05$) of DHA + TXT compared to Control + TXT and **e** excised tumor weight of MAXF574 tumor bearing mice. Kruskal–Wallis test for non-parametric analysis was employed to compare tumor weight between groups. The P value for the main effect of the diet = $P < 0.001$; comparison of Control to DHA + TXT = $P < 0.004$; comparison of Control to DHA = $P < 0.02$; comparison of Control + TXT to DHA = $P < 0.03$ and comparison of Control to Control + TXT = $P < 0.48$

week and then further randomized for chemotherapy treatments. As six rounds of neoadjuvant chemotherapy is standard of care for breast cancer patients prescribed neoadjuvant therapy, the mice received 5 mg/kg docetaxel chemotherapy or 0.9% saline (placebo) injection twice weekly for 6 week and then the experiment was ended. Experimental groups are defined as: control, control + TXT, DHA, and DHA + TXT. Body weights and food intake were monitored three times per week throughout the experiment. Mice were euthanized, tumors carefully excised and weighed; one piece was formalin fixed for immunohistochemistry and another was homogenized for protein analysis. Individuals performing the excision and weighing of the tumor; and all subsequent assays were blinded to the diet/chemotherapy treatments.

Western blot analysis

Tumors were minced, and protein lysates were prepared and Western blots were performed as previously described [32, 33]. Primary antibodies to β Catenin, BCL-2, BCLXL, Bad, Bax, Bid, Caspase 3, Caspase 7, Caspase 8, CD95, Cyclin B1, Cdc25C, CDC2, DR5, EGFR, FADD, MAPK, MCM2, pAKT, PARP, pBCL-2, PI3 K, PCNA, PLK1, Ras (Cell Signaling Technology, New England Biolabs, Whitby, ON), Caspase 10, GPX4, Ripk1, Survivin and Wee1 (Abcam, Cedarlane, Burlington, ON) were diluted 1:1000 in 5% w/v BSA-TBST. GAPDH (Cell Signaling Technology, New England Biolabs, Whitby, ON) was used as a loading control at a concentration of 1:5000 in 5% w/v BSA-TBST and the secondary antibody, Anti-rabbit IgG HRP (Cell Signaling Technology, Whitby ON) or Goat Anti-Mouse IgG (Abcam, Cedarlane, Burlington, ON). Subsequently, membranes were developed using Pierce ECL 2 Western Blotting Substrate (Fisher Scientific, Edmonton AB) and visualized on a Typhoon™ Trio + variable mode imager (GE Life Sciences, Baie d'Urfe, PQ). The relative intensities of band signals were quantified using ImageQuant TL software.

Immunohistochemistry

Tumor sections were incubated with antibodies for CD95, Cyclin B1 and Ki67 (Cell Signaling Technology, Whitby ON), and positively stained cells were visualized using ImmunoDetector liquid 3,3′-Diaminobenzidine (DAB, Bio SB Inc., Santa Barbara, CA, USA). Apoptosis in tumor sections was assessed using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore, Temecula, CA) according to manufacturer's instructions. Sections were imaged using AxioCam and the proportion of positive cells was determined using MetaMorph software (Carl Ziess Canada Ltd, North York ON).

Statistical analysis

Statistical analyses were carried out using SAS, version 9.4 (SAS Institute Inc. Cary, NC). Data were analyzed for normal distribution and if normally distributed analyzed by 2-way ANOVA followed by post hoc Duncan analysis using to identify significant differences between treatments ($P < 0.05$). Non-parametric data (tumor weights and volume) were analyzed using the Kruskal–Wallis to identify differences followed by post hoc analysis using the Mann–Whitney test to identify differences between treatments. All statistical tests were two sided, and P values less than 0.05 were considered statistically significant.

Results

Effect of DHA dietary supplementation on MAXF574 TNBC PDX tumor growth

We first assessed whether DHA in conjunction with TXT chemotherapy could reduce tumor growth in a TNBC PDX model compared to control + TXT. Mice bearing MAXF574 PDX tumors did not differ in food intake, and final body weight did not differ amongst treatments (Fig. 1b, c). Final tumor volumes after 6 weeks of chemotherapy were lower in the DHA ($P < 0.02$) and DHA + TXT ($P < 0.002$) groups compared to control + TXT (Fig. 1d). Excised tumor weight was significantly lower in the DHA + TXT group compared to control + TXT ($P < 0.01$), as well as control ($P < 0.004$) and DHA alone ($P < 0.04$; Fig. 1e). There was no significant difference in final tumor weight in control compared to control + TXT ($P < 0.5$; Fig. 1e).

Effect of DHA dietary supplementation on apoptotic signaling

To assess the ability of DHA dietary supplementation to enhance apoptotic signaling, MAXF574 tumors were assessed for markers of apoptosis by Western blot analysis and immunohistochemistry. While there were no changes in whole cell protein expression of apoptotic markers CD95, DR5, FADD, Caspase 8, Caspase 7, Bad, Bax or GPX4 (Fig. 2a), DHA + TXT tumor extracts were found to have differential expression of TXT-mediated apoptotic pathway proteins, specifically a lower expression of Bcl-2, pro-Caspase 3, pAKT, and Parp as well as a higher expression of ASK1, Bid, and Ripk1 compared to control ($P < 0.05$) but not control + TXT (Fig. 2a, b). Immunohistochemical analysis of tumors from DHA + TXT mice determined CD95 expression (Fig. 2c, d) to be higher than control and control + TXT tumors ($P < 0.05$). DHA + TXT tumors had higher TUNEL expression (Fig. 2d, e)

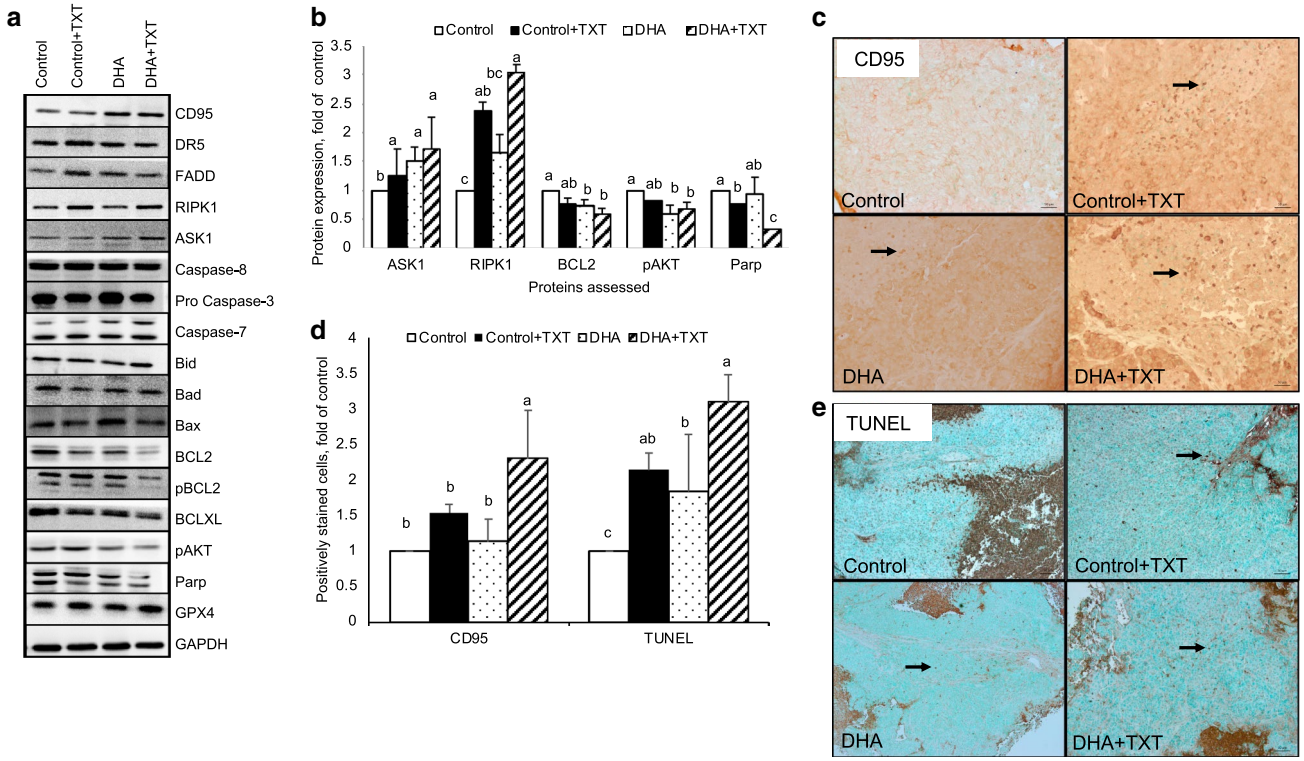

Fig. 2 Apoptotic protein expression in MAXF574 PDX tumor extracts from NSG mice. **a** Representative Western blot analysis of apoptosis proteins and **b** densitometric quantification of blots from (**a**). Values represent the mean ± SEM ($n = 8$ mice per group). Labeled means without a common letter differ ($P < 0.05$) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: ASK1: $P = 0.05$; RIPK1: $P = 0.1$; BCL2: $P = 0.08$; pAKT: $P = 0.04$; Parp: $P = 0.13$; the main effect of the treatment: ASK1: P = 0.29; RIPK1: P = 0.002; BCL2: P = 0.19; pAKT: P = 0.71; Parp: $P = 0.03$ and P interaction: ASK1: $P = 0.93$; RIPK1: $P = 0.98$; BCL2: $P = 0.75$; pAKT: $P = 0.21$; Parp: $P = 0.24$. **c** Immunohistochemical staining of CD95, **d** relative quantification of CD95 and TUNEL staining and **e** apoptosis by TUNEL assay. Positive staining is dark brown color and indicated by arrows

compared to control but not significantly different control + TXT tumor groups ($P < 0.05$).

Effect of DHA supplementation on markers of proliferation

Changes in proliferation of MAXF574 tumors from mice fed DHA in combination with TXT compared to tumors from control fed mice with or without TXT were assessed by Western blot analysis and immunohistochemistry. There were no differences in proliferation or growth associated markers PCNA, EGFR, Ras, p38 MAPK, p44/42 MAPK, or β Catenin amongst treatments. MCM2 was higher in both control + TXT and DHA + TXT groups, and PI3K was lower in all experimental groups compared to control (Fig. 3a, b, $P < 0.05$). Tumors from mice fed DHA were found to have lower Ki67 staining compared to control and control + TXT groups and tumors from mice in the DHA + TXT had lower Ki67 staining compared to all groups (Fig. 3c, d) ($P < 0.05$).

Effect of DHA dietary supplementation on markers of cell cycle progression

To assess the ability of DHA dietary supplementation to change signals associated with the cell cycle, MAXF574 tumors were assessed for markers of cell cycle progression by Western blot analysis (Fig. 4a, b) and immunohistochemistry (Fig. 4c). No differences were observed in CDC2, PLK1 or Wee1 (Fig. 4a). However, Survivin expression was lower in DHA + TXT compared to control and control + TXT. In addition, cdc25c expression was lower in DHA + TXT compared to control + TXT but did not differ from the control ($P < 0.05$, Fig. 4b). While Cyclin B1 protein expression trended higher in DHA + TXT samples ($P < 0.1$, Fig. 4a, b), immunohistochemical staining was not significantly different amongst samples (Fig. 4c).

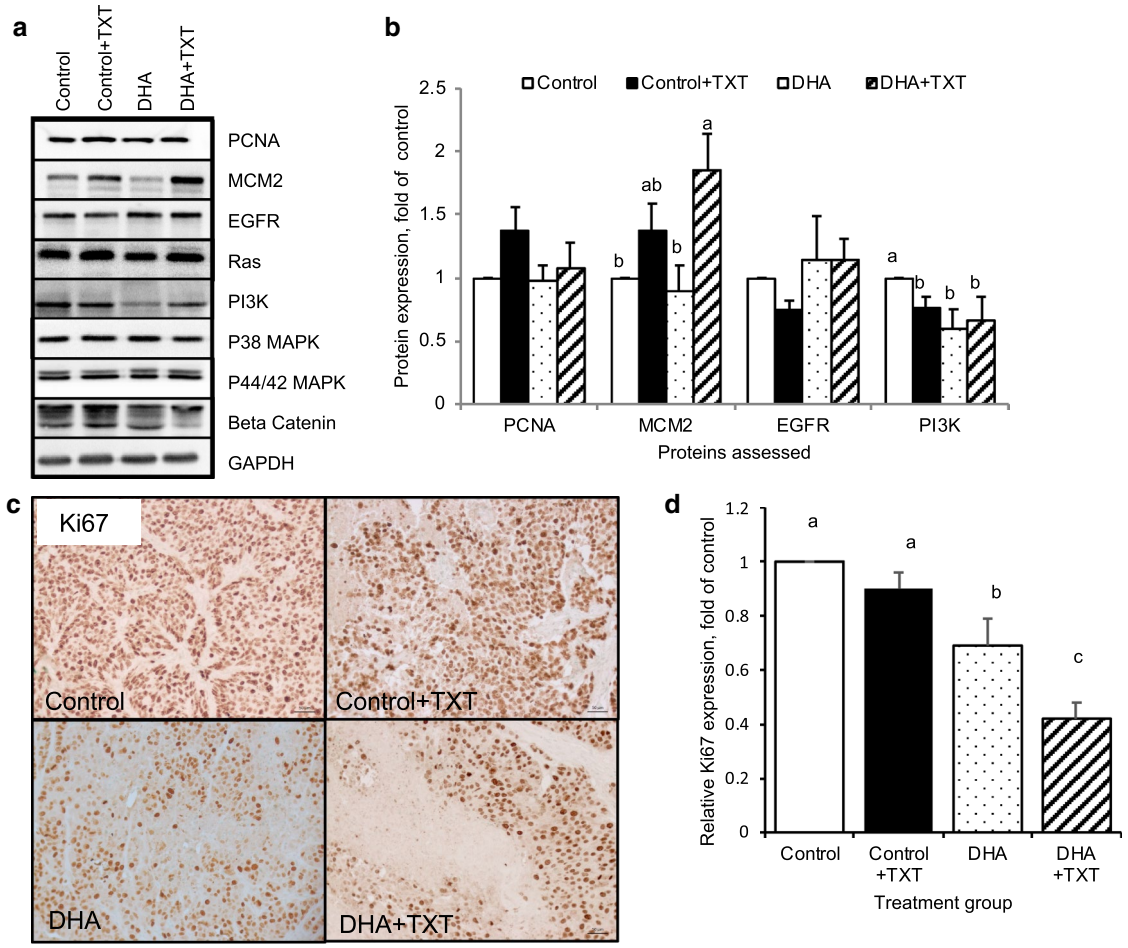

Fig. 3 Proliferation protein expression in MAXF574 PDX tumor extracts from NSG mice. **a** Representative Western blot analysis of proliferation proteins and **b** densitometric quantification of blots from (**a**). Values represent the mean ± SEM ($n=8$). Labeled means without a common letter differ ($P<0.05$) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: PCNA: $P=0.31$; MCM2: $P=0.36$; EGFR: $P=0.19$; PI3 K: $P=0.05$; the main effect of the treatment: PCNA: $P=0.1$; MCM2: $P=0.003$; EGFR: $P=0.51$; PI3 K: $P=0.56$ and P interaction: PCNA: $P=0.4$; MCM2: $P=0.17$; EGFR: $P=0.54$; PI3 K: $P=0.25$. **c** Representative immunohistochemistry of Ki67. Positive staining is dark brown color and **d** quantification of Ki67 ($P<0.05$)

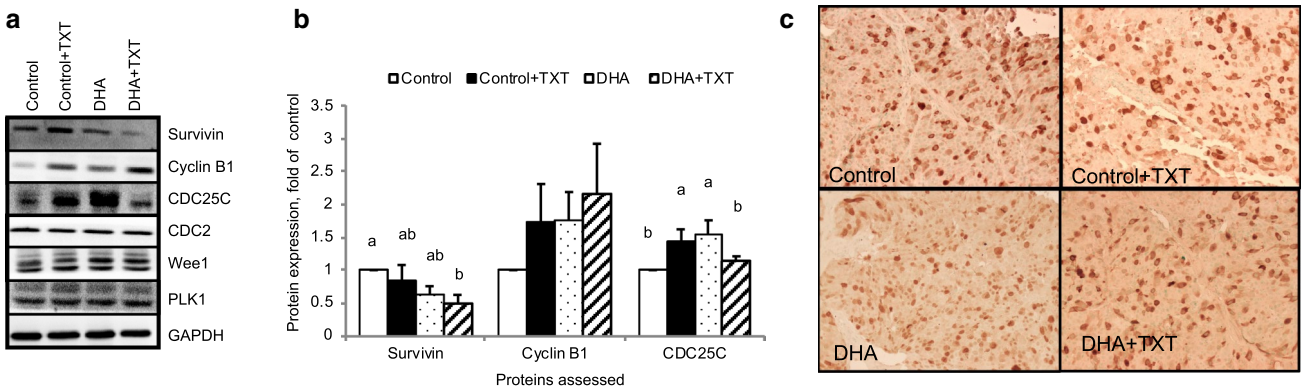

Fig. 4 Cell cycle protein expression in MAXF574 PDX tumor extracts from NSG mice. Representative Western blot analysis of **a** cell cycle proteins, **b** with densitometric quantification of blots from (**a**). Values represent the mean ± SEM ($n=8$ mice per group). Labeled means without a common letter differ ($P<0.05$) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: Survivin: $P=0.0.02$; Cyclin B1: $P=0.28$; CDC25C: $P=0.38$; the main effect of the treatment: Survivin: $P=0.4$; Cyclin B1: $P=0.29$; CDC25C: $P=0.96$ and P interaction: Survivin: $P=0.95$; Cyclin B1: $P=0.76$; CDC25C: $P=0.01$. **c** Representative immunohistochemistry of Cyclin B1. Positive staining is dark brown color and nuclei are stained green (methyl green)

Effect of DHA dietary supplementation on MAXF401 tumor growth

To confirm if the effects observed were applicable to other triple negative human tumors, a second TNBC PDX experiment was conducted. We sought to answer two key questions with this experiment: (1) Reproducibility: can we confirm efficacy in a second model and (2) Does DHA enhance the anticancer actions of docetaxel? Therefore, we employed a clinically relevant experimental design where our comparison was between control + TXT and DHA + TXT and there was no DHA alone group (Experimental Layout Supplemental Fig. 1a). Mice bearing MAXF401 xenografts fed DHA had reduced average daily food intake compared to those fed the control diet (Online Resource: Supplemental Fig. 1b), but there were no differences in body weight before (data not shown) or after chemotherapy amongst groups (Online Resource: Supplemental Fig. 1c). Excised tumor weight was lower for the DHA + TXT group compared to control + TXT ($P < 0.05$) and control alone ($P < 0.004$). For mice fed the control diet, TXT treatment did not significantly reduce tumor weight ($P < 0.44$) (Fig. 5a).

Excised tumors were then assessed by Western blot analysis for select markers that were found to be differentially expressed in the first PDX experiment (Fig. 5b, c). DHA + TXT tumor protein extracts were found to have a higher expression of Ripk1, Bid, and Cyclin B1 compared to control and control + TXT ($P < 0.05$); lower expression of Bcl-2 and Parp compared to control and control + TXT ($P < 0.05$); and lower expression of Survivin compared to control ($P < 0.05$) but not significantly different from control + TXT.

Discussion

To our knowledge, the current study is the first to report the anticancer effects of DHA supplementation concomitant with chemotherapy in a PDX model of BC. Previous studies by our group and others have determined the efficacy and potential mechanisms of action of DHA in conjunction with chemotherapy in vitro and in vivo models of breast cancer (Supplemental Table 3) [15, 19–21, 29, 34–38]. While these studies have elucidated mechanisms of action for DHA in conjunction with chemotherapy, they have relied on immortalized cell lines that do not reflect the heterogeneity of a primary patient tumor. For this reason, we have moved to the more clinically translatable heterogenic PDX model to determine if DHA had efficacy in this model. We observed, in two different PDX models of TNBC, that feeding a diet supplemented with DHA results in a significant reduction in tumor growth, and a greater response to first line docetaxel therapy. In fact, for control fed mice, docetaxel alone did not result in smaller tumors in either PDX model, rather resulted in greater variation in tumor sizes in the control + TXT group (although not significantly different than control alone). Interestingly, MAXF574 tumors excised from mice fed a DHA diet without chemotherapy were significantly smaller than tumors from mice fed control diet with or without chemotherapy. This has been observed in other preclinical models of breast cancer [39–42], and these results suggest

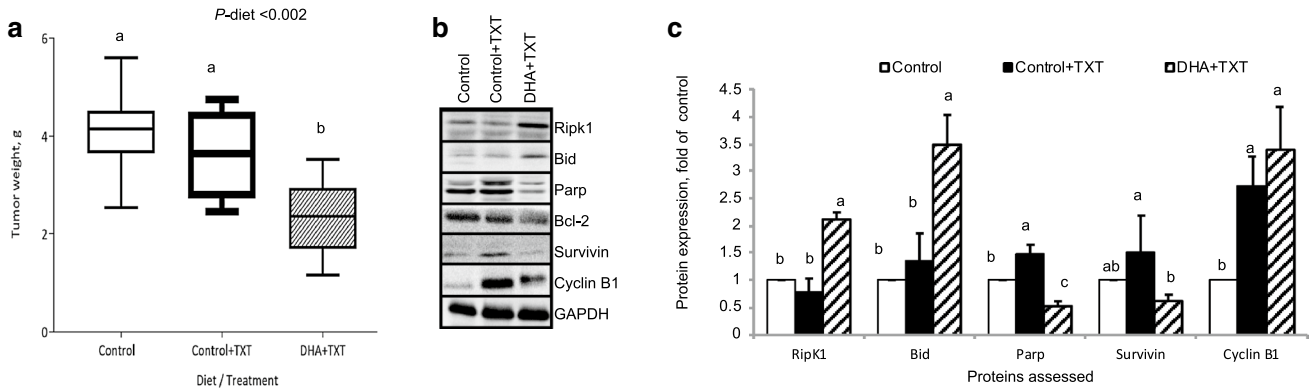

Fig. 5 Effect of dietary DHA with TXT on MAXF401 TNBC PDX growth in NSG mice. **a** Excised tumor weight of MAXF401 tumor bearing mice **b** Representative Western blot analysis of proteins and **c** densitometric quantification of blots from (**b**). Kruskal–Wallis test for non-parametric analysis was employed for tumor weights. The P value for the main effect of the diet = $P < 0.002$; comparison of Control to DHA + TXT = $P < 0.004$; comparison of Control + TXT to DHA + TXT = $P < 0.049$; and comparison of Control to Control + TXT = $P < 0.44$. For the Western blots, values represent the mean ± SEM ($n = 8$). Labeled means without a common letter differ ($P < 0.05$) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: RIPK1: $P = 0.004$; Bid: $P = 0.002$; Parp: $P = 0.0004$; Survivin: $P = 0.1$; Cyclin B1: $P = 0.48$ the main effect of the treatment: RIPK1: $P = 0.001$; Bid: $P = 0.35$; Parp: $P = 0.16$; Survivin: $P = 0.55$; Cyclin B1: $P = 0.04$ and P interaction RIPK1: $P = 0.01$; Bid: $P = 0.09$; Parp: $P = 0.05$; Survivin: $P = 0.45$; Cyclin B1: $P = 0.78$

that future studies exploring the effect of DHA alone in the PDX model would be warranted. However, this will not be translatable to medical treatment as women with TNBC are prescribed chemotherapy in clinic. For this reason, we focused on the comparison of tumors from mice fed DHA in conjunction with TXT to control + TXT that was assessed in this study. MAXF574 tumors excised from mice fed a DHA diet in conjunction with TXT were 64% smaller than tumors from mice fed a control diet concomitant with chemotherapy. Assessment of the second TNBC PDX, MAXF401, confirmed the positive synergism between DHA and TXT with a 34% reduction of tumor growth. The beneficial effects of DHA on docetaxel, in two different PDXs occurred without altering body weight; an important measure for improved clinical outcomes in some cancers [43–45].

TNBC is known to be aggressive. It has a less favorable prognosis [7, 8] and does not respond well to targeted therapies. The ability of neoplasms to employ multiple hallmarks of cancer as a means to survive and proliferate, creates a complex, continuously evolving environment that proves difficult to treat with standard or targeted chemotherapeutics [4, 5]. The efficacy of a treatment may rely on its ability to elicit a response from numerous pathways within the tumor that are involved in apoptosis and proliferation, a phenomenon that was observed in the present study. We found that DHA improved the action of TXT in a multi-faceted way by simultaneously increasing apoptotic cell death through changes in ASK1, Ripk1, BCL2, pAKT, and Parp; disrupting proliferation as indicated by changes in Ki67, and suppressing the progression of the cell cycle through changes in Survivin. Collectively, these alterations could contribute to substantially reduced tumor burden. These specific pathways are key hallmarks known to be implicated in cancer progression [4, 5] and although they were not completely abrogated by the combined effects of DHA and docetaxel, the cumulative effects of differentially expressed proteins, in overlapping pathways, likely contributed to a striking reduction in tumor weight with combined therapies.

The apoptotic response, known to be triggered by both docetaxel [46] and DHA [15, 32, 41, 47–50], is consistent with the higher CD95 and TUNEL staining in DHA + TXT tumor sections and with the observed upregulation of pro-apoptotic and decreased anti-apoptotic protein expression in DHA + TXT tumors compared to control + TXT tumors. In DHA + TXT protein extracts, there was a reduction in pro-caspase 3 suggesting cleavage and activation for initiation of apoptosis [51]. Parp, one of the earliest substrates to be cleaved by caspase-3 during apoptosis [52], was found to be substantially diminished in DHA + TXT protein extracts in both PDX models. Also, of note was the lower expression of Bcl-2, a potent inhibitor of apoptosis. This protein has been identified as a candidate biomarker of clinical response to docetaxel [10]. Additionally, a systematic review concluded that the negative expression of Bcl-2 predicted favorable response to chemotherapy and predicted remission post-neoadjuvant chemotherapy [53]. Taken together, these results suggest that feeding DHA facilitates apoptosis in PDX tumors treated with TXT compared to mice fed a diet with a fat composition similar to that of the North American diet. We also tested whether feeding DHA could improve the anti-proliferative effects of TXT. The nuclear antigen Ki67 is considered a marker of cellular proliferation/cellular mitosis in breast tumor biopsies [54, 55] and is used in clinic to assess efficacy of neoadjuvant chemotherapy prior to surgical removal (pre- and post-measurements of Ki67) [56]. In the present study, we found a that TXT significantly lowered Ki67 expression in tumors from animals fed DHA, the combined effects of TXT and DHA resulted in an even lower Ki67 content.

Within the cell, TXT binds to β tubulin preventing depolymerization of microtubules thereby leading to cell cycle arrest [57] with an increase in Cyclin B1 expression [58, 59] as cells become paused in the G2M phase. In both PDX experiments, DHA + TXT tumor extracts trended ($P = 0.1$) toward increased Cyclin B1 protein expression compared to control although the localization and distribution of cyclin B1 was not found to be different in immunohistochemical analysis. Survivin reaches its peak expression in G2M [60] and is overexpressed in many cancers [57]. Positive expression of Survivin correlates with poorer patient prognosis [61] and in the current study, feeding DHA with TXT treatment was found to reduce Survivin expression. This is consistent with facilitating the effects of TXT on cell cycle arrest.

There are limitations to our study. Other $n-3$ LCPUFA have been shown to exert anti-cancer effects, including EPA alone in vitro [31, 65] and EPA in combination with DHA [31] but more studies are needed to investigate the efficacy of these combinations in conjunction with chemotherapy. Additionally, as the response to docetaxel alone did not result in a reduction in tumor size, the mode of action through which DHA overcomes chemotherapy resistance in the PDX model should be investigated in future studies. Finally, the translational applicability of the immunocompromised mouse model used in this study should be considered. It is well established that DHA is a beneficial modulator on the immune system [62] and it has been shown in other mammary cancer models that it can reduce inflammation and improve anti-tumor immune function [63, 64]. A clinical trial to determine the efficacy of DHA concomitant with chemotherapy is currently undergoing the approval process with the objective to assess the impact of supplementing women with DHA during neoadjuvant chemotherapy. This study will specifically quantify the effects of DHA on the Ki67 index in the tumor and will determine whether it can

counteract the negative effects of chemotherapy on immunity (ClinicalTrials.gov Identifier: NCT03831178).

Conclusions

In summary, our findings provide clear evidence that supplementing the diet with DHA improves the efficacy of the neoadjuvant cytotoxic drug, TXT, in two chemo-resistant preclinical PDX models. A single mechanism could not be identified but analysis of cellular proteins suggest that this occurs by effects on apoptosis and cell cycle regulation concomitant with proliferation.

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Author contributions MN and CJF: designed the research and wrote the manuscript; MN: conducted the research; MN, SG, LP, VM and CJF: analyzed the data; CJF: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Alberta Animal Policy and Welfare Committee and were in accordance with the Canadian Council on Animal Care guidelines. Animal Protocol Number AUP00000134.

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