RESEARCH ARTICLE

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Effects of Omega-3 Supplementation on *Ki-67* and *VEGF* Expression Levels and Clinical Outcomes of Locally Advanced Breast Cancer Patients Treated with Neoadjuvant CAF Chemotherapy: A Randomized Controlled Trial Report

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Abstract

Background: Omega-3 is a polyunsaturated fatty acid with an ability to regulate cell proliferation and apoptosis through interaction with inflammatory mediators. The potential additional beneficial effects of Omega-3 on chemotherapy patients with breast cancer is not yet completely revealed. **Methods:** A double-blind randomized control trial (RCT) involving a total of 48 locally advanced breast cancer patients was conducted. *Ki-67* and *VEGF* expressions, as well as overall survival of patients receiving neoadjuvant cyclophosphamide-doxorubicin-5'fluorouracyl (CAF) chemotherapy plus Omega-3 (intervention group) or placebo (control group), were compared. Kaplan-Meier curve and Cox-regression tests were used to assess conditional disease-free survival (DFS) and overall survival (OS) between the two groups. **Results:** Decreased *Ki-67* expression was observed in the intervention group compared to control (42.4±4.8 versus 39.2±5.3; T-test p=0.032). Decreased *Ki-67* expression was observed in intervention compared to control group (42.4±4.8 versus 39.2±5.3; T-test p=0.032). Decreased *VEGF* expression was also seen in the intervention group compared to control (32.7±5.2 versus 29.5±5.4; T-test p=0.041). *VEGF* expression positively correlated with *Ki-67* expression (Spearman's test p<0.001, R^2=0.541). Overall survival in the intervention group was significantly longer in comparison to the control group (mean survival: 30.9 ± 3.71 versus 25.9 ± 3.6 weeks, Mantel-Cox test p=0.048; HR=0.411, 95%CI: 0.201-0.840). Disease-free survival was significantly longer in the intervention group compared to the control group (mean survival: 28.5 ± 3.3 versus 23.7 ± 3.6, respectively; Mantel-Cox test p=0.044, HR= 0.439, 95%CI: 0.222-0.869). **Conclusion:** Omega-3 fatty acid supplementation improved overall survival and progression-free survival of locally advanced breast cancer treated with CAF neoadjuvant chemotherapy and mastectomy.

Keywords: Omega-3- breast cancer- *VEGF*, *Ki-67*- survival- chemotherapy

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Introduction

Strategies to improve clinical management of breast cancer, as the most common cancer and the leading cause of cancer-associated mortality among women worldwide, is a high priority (El Saghir et al., 2008; Warrier et al., 2015). More than 50,000 women per year are diagnosed with breast cancer in Indonesia with relatively higher mortality rates (annual breast cancer-related mortality 20,000 per year) (Bray et al., 2018; WHO, 2014). Most patients with breast cancer in Indonesia are diagnosed in late stages causing higher proportions of mortality rates and shorter disease-free progression (Akhsan and Aryandono, 2010; Anwar et al., 2018a). In addition, various factors are associated with low awareness and medication adherence that might contribute to the late presentation at diagnosis and poorer outcomes (Anwar et al., 2018b; Anwar et al., 2018c). Limited health facilities and manpower to perform cancer prevention, early diagnosis screening, as well as limited insurance coverage (Strasser-Weippl et al., 2015; Anwar et al., 2018b; Anwar et al., 2018c) might also contribute to the high proportion of advanced and metastatic cancer cases.

In addition to public health policy, improvement of clinical management for advanced and metastatic cancer in Indonesia is warranted. Current approaches for

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locally-advanced breast cancer are mainly focused on the integration of multimodal therapies particularly surgical resection followed by suitable adjuvant chemotherapy and radiotherapy (Anderson et al., 2008; El Saghir et al., 2008). However, 3-year survival rates in locally advanced breast cancer using this approach are only 50-80% and the 10-year survival rates are relatively low ranging between 30-40% (El Saghir et al., 2008; Warrier et al., 2015). Therefore, new strategies to address the clinical management of locally advanced breast cancer are required.

Accumulating evidence has shown that Omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), play an essential role in the regulation of inflammatory responses (Mori and Beilin, 2004). In the lipid rafts of phospholipid membranes, Omega-3 is involved in the biological signaling affecting breast carcinogenesis (Rogers et al., 2010; Ravacci et al., 2013). With the ability to modulate inflammatory responses and inter-cellular signaling including cell proliferation and apoptosis, Omega-3 fatty acids are a potential substance for supplementation during standard chemotherapy to improve clinical outcomes. In this study, we performed a randomized clinical trial involving 48 patients with locally advanced breast cancer receiving standard neoadjuvant cyclophosphamide, doxorubicin, and fluorouracil (CAF) chemotherapy. Patients were assigned to either the intervention group that received supplementation of Omega-3 or the control group (without supplementation of Omega-3) concomitant with neoadjuvant CAF chemotherapy. We observed lower *Ki-67* and *VEGF* expression as well as better overall survival and progression-free survival in the intervention group.

Materials and Methods

Patient and Selection Criteria

Patients fulfilling inclusion criteria (confirmed diagnosis of breast cancer, locally advanced stage IIIB breast cancer, ductal invasive, age 25-60 years old, who received neoadjuvant chemotherapy CAF in Dr. Kariadi, RSI Sultan Agung, and Roemani hospitals in 2015-2016) were assigned to receive or not to receive supplementation of Omega-3 fatty acids in a double-blind randomized control trial. Only patients who were eligible to receive neoadjuvant chemotherapy, including Karnofsky index ≥80, hemoglobin levels ≥10 g/dl, thrombocyte count ≥ 100,000/mm^3, leucocytes count ≥ 35,000/mm^3 were recruited. Excluding criteria were an allergy to Omega-3 fatty acids, serum creatinine ≥ 1.5 mg/dL, liver function tests (AST/SGOT and ALT/SGPT) ≥ 100 IU/L, history of chest radiotherapy, history of cardiac diseases including myocarditis, valve abnormalities, hypertension, and history of other malignancies. Written informed consent of patients eligible to this study was obtained from each participant.

All participants received standard neoadjuvant CAF chemotherapy (cyclophosphamide 600 mg/m^2, doxorubicin 60 mg/m^2, and 5-FU 600 mg/m^2). In addition, supplementation of 1 g/day Omega-3 fatty acids or placebo along with administration of 3 cycles of neoadjuvant chemotherapy (for 51 days) was given to intervention (N=24) and control groups (N=24), respectively.

Ethical Clearance

The study protocol was reviewed and approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine Universitas Diponegoro, Semarang – Indonesia (2014). All participants provided written informed consent before participating in this study.

Expression of Ki-67 and VEGF

Expression levels of *Ki-67* and *VEGF* were semi-quantified analyzed using immunohistochemistry (IHC) from paraffin blocks of tumor materials obtained from mastectomy after neoadjuvant chemotherapy. Briefly, around 4 μm-thick tissue sections were cut, deparaffinized, and rehydrated according to the standard protocols. Immunostaining was done according to the standard procedures using antibody clone MIB-1 clone (1:500, DAKO, Denmark) for *Ki-67* and C-1 clone (1:500, Santa Cruz sc-7269, California) for *VEGF*. Quantification of the immune-reactivity was examined by two independent and experienced pathologists using the standard scoring system (4 fields of 100 cells each) (Leung et al., 2016). Semi-quantification calculation was performed, and the data were then compared between the intervention and control groups.

Omega-3 supplementation

Supplementation of Omega-3 fatty acids (1 g/day) in an oil-fish capsule or placebo to the intervention or control group was administrated once a day along with administration of 3 cycles of neoadjuvant chemotherapy (for 51 days). Randomization was performed by assigning random numbers according to the random number tables to the intervention or control group.

Follow-up

Participants were regularly followed up starting from the date of diagnosis until any tumor progression or mortality was recorded. Follow-up visits were structurally scheduled according to the hospital guidelines, namely every month for the first 6 months and every 6 months after completion of therapy unless any non-scheduled visits were indicated. For all visits, a thorough clinical examination was performed and breast sonography and/or mammography, abdominal ultrasonography, chest X-ray, and bone scan were performed as indicated/scheduled. Progression-free survival (PFS) and overall survival (OS) were determined to start from the time elapsed between diagnosis to any evidence of tumor progression and death from any cause, respectively.

Statistical Analysis

Clinical and pathological variables were presented in means/medians ± standard deviation (SD). Continuous and categorical variables between groups were compared using the Mann-Whitney-U tests and χ^2 tests, respectively. Kaplan-Meier survival curve and log-rank Mantel-Cox tests were used to compare OS and PFS between the two groups. Cox regression analysis was used to identify

factors influencing OS and PFS. For all comparisons, $p<0.05$ was considered as statistically significant. Statistical analysis was performed using SPSS Statistics for Windows version 17.0 (SPSS Inc., Chicago, USA).

Results

A total of 94 locally advanced breast cancer (stage IIIB) patients consented to participate in the study and initially 81 of them met inclusion criteria but 33 of those were excluded due to exclusion criteria. In total, 48 patients were included in the study and randomized to receive or not to receive supplementation of Omega-3 fatty acids (1gram/day for 51 days) during the period of 3 cycles of neoadjuvant CAF chemotherapy. During the treatment, 3 patients suffered from diarrhea and the supplementation of Omega-3 was paused for 5-7 days. Drop-out was found in this study in neither intervention nor control groups. Characteristics of study participants and comparisons between the sub-categories are shown in the Table 1. Age, histopathological grades, hormonal status, and HER2 expression were not significantly different between intervention and control groups (Table 1).

Table 1. Clinical and Pathological Characteristics of Intervention (N=24) and Control (N=24) Groups

Characteristics	Intervention group (N=24)	Control/Placebo (N=24)	P
Age	46.5±8.07 year	48.5±8.77 year	0.420 [1)]
Histopathological grade			0.771[2)]
Grade 1	2 (8.3%)	3 (12.5%)	
Grade 2	8 (33.3%)	6 (25%)	
Grade 3	14 (58.3%)	15 (62.5%)	
Axillary lymph node			1.000[3)]
Positive	21 (88%)	22 (92%)	
Negative	3 (12%)	2 (8%)	
Estrogen receptor (ER)			0.365[2)]
Positive	17 (71%)	14 (58%)	
Negative	7 (29%)	10 (42%)	
Progesterone receptor (PR)			0.771[2)]
Positive	10 (42%)	11 (46%)	
Negative	14 (58%)	13 (54%)	
HER-2 expression			1.000[3)]
Positive	2 (8%)	1 (4%)	
Negative	22 (92%)	23 (96%)	

1, Independent t-test; 2, Chi-square test; 3, Fisher Exact test

Expression levels of Ki-67 and VEGF at baseline and after neoadjuvant chemotherapy and supplementation of Omega-3 or placebo

Representative image of immune-staining of *Ki-67* and *VEGF* at baseline and after neoadjuvant chemotherapy with or without supplementation of Omega-3 is presented in Figure 1. Quantitative analysis of IHC was performed as described in the Materials and Methods. All normality tests using Shapiro-Wilk analyses of different clinical and pathological variables as well as survival rates between groups was performed and no statistical difference was shown ($p>0.05$). Expression levels of proliferation index (*Ki-67*) at baseline (before neoadjuvant and supplementation of Omega-3 or placebo) in the intervention and control groups were 54.9 ± 9 versus 52.5 ± 5.3, respectively (t-test, p=0.265). Decreased means of *Ki-67* expression levels after neoadjuvant chemotherapy and supplementation of Omega-3 or placebo were 42.4±4.8 and 39.2±5.3 in intervention and control groups, respectively (t-test, p=0.032).

VEGF expression levels at baseline were not significantly different between the intervention and control group (50.3 ± 9.7 and 48.3 ± 8.4, respectively; t-test p=0.469). After CAF neoadjuvant chemotherapy and supplementation of Omega-3 or placebo, decreased means of *VEGF* expression levels were 32.7±5.2 and 29.5±5.4 in the intervention and control group, respectively (t-test

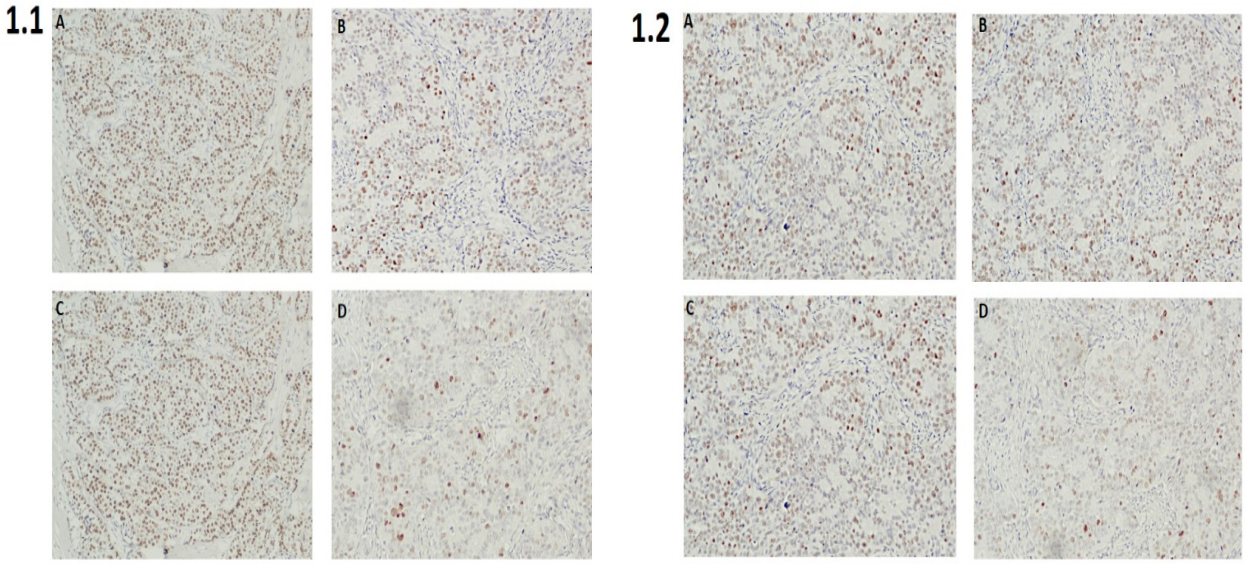

Figure 1. Representative Images of Immune-Staining of *Ki-67* (1.1) and *VEGF* (1.2) in the intervention and control groups at the baseline and after neoadjuvant chemotherapy plus supplementation of Omega-3 or placebo. A: baseline – control group, B: after therapy – control group, C: baseline - intervention group, D: after therapy - intervention group.

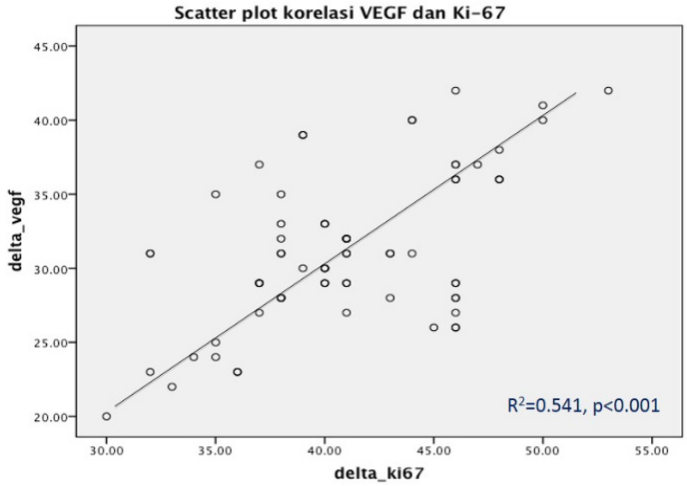

Figure 2. Correlation of *Ki-67* and *VEGF* expression levels after CAF neoadjuvant chemotherapy and supplementation of Omega-3 or placebo in the intervention and control group. Decreased expression levels of *Ki-67* and *VEGF* after therapy were positively correlated (Spearman test: R2=0.541, p<0.001

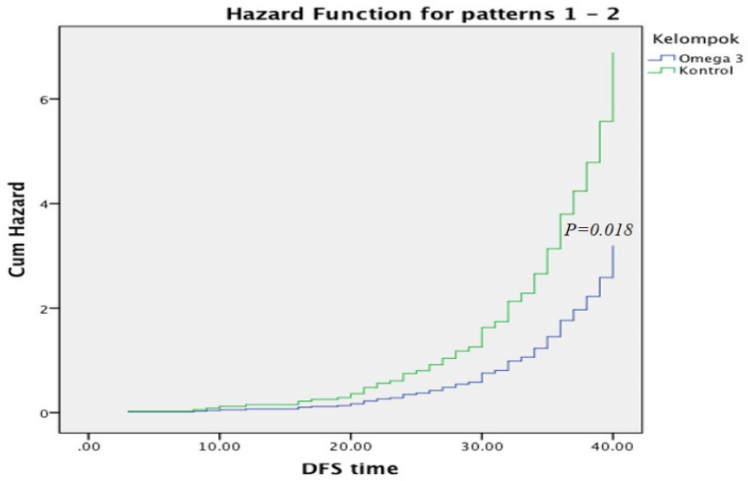

Figure 3. Cumulative Hazard Function of Disease-Free Survival (DFS) Comparing Intervention and Control Groups. Hazard Ratio of DFS in the Intervention Group Compared to Control Group HR= 0.439, 95%; CI: 0.222-0.869, *p=0.018.*

p=0.041). Decreased expression levels of *Ki-67* and *VEGF* after therapy were positively correlated (Spearman test: R2=0.541, p<0.001, Figure 2).

Progression-free survival (PFS) and Overall survival (OS)

After median follow-up for 48 weeks, means of PFS were 28.5 ± 3.3 and 23.7 ± 3.6 weeks in the

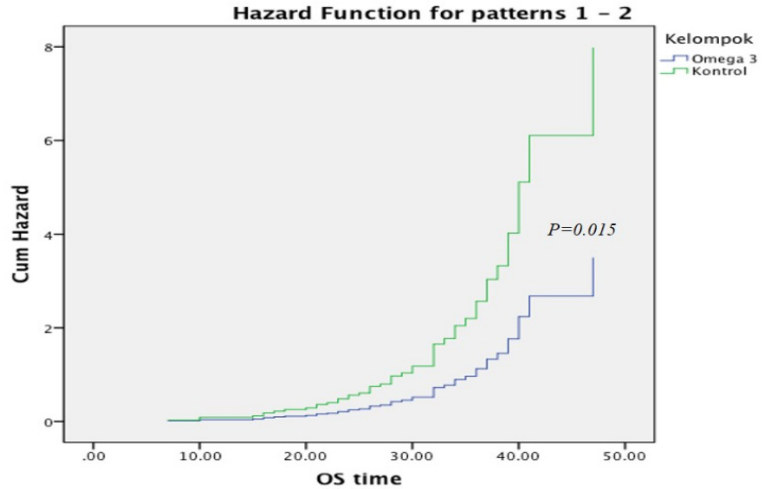

Figure 4. Cumulative Hazard Function of Overall Survival (OS) Comparing Intervention and Control Groups. Hazard Ratio of OS in the intervention group compared to control group HR= 0.411; 95%, CI: 0.201-0.840, *p=0.015.*

intervention and control groups, respectively (log-rank Mantel-Cox test, p=0.044, HR=0.44, 95%CI: 0.222-0.869). In addition, *Ki-67* and *VEGF* expression levels at baseline were significant predictors for shorter DFS (HR 1.44, 95%CI: 1.05-2.21, p=0.039; HR 1.33 95%CI: 1.045-2.115, p=0.043; respectively, Figure 3).

Means of overall survival were 30.9 ± 3.7 weeks and 25.9 ± 3.6 weeks for intervention and control groups, respectively (log-rank Mantel-Cox test p=0.048, HR=0.411, 95%CI: 0.201-0.840). In addition, expression levels of *Ki-67* and *VEGF* were significant predictive markers for shorter overall survival (HR=1.46. 95%CI: 1.052-2.120, p=0.042 and HR=1.32 (CI 95%=1.091-2.083, respectively, Figure 4).

Discussion

Breast carcinogenesis has been previously viewed as a result of the accumulation of genetic and epigenetic changes regulating cell proliferation, apoptosis, and hormonal signaling (Coasar et al., 2015; Locatelli and Curigliano, 2017). Recent studies have also demonstrated the important roles of cytokine and inflammatory responses in the development and progression of breast cancer (Bhatelia et al., 2014; Deng et al., 2016). Omega-3 fatty acids are essential elements in the production of bioactive mediators during inflammatory responses (Mori and Beilin, 2004; Calder, 2010). In addition, Omega-3 fatty acids also regulate cellular responses and signaling through phospholipid membranes and lipid rafts (Mori and Beilin, 2004; Calder, 2010). Several case-control studies showed lower breast cancer risk in premenopausal women consuming higher amounts of Omega-3 fatty acids (Goodstine et al., 2003; Chajès et al., 2012). A cohort study demonstrated correlations between high fish consumption with Omega-3 levels in red cells and lower risk of breast pathology and breast cancer (Shannon et al., 2007; Shannon et al., 2009). A meta-analysis involving 16 prospective cohort studies also showed lower breast cancer risk in individuals with higher intake of Omega-3 (Zheng et al., 2013). Dose-response analysis has shown 5% reduction of breast cancer risk for consumption of 0.1g/day of Omega-3 (Zheng et al., 2013). Omega-3 fatty acids have also been associated with significant improvement of breast cancer clinical outcomes including reduction of cancer recurrence, adverse cardiovascular events, obesity, osteoporosis, and chemotherapy-related neuropathy (Fabian et al., 2015).

Omega-3 acids modulate several biological pathways that might contribute to the initiation and progression of cancer (Fabian et al., 2015). In addition, immune response through tumor microenvironments has also been shown as an essential factor in tumor growth, invasiveness, and resistance to therapy (Grivennikov et al., 2010). An in vitro study showed that exposure to Omega-3 resulted in dose-dependent colorectal cell apoptosis after irradiation (Benais-Pont et al., 2006). An in vivo study using athymic nude mice revealed that food supplementation with Omega-3 was correlated with slower growth of human pancreatic xenografts (Fukui et al., 2013). In advanced stage breast cancer patients treated with anthracycline-based chemotherapy, supplementation of 1.8g DHA daily showed benefits in terms of progression-free and overall survival rates particularly in patients with a higher proportion of cell membrane-incorporated DHA (Bougnoux et al., 2009). Therefore, enrichment of DHA into cell membranes through Omega-3 supplementation is associated with sensitization of chemotherapy by induction of oxidative stress response and other cell signaling to induce apoptosis (Bougnoux et al., 2009).

We showed that supplementation of Omega-3 fatty acid in patients with locally advanced breast cancer (Stage IIIB) treated with standard CAF neoadjuvant chemotherapy and mastectomy was significantly associated with better PFS and OS. We measured *Ki-67* and VEGF protein expressions to monitor cell proliferation and angiogenesis upon Omega-3 supplementation. *Ki-67* proliferation index has emerged as an independent marker for breast cancer PFS and OS (Inwald et al., 2013). Meanwhile, VEGF is an essential biological marker to evaluate angiogenesis and lymphangiogenesis (Fox et al., 2007; Widodo et al., 2018) that has been closely associated with breast cancer survival and therapeutic responses (Gasparini, 2000; Liu et al., 2011). In this study, we also demonstrated significantly decreased expression levels of *Ki-67* and *VEGF* in the patients supplemented with Omega-3 compared to the placebo. This initial RCT indicates the advantages of Omega-3 supplementation in neoadjuvant chemotherapy in locally advanced breast cancer. However, there are some limitations in this study that need to be further addressed including the optimal dose, bioavailability, and circulating levels and lipid raft percentage of Omega-3 upon the supplementation. In addition, other metabolic factors including body mass index, blood lipid profile, diet, and physical inactivity that might influence the effects of Omega-3 supplementation have not yet been evaluated. We might also have to consider that breast cancer is a heterogeneous disease consisting of several pathological and molecular subtypes which might contribute in the natural course and prognosis of the disease (Widodo et al., 2017).

Conflict of interest

All authors declared no financial nor professional competing interests.

Acknowledgments

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