Omega-3 fatty acids, cytokines and lymphocyte proliferation in young and older women[1]

Philip C. Calder

[1]From Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton SO16 6YD, United Kingdom

Address correspondence to Philip Calder, Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Institute of Developmental Sciences Building, MP887 Southampton General Hospital, Tremona Road, Southampton SO16 6YD, United Kingdom
Email: pcc@soton.ac.uk
Phone: + 44 2381205250

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[2]Abbreviations used: Con A, concanavalin A; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; TNF, tumor necrosis factor.

In 1991 Simin Meydani and co-workers published a paper in *Journal of Nutrition* that is considered to be a landmark study in the field of omega-3 fatty acids, inflammation and immunity (1). According to Web of Science, the paper has now been cited 537 times (search conducted on May 10 2018); it is easily the most highly cited paper published in the journal in 1991. The pattern of citations of this paper continues almost unchecked, with 16 citations in 2017 and 5 already in 2018. This is a special paper that has had significant impact in its field. It reports a study with omega-3 fatty acid ("fish oil") supplements (providing 1.68 g eicosapentaenoic acid (EPA)[2] plus 0.72 g docosahexaenoic acid (DHA)) being given daily for 12 wk. The subjects studied were six healthy young women aged 23 to 33 y and six healthy older (*sic*) women aged 51 to 68 y. All received the omega-3 fatty acid supplements: there was no control group and hence the study was unblinded to both subjects and researchers. Blood samples were collected at baseline and after 1, 2 and 3 mo of omega-3 fatty acid supplementation. The focus of the research was the response of isolated peripheral blood mononuclear cells (PBMCs) to stimulation *ex vivo*. PBMCs are a mix of lymphocytes (about 85% of cells present) and monocytes (about 15% of cells present) and can be used to assess T lymphocyte (T-cell), B lymphocyte (B-cell) and monocyte responses by using agents that are known to stimulate only (or mainly) one of these cell types. Meydani *et al*. used heat-killed *Staphylococcus aureus* and endotoxin (aka lipopolysaccharide) from *Escherichia coli* 1335 to stimulate monocytes and measured tumor necrosis factor (TNF) and interleukin (IL)-1β in the culture medium after 24 h. They used the mitogenic plant lectins concanavalin A (Con A) and phytohemagglutinin (PHA) to stimulate T-cells and measured IL-2 and IL-6 in the culture medium after 48 hr and the proliferative response of T-cells.

What were the key findings? Omega-3 fatty acids decreased the concentration of IL-β in the medium of endotoxin-stimulated PBMCs from both young and older women in a time-dependent manner with a greater effect seen with cells from the older women. Omega-3 fatty acids decreased the concentration of TNF in the medium of endotoxin-stimulated PBMCs from both young and older women in a time-dependent manner with no difference between the two age groups. Omega-3 fatty acids decreased the concentration of TNF in the medium of *S. aureus*-stimulated PBMCs from the older women in a time-dependent manner; there appeared to be a smaller and time-dependent decrease in TNF concentration for PBMCs from young women but this was not statistically significant perhaps due to the small number of subjects studied. Omega-3 fatty acids decreased the concentration of IL-6 in the medium of

Con A-stimulated PBMCs from both young and older women in a time-dependent manner with a greater effect seen with cells from the older women. The paper states that omega-3 fatty acids "reduced IL-2 production [by Con A-stimulated PBMCs] in both young and older women" but the effect was not actually statistically significant in either age group, again most likely due to the small sample size. Finally, omega-3 fatty acids decreased T-cell proliferation in PHA-simulated cultures of PBMCs from older women in a time-dependent manner, with no effect seen for cells from young women. It is concluded that omega-3 fatty acids from fish oil suppress production of several cytokines involved in inflammatory and immune responses with a greater effect in older than young women and that omega-3 fatty acids suppress T-cell proliferation in older but not young women.

In the current age of evidence-based medicine, with its demand for adequately powered, double-blind, randomized, placebo controlled trials, how can an uncontrolled, unblinded study in two groups of six women with no power calculation and probably less than adequate statistical analysis retain its relevance and high level of citation? The answer to this question lies in the context of the study and its historical significance to the field. It seems likely that this study was conducted in 1989 (an abstract reporting some of the findings had been published in 1990 (2)). At that time, reports of the influence of omega-3 fatty acids on immunity and inflammation, beyond effects on leukocyte chemotaxis and on the key arachidonic acid-derived eicosanoids prostaglandin E_2 and leukotriene B_4, were very few (see (3) for a comprehensive review of studies up to 1995) and there had only been one human study reporting effects on cytokines (4). Meydani *et al*. were the first to report from a human study of increasing intake of EPA and DHA, effects on IL-2 and IL-6 production and on T-cell proliferation. They were the second, after Endres *et al*. (4), to report on omega-3 fatty acids and TNF and IL-1β from a human study. The first report from an animal study of dietary omega-3 fatty acids decreasing IL-1 and TNF production (by rat Kupffer cells (liver macrophages)) was published in 1988 (5), while the first report from an animal study of dietary omega-3 fatty acids decreasing IL-2 production (by pig alveolar lymphocytes) was not published until 1994 (6). The earliest reports from animal studies of dietary omega-3 fatty acids decreasing T-cell proliferation were published in the late1980s (7-9). *In vitro* studies reporting direct effects of EPA and DHA on mitogen-induced proliferation of cultured T-cells were published in the early 1990s (10-15) while the first *in vitro* studies of EPA and DHA directly affecting IL-2 production by mitogen-stimulated rat T-cells and human PBMCs were

published in 1992 (13, 14). This historical overview puts the study of Meydani et al. (1) clearly into context: it was essentially "first in man" and one of the "first in field", signifying its major impact at the time and in the years thereafter and its importance to the field so explaining its citation longevity.

By studying the effect of increased intake of EPA and DHA on cytokines and T-cell responses, Meydani *et al*. were conducting highly novel, state-of-the-art research. This was only possible because of the technological advances in immunology that were made in the mid-to-late 1980s. The cytokines Meydani *et al*. studied had been discovered only in the 1970s and early 1980s and techniques to easily and reliably measure those cytokines had only recently become available. A bioassay was used to measure IL-2 concentration and newly-available radioimmunoassays were used to measure IL-1β, IL-6 and TNF concentrations. In this sense Meydani *et al*. were applying the cutting-edge immunologic technologies of the time to a timely nutritional question.

Meydani *et al.* (1) were following up on the study of Endres *et al*. (4). This study, published in 1989 and cited 1488 times (Web of Science accessed on May 10 2018), involved nine healthy volunteers who consumed 4.6 g of EPA plus DHA daily for 6 wk. Again the study was small, uncontrolled and unblinded. Endres *et al*. used radioimmunoassays to measure IL-1α, IL-1β and TNF concentrations in the supernatants of endotoxin-stimulated PBMCs at baseline and at the end of omega-3 fatty acid supplementation. The concentrations of all three cytokines were decreased after omega-3 fatty acid supplementation compared to at baseline. Curiously, 10 wk after stopping supplementation the concentrations of all three cytokines were even lower although they returned to pre-supplementation levels 20 wk after stopping supplementation. These two studies (1, 4) suggest that the combination of EPA and DHA can be used to diminish the production of major pro-inflammatory cytokines, like TNF and IL-1β, and that such an effect is likely to be part of the anti-inflammatory action of these fatty acids; this conclusion remains relevant today (16, 17) and partly explains the efficacy of high dose EPA and DHA in inflammatory conditions like rheumatoid arthritis (18-20).

An important question relates to the extent to which the findings of Meydani *et al.* (1) have stood the test of time. Others have replicated the findings. For example, Caughey et al. (21) showed that fish oil providing 2.7 g EPA+DHA/d for 4 wk decreased endotoxin-induced

production of TNF and IL-1β by PBMCs. Likewise Trebble et al. (22) reported an omega-3 fatty acid dose-dependent decrease in TNF and IL-6 production by endotoxin-stimulated PBMCs taken from young men supplemented with up to 2 g EPA + DHA daily for 4 wk. Thies *et al*. showed that fish oil providing only 1 g EPA+DHA/d for 12 wk decreased mitogen-stimulated T-cell proliferation in PBMCs from subjects aged 60 to 68 y (23), although there was no effect on IL-2 production (23) or on endotoxin-induced production of TNF, IL-1β and IL-6 by PBMCs (24). However, in contrast to what Meydani *et al.* (1) had seen, Trebble *et al*. reported that 2 g EPA+DHA/d for 4 wk increased mitogen-induced T-cell proliferation and production of interferon (IFN)-γ by cultured PBMCs collected from young men (25). Rees *et al*. (26) saw no effect of EPA up to a dose of 4.05 g/d for12 wk on endotoxin-stimulated production of TNF, IL-1β or IL6 by PBMCs collected from young (mean age ~25 y) and older (mean age ~60 y) men. In the young men there was no effect of EPA on Con A-induced T-cell proliferation or on IL-2 or IFN-γ production (27); these were not studied in the older men. Yaqoob *et al*. (28) saw no effect of fish oil providing 3.2 g EPA+DHA/d for 12 wk on the proliferation of Con A-stimulated PBMCs, or on the *ex vivo* production of a range of cytokines by PBMC cultures stimulated by either Con A (IL-2, IFN-γ) or endotoxin (TNF, IL-1α, IL-1β). A later study by the Meydani group (29) reported no effect of 2.5 g EPA+DHA/d for 12 wk on mitogen-stimulated T-cell proliferation in cultures of PBMCs from elderly (age > 65 y) men and women. Thus, the early findings of Meydani *et al*. (1) are replicated only by some subsequent studies. There may be explanations for this. Dose of omega-3 fatty acids used, duration of supplementation and age of the subjects studied are each likely to be important, but these do not seem to be the sole explanation, because the study of Rees *et al*. (26) used several doses up to 4.05 g EPA/d for the same duration as Meydani et al. (12 wk) and in both young and older subjects and did not identify the same effects as Meydani *et al*. Sex may be important as Meydani *et al*. (1) studied females while Rees *et al*. (26) studied males. It is also important to note that the other studies referred to herein had a larger sample size than that of Meydani *et al*. and included a control group, both of which would help mitigate against chance findings. One other factor that has been identified as a determinant of the effect of omega-3 fatty acids on pro-inflammatory cytokine production by endotoxin-stimulated PBMCs is genetics. While fish oil providing 1.8 g EPA+DHA/d for 12 wk had no overall effect on production of TNF by endotoxin-stimulated PBMCs from men aged 20 to 57 y, TNF production was lowered in those individuals with certain polymorphisms in the *TNF* and *TNFB* genes (30). This suggests a

genetic determinant to the anti-inflammatory effects of omega-3 fatty acids. Together these observations indicate the need for a large, controlled study systematically evaluating the effect of different doses of omega-3 fatty acids on inflammatory cytokine production and T-cell function according to subject age, sex and genotype. Even so there are other important considerations. These include whether EPA and DHA have different effects on inflammation and immunity (31), and therefore the extent to which the different combinations of these two omega-3 fatty acids that have been used might have influenced the outcomes of the studies in this area; the role of the background dietary intake of omega-6 fatty acids, which might act to oppose the anti-inflammatory actions of omega-3 fatty acids (32); and the degree of oxidative stress induced by supplemental intakes of high doses of EPA and DHA. Oxidative stress induces inflammation and can suppress T-cell function. Therefore, omega-3 fatty acids and oxidative stress may oppose one another, although the highly unsaturated nature of EPA and DHA makes them good substrates for oxidative damage. In this regard, a companion paper to that of Meydani *et al*. (1), also published in *Journal of Nutrition* in 1991 (33), reported that the older women had higher levels of lipid peroxides in their plasma after 2 mo than the young women. This might have been expected to increase inflammation, which was not seen according to the markers measured, but may account for why T-cell function declined in the older but not the young women (1). Wu *et al*. (29) identified an omega-3 fatty acid-vitamin E interaction in determining T-cell proliferation in the elderly, supporting the notion that the effects of omega-3 fatty acids and oxidative stress oppose one another at least as far as T-cell function is concerned.

The paper of Meydani *et al.* (1) offers valuable information on two other important aspects. First, they demonstrated that endotoxin-induced production of IL-1β was higher and mitogen-stimulated production of IL-2 by T-cells and T-cell proliferation were lower in PBMC cultures from older compared to young women. These observations suggest an exaggerated inflammatory response with ageing combined with a suppression of T-cell-mediated immunity, consistent with current ideas around inflammaging (34, 35) and immunosenescence (36, 37). Consistent with Meydani *et al*., Rees *et al*. (26) observed that endotoxin-stimulated production of TNF, IL-1β and IL-6 was higher for PBMCs from older men than from young men, although the comparison for T-cell functions was not made. The other piece of valuable information offered by Meydani *et al*. (1) is that when given the same oral dose of EPA and DHA, the increase in both fatty acids in plasma was greater in the older

compared with the young women. EPA in plasma increased about twice as much in the older compared with young women while the increase in DHA was about 50% higher. There was no clear explanation for this as both compliance to the supplements and the intake of fat and different fatty acids from the background diet were not different between the two groups of women. Interestingly, Rees *et al.* (26) reported similar findings for the increment in EPA in both plasma phospholipids and PBMCs: in subjects given 4.05 g EPA/d for 12 wk the increment in EPA in plasma phospholipids was 70% higher in older men compared with younger while for PBMCs the increment was 60% higher. Again a clear explanation for these findings was not made, but the general consistency between the findings of these two studies (1, 26) suggests a real effect that is indicative of a change in whole-body handling of oral omega-3 fatty acids in older compared with young adults. This is worthy of further investigation.

In summary, the paper of Meydani *et al*. (1) used what were at the time state-of-the-art immunologic techniques to evaluate the impact of oral omega-3 fatty acids on markers of inflammation and T-cell immunity. The paper reports a series of novel findings related to immunologic differences between adults in different age groups, the effects of omega-3 fatty acids on the outcomes reported, and the differential impact of omega-3 fatty acids in young and older women. Many of these observations have been replicated, although not consistently. The findings of the study remain relevant, but it has several limitations including a small sample size, lack of a control group and absence of blinding.

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