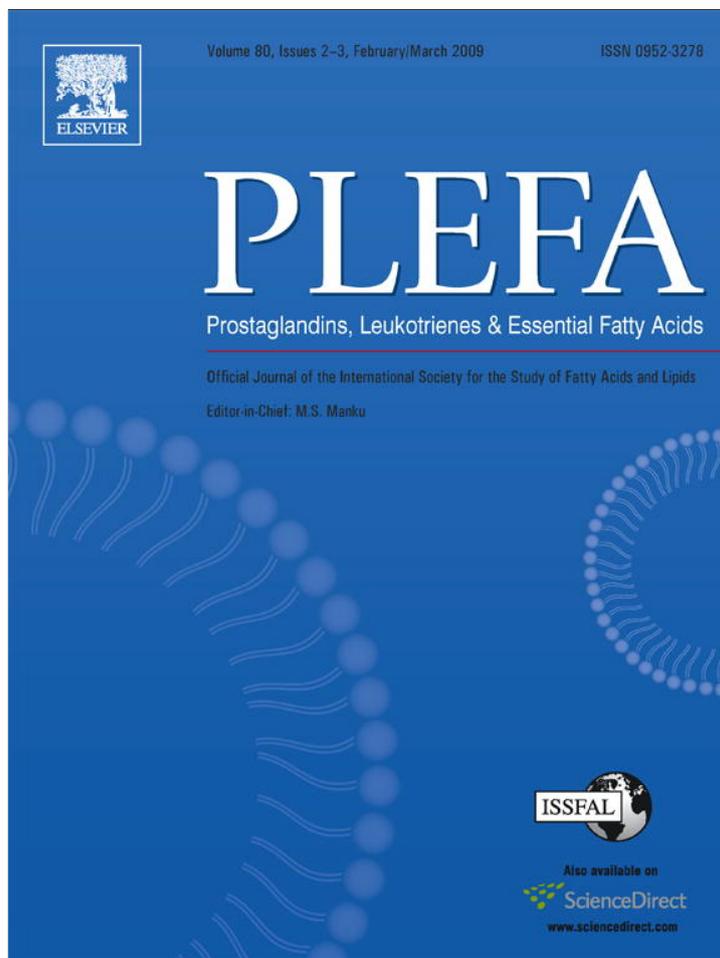


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# Prostaglandins, Leukotrienes and Essential Fatty Acids

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## Omega-3 fatty acid supplementation decreases matrix metalloproteinase-9 production in relapsing-remitting multiple sclerosis<sup>☆, ☆ ☆</sup>

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## ABSTRACT

**Objectives:** The primary objective was to evaluate the effect of omega-3 fatty acids (omega-3 FA) on matrix metalloproteinase-9 (MMP-9) production by immune cells in multiple sclerosis (MS). Quality of life, fatty acid levels, and safety were also evaluated.

**Materials and Methods:** Ten participants with relapsing-remitting MS (RRMS) received omega-3 FA supplementation (9.6 g/day fish oil) in an open-label study. Participants were evaluated at four time points, baseline, after 1 month of omega-3 FA supplementation, after 3 months of omega-3 FA supplementation, and after a 3-month wash out.

**Results:** Immune cell secretion of MMP-9 decreased by 58% after 3 months of omega-3 FA supplementation when compared with baseline levels ( $p < 0.01$ ). This effect was coupled with a significant increase in omega-3 FA levels in red blood cell membranes.

**Conclusions:** Omega-3 FA significantly decreased MMP-9 levels in RRMS and may act as an immunomodulator that has potential therapeutic benefit in MS patients.

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### 1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) in which T lymphocytes, macrophages, and antibodies are believed to be involved in disease pathogenesis [1,2]. Although the cause of MS is unknown, there is general agreement that MS results from an acquired immunodysregulation and aberrant activation leading to T cell-driven inflammatory processes in the CNS that result in demyelination and axonal damage. About 85% of patients begin with a relapsing-remitting clinical course in which there are relapses or attacks of MS lasting days to weeks followed by improvement and stability lasting months to years. About 50% of patients with relapsing-remitting MS (RRMS) develop secondary progressive MS in which there is progressive worsening of the disease. Although there are heterogeneous mechanisms to disease pathology, active inflammatory processes in the CNS are more highly associated with RRMS than with the progressive types of MS [3].

Current pharmacological therapies for MS include: interferon beta, glatiramer acetate, natalizumab, and mitoxantrone. These are immunomodulatory therapies and act by altering the T cell-driven inflammatory processes in the CNS [4–7]. In large randomized, double-blind, placebo-controlled trials, these therapies decrease the frequency and severity of relapses and have a modest effect on lengthening time to disability [4–9]. Although the availability of these therapies has improved the disease course in MS, they all require injection rather than an oral route of administration and there are significant side effects which include injection site reactions, flu-like symptoms, and muscle aches [10]. A subset of MS patients produce anti-interferon-beta neutralizing antibodies in response to interferon beta (IFN-beta) therapy which decreases the effectiveness of this therapy and can increase the risk of subsequent relapses in these patients [11]. The high cost of MS immunomodulatory therapies, which can cost \$25,000 or more per year, may prohibit their use in some patients. Considering the limitations of current MS immunomodulatory therapies, identifying a cost-effective oral therapy that has the potential to alter the MS disease course with minimal side effects is warranted.

The immunomodulatory effects of omega-3 fatty acids have been well documented. Numerous studies have reported a decrease in protein levels of inflammatory cytokines including, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-1 (IL-1), interleukin-2 (IL-2), and vascular cell adhesion molecule-1 (VCAM-1) [12]. Omega-3 fatty acids (omega-3 FA) have been

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evaluated in a number of inflammatory diseases such as rheumatoid arthritis and multiple sclerosis with proven benefit [13,14,15]. In an open-label study ( $n = 20$  MS and 15 healthy participants), Gallai et al. [15] found a significant decrease from baseline, in the levels of interleukin- $1\beta$  ( $p < 0.03$ ), TNF- $\alpha$  ( $p < 0.02$ ), IL-2 ( $p < 0.002$ ), and IFN- $\gamma$  ( $p < 0.01$ ) produced from unstimulated and stimulated peripheral blood mononuclear cells (PBMC) after 3 months of omega-3 FA supplementation containing 3.0 g/day of eicosapentaenoic acid (EPA) and 1.8 g/day docosahexaenoic acid (DHA). Weinstock-Guttman et al. [16] conducted a double-blind, placebo-controlled study in RRMS patients ( $n = 31$ ) and found a significant improvement in quality of life (physical and mental health components) favoring participants receiving a low saturated fat diet and omega-3 FA supplementation at 6 months. This study also examined changes in plasma inflammatory cytokine levels and found no difference between groups. The difference in outcomes on inflammatory cytokine levels between these two studies may reflect differences in the concentrations of EPA and DHA evaluated (Weinstock-Guttman et al. evaluated a lower concentration than Gallai et al.) and differences in cytokine measures (plasma versus immune cell secreted). Despite these differences, both studies reported positive effects of omega-3 FA supplementation in MS.

In addition to the omega-3 FA effect on decreasing proinflammatory cytokines levels, there is evidence supporting an omega-3 FA effect in decreasing matrix metalloproteinase (MMP) levels. There are a number of published in vitro studies that report a significant omega-3 FA effect in decreasing genetic expression, protein levels, and activity of MMP-2, -3, -9, -13 [17–19]. One in vitro study reports a significant and dose-dependent decrease in MMP-9 protein levels secreted from LPS-activated microglial cells that were incubated with either fish oil or an omega-3 FA mixture (53% EPA and 27% DHA) [20].

In MS, MMP-9 is thought to have a significant role in the transmigration of inflammatory cells into the CNS by aiding in the disruption of the blood–brain barrier [21]. Several studies have reported higher MMP-9 levels in MS subjects when compared with control subjects [22–24]. RRMS patients are reported to have an increase in immune cell expression of MMP-9 (measured by messenger ribonucleic acid (mRNA) levels), compared with healthy controls [25]. Interferon beta has the ability to inhibit MMP-9 levels produced from T-lymphocytes and CD4+T cells [26–28] which is thought to be one mechanism by which this therapy acts to alter the disease course [23]. To date the immunomodulatory effects of omega-3 FA on MMP-9 levels in MS patients have not been evaluated.

The primary objective of the present study was to evaluate the ability of omega-3 FA supplementation in decreasing MMP-9 protein levels secreted from peripheral blood mononuclear cells (PBMC) and from serum in RRMS.

## 2. Materials and methods

This was an open-label study in 10 relapsing remitting MS patients to determine the immunomodulatory effects of omega-3 FA supplementation. The study was approved by the Institutional Review Board at Oregon Health & Science University (OHSU). All participants gave informed consent before entering the study. The study duration was 6 months with 3 months of omega-3 FA supplementation followed by a 3-month wash out period. After baseline assessments, all 10 participants were given omega-3 FA in the form of fish oil concentrate (9.6 g/day containing 2.9 g EPA and 1.9 g DHA) (Vital Nutrients, Middletown, CT, USA). To account for pretreatment variations in MMP-9 levels, blood was collected at three baseline time points at 1-week intervals. Once omega-3

FA supplementation was started only one MMP-9 blood draw was performed to accurately reflect treatment timepoints, after 1 month of omega-3 FA supplementation; after 3 months of omega-3 FA supplementation; after a 3-month wash out.

### 2.1. Inclusion and exclusion criteria

Participants with a definite diagnosis of MS using the McDonald criteria [29]; relapsing-remitting type of MS; ages 18–65 years were enrolled. Exclusions to participation included the following: other types of MS; omega-3 FA supplementation within 30 days of enrollment; eating more than 6 ounces per week of fish within 30 days of enrollment; pregnancy; MS exacerbation or corticosteroid treatment within 60 days of enrollment; significant health condition (e.g. coronary heart disease, uncontrolled diabetes mellitus, liver disease). Participants were allowed to continue their MS disease modifying therapies, therapies for MS symptoms, and dietary supplements other than omega-3 fatty acids.

### 2.2. Primary outcome measure

The primary outcome measure was MMP-9 levels secreted from PBMC and MMP-9 levels in serum. Blood samples for PBMC isolation were collected by venipuncture into heparinized Vacutainer<sup>®</sup> tubes. PBMC were isolated within 4 h of blood draw (Lymphocyte separation media; Mediatech) and were incubated in PBS at  $2 \times 10^6$  cells/ml at 37 °C for 48 h. Cell supernatants were collected and stored at –80 °C until assayed. Blood samples for serum were collected by venipuncture into untreated Vacutainer<sup>®</sup> tubes. Serum was separated from the clot by centrifugation, aliquoted (0.5 ml) and stored at –80 °C until assayed. MMP-9 levels from cell supernatants and serum were measured by enzyme-linked immunosorbent assay (R&D Systems Inc., Minneapolis, MN).

### 2.3. Secondary outcome measure

Quality of life was assessed by the MS Quality of Life Inventory (MSQLI) as a secondary outcome measure. The MSQLI is a modular MS-specific health-related quality of life (HRQL) instrument consisting of the Short Form-36 questions (SF-36) supplemented by nine symptom specific measures (fatigue, pain, bladder function, bowel function, emotional status, perceived cognitive function, visual function, sexual satisfaction, and social relationships) [30].

### 2.4. Other measures

Incorporation of fatty acids into cell membranes was measured by red blood cell (RBC) membrane fatty acid analysis. Erythrocytes were separated from plasma by centrifugation. Lipids were extracted from erythrocytes and fatty acids were measured by capillary gas/liquid chromatography. This assay was performed at Oregon Health & Science University's Lipid Laboratory using methods that have been validated and previously published [31,32].

Neurologic impairment was measured by the Expanded Disability Status Scale (EDSS). This is an ordinal scale giving a measure between 0 (normal) to 10.0 (dead). Patients with scores in the 0.5–4.0 range have mild impairment and are able to walk at least 500 m without aid or rest. Patients with scores in the 4.5–7.0 range have increasing limitations in their ability to walk. Patients with scores of  $\geq 7.5$  cannot walk and have upper extremity dysfunction [33].

Safety was assessed monthly by adverse event reports and by laboratory measures (comprehensive metabolic panel and prothrombin time). Compliance was measured by pill count.

### 2.5. Data analysis

Statistical analysis was performed using SPSS version 14.0. Descriptive statistics was used to summarize participant characteristics information. Linear mixed model was used to compare baseline MMP-9 levels from PBMC and serum to the three other time points (1 month of omega-3 FA supplementation, 3 months of omega-3 FA supplementation, and after a 3-month wash out). This model was used to adjust for increased variability in the baseline measurement as a result of comparing three baseline measurements for MMP-9 levels to one measurement at each time of the other time points. PBMC or serum MMP-9 levels were entered as the dependent variable and intervention time (baseline, 1 month of omega-3 FA, 3 months of omega-3 FA, after a 3-month wash out) was entered as both fixed and random variables. Age, education, MS duration, disability (EDSS), and MS disease-modifying therapy use were entered into the model to evaluate their effects on MMP-9 levels. Repeated measures were used to compare EPA and DHA levels and quality of life scores.

## 3. Results

All 10 subjects completed the 6-month study. Subject characteristics are summarized in Table 1. All of the subjects were Caucasian, most were female (90%) with an education level of high school or greater (70%). Mean age was 47.6 years, mean MS duration was 15.3 years with a mean EDSS of 3.2. Half of the subjects were on a MS immunomodulatory therapy (IFN-beta or glatiramer acetate).

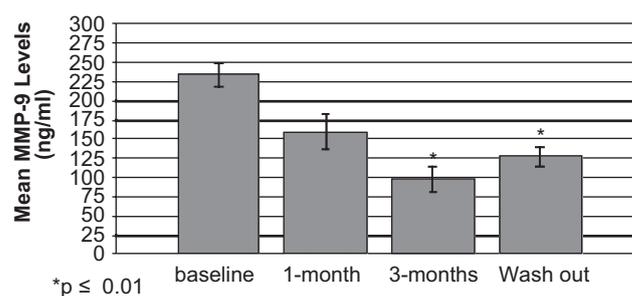
There was a 58% decrease from mean baseline MMP-9 levels secreted from PBMC after 3 months of omega-3 FA supplementation ( $p = 0.002$ ) and a 45% decrease from mean baseline levels after a 3-month wash out ( $p = 0.01$ ). The decrease from mean baseline MMP-9 levels was significant at both time points (Fig. 1). This difference persisted even after adjusting for age, education level, MS duration, disability, and IFN-beta and glatiramer acetate use. All 10 subjects showed a decrease in MMP-9 levels after 3 months of omega-3 FA supplementation (Fig. 2). There was no significant difference from mean baseline MMP-9 levels in serum after 1 month or 3 months of omega-3 FA supplementation ( $p = 0.61$ ,  $p = 0.68$ ) or after a 3-month wash out ( $p = 0.95$ ) (Table 2).

**Table 1**  
Subject characteristics ( $n = 10$ ).

Characteristic	Mean (SD) or %
Age	47.6 (9.0)
Gender	
Female	90%
Male	10%
Race	
Caucasian	100%
Education	
High school grad/some college	70%
Bachelors degree or greater	30%
MS duration in years	15.3 (9.4)
EDSS <sup>a</sup>	3.2 (0.23)
MS drug therapy use <sup>b</sup>	50%

<sup>a</sup> Expanded disability status scale.

<sup>b</sup> Disease modifying therapy (IFN-beta or glatiramer acetate).



**Fig. 1.** Mean MMP-9 levels secreted by unstimulated PBMC: comparison to baseline. Mean MMP-9 levels measured from unstimulated PBMC at baseline, after 1 month of omega-3 FA supplementation, after 3 months of omega-3 FA supplementation, and after a 3-month wash out.

After 3 months of omega-3 FA supplementation, there was a 6.3-fold increase from mean baseline levels in RBC membrane EPA levels ( $p = 0.001$ ) and a 1.7-fold increase in DHA levels ( $p = 0.04$ ) (Fig. 3). Although the mean EPA and DHA levels had decreased after a 3-month wash out, they remained significantly higher than baseline levels, EPA ( $p = 0.02$ ), DHA ( $p = 0.05$ ).

### 3.1. Quality of life

No significant differences were found from mean baseline physical or mental components scores of the SF-36 or from mean baseline values on any of the MS-specific components of the MSQLI (data not shown, all  $p$ -values  $> 0.20$ ).

### 3.2. Safety and compliance

All of the subjects (10/10) reported “fish burps” and 3/10 subjects reported fatigue within the first month of omega-3 FA supplementation (two of these subjects reported that hot weather was bothering them). No MS relapses occurred during the study. All mean baseline measures on the comprehensive metabolic panel were within normal limits and there was no significant change from mean baseline levels on any of these measures. Mean baseline prothrombin levels were 0.96 INR (SD 0.05) and mean levels after 3 months of omega-3 FA supplementation were 0.99 INR (SD 0.08). There was no significant difference between these two time points ( $p = 0.308$ ). Mean percent compliance by pill count was 88.6% with a compliance range of 75–100%.

## 4. Discussion

This is the first study to demonstrate the ability of omega-3 FA supplementation to alter MMP-9 production by immune cells in RRMS. The study demonstrated that omega-3 FA with 9.6 g of fish oil concentrate per day (containing 2.9 g EPA and 1.9 g DHA) resulted in a significant decrease in MMP-9 levels secreted from PBMC in people with MS after 3 months of supplementation. This outcome was independent of age, MS duration, neurologic impairment (EDSS), and IFN-beta or glatiramer acetate use. All 10 subjects showed a decrease in immune cell secreted MMP-9 levels after 3 months of supplementation which suggests that the observed effect may be independent of IFN-beta use, glatiramer acetate use, or over the counter supplement use (Fig. 2). Omega-3 FA at this dose was well tolerated and did not significantly alter basic metabolic function or prothrombin time over a period of 3 months. Our study did not show a significant effect of omega-3 FA supplementation on serum MMP-9 levels. As increased MMP-9 levels may have an impact on MS disease pathogenesis, the ability

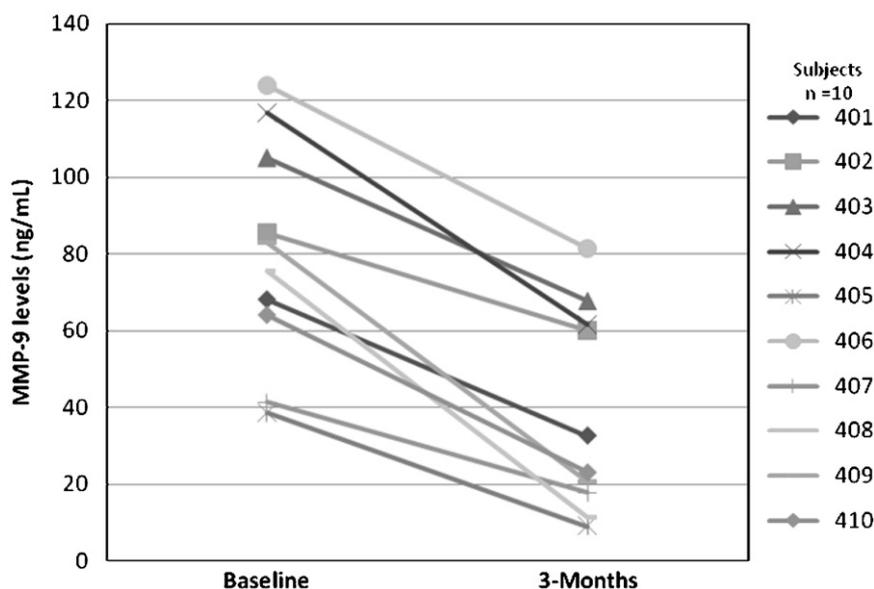


Fig. 2. Change in unstimulated PBMC secreted MMP-9 levels from baseline to 3 months of omega-3 FA supplementation in all 10 relapsing remitting MS subjects. Baseline MMP-9 levels reflect the mean of three baseline measures.

Table 2 Mean serum MMP-9 levels in MS subjects: comparison to baseline (n = 10).

Timepoint	Mean	Standard error	p-Value
Baseline	532.3	18.7	
1 Month	562.7	30.6	0.61
3 Months	553.2	38.3	0.68
Wash out	527.1	61.4	0.95

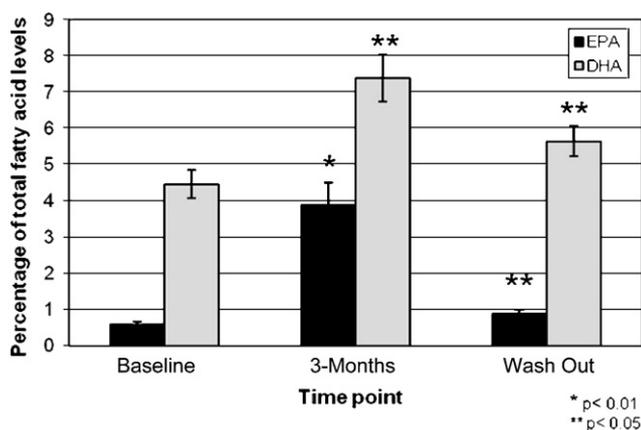


Fig. 3. Mean EPA and DHA levels in MS subjects measured from RBC membranes: comparison to baseline mean EPA and DHA levels measured from red blood cell membranes at baseline, after 3 months of omega-3 FA supplementation and after a 3-month wash out.

of omega-3 FA to decrease the levels secreted by immune cells is a significant observation.

In the periphery, MMP-9 is produced by several cells types including monocytes, macrophages, T-lymphocytes, neutrophils and endothelial cells [34]. Our study was interested in examining the ability of omega-3 FA in modulating MMP-9 secretion from PBMC because the migration of activated T-lymphocytes into the CNS is one mechanism of disease pathogenesis. PBMC are

composed of both T-lymphocytes (CD4+ and CD8+), B cells, and natural killer cells (NK cells). PBMC from MS patients are reported to have significantly elevated MMP-9 mRNA levels when compared to healthy controls, the increased MMP-9 expression in PBMC may enable these cells to migrate into the CNS [35,36]. Stuve et al. [27] observed that activated T-lymphocytes isolated from untreated MS patients were able to migrate through a fibronectin barrier due to increased MMP-9 activity. T-lymphocytes treated with IFN-beta decreased the migratory ability of these cells. Dressel et al. [26] observed that the migration of CD4+T cells through a fibronectin barrier, but not CD8+cells, was enhanced in untreated MS patients compared with controls and that CD4+migration and MMP-9 production was reduced by IFN-beta treatment. Decreasing MMP-9 expression and MMP-9 protein levels in T-lymphocytes is one proposed mechanism for the therapeutic action of IFN-beta therapy in MS [37]. The results from our study suggest that omega-3 FA may act by a similar mechanism.

How omega-3 FA decrease MMP-9 production in immune cells remains uncertain. It is possible that the effects of omega-3 FA on MMP-9 levels are secondary to decreasing proinflammatory cytokine levels. Release of MMP-9 by monocytes can be induced by autocrine secretion of IL-1 or TNF- $\alpha$  and it has been reported that both proinflammatory cytokines increase MMP-9 mRNA levels [38]. Omega-3 FA are reported to decrease both IL-1 and TNF- $\alpha$  in MS subjects and healthy controls [15] and may decrease MMP-9 levels by decreasing levels of both cytokines. Omega-3 FA also have the ability to decrease nuclear factor-kappa B (NF- $\kappa$ B) and activator protein-1 (AP-1) binding activity, both of which have binding sites on the MMP-9 gene promoter [38–41]. Omega-3 FA may inhibit MMP-9 expression by modulating NF- $\kappa$ B and AP-1 binding activity which could lead to decreased MMP-9 protein levels.

Our study did not evaluate the effect of omega-3 FA on TIMP-1 and TIMP-2 levels as a possible mechanism for regulating MMP-9 levels in RRMS. TIMP-1 and TIMP-2 form complexes with soluble MMP-9 that result in an inhibition of MMP-9 activity. There are five pilot studies that have reported significant increases in TIMP-1 levels in MS patients after IFN-beta therapy [42–46]. Collectively these studies suggest that one mechanism through

which IFN-beta therapy may act is by simultaneously decreasing MMP-9 while significantly increasing TIMP-1 levels. In vitro, there is limited evidence that omega-3 FA have the ability to decrease MMP levels while increasing TIMP levels. Renal cell carcinoma cells (Caki-1) exposed to EPA, but not arachidonic acid (an omega-6 fatty acid) showed a dose-related decrease in MMP-2 expression and a dose-related increase in TIMP-2 expression [19]. It will be of interest in future studies to determine if omega-3 FA have the ability to increase TIMP-1 levels while decreasing MMP-9 levels in MS.

A small sample size and not having a placebo-control group limits the interpretation of the study results. Although our study had a small sample size, all 10 subjects showed a decrease in MMP-9 levels after 3 months of omega-3 FA supplementation, decreasing the likelihood that the effect was a random event. In support of the present study's findings, our group has observed a significant decrease in MMP-9 activity in PMBC treated with EPA or DHA at a concentration of 5 mg/dl with no effect observed in PBMC treated with oleic acid at the same concentration (unpublished in vitro data). The in vitro concentration of EPA and DHA at 5 mg/dl would be achievable in whole blood following a daily oral dose of 0.6 g EPA and 0.50 g DHA [47], an oral dose that is significantly lower than the dose used in our clinical study (daily dose of 2.9 g EPA and 1.9 g DHA). Our unpublished in vitro results suggest that it may be possible to decrease MMP-9 levels secreted from immune cells at a lower oral dose of omega-3 FA than used in our clinical study and that evaluation of lower doses of omega-3 FA in MS should be explored. Although not done in the present study, future studies evaluating the effects of omega-3 FA supplementation on MMP-9 levels in MS warrant the inclusion of a placebo-control group. Olive oil contains up to 75% oleic acid and may serve as a good placebo for future clinical studies evaluating omega-3 FA effects on MMP-9 in MS as oleic acid did not affect MMP-9 activity in vitro (our unpublished data). Careful attention to blinding in the design of omega-3 FA supplementation studies is also warranted, as all 10 participants in our study reported 'fish burps' during omega-3 FA supplementation.

No changes in quality of life as measured by the MSQI from baseline values after 3 months of omega-3 FA supplementation or after a 3-month wash out were observed. Because MS patients score significantly lower on health-related quality of life measures (HRQL) than those in the general population [48,49] and drug therapy side effects can decrease HRQL in MS [50], it is important to identify therapies that are effective but do not significantly decrease quality of life. Omega-3 FA was given at a fairly high dose, 9.6 g of fish oil concentrate per day for 3 months, yet quality of life was not significantly decreased.

## 5. Conclusions

**Our data showed that omega-3 FA has the ability to significantly decrease immune cell secreted MMP-9 levels and was well tolerated at a dose of 9.6 g/day over 3 months.** Because increased MMP-9 levels may facilitate the migration of activated immune cells into the CNS, **omega-3 FA may have a potential therapeutic role in RRMS patients.** Future studies evaluating the effects of omega-3 FA on MMP-9 and TIMP-1 levels and the evaluation of longer-term effects of omega-3 FA supplementation on clinical outcomes in MS are warranted.

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