

# Effect of Dietary Fatty Acids on Inflammatory Gene Expression in Healthy Humans\*

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Over the past 100 years, changes in the food supply in Western nations have resulted in alterations in dietary fatty acid consumption, leading to a dramatic increase in the ratio of omega-6 ( $\omega 6$ ) to  $\omega 3$  polyunsaturated fatty acids (PUFA) in circulation and in tissues. Increased  $\omega 6/\omega 3$  ratios are hypothesized to increase inflammatory mediator production, leading to higher incidence of inflammatory diseases, and may impact inflammatory gene expression. To determine the effect of reducing the  $\omega 6/\omega 3$  ratio on expression of inflammatory pathway genes in mononuclear cells, healthy humans were placed on a controlled diet for 1 week, then given fish oil and borage oil for an additional 4 weeks. Serum and neutrophil fatty acid composition and *ex vivo* leukotriene B<sub>4</sub> production from stimulated neutrophils were measured at the start and end of the supplementation period and after a 2-week washout. RNA was isolated from mononuclear cells and expression of PI3K, Akt, NF $\kappa$ B, and inflammatory cytokines was measured by real-time PCR. A marked increase was seen in serum and neutrophil levels of long-chain  $\omega 3$  PUFA concomitant with a reduction in the  $\omega 6/\omega 3$  PUFA ratio (40%). The *ex vivo* capacity of stimulated neutrophils to produce leukotriene B<sub>4</sub> was decreased by 31%. Expression of PI3K $\alpha$  and PI3K $\gamma$  and the quantity of PI3K $\alpha$  protein in mononuclear cells was reduced after supplementation, as was the expression of several proinflammatory cytokines. These data reveal that PUFA may exert their clinical effects via their capacity to regulate the expression of signal transduction genes and genes for proinflammatory cytokines.

Since the beginning of the 20th century, the fatty acid composition of complex lipids (such as triglycerides) in Western diets has changed dramatically, largely due to a marked increase in the consumption of omega-6 ( $\omega 6$ ) polyunsaturated fatty acids (PUFA)<sup>2</sup> and a concomitant decrease in the consumption of omega-3 ( $\omega 3$ ) PUFA (1, 2). The increased ingestion of  $\omega 6$  PUFA is due in large part to growth in the production and consumption of vegetable oils, beef, pork, and poultry and

has been exacerbated by changes in livestock husbandry and feeding practices (3). This together with a reduced consumption of wild, fatty fish containing high concentrations of  $\omega 3$  PUFA has resulted in lower  $\omega 3$  intake and in  $\omega 6/\omega 3$  ratios in the United States diet of greater than 10:1. Anthropological evidence suggests that our hunter-gatherer ancestors maintained a ratio closer to 2:1 for  $\sim 100,000$  generations (3, 4). This dietary change is postulated to enhance circulating and cellular pro-inflammatory mediators (eicosanoids and cytokines) and reduce anti-inflammatory mediators, resulting in an overall increase in systemic inflammation and a higher incidence of allergic and inflammatory disease including asthma, allergies, diabetes, cardiovascular disease, and arthritis.

However, despite 50 years of research supporting the efficacy of long chain PUFA, such as eicosapentaenoic acid (EPA, 20:5,  $\omega 3$ ) and docosahexaenoic acid (DHA, 20:6,  $\omega 3$ ) in treating inflammatory diseases, a great deal remains unknown regarding the underlying molecular mechanisms responsible for their potent biological effects. The most studied mechanisms center around the observation that shifts in dietary consumption of  $\omega 6$  and  $\omega 3$  PUFA lead to alterations in the quantities of  $\omega 6$  and  $\omega 3$ -derived eicosanoids produced in animals and humans, thereby disturbing the balance of lipid-based pro- and anti-inflammatory mediators produced at sites of inflammation. For example, the 4-series leukotrienes (LT) (e.g. LT<sub>B4</sub>) produced by the action of 5-lipoxygenase (5-LO) on the  $\omega 6$  PUFA arachidonic acid (AA, 20:4) are highly inflammatory, while the 5-series LT (e.g. LT<sub>B5</sub>) produced from the  $\omega 3$  PUFA EPA are 10–100-fold less active (5). Also, Serhan *et al.* (6) have demonstrated that increasing cellular uptake of  $\omega 3$  fatty acids causes an enhancement in the production of resolvins and protectins, which are proposed to dampen and resolve inflammatory responses.

Alternatively, the presence of high concentrations of  $\omega 3$  PUFA, or shifts in  $\omega 6/\omega 3$  ratios may modulate the expression of genes known to be critical to inflammatory processes. Curtis *et al.* (7) have shown that  $\alpha$ -linolenic acid (LNA, 18:3,  $\omega 3$ ), EPA, or DHA reduce the expression of genes for TNF $\alpha$  and IL-1 $\beta$  in bovine chondrocytes. Similarly, mice fed fish oil have decreased mRNA levels for numerous inflammatory cytokines including IL-1 $\beta$ , IL-6, and TNF $\alpha$  in kidney, spleen, and peritoneal macrophages (8, 9). *In vitro* studies from our laboratory suggest that AA alters cell cycle progression and apoptosis via its capacity to regulate several members of the AP1 family of transcription factors (10).

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<sup>2</sup> The abbreviations used are: PUFA, polyunsaturated fatty acid; PBMC, peripheral blood mononuclear cell; FAME, fatty acid methyl ester; LT, leukotriene; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; IL, interleukin; TNF, tumor necrosis factor; AA, arachidonic acid; DGLA, dihomo- $\gamma$ -linolenic acid; PI3K, phosphatidylinositol 3-kinase.

To date, the majority of studies examining PUFA and gene expression have been carried out in isolated cell systems and a few animal studies. This raises the question of whether the observed alterations in gene expression apply to humans. The current study has utilized a dietary intervention strategy in which healthy humans were fed controlled diets including dietary supplements containing specific dosages of short chain (18 carbon) PUFA and long chain (>20 carbon) PUFA in a manner, which is consistent with our understanding of early human diets. These experiments suggest that altering circulating levels of  $\omega 6$  and  $\omega 3$  PUFA likely influences inflammatory responses in part by the capacity of these fatty acids or their metabolites to regulate the expression of early signal transduction genes and to block the expression of pivotal cytokines and chemokines at a transcriptional level.

## EXPERIMENTAL PROCEDURES

**Materials**—Triheptadecanoin internal standard was purchased from Nu-Check Prep (Elsyan, MN). Isolymp was purchased from Gallard-Schlesinger (Plainview, NY). Ionophore A23187 was purchased from Calbiochem. TRIzol reagent was purchased from Invitrogen. All reverse transcription reagents and Sybr Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). All forward and reverse primer pairs were purchased from SuperArray (Frederick, MD). Protran nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH). Anti- $\beta$ -actin and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA) and anti-P13K p110 $\alpha$  antibody was purchased from US Biological (Swampscott, MA). All other chemicals and solvents were purchased from Sigma or Fisher Scientific.

**Study Design**—Healthy human volunteers ( $n = 27$ ) were screened for exclusion criteria and enrolled in the study in groups of three to five. The volunteers were provided with a background diet for 5 weeks. After 1 week on the diet they were given dietary supplements containing fish oil (775 mg EPA/day) and borage oil (831 mg GLA/day) for 4 weeks. The study period was followed by a two-week washout period during which the volunteers resumed their normal diets. Fasting blood draws were performed once per week, and urine samples were collected.

**Isolation of Mononuclear Cells and Neutrophils from Whole Blood**—Approximately 30 ml of whole blood was mixed with 6 ml of Isolymp and allowed to settle at room temperature for 1 h. The plasma layer was spun, and the cell pellet was resuspended in phosphate-buffered saline (PBS) to separate red blood cells, neutrophils, and peripheral blood mononuclear cells (PBMCs). The PBMCs were removed, washed several times in cold PBS to remove all traces of Isolymp, then counted and resuspended in PBS. Contaminating red blood cells were removed from the neutrophil pellet via hypotonic lysis with 0.2% NaCl, and neutrophils were resuspended in Hank's buffer plus  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at a concentration of  $1 \times 10^7$  cells/ml.

**Ionophore-stimulated Leukotriene Production**—1-ml aliquots of neutrophils were preincubated in a 37 °C water bath, then stimulated for 5 min with 2  $\mu\text{M}$  or 5  $\mu\text{M}$   $\text{Ca}^{2+}$  ionophore

A23187 (2.5 mM in DMSO). The stimulation was stopped by adding 1 ml of ice cold Hank's buffer followed by centrifugation at  $200 \times g$  at 4 °C for 10 min. The neutrophil pellet was used for fatty acid analysis, and the supernatant was combined with 250 ng of PGB<sub>2</sub> internal standard, acidified with two drops of 9% formic acid and extracted twice with ethyl acetate. The extracts were dried under  $\text{N}_2$  and resuspended in methanol/water (0.45:0.55) for HPLC analysis. Leukotrienes were separated on an Agilent Zorbax ODS analytical column (4.6  $\times$  250 mm) using a Hewlett Packard Series 1050 instrument equipped with a Hewlett Packard 1040M diode array detector and an Agilent 1200 series autosampler. The solvent system was as described previously (11). Data were processed using a Hewlett Packard Compac computer and Agilent Chemstation software. Quantification of the leukotrienes was by reference to the internal standard.

**GC Analysis of Serum Fatty Acids**—Fatty acid methyl esters (FAME) were prepared following a modification of the protocol by Metcalfe *et al.* (12). Serum samples (100  $\mu\text{l}$ ) were added to tubes containing 25  $\mu\text{g}$  of triheptadecanoin (C17:0) as internal standard, and saponified with EtOH and 50% KOH at 60 °C for 30 min. Neutral lipids were then extracted and discarded. The samples were acidified, and the fatty acids were extracted into hexane. Samples were brought to dryness under nitrogen, incubated for 5 min at 100 °C with 0.5 N NaOH in methanol and FAME were prepared by incubation with 12% boron trifluoride for an additional 5 min at 100 °C. The FAME were extracted into hexane, blown to dryness under nitrogen, and resuspended in a small volume of iso-octane for the GC analysis. The FAME were separated using a CP Select CB for FAME capillary GC column (100 m  $\times$  0.25 mm ID, film thickness 0.25  $\mu\text{m}$ ) and a HP 5890 temperature-programmed GC with  $\text{H}_2$  as the carrier gas and helium as the make-up gas. The fatty acid distribution was determined via reference to the internal standard.

**RNA Isolation and Reverse Transcription-Polymerase Chain Reaction**—RNA was isolated from peripheral blood mononuclear cells using TRIzol reagent following the manufacturer's directions. The RNA concentrations were assessed by measuring the absorbance at 260 nm. Approximately 1  $\mu\text{g}$  of total RNA was reverse-transcribed using 2.5 units/ $\mu\text{l}$  MuLV reverse transcriptase in a 20- $\mu\text{l}$  reaction mixture containing 1 mM of each deoxyribonucleotide, 5 mM  $\text{MgCl}_2$ , 1 unit/ $\mu\text{l}$  RNase inhibitor, and 2.5  $\mu\text{M}$  random hexamers in PCR Buffer II. The reaction mixture was heated to 42 °C for 15 min, then 99 °C for 5 min, then held at 5 °C for at least 5 min.

**Quantitative Real-time Polymerase Chain Reaction**—Real-time quantitative PCR of the cDNA template was performed in an ABI Prism 7000 SDS (Applied Biosystems). The PCR reaction contained 150 ng of cDNA, 10  $\mu\text{M}$  forward and reverse primers, and 12.5  $\mu\text{l}$  of 2 $\times$  RT<sup>2</sup> Real-Time Sybr Green PCR Master Mix in a total volume of 25  $\mu\text{l}$ . The PCR cycling conditions were: hold for 10 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Results were calculated as expression of the target gene relative to expression of the reference gene (GAPDH).

**Western Blot**—PBMCs were pelleted and lysed by addition of 1 ml of TRIzol reagent per  $10^6$  cells. Protein was extracted fol-

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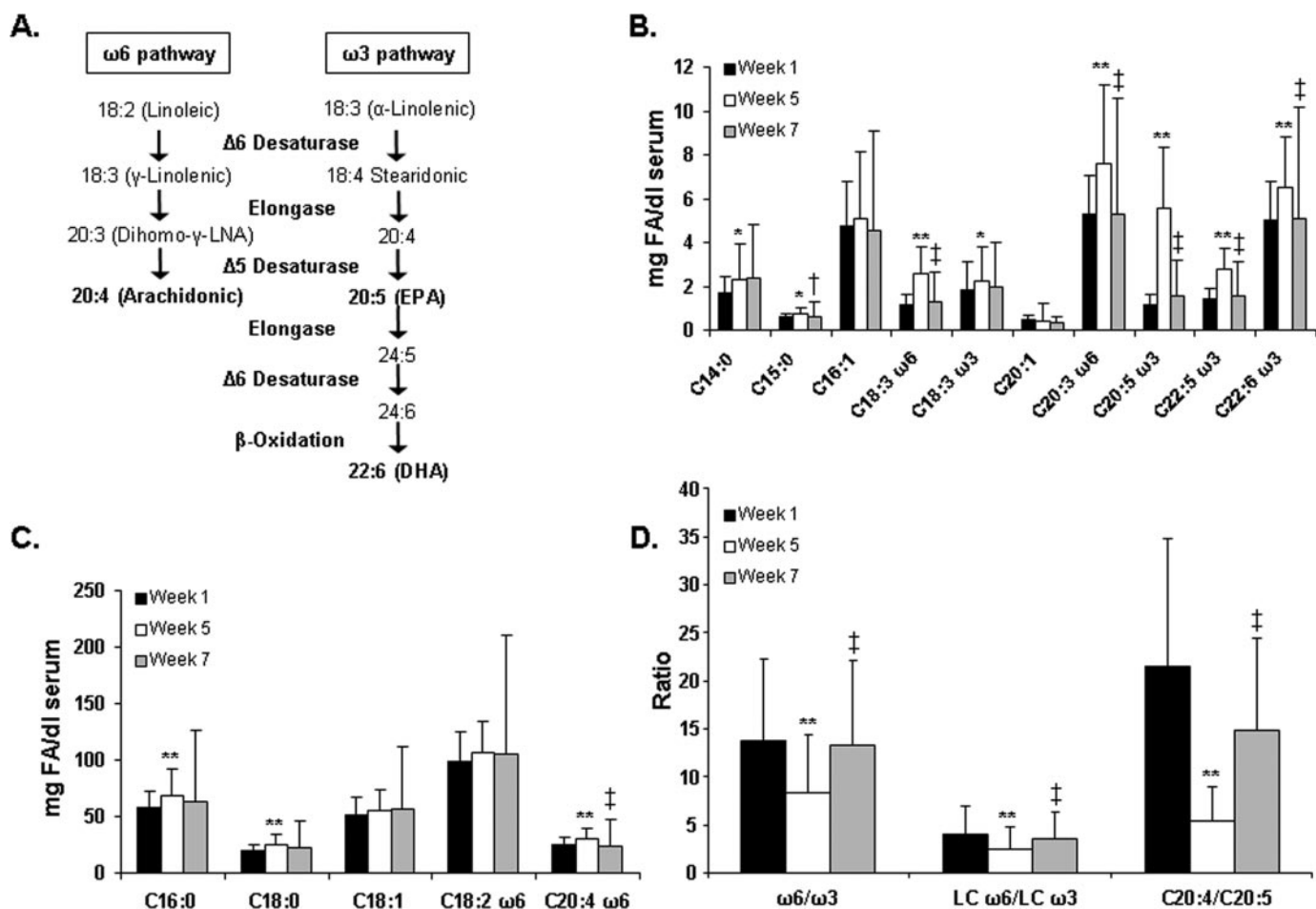


FIGURE 1. **Total serum fatty acids.** Fatty acid methyl esters were derived from serum of study participants at the start of supplementation (week 1), at the end of supplementation (week 5), and after a 2-week washout period (week 7), and total fatty acids were measured by GC (B and C). Ratios of  $\omega$ 6 and  $\omega$ 3 PUFA were calculated based on the total serum values (D). A shows the  $\omega$ 6 and  $\omega$ 3 fatty acid metabolic pathways. Data are expressed as mean  $\pm$  S.D. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  week 1 versus week 5; †,  $p < 0.05$ ; ‡,  $p < 0.01$  week 5 versus week 7,  $n = 27$ .

lowing the manufacturer's protocol. 10  $\mu$ g of protein was electrophoresed on 8% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with primary antibodies that recognize Akt, PI3K, and  $\beta$ -actin, followed by infrared fluorophore-conjugated secondary antibodies for detection and quantification using the Odyssey Infrared Imaging System (Licor, Lincoln, NE).

**Statistical Analysis**—All data were analyzed using two-tailed paired Student's *t*-tests to compare the week 1 value to the week 5 value, or the week 5 value to the week 7 value, with a significance level of 0.05.

## RESULTS

**Serum Fatty Acid Distribution**—Healthy human volunteers were placed on a background control diet for 5 weeks, followed by a 2-week washout period. This diet contained 27% fat, 18% protein, and 55% carbohydrate and maintained dietary AA, EPA, and DHA levels below 100 mg/day. After 1 week on the diet, study participants were placed on two supplements: borage oil (containing the short [18 carbon],  $\omega$ 6 fatty acid,  $\gamma$ -linolenic acid [GLA], and other 18 carbon  $\omega$ 6 fatty acids) and fish oil (containing the long [ $\geq$ 20 carbon] chain  $\omega$ 3 fatty acids, EPA and DHA). Serum was collected and circulating fatty acids were measured at week 1 and after 4

weeks (week 5) of supplementation with fish oil and borage oil. Fig. 1 shows the change in the levels of key serum fatty acids from week 1 to week 5 and following the 2-week washout (week 7). Fig. 1A shows the biochemical pathway responsible for the conversion of short chain  $\omega$ 6 and  $\omega$ 3 PUFA to long chain PUFA. There was a significant increase in GLA and its elongation metabolite dihomogamma-linolenic acid (DGLA, 20:3,  $\omega$ 6) after supplementation; both decreased significantly during the washout (Fig. 1B). Similarly, EPA and its elongation metabolites DPA (22:5,  $\omega$ 3) and DHA increased significantly after dietary supplementation with borage and fish oil. All three  $\omega$ 3 PUFA decreased to essentially starting values during the washout. Fig. 1C shows a small but statistically significant increase in AA, possibly due to a portion of GLA in the borage oil supplement being converted to AA via elongation and desaturation by the  $\Delta$ 5 desaturase; however, AA levels also returned to baseline following the washout. The ratios of all  $\omega$ 6 to  $\omega$ 3 PUFA, long chain  $\omega$ 6 to  $\omega$ 3 PUFA, and AA to EPA are shown in Fig. 1D. From week 1 to week 5, the overall  $\omega$ 6 to  $\omega$ 3 PUFA ratio decreased by 40%, from a ratio of  $\sim$ 15:1 typical of a Western diet to less than 9:1, followed by a return to the starting level after the washout. The ratio of long-chain  $\omega$ 6 to long-chain  $\omega$ 3 PUFA decreased by 39%, while the ratio of AA to EPA decreased by 75%, and both increased from week 5 to week 7.

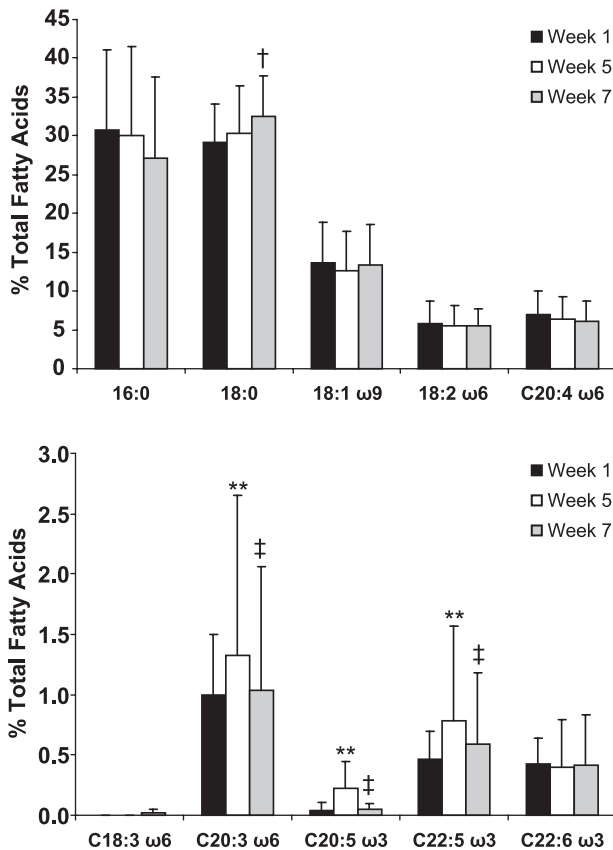


FIGURE 2. **Fatty acid composition of neutrophil glycerolipids.** Fatty acid methyl esters were derived from neutrophils isolated from study participants at the start of supplementation (week 1), at the end of supplementation (week 5), and after a 2-week washout period (week 7) and total fatty acids were measured by GC. Data are expressed as mean  $\pm$  S.D. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  week 1 versus week 5; †,  $p < 0.05$ ; ‡,  $p < 0.01$  week 5 versus week 7,  $n = 27$ .

**Neutrophil Fatty Acid Distribution and Leukotriene Production**—Similar to serum, the fatty acid composition of circulating neutrophils reflected the changes in the diet (Fig. 2). Both EPA and its elongation metabolite DPA increased significantly from week 1 to week 5, followed by a decrease to near baseline values 2 weeks after completing the supplementation (washout). Additionally, there was a statistically significant increase in DGLA, the elongation product of GLA from the borage oil, from week 1 to week 5. Levels of DGLA were returning to baseline values 2 weeks after completion of the supplementation. There was no difference (from baseline) in AA levels within neutrophil glycerolipids after the supplementation.

Previous experiments from our laboratory and others have demonstrated that the *ex vivo* capacity of neutrophils to produce leukotrienes is a reliable pharmacodynamic marker to gauge the effect of specific diets on systemic eicosanoid production (11, 13, 14). Neutrophils were isolated from whole blood and stimulated before supplementation (week 1), after supplementation (week 5), and again after a 2-week washout period (week 7).  $LTB_4$ , 20-hydroxy and 20-carboxyl  $LTB_4$ , as well as the two non-enzymatic isomers of  $LTB_4$  were measured by reverse phase HPLC from 11 of the initial study subjects (Fig. 3).  $LTB_4$  production decreased by 31% after 4 weeks of supplementation with fish oil and borage oil, and ten out of eleven subjects (the responders) responded to the diet with an average decrease

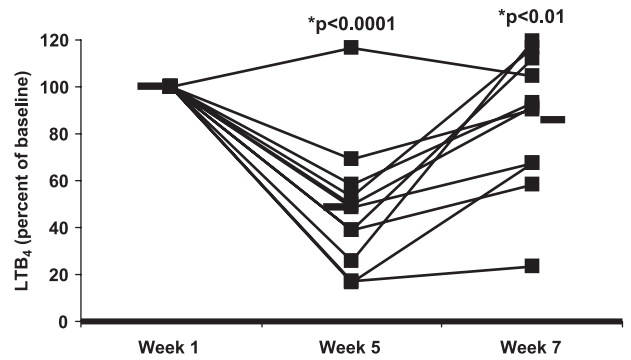


FIGURE 3.  **$LTB_4$  production from stimulated neutrophils.** Neutrophils were isolated from whole blood of study participants at the start of supplementation (week 1), at the end of supplementation (week 5), and after a 2 week washout period (week 7). Cells were stimulated *ex vivo* with A23187 for 5 min and  $LTB_4$  production was measured by HPLC. Week 1 data were normalized to 100%. Black bars represent the mean  $LTB_4$  production.  $n = 11$ .

TABLE 1  
**White blood cell differential**

White blood cell differential was measured in study participants at the start of the study (week 1), after 4 weeks of supplementation (week 5), and after a 2-week washout period (week 7). Values are expressed as mean  $\pm$  S.E.  $n = 27$ .

	Week 1	Week 5	Week 7
Lymphocytes (per $mm^3$ )	2.06 $\pm$ 0.08	2.21 $\pm$ 0.01 <sup>a</sup>	2.19 $\pm$ 0.12
Monocytes (per $mm^3$ )	0.54 $\pm$ 0.03	0.52 $\pm$ 0.03	0.53 $\pm$ 0.04
Eosinophils (per $mm^3$ )	0.18 $\pm$ 0.04	0.2 $\pm$ 0.03	0.2 $\pm$ 0.03
Basophils (per $mm^3$ )	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01
Neutrophils ( $\times 10^6$ /dL serum)	3.69 $\pm$ 0.39	4.43 $\pm$ 0.49 <sup>a</sup>	4.39 $\pm$ 0.38

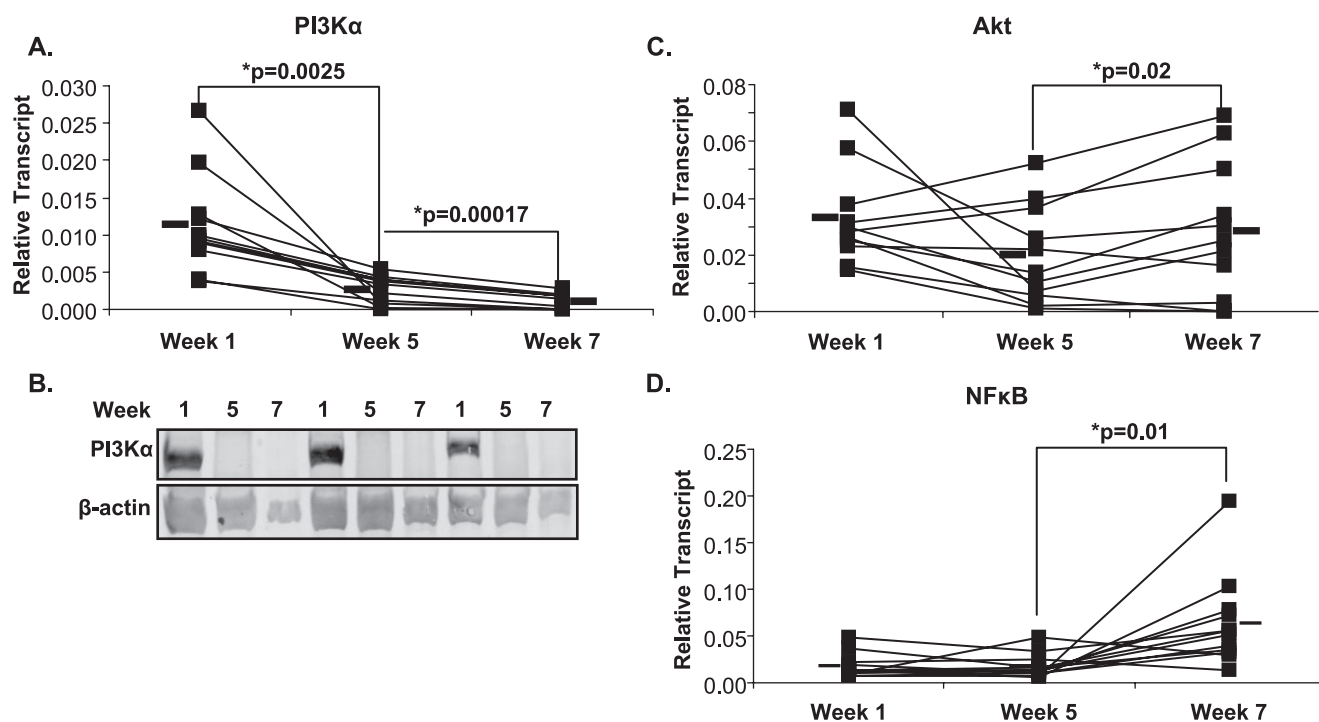
<sup>a</sup>  $p < 0.05$  Week 1 vs. Week 5.

of 55%. Two weeks after returning to individual diets, leukotriene levels returned to baseline values, on average. These data indicate that altering the  $\omega 6$  and  $\omega 3$  PUFA intake in healthy subjects with fish oil and borage oil supplementation markedly alters the composition of supplementation fatty acids in neutrophil glycerolipids and inhibits the capacity of neutrophils to produce leukotrienes, a putative marker of inflammation.

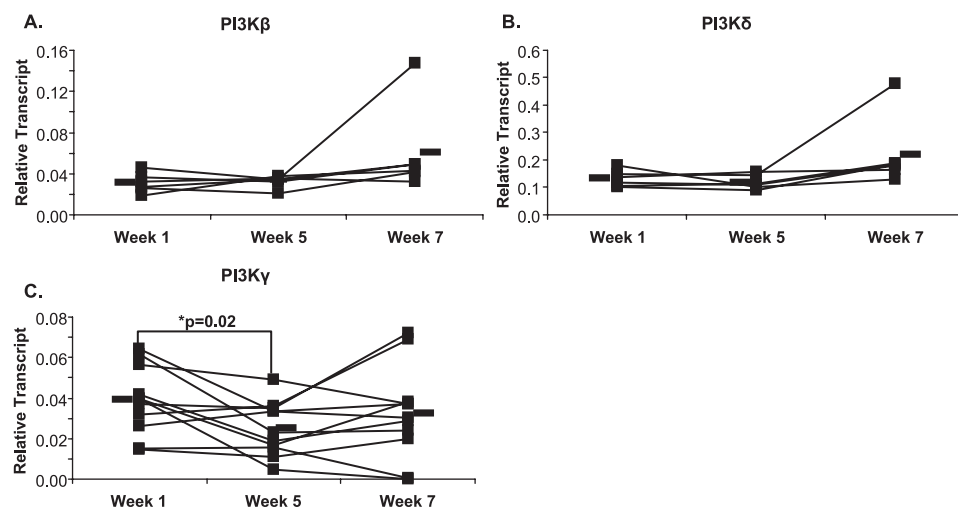
**Expression of Genes That Participate in Eicosanoid Generation**—The next series of experiments were designed to determine whether leukotrienes may be inhibited at least in part by the effects of dietary fatty acids on the expression of enzymes that participate in leukotriene generation. Studies to date indicate that it is difficult to monitor global gene expression in terminal-differentiated, circulating neutrophils; therefore, to test this hypothesis, mRNA was isolated from a second type of circulating inflammatory cell, peripheral blood mononuclear cells. White blood cell differentials measured for each subject showed only a small, though significant, increase in the number of lymphocytes and neutrophils after supplementation (Table 1). Initial experiments utilizing an inflammation-specific microarray demonstrated that supplementation caused no effect on message levels of enzymes that participate in leukotriene generation, including cytosolic group IV  $PLA_2$ , 5-lipoxygenase (5-LO),  $LTA_4$  hydrolase (also known as  $LTB_4$  synthase), and  $LTC_4$  synthase (data not shown).

Numerous studies have implicated the PI 3-kinase (PI3K)/Akt pathway as having an important role in initiating early eicosanoid generation in response to several immunologic stimuli. Accordingly, the mRNA expression of the proximal signaling genes  $PI3K\alpha$ , Akt, and  $NF\kappa B$  was measured by quantitative

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**FIGURE 4. Gene expression in mononuclear cells.** Mononuclear cells were isolated from whole blood before supplementation (week 1), after supplementation (week 5), and after a 2-week washout (week 7). Gene expression was measured by real-time PCR for (A) PI3K $\alpha$ , (C) Akt, and (D) NF $\kappa$ B. Black bars represent mean expression. Protein was isolated from mononuclear cells and immunoblot analysis was performed using an antibody to PI3K $\alpha$  (B).  $n = 12$  for real-time analysis,  $n = 3$  for immunoblot analysis.



**FIGURE 5. Gene expression in mononuclear cells.** Mononuclear cells were isolated from whole blood before supplementation (week 1), after supplementation (week 5), and after a 2-week washout (week 7). Gene expression was measured by real-time PCR for PI3K $\beta$  (A), PI3K $\delta$  (B), and PI3K $\gamma$  (C). Black bars represent mean expression.  $n = 6$  for A and B,  $n = 12$  for C.

real-time RT-PCR (Fig. 4). Fig. 4A shows that supplementation induced a large decrease in the expression of PI3K $\alpha$ , which was maintained after the washout. Immunoblot analysis of protein from mononuclear cells supported the PCR results, with PI3K $\alpha$  abundantly present at week 1 and present at much lower amounts at week 5 and week 7 (Fig. 4B). Downstream of PI3K $\alpha$ , the expression of Akt (Fig. 4C) and NF $\kappa$ B (Fig. 4D) in circulating mononuclear cells was not significantly altered during supplementation, although expression of both increased upon washout. Akt protein remained constant at all time points (data not shown). The aforementioned experiments raised the question

of whether the effects of supplementation observed with PI3K $\alpha$  were observed with other PI3K isoforms. To address this, PI3K $\beta$ , PI3K $\delta$ , and PI3K $\gamma$  were measured by real-time PCR. PI3K $\gamma$  mRNA was significantly decreased following supplementation while PI3K $\beta$  and PI3K $\delta$  remained unchanged following supplementation and washout (Fig. 5).

**Inflammatory Cytokine Expression**—PI3K not only plays an important role in eicosanoid formation, but also in cell growth, survival, and inflammation by utilizing downstream effectors including Akt and NF $\kappa$ B, which in turn influence the production of a variety of signaling molecules such as cytokines. To better understand the potential overall

effect of these dietary PUFA, the expression of eight cytokines known to play an important role in the inflammatory response was measured by real-time PCR. The expression of six cytokines involved in the immune response is shown in Fig. 6. A significant decrease in expression was observed for IL-1 $\beta$ , IL-10, and IL-23. Additionally, IL-5 and IL-17 showed strong trends toward decreased expression after supplementation. Changes were not seen for TNF $\alpha$  ( $p = 0.11$ ) and IL-6 ( $p = 0.27$ ) (data not shown). Only IL-1 $\beta$  showed a significant continued decrease from week 5 to week 7, while expression of the other

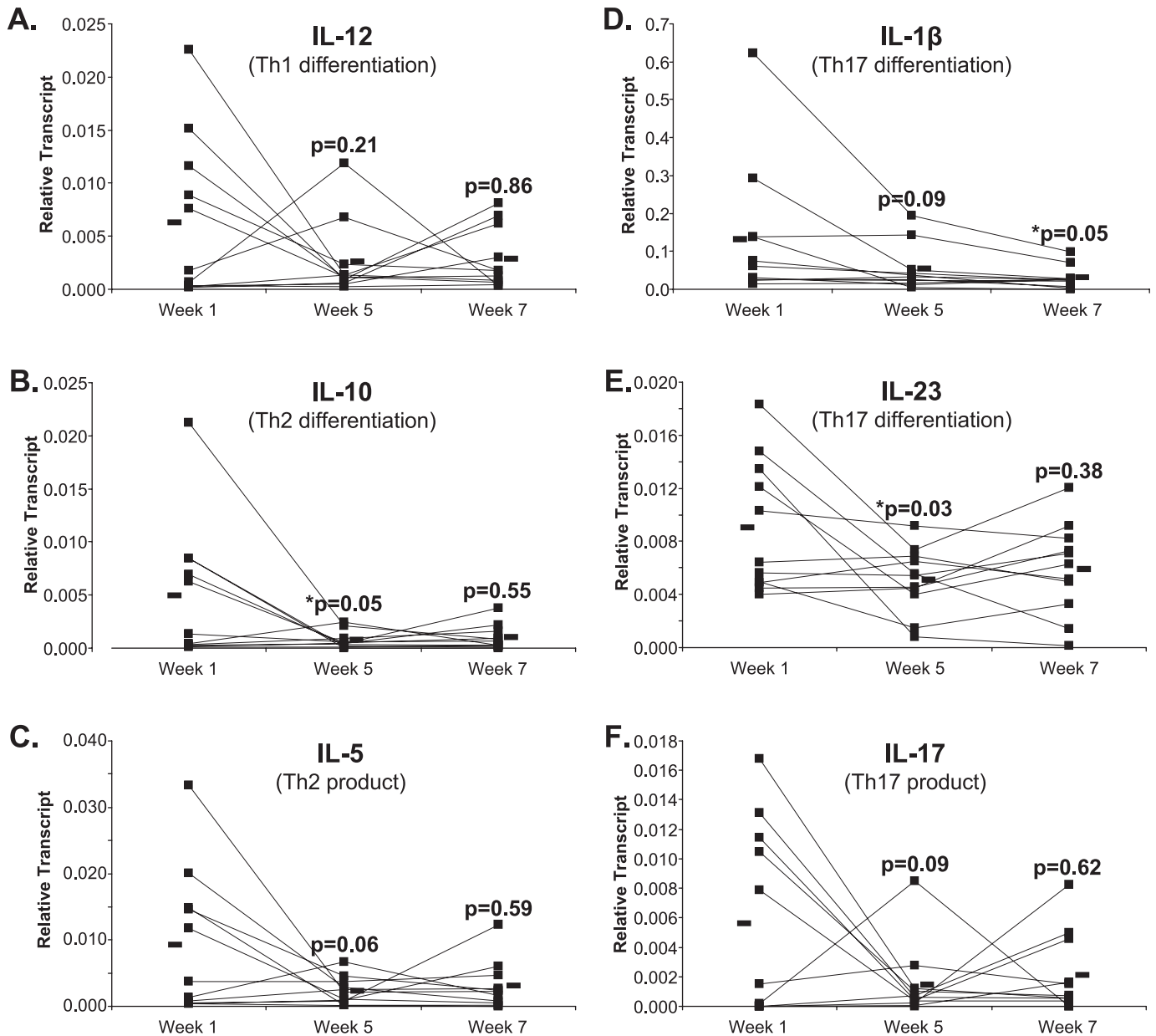


FIGURE 6. **Expression of inflammatory cytokines in mononuclear cells.** Mononuclear cells were isolated from whole blood before supplementation (week 1), after supplementation (week 5), and after a 2-week washout period (week 7) and real-time PCR was used to measure cytokine expression. A, IL-12, a Th1 differentiation cytokine; B, IL-10, a Th2 differentiation cytokine; C, IL-5, a Th2 product; D, IL-1 $\beta$ , a Th17 differentiation cytokine; E, IL-23, a Th17 differentiation cytokine; F, IL-17, a Th17 product. Black bars represent mean expression. The *p* value above the week 5 values represents the difference between the week 1 values and the week 5 values; the *p* value above the week 7 values represents the difference between the week 5 values and the week 7 values, *n* = 11.

cytokines showed trends toward increasing back to the baseline week 1 values during the washout. These washout results were not statistically significant, possibly due to the shorter duration of the washout period (2 weeks) compared with the treatment period (4 weeks), or due to metabolic differences between mononuclear cells and neutrophils.

## DISCUSSION

Research over the past 50 years has shown that  $\omega$ 3 PUFA have potent therapeutic effects in a wide variety of inflammatory diseases, including cardiovascular disease (15), rheumatoid arthritis (16, 17), allergic disorders, and depression (18). It is not clear at this time whether it is the ratio of total  $\omega$ 6 to  $\omega$ 3

PUFAs, the ratio of long chain  $\omega$ 6 to  $\omega$ 3 PUFAs, or merely the presence of high concentrations of  $\omega$ 3 PUFAs (long or short chain) that is most important in determining the clinical effectiveness of these dietary oils. It is also uncertain how the PUFA and their metabolites exert their effects to alter the course of inflammation in such diseases. The aim of this study was to provide both long-chain  $\omega$ 3 fatty acids (EPA and DHA) in fish oil and a short-chain  $\omega$ 6 fatty acid (GLA) in borage oil to identify the potential anti-inflammatory mechanisms by which these fatty acids exert their effects. Two recent studies suggest that EPA/GLA combinations are especially effective in critical care patients suffering from sepsis (19, 20). Specifically, addition of relatively high concentrations of these oils decreased the

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amount of time patients were on the ventilator, decreased the number of days patients were in the ICU, and increased overall survival.

An important aspect of the current study was that all participants were fed a controlled diet. This diet was provided at a caloric content necessary to maintain the body weight of each study subject while keeping the fat content equal for all individuals. A previous study from our laboratory which measured changes in serum fatty acid content with age determined that a controlled diet which normalizes background ingestion of fatty acids is necessary to observe consistent changes in circulating fatty acids in relatively small numbers of people (21).

Our studies as well as others have demonstrated that *ex vivo* leukotriene production by neutrophils can serve as an important pharmacodynamic marker to determine the effects of leukotriene blockers in humans (11, 13, 14). Consequently leukotriene production was measured in eleven initial subjects, and we found that the fish oil/borage oil combination was sufficient to decrease leukotriene production. Moreover, this effect was reversed after the 2-week washout period (Fig. 3), similar to the reversal of  $\omega 3$  and  $\omega 6$  PUFA levels in serum and PMN (Figs. 1 and 2). We next examined the influence of the supplements on the expression of enzymes directly responsible for leukotriene generation in circulating mononuclear cells, including group IV cPLA<sub>2</sub>, 5-LO, LTA<sub>4</sub> hydrolase, and LTC<sub>4</sub> synthase utilizing a combination of microarray technology and real-time PCR. These studies revealed no changes in the expression of any of the examined enzymes associated with leukotriene generation. This result suggests that the observed leukotriene inhibition was a consequence of the well-described effects of this type of supplementation on AA substrate availability and/or the production of alternative fatty acid substrates such as those from DGLA or EPA and not due to expression of key enzymes of leukotriene synthesis, consistent with the 2-week metabolic washout.

Next, we evaluated whether the *in vivo* changes in eicosanoids could be explained by supplement-induced alterations of key signaling enzymes or transcription factors in the signal transduction pathways that lead to their biosynthesis. Surprisingly, altering PUFA ratios caused a dramatic decrease in PI3K expression and, in particular, the expression of PI3K $\alpha$  and PI3K $\gamma$ . In contrast, there was no change in expression of the other PI3K isoforms, PI3K $\beta$  or PI3K $\delta$ , nor were there changes in message levels of the downstream effectors, Akt and NF $\kappa$ B. Although there was an increase in the number of lymphocytes from week 1 to week 5, this change was very small (<8%), suggesting that the changes we saw in gene expression were not due to a change in the composition of the white blood cells.

PI3K is the initial step in a wide array of signaling pathways, controlling cellular processes from apoptosis to cell growth and differentiation, glucose transport to cell migration, and from eicosanoid and cytokine production to the leukocyte oxidative burst. Four distinct PI3K isoforms appear to be responsible for these activities. PI3K $\delta$  and PI3K $\gamma$  are thought to play important roles in the inflammatory response (22–24). The specific functions of PI3K $\alpha$  and PI3K $\beta$  are just beginning to be understood, with studies to date showing they are involved in control of

glucose uptake and metabolism in muscle, liver, and adipose tissue (25).

Only a few studies have examined the regulation of PI3K expression. PUFA such as AA have been demonstrated to affect PI3K activity and phosphorylation. Addition of AA to PC-3 prostate cancer cells has been shown to induce at least ten NF $\kappa$ B-regulated genes, including IL-1 $\beta$ , IL-6, and IL-8. Also, PI3K and Akt, both upstream regulators of the NF $\kappa$ B pathway, have been shown to be activated after AA addition (26). To our knowledge, this is the first paper to demonstrate that altering either concentrations or ratios of circulating  $\omega 6$  and  $\omega 3$  PUFA directly affects the expression of specific PI3K isoforms.

To better understand the effect of this combination of long-chain  $\omega 3$  PUFA and short-chain  $\omega 6$  PUFA on the immune response, message levels of several T helper 1 (Th1), Th2, and Th17 cytokines were measured from circulating mononuclear cells before and after supplementation. This area of immune regulation was examined because several studies have highlighted the connection between T cell phenotypes and PI3K expression/activation. Class IA PI3K has been shown to control the balance of the Th response by inducing the Th2 response (*i.e.* humoral immunity) and/or repressing the Th1 response (27, 28). The Th1 response is thought to be suppressed because PI3K inhibits IL-12 production from dendritic cells. Agonists that induce the production of IL-12 (*e.g.* TLR ligands such as LPS) typically activate PI3K, creating a negative feedback loop to encourage the Th2 antibody-mediated response (29, 30). In addition, PI3K negatively regulates TLR signaling at the first encounter with a pathogen, thereby turning off the Th1 response that is initiated by TLRs upon endotoxin binding (31, 32).

The current study revealed that dietary supplementation had no effect on the Th1 cytokine IL-12 (Fig. 6A) but reduced the Th2 cytokines IL-5 and IL-10 (Fig. 6B). It has been shown that allergy is characterized by an imbalance toward the Th2 response (33); therefore, the current results suggest that altering cytokine profiles is a potential mechanism by which this fish oil/borage oil combination may influence allergic responses.

The Th17 axis was also examined in this study. The current literature suggests Th17 immunity plays an important role in autoimmune diseases including irritable bowel disease, multiple sclerosis, and psoriasis and blocking this cytokine network protects against autoimmune disease. Fig. 6, D and E show that supplementation blocks the expression of IL-1 $\beta$  and IL-23, both cytokines shown to enhance Th17 differentiation. Additionally, supplementation inhibits IL-17, a product of Th17 cells (Fig. 6F). These data suggest that in healthy populations, supplementation alters the profile of cytokines known to participate in inflammatory and autoimmune responses. In combination, changes in signaling molecule expression and cytokine production may provide prolonged changes in inflammatory responses relative to the rapidly re-equilibrating levels of PUFA and their metabolites. This suggests that effects of dietary PUFA differentially impact inflammatory leukocyte functions, as LTB<sub>4</sub> production in neutrophils was more rapidly reversed than kinase levels and cytokine production from mononuclear cells during the washout.

It has been hypothesized that the marked shift in  $\omega 6/\omega 3$  fatty acids in the Western diet over the past three generations may be responsible, in part, for the increase in the risk and incidence of numerous inflammatory diseases including asthma, allergic rhinitis, diabetes, and inflammatory joint diseases. Further it has been suggested that returning PUFA concentrations and ratios in human diets to those that were found during early human development or those found in less developed populations would have anti-inflammatory effects and reduce the incidence of inflammatory diseases in the population. Several mechanisms have been postulated to explain the apparent relationship between altered PUFA ratios and increased inflammation. This report demonstrates, for the first time in humans, that the expression of an early step (PI3K) in signal transduction, as well as several important downstream effectors, are significantly reduced by altering ingestion of PUFA to shift circulating  $\omega 6$  to  $\omega 3$  ratios. Whether this effect is due to higher overall levels of long chain  $\omega 3$  PUFA or short chain  $\omega 6$  PUFA or the ratio of  $\omega 6$  to  $\omega 3$  PUFA is beyond the scope of this *in vivo* study and is presently being examined in our laboratory. However, these data provide evidence that large changes in gene expression are likely an important mechanism by which PUFA exert their potent effects in clinical conditions.

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