

**Plasma levels of the n-3 polyunsaturated fatty acid eicosapentaenoic acid are associated with anti-TNF responsiveness in rheumatoid arthritis, and inhibit the etanercept driven rise in Th17 cell differentiation *in vitro***

*Louisa Jeffery, Helena L Fisk, Philip C Calder, Andrew Filer, Karim Raza, Christopher D Buckley, Iain McInnes, Peter C Taylor and Benjamin A Fisher*

**ABSTRACT**

**Objective**

To determine whether levels of plasma n-3 PUFAs are associated with response to anti-TNF agents in RA, and whether this putative effect may have its basis in altering anti-TNF driven Th17 cell differentiation

**Methods**

Plasma was collected at baseline and after three months of anti-TNF treatment in 22 patients with established RA, and fatty acid composition of the phosphatidylcholine (PC) component measured. CD4+CD25- T cells and monocytes were purified from the blood of healthy donors and co-cultured in the presence of anti-CD3, with or without etanercept, EPA or the control fatty acid, linoleic acid (LA). Expression of IL-17 and IFN $\gamma$  was measured by intracellular staining and flow cytometry.

**Results**

Plasma PC EPA levels, and the EPA/arachidonic acid ratio, correlated inversely with change in DAS28 scores at 3 months (-0.51; p=0.007, and -0.48; p=0.01 respectively), indicating that higher plasma EPA was associated with a greater reduction in DAS28. Plasma PC EPA was positively associated with EULAR

response (P=0.02). An increase in Th17 cells post-therapy has been associated with non-response to anti-TNF. Etanercept increased Th17 frequencies in vitro. Physiological concentrations of EPA, but not LA, prevented this.

## **Conclusion**

EPA status was associated with clinical improvements to anti-TNF therapy in vivo and prevented the effect of etanercept on Th17 cells in vitro. EPA supplementation might be a simple way to improve anti-TNF outcomes in RA patients by suppressing Th17 frequencies.

## **Key Words**

Rheumatoid arthritis, Anti-TNF, polyunsaturated fatty acids, eicosapentaenoic acid, omega-3, Th17

*L Jeffery PhD, A Filer PhD, K Raza PhD, CD Buckley PhD and BA Fisher MD(Res), Rheumatology Research Group and Arthritis Research UK Rheumatoid Arthritis Pathogenesis Centre of Excellence (RACE), University of Birmingham, Birmingham, UK.*

*HL Fisk BSc, PC Calder PhD, Human Development & Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton UK and NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust and University of Southampton, Southampton, UK*

*I McInnes PhD, Glasgow Biomedical Research Centre, University of Glasgow, Glasgow UK*

*PC Taylor PhD, Kennedy Institute of Rheumatology, University of Oxford, Oxford, UK.*

## **Correspondence and reprints:**

Dr Benjamin Fisher

Rheumatology Research Group, Centre for Translational Inflammation Research, Queen Elizabeth Hospital Birmingham, Birmingham, B15 2WB, UK

[b.fisher@bham.ac.uk](mailto:b.fisher@bham.ac.uk)

## Introduction

Anti-TNF agents have revolutionised the management of severe rheumatoid arthritis (RA), but a substantial proportion of patients either fail to respond or do so only partially.<sup>1</sup> The reasons for this remain to be fully elucidated but are likely to include genetic, genomic and environmental factors.<sup>2</sup> In some patients, inadequate response may be mediated by an anti-TNF driven rise in pro-inflammatory Th17 cell differentiation.<sup>3</sup> Understanding the reasons for inadequate response may guide choice of an alternative biological agent for some patients, or optimization of anti-TNF therapy for others, through alteration of environmental factors or selection of synergistic agents.

Long-chain n-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), have long-been considered to have anti-inflammatory and immunomodulatory actions and a number of explanatory mechanisms have been proposed. These include modulation of eicosanoid metabolites by competition with arachidonic acid (AA) and inhibition of cyclooxygenase, alteration of lipid rafts, signaling through receptors such as GPR120 and PPAR $\gamma$ , and generation of pro-resolution mediators.<sup>4:5</sup> N-3 (also known as omega 3) PUFA derive their name from having their first double-bond at the third carbon atom from the methyl end of their fatty acyl chain.<sup>6</sup> They cannot be synthesized *de novo* by animals and most human dietary intake is in the form of plant-derived  $\alpha$ -linolenic acid (18:3n-3).<sup>7:8</sup>  $\alpha$ -linolenic acid is metabolised by a series of desaturation and elongation steps to the longer chain EPA and DHA. This process of conversion into longer-chain PUFA is

poor in humans,<sup>9</sup> and is in direct competition with the desaturation and elongation of the considerably more abundant n-6 (omega 6) PUFAs. Therefore in humans, most EPA and DHA is of dietary origin, with marine foods being especially good sources but having a highly variable intake.

Fish oils contain EPA and DHA. Fish oils ameliorate collagen-induced arthritis,<sup>10-12</sup> and demonstrate modest effects on RA with a consistent reduction in tender joint count, morning stiffness and non-steroidal anti-inflammatory drug (NSAID) usage being observed in multiple small clinical trials when added to conventional DMARD therapy.<sup>13-25</sup> Studies suggest that higher doses of fish oil (e.g. 3-6 g/day) are more effective than lower doses. Beneficial effects continue to be seen when fish oil is added to triple DMARD therapy with a treat-to-target approach in early RA.<sup>26</sup>

In this study we sought to determine whether plasma levels of n-3 fatty acids correlated with response to anti-TNF in RA, and propose and test a mechanistic hypothesis for the heightened responsiveness we observed in association with higher EPA levels in the absence of high dose fish oil supplementation.

## **Materials and Methods**

### **Patients**

Plasma was available for 22 out of 23 patients with established RA who participated in a comparative study of etanercept and infliximab.<sup>27</sup> Etanercept is a p75 TNF receptor-Fc fusion protein and infliximab is a chimeric monoclonal antibody to TNF. Eligible patients were aged  $\geq 18$  years, fulfilled 1987 ACR

classification criteria for RA, were positive for rheumatoid factor or anti-CCP antibodies, had disease duration >6 months and DAS28 >4.0, had previously failed at least one DMARD and were on a stable dose of at least 7.5 mg weekly of methotrexate. No other DMARDs were allowed within the 4 weeks prior to commencing treatment. Participants were randomised to standard dosing of either infliximab 3 mg/kg intravenously at weeks 0, 2, 6 and 10, or subcutaneous etanercept 25 mg twice weekly. Therapy was kept stable for the first 3 months of treatment. The demographics of these patients are presented in Table 1. The study was conducted in compliance with the Helsinki declaration and ethical approval was obtained from the West Glasgow Ethics Committee (06/S0703/64). All subjects gave written informed consent. Clinical trial registration number is ISRCTN44880063.

### **Plasma phosphatidylcholine fatty acids**

Plasma samples from baseline and 3 months were stored at -80°C prior to analysis. After addition of dipentadecanoyl phosphatidylcholine as an internal standard, total lipids were extracted with chloroform and methanol (2:1 vol/vol) and butylated hydroxytoluene added as an antioxidant. Phosphatidylcholine (the major phospholipid in human plasma) was isolated by solid-phase extraction on aminopropyl silica cartridges using chloroform to elute triacylglycerol and cholesteryl ester fractions prior to elution of phosphatidylcholine with chloroform/methanol (60:40 vol:vol). Purified phosphatidylcholine was dissolved in toluene and fatty acid methyl esters were produced by reaction with methanol containing 2% (vol/vol) sulphuric acid at 50°C for two hours. After cooling and neutralization, fatty acid methyl esters were extracted into hexane and separated

on a BPX-70 column fitted to a HP6980 gas chromatograph (Hewlett-Packard, Palo Alto, US). Helium was used as the running gas and fatty acid methyl esters were detected by flame ionization, identified by comparison with previously run standards, and quantified using ChemStation software (Agilent Technologies, Palo Alto, USA). Data were expressed as absolute concentration ( $\mu\text{g/ml}$  plasma), which is related to fatty acid consumption.<sup>28</sup> Long-chain n-3 PUFA were considered to be 20:5n-3 (EPA), docosapentaenoic acid (DPA; 22:5n-3) and 22:6n-3 (DHA).

Activities of desaturase enzymes, and in particular delta-6 desaturase (D6D), are considered to be the rate-limiting steps in the conversion of the precursor n-3 PUFA  $\alpha$ -linolenic acid to the longer chain EPA and DHA, as well as in the parallel metabolism of n-6 PUFA. Activity of desaturase enzymes can be inferred from product to precursor ratios<sup>29</sup>. Product to precursor ratios were used to estimate the activity of the delta-5 (20:4n-6/20:3n-6) and delta-6 18:3n-6/18:2n-6) desaturases.

### **Th17 cell differentiation**

Human CD4+CD25- T cells and autologous monocytes were isolated from peripheral blood mononuclear cells obtained from fresh leukocyte reduction system cones (National Blood Service, Birmingham UK) by ficoll gradient centrifugation. Negative selection reagents (StemCell technologies) were used for the enrichment of monocytes and CD4+CD25- T cells and greater than 95% cell purity attained as confirmed by flow cytometry using antibodies against CD3, CD4 and CD14 (BD). CD25 expression on T cells could not be assessed due to interference of the anti-CD25-TAC reagent used for the cell selection. Efficient depletion of regulatory T cells was therefore determined by staining for FoxP3 and

CD127.

To assess Th17 differentiation, T cells and monocytes were cultured at a ratio of one monocyte to five T cells in the presence of anti-CD3 (OKT3 clone) (0.5 µg/ml). The cultures were prepared in RPMI 1640 medium supplemented with 50 U/ml Penicillin and Streptomycin and 2 mM L-Glutamine (Gibco, Life Technologies) and incubated at 37°C, 95% humidity and 5% CO<sub>2</sub>. After seven days, T cell expression of IL-17 and IFN $\gamma$  was assessed by intracellular cytokine staining and flow cytometry. In brief, T cells were re-stimulated for five hours with PMA (50 ng/ml) and ionomycin (1 µM). Brefeldin A (10 µg/ml) was added during the final four hours to promote cytokine accumulation. Cells were washed with PBS and dead cells labeled with a fixable live/dead discrimination dye (Life technologies) before fixation for 12 minutes in 3% paraformaldehyde. Following one wash with PBS, cells were permeabilised with 0.1% saponin-PBS and labeled with antibodies against CD3 (BD), IL-17 (ebiosciences) and IFN $\gamma$  (BD). To assess the effect of etanercept, EPA and the control n-6 fatty acid, LA, upon Th17 differentiation, the cultures were also supplemented with EPA or LA at 5 µg/ml, with or without etanercept.

### **Statistical analysis**

Comparison of baseline data between subjects randomized to infliximab versus etanercept used the Mann Whitney U test or the independent samples T test depending on distribution of data. Subsequent analyses pooled both groups for a population size of 22. Comparison of fatty acid levels and product/precursor ratios between baseline and week 12 were undertaken using the Wilcoxon Signed Rank test. Correlations of plasma fatty acid levels at baseline, week 12 and the mean of these time points (mean value considered the primary dependent variable of

interest) with change in DAS28 were analysed using Spearman's rho. The relationship of EULAR outcomes to EPA levels, and of EPA tertiles with change in DAS28 and its constituents, was tested by one-way ANOVA. Analyses of the clinical and *in vitro* data were undertaken using SPSS 20 and Graphpad Prism 5 respectively.

## **Results**

### **Baseline and 12 weeks**

Baseline demographics are shown in Table 1 and the two treatment groups were not statistically different. In order to determine the effect of anti-TNF on desaturase activity, we compared fatty acid profiles from baseline and week 12 of treatment, with a view to using mean values across time points to give a more robust biomarker of PUFA status over the time course of the study. Interestingly there was a trend towards a reduction in the 18:3n-6/18:2n-6 ratio following treatment with anti-TNF ( $p=0.05$ ) which might indicate a reduction in D6D activity after 12 weeks, being accompanied by a lower mean 18:3n-6 ( $p=0.08$ ) product, 0.60  $\mu\text{g/ml}$  (IQR 0.52, 1.15) versus 0.51 (IQR 0.47, 0.62), and a higher level of 22:4n-6 (adrenic acid;  $p=0.039$ ); 0.15  $\mu\text{g/ml}$  (IQR 0.11, 0.21) versus 0.19 (IQR 0.12, 0.27). Adrenic acid was the last measurable n-6 PUFA prior to the second delta-6 desaturase catalysed step. However no significant differences were observed in any other fatty acid after anti-TNF treatment, including all the longer chain n-3 fatty acids (data not shown), and therefore subsequent analyses used the average of baseline and week 12 measurements.

### **Relationship of plasma phosphatidylcholine fatty acids with change in DAS28**



Table 2 shows the correlations between individual fatty acids, total n-3, n-3/n-6 and EPA/AA ratios, and desaturase product/precursor ratios with change in DAS28 after 3 months of anti-TNF therapy. The most significant correlations with time-averaged levels were seen with EPA (-0.51; p=0.007) and the EPA/AA (20:4n-6) ratio (-0.48; p=0.01) indicating that higher plasma levels of EPA were associated with a greater reduction in DAS28 at 3 months after anti-TNF. This association with EPA was still statistically significant when restricting the analysis to subjects treated with etanercept (r=-0.54; p=0.04), with a trend seen in the infliximab group (r=-0.42; p=0.10).

### **Relationship of plasma phosphatidylcholine EPA levels with EULAR response and components of DA28**

At 12 weeks, 6 subjects were EULAR non-responders,<sup>30</sup> and 13 and 3 were moderate and good responders, respectively. Median EPA levels in these groups were 14.8, 19.1 and 29.4 µg/ml respectively (p=0.02; Figure 1). EPA levels were next divided into tertiles and the highest tertile was associated with a larger fall in DAS28 scores at 12 weeks (p=0.03; Figure 1). Figure 1 also shows the four components that comprise the DAS28 score, in relation to tertiles of EPA, with trends being observed for ESR (p=0.07) and tender joint count (p=0.15).

### **Effect of etanercept and EPA on Th17 differentiation**

Poor responses to anti TNF therapy have been associated with increased IL-17 production and the frequency of Th17 cells.<sup>3;31</sup> Therefore, using our established monocyte-driven T cell stimulation system to study Th17 differentiation *in vitro*,<sup>32</sup> we investigated the effect of anti-TNF upon Th17 differentiation. At

pharmacologically-relevant concentrations, etanercept promoted a significant, 1.6 fold increase in the frequency of cells expressing IL-17 (Figure 2A and B). This increase in frequency included cells that expressed IL-17 alone, as well as those that expressed IL-17 together with IFN $\gamma$ . By contrast the frequency of cells that expressed IFN $\gamma$  alone was not affected (figure 2B). Treatment with infliximab did not significantly increase Th17 frequencies in this assay (data not shown). Given that elevated levels of EPA were associated with improved responses to anti-TNF, we were interested to see if this could involve downregulation of IL-17 by EPA. Thus we repeated monocyte-driven T cell stimulations in the presence of EPA or the control n-6 fatty acid, LA. Neither EPA nor LA affected the frequency of Th17 cells relative to ethanol carrier control. Importantly, EPA but not LA prevented the etanercept induced increase in Th17 frequency (figure 3).

## **Discussion**

In this cohort of RA patients receiving either etanercept or infliximab, higher plasma levels of EPA were associated with a greater reduction in DAS28 scores following treatment compared to patients with lower plasma EPA. These differences were more significant in patients receiving etanercept, and it is possible that differences in construct, avidity and immunogenicity may influence these findings<sup>33</sup>. Our observed association may relate to the modest benefits previously seen with n-3 PUFA supplementation in RA patients, although a key difference is that there was no n-3 PUFA supplementation in our study.<sup>13-15</sup> We therefore hypothesized that higher levels of EPA may have additional mechanisms relevant to anti-TNF treatment. One potentially detrimental effect of anti-TNF

therapy is a variable increase in Th17 cells. This is thought to result from the reversal of TNF-mediated p40-suppression, p40 being a subunit of IL-23 which is important in Th17 cell differentiation.<sup>34</sup> Higher production of IL-17 has been associated with non-response to anti-TNF.<sup>3;31</sup> and there is currently great interest in dual targeting of TNF and IL-17, to optimize biological responses in the face of cytokine compensation.<sup>35</sup> Were EPA to suppress Th17 differentiation in the context of anti-TNF therapy, supplementation with fish oil might present an alternative, and potentially safer, combination approach.

We were able to replicate this anti-TNF-mediated effect on Th17 frequencies *in vitro*, using a co-culture assay of CD4<sup>+</sup>CD25<sup>-</sup> T cells stimulated with autologous monocytes. Addition of etanercept, a TNF-receptor fusion protein, resulted in increased frequencies of IL-17<sup>+</sup> cells in a dose-dependent manner. Addition of EPA, but not the n-6 LA control, prevented this etanercept-driven increase in Th17 frequency.

The mechanism behind this observation has not been established, but eicosanoid metabolites of the n-6 fatty acid AA have also been associated with promotion of Th17 generation. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) stimulates IL-23 and IL-1 $\beta$  production by macrophages and dendritic cells whilst inhibiting IL-12, and it also increases the expression of IL-23 and IL-1 $\beta$  receptors on T cells, so regulating Th17 differentiation.<sup>36</sup> EPA competes with AA for the prostaglandin-generating COX enzymes, has an inhibitory effect on this enzyme, and yields the generally less pro-inflammatory PGE<sub>3</sub>.<sup>37</sup> If alteration of eicosanoid metabolites does influence response to anti-TNF therapy, then one might also expect synergism between anti-TNF drugs and non-steroidal anti-inflammatory drugs (NSAIDs). Notably, some

registry studies have indeed found the use of NSAIDs to predict better response to anti-TNF,<sup>38;39</sup> although this association was not considered at the time to be causal, and other studies have not confirmed this.<sup>40</sup> Against this eicosanoid hypothesis, might be the observation that inhibition of PGE<sub>2</sub> production by monocytes *ex vivo* required a relatively high intake of EPA in a dose-response study, although this was conducted in healthy volunteers rather than patients with RA<sup>41</sup>

Other mechanisms may also be important. In mouse models of colitis, n-3 fatty acids have recently been demonstrated to reduce Th17 cell numbers,<sup>42;43</sup> thought in part to reflect reduced membrane-raft responsiveness to the Th17 polarising cytokine IL-6.<sup>44</sup> Indeed, it is possible that the ability of Methotrexate to reduce IL-6 levels may in part explain the benefits of combining this drug with anti-TNF<sup>45</sup>.

Plasma phosphatidylcholine fatty acids are a better indicator of dietary intake in comparison with dietary questionnaires due to the measurement error inherent in the latter<sup>46</sup>, but only reflect dietary intake over the preceding few days or weeks<sup>47-50</sup>. We therefore used the average of baseline and week 12 measurements in order to obtain a more robust biomarker of PUFA status. It should be noted that an individual's long-chain n-3 PUFA status may be influenced not just by dietary n-3 intake, but also by the amount of n-6 PUFA in the diet, as these compete for the same desaturase and elongase enzymes, and by polymorphisms in the desaturase enzymes<sup>29</sup>. Although our primary analysis focused on time-averaged plasma fatty acids, it is of interest that week 12 levels of docosapentaenoic acid (DPA) were also negatively correlated with DAS28 levels. The effect of DPA on inflammation is relatively under investigated compared with EPA and DHA, but a recent study of complete Freund's adjuvant-induced arthritis in rats found that monoglyceride

EPA or DPA, but not DHA, reduced paw swelling, arthritis score and levels of pro-inflammatory cytokines<sup>51</sup>. Further investigation of DPA anti-inflammatory effects would be warranted.

D6D is considered a rate-limiting step in long-chain PUFA metabolism. The transcription and therefore activity of D6D may be upregulated by insulin and statins<sup>52</sup> and downregulated by long-chain PUFA,<sup>53</sup> glucagon, adrenaline, steroids and smoking.<sup>54;55</sup> Interestingly, there is a recognized association of D6D activity and later development of type 2 diabetes, independent of disturbances in glucose metabolism.<sup>56</sup> The reason for this is not clear, but inflammation is a risk factor for insulin resistance<sup>57</sup> and our data implies that inflammation may also increase D6D activity, providing a possible explanation for this association. Our findings are consistent with the observation that TNF $\alpha$  increases hepatic expression of sterol regulatory element binding protein-1c (SREBP-1c) in mice,<sup>58</sup> since SRBP-1c plays a key role in upregulating D6D gene transcription.<sup>53</sup> The alteration in D6D activity we observed does not explain the association of anti-TNF response with EPA levels, as this would predict an opposite relationship, baseline levels of EPA are also predictive of response, and D6D activity plays only a small role in the conversion of longer chain n-3 fatty acids,<sup>9;56</sup> as confirmed by the comparison of fatty acid levels pre- and post-anti-TNF. Furthermore baseline levels of EPA were also predictive of response. The desaturase activity inferred from these fatty acid ratios generally reflects conversion in the liver,<sup>4</sup> however monocytes also demonstrate D6D and D5D activity,<sup>55;59;60</sup> and we cannot rule out the possibility that an alteration of desaturase activity may be more pronounced in these cells,

which rely on AA, a long-chain n-6 PUFA, for eicosanoid production following stimulation.

There are some limitations in this study. The sample size is small and therefore the study requires replication. In view of the sample size and the pilot nature of this data, we did not apply a Bonferroni correction. We did not measure the fatty acid profile in mononuclear cell membranes, which arguably may be more physiologically relevant, although these correlate well with plasma levels.<sup>61</sup> Finally, a causal relationship cannot be assumed from this data. The strengths are a well-characterised population with a biomarker of fatty acid intake at more than one time point.

In summary, we have presented an association of EPA levels with anti-TNF response in RA. If replicated in further studies and causality confirmed, dietary modulation may provide a simple method for improving outcomes with this therapy.

**Funding:** Unrestricted grant from Schering-Plough (now Merck) for the clinical trial upon which this study is based.

**Conflicts of Interest:** None to declare

## Reference List

- (1) Nam JL, Ramiro S, Gaujoux-Viala C, Takase K, Leon-Garcia M, Emery P et al. Efficacy of biological disease-modifying antirheumatic drugs: a systematic literature review informing the 2013 update of the EULAR recommendations for the management of rheumatoid arthritis. *Ann Rheum Dis* 2014; 73:516-528.
- (2) van den Broek M, Visser K, Allaart CF, Huizinga TW. Personalized medicine: predicting responses to therapy in patients with RA. *Curr Opin Pharmacol* 2013; 13:463-469.
- (3) Alzabin S, Abraham SM, Taher TE, Palfreeman A, Hull D, McNamee K et al. Incomplete response of inflammatory arthritis to TNFalpha blockade is associated with the Th17 pathway. *Ann Rheum Dis* 2012; 71:1741-1748.
- (4) Calder PC. Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? *Br J Clin Pharmacol* 2013; 75:645-662.
- (5) Calder PC. Fatty acids and inflammation: the cutting edge between food and pharma. *Eur J Pharmacol* 2011; 668 Suppl 1:S50-8.
- (6) Ratnayake WM, Galli C. Fat and fatty acid terminology, methods of analysis and fat digestion and metabolism: a background review paper. *Ann Nutr Metab* 2009; 55:8-43.
- (7) Kris-Etherton PM, Taylor DS, Yu-Poth S, Huth P, Moriarty K, Fishell V et al. Polyunsaturated fatty acids in the food chain in the United States. *Am J Clin Nutr* 2000; 71 Suppl 1:179S-188S.
- (8) Williams CM, Burdge G. Long-chain n-3 PUFA: plant v. marine sources. *Proc Nutr Soc* 2006; 65:42-50.
- (9) Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am J Clin Nutr* 2006; 83 Suppl 6:1467S-1476S.
- (10) Leslie CA, Gonnerman WA, Ullman MD, Hayes KC, Franzblau C, Cathcart ES. Dietary fish oil modulates macrophage fatty acids and decreases arthritis susceptibility in mice. *J Exp Med* 1985; 162:1336-1349.
- (11) Leslie CA, Conte JM, Hayes KC, Cathcart ES. A fish oil diet reduces the severity of collagen induced arthritis after onset of the disease. *Clin Exp Immunol* 1988; 73:328-332.
- (12) Ierna M, Kerr A, Scales H, Berge K, Griinari M. Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. *BMC Musculoskelet Disord* 2010; 11:136.

- (13) Fortin PR, Lew RA, Liang MH, Wright EA, Beckett LA, Chalmers TC et al. Validation of a meta-analysis: the effects of fish oil in rheumatoid arthritis. *J Clin Epidemiol* 1995; 48:1379-1390.
- (14) Kremer JM. n-3 fatty acid supplements in rheumatoid arthritis. *Am J Clin Nutr* 2000; 71 Suppl 1:349S-351S.
- (15) Cleland LG, James MJ, Proudman SM. The role of fish oils in the treatment of rheumatoid arthritis. *Drugs* 2003; 63:845-853.
- (16) Kremer JM, Lawrence DA, Petrillo GF, Litts LL, Mullaly PM, Rynes RI et al. Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal antiinflammatory drugs. Clinical and immune correlates. *Arthritis Rheum* 1995; 38:1107-1114.
- (17) Kremer JM, Lawrence DA, Jubiz W, DiGiacomo R, Rynes R, Bartholomew LE et al. Dietary fish oil and olive oil supplementation in patients with rheumatoid arthritis. Clinical and immunologic effects. *Arthritis Rheum* 1990; 33:810-820.
- (18) Cleland LG, French JK, Betts WH, Murphy GA, Elliott MJ. Clinical and biochemical effects of dietary fish oil supplements in rheumatoid arthritis. *J Rheumatol* 1988; 15:1471-1475.
- (19) Kremer JM, Bigauoette J, Michalek AV, Timchalk MA, Lininger L, Rynes RI et al. Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. *Lancet* 1985; 1:184-187.
- (20) Kremer JM, Jubiz W, Michalek A, Rynes RI, Bartholomew LE, Bigauoette J et al. Fish-oil fatty acid supplementation in active rheumatoid arthritis. A double-blinded, controlled, crossover study. *Ann Intern Med* 1987; 106:497-503.
- (21) Adam O, Beringer C, Kless T, Lemmen C, Adam A, Wiseman M et al. Anti-inflammatory effects of a low arachidonic acid diet and fish oil in patients with rheumatoid arthritis. *Rheumatol Int* 2003; 23:27-36.
- (22) Galarraga B, Ho M, Youssef HM, Hill A, McMahon H, Hall C et al. Cod liver oil (n-3 fatty acids) as a non-steroidal anti-inflammatory drug sparing agent in rheumatoid arthritis. *Rheumatology (Oxford)* 2008; 47:665-669.
- (23) Volker D, Fitzgerald P, Major G, Garg M. Efficacy of fish oil concentrate in the treatment of rheumatoid arthritis. *J Rheumatol* 2000; 27:2343-2346.
- (24) Geusens P, Wouters C, Nijs J, Jiang Y, Dequeker J. Long-term effect of omega-3 fatty acid supplementation in active rheumatoid arthritis. A 12-month, double-blind, controlled study. *Arthritis Rheum* 1994; 37:824-829.
- (25) Nielsen GL, Faarvang KL, Thomsen BS, Teglbjaerg KL, Jensen LT, Hansen TM et al. The effects of dietary supplementation with n-3 polyunsaturated fatty acids in patients with rheumatoid arthritis: a randomized, double blind trial. *Eur J Clin Invest* 1992; 22:687-691.



- (26) Proudman SM, James MJ, Spargo LD, Metcalf RG, Sullivan TR, Rischmueller M et al. Fish oil in recent onset rheumatoid arthritis: a randomised, double-blind controlled trial within algorithm-based drug use. *Ann Rheum Dis* 2015;74:89-95.
- (27) Fisher BA, Donatien P, Filer A, Winlove CP, McInnes IB, Buckley CD and Taylor PC. Decrease in articular hypoxia and synovial blood flow at early time points following infliximab and etanercept treatment in rheumatoid arthritis. 2016 Sep 8.
- (28) Browning LM, Walker CG, Mander AP, West AL, Madden J, Gambell JM et al. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. *Am J Clin Nutr* 2012; 96:748-758.
- (29) Tosi F, Sartori F, Guarini P, Olivieri O, Martinelli N. Delta-5 and delta-6 desaturases: crucial enzymes in polyunsaturated fatty acid-related pathways with pleiotropic influences in health and disease. *Adv Exp Med Biol* 2014; 824:61-81.
- (30) Fransen J, van Riel PL. The Disease Activity Score and the EULAR response criteria. *Clin Exp Rheumatol* 2005; 23 Suppl 39:S93-S99.
- (31) Chen DY, Chen YM, Chen HH, Hsieh CW, Lin CC, Lan JL. Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF-alpha therapy. *Arthritis Res Ther* 2011; 13:R126.
- (32) Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J Immunol* 2009; 183:5458-5467.
- (33) Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther* 2008; 117:244-279.
- (34) Notley CA, Inglis JJ, Alzabin S, McCann FE, McNamee KE, Williams RO. Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells. *J Exp Med* 2008; 205:2491-2497.
- (35) Taylor PC, Williams RO. Editorial: combination cytokine blockade: the way forward in therapy for rheumatoid arthritis? *Arthritis Rheumatol* 2015; 67:14-16.
- (36) Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK et al. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med* 2009; 206:535-548.

- (37) Calder PC. Polyunsaturated fatty acids and inflammation. *Prostaglandins Leukot Essent Fatty Acids* 2006; 75:197-202.
- (38) Hyrich KL, Watson KD, Silman AJ, Symmons DP. Predictors of response to anti-TNF-alpha therapy among patients with rheumatoid arthritis: results from the British Society for Rheumatology Biologics Register. *Rheumatology (Oxford)* 2006; 45:1558-1565.
- (39) Kristensen LE, Kapetanovic MC, Gulfe A, Soderlin M, Saxne T, Geborek P. Predictors of response to anti-TNF therapy according to ACR and EULAR criteria in patients with established RA: results from the South Swedish Arthritis Treatment Group Register. *Rheumatology (Oxford)* 2008; 47:495-499.
- (40) Kleinert S, Tony HP, Krause A, Feuchtenberger M, Wassenberg S, Richter C et al. Impact of patient and disease characteristics on therapeutic success during adalimumab treatment of patients with rheumatoid arthritis: data from a German noninterventional observational study. *Rheumatol Int* 2012; 32:2759-2767.
- (41) Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. *Am J Clin Nutr* 2006; 83:331-342.
- (42) Monk JM, Jia Q, Callaway E, Weeks B, Alaniz RC, McMurray DN et al. Th17 cell accumulation is decreased during chronic experimental colitis by (n-3) PUFA in Fat-1 mice. *J Nutr* 2012; 142:117-124.
- (43) Monk JM, Hou TY, Turk HF, Weeks B, Wu C, McMurray DN et al. Dietary n-3 polyunsaturated fatty acids (PUFA) decrease obesity-associated Th17 cell-mediated inflammation during colitis. *PLoS One* 2012; 7:e49739.
- (44) Allen MJ, Fan YY, Monk JM, Hou TY, Barhoumi R, McMurray DN et al. n-3 PUFAs reduce T-helper 17 cell differentiation by decreasing responsiveness to interleukin-6 in isolated mouse splenic CD4(+) T cells. *J Nutr* 2014; 144:1306-1313.
- (45) Kremer JM, Lawrence DA, Hamilton R, McInnes IB. Long-term study of the impact of methotrexate on serum cytokines and lymphocyte subsets in patients with active rheumatoid arthritis: correlation with pharmacokinetic measures. *RMD Open* 2016; 2:e000287.
- (46) Arab L. Biomarkers of fat and fatty acid intake. *J Nutr* 2003; 133 Suppl 3:925S-932S.
- (47) Kohlmeier L. Future of dietary exposure assessment. *Am J Clin Nutr* 1995; 61 Suppl 3:702S-709S.
- (48) Corrocher R, Pagnan A, Ambrosio GB, Ferrari S, Olivieri O, Guarini P et al. Effects induced by olive oil-rich diet on erythrocytes membrane lipids and

- sodium-potassium transports in postmenopausal hypertensive women. *J Endocrinol Invest* 1992; 15:369-376.
- (49) Dougherty RM, Galli C, Ferro-Luzzi A, Iacono JM. Lipid and phospholipid fatty acid composition of plasma, red blood cells, and platelets and how they are affected by dietary lipids: a study of normal subjects from Italy, Finland, and the USA. *Am J Clin Nutr* 1987; 45:443-455.
- (50) Hodson L, Skeaff CM, Fielding BA. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog Lipid Res* 2008; 47:348-380.
- (51) Morin C, Blier PU, Fortin S. Eicosapentaenoic acid and docosapentaenoic acid monoglycerides are more potent than docosahexaenoic acid monoglyceride to resolve inflammation in a rheumatoid arthritis model. *Arthritis Res Ther* 2015; 17:142.
- (52) Rise P, Colombo C, Galli C. Effects of simvastatin on the metabolism of polyunsaturated fatty acids and on glycerolipid, cholesterol, and de novo lipid synthesis in THP-1 cells. *J Lipid Res* 1997; 38:1299-1307.
- (53) Nakamura MT, Nara TY. Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu Rev Nutr* 2004; 24:345-376.
- (54) Brenner RR. Hormonal modulation of delta6 and delta5 desaturases: case of diabetes. *Prostaglandins Leukot Essent Fatty Acids* 2003; 68:151-162.
- (55) Ghezzi S, Rise P, Ceruti S, Galli C. Effects of cigarette smoke on cell viability, linoleic acid metabolism and cholesterol synthesis, in THP-1 cells. *Lipids* 2007; 42:629-636.
- (56) Kroger J, Zietemann V, Enzenbach C, Weikert C, Jansen EH, Doring F et al. Erythrocyte membrane phospholipid fatty acids, desaturase activity, and dietary fatty acids in relation to risk of type 2 diabetes in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Am J Clin Nutr* 2011; 93:127-142.
- (57) Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. *J Clin Invest* 2006; 116:1793-1801.
- (58) Endo M, Masaki T, Seike M, Yoshimatsu H. TNF-alpha induces hepatic steatosis in mice by enhancing gene expression of sterol regulatory element binding protein-1c (SREBP-1c). *Exp Biol Med (Maywood)* 2007; 232:614-621.
- (59) Arbo I, Halle C, Malik D, Brattbakk HR, Johansen B. Insulin induces fatty acid desaturase expression in human monocytes. *Scand J Clin Lab Invest* 2011; 71:330-339.
- (60) Rise P, Ghezzi S, Carissimi R, Mastromauro F, Petroni A, Galli C. Delta5 desaturase mRNA levels are increased by simvastatin via SREBP-1 at early

stages, not via PPARalpha, in THP-1 cells. *Eur J Pharmacol* 2007; 571:97-105.

- (61) Kew S, Banerjee T, Minihane AM, Finnegan YE, Williams CM, Calder PC. Relation between the fatty acid composition of peripheral blood mononuclear cells and measures of immune cell function in healthy, free-living subjects aged 25-72 y. *Am J Clin Nutr* 2003; 77:1278-1286.

**Figure 1.** Time-averaged plasma EPA levels in patients who were EULAR non-responders, moderate and good responders at 3 months. Relationship between tertiles of time-averaged plasma EPA, and change in Disease Activity Score 28 (DAS28) and its four constituents [28 swollen and tender joint count (28SJC and 28TJC), patient visual analogue scale for global disease activity (VAS), and erythrocyte sedimentation rate (ESR)], and Health Assessment Questionnaire (HAQ) at 3 months is also shown.

**Figure 2:** Etanercept increases the frequency of IL-17+ T cells. CD4+CD25- T cells were stimulated with autologous monocytes for seven days, under increasing concentrations of etanercept and the frequency of cells expressing IL-17 and IFN $\gamma$  measured by flow cytometry. Representative data for control and 2.5 $\mu$ g/ml etanercept are shown in **A** and relative frequencies of IL-17+ and IFN $\gamma$ + cells across etanercept concentrations 0-10  $\mu$ g/ml are summarised in **B** for five donors. Significance was tested by ANOVA with Bonferroni post-hoc analysis. Stars and crosses indicate significance with respect to minus etanercept. Stars are used for IL-17+IFN $\gamma$ - and crosses for IL-17+IFN $\gamma$ + cells \* P<0.05, \*\* P<0.01, \*\*\*P<0.001.

**Figure 3:** Eicosapentaenoic acid inhibits induction of IL-17+ T cells by Etanercept. CD4+CD25- T cells were stimulated with autologous monocytes for seven days in the presence of ethanol (carrier control), 5 µg/ml Linoleic acid (LA) (control fatty acid) or 5 µg/ml Eicosapentaenoic acid (EPA) with or without 2.5 µg/ml Etanercept (Et). The frequency of IL-17+ T cells was measured by flow cytometry. Bars show the mean frequencies for seven donors. Error bars indicate standard deviation. Significance was tested using paired T tests.

Figure 1

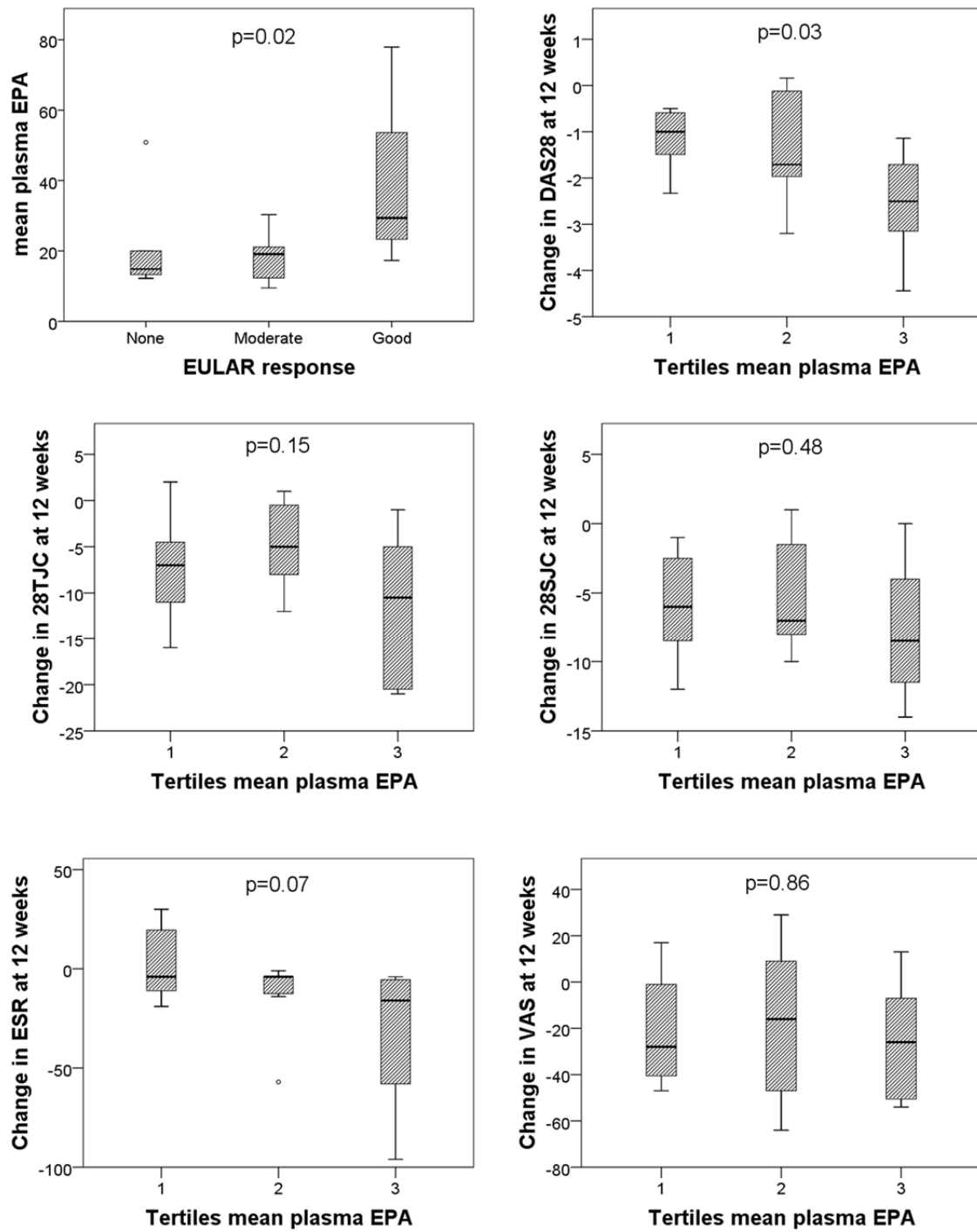


Figure 2

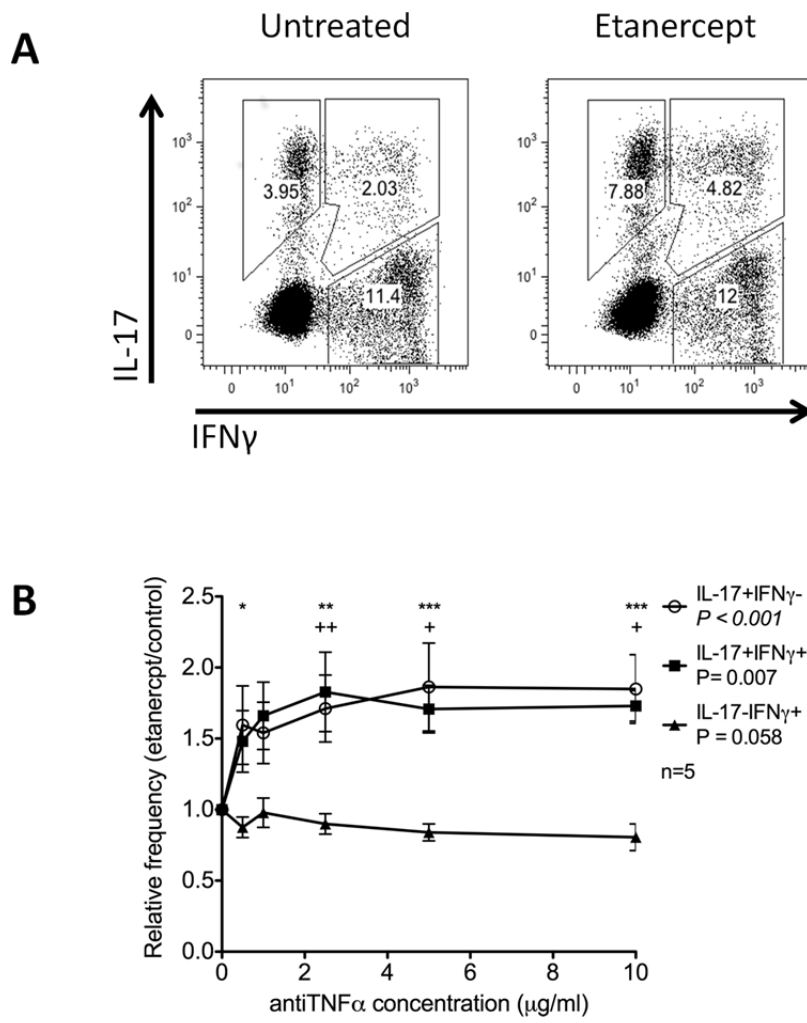


Figure 3

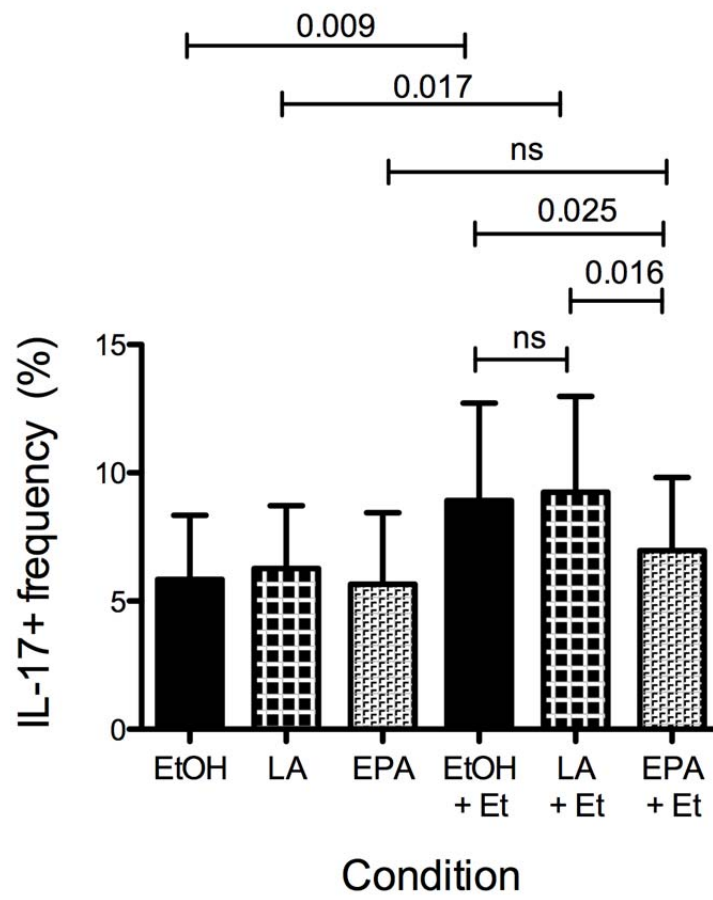




Table 1. Patient demographics at study entry. P-values compare the infliximab and etanercept arms. RF; rheumatoid factor. CCP; cyclic citrullinated peptides. DAS28; Disease Activity Score 28. HAQ; Health Assessment Questionnaire. ESR; erythrocyte sedimentation rate.

	Etanercept	Infliximab	All	p value
N	11	11	22	
Age (y) <sup>1</sup>	53.5 (15.3)	52.5 (9.6)	53.0 (12.5)	0.85
Disease duration (y) <sup>2</sup>	12 (7,15)	10 (2,20)	10 (4, 16.3)	0.22
Methotrexate dose (mg/week) <sup>1</sup>	14.3 (6.0)	16.6 (4.5)	15.5 (5.3)	0.33
RF <sup>3</sup>	9 (82)	8 (73)	17 (77)	1.00
RF (dilution) <sup>2</sup>	160 (40,640)	80 (0,640)	120 (30, 640)	0.75
Anti-CCP <sup>3</sup>	10 (91)	8 (73)	18 (82)	0.59
Baseline DAS28 <sup>1</sup>	6.38 (0.65)	6.00 (1.45)	6.19 (1.12)	0.44
Change in DAS28 at 12 weeks <sup>1</sup>	-1.80 (1.31)	-1.59 (1.12)	-1.69 (1.19)	0.69
HAQ <sup>1</sup>	1.73 (0.68)	1.61 (0.42)	1.67 (0.56)	0.12
ESR (mm/hr) <sup>2</sup>	26 (15, 52)	31 (16, 48)	27.5 (15.8, 49)	0.70
<sup>1</sup> mean (sd); <sup>2</sup> median (IQR); <sup>3</sup> number of subjects positive (%)				

Table 2. Correlations (Spearman's rho) between plasma phosphatidylcholine n-3 and n-6 fatty acids ( $\mu\text{g/ml}$ ) at baseline, week 12 and the averaged concentration from both time points, with change in DAS28 score following 12 weeks of anti-TNF therapy. Long-chain n-3 PUFA were considered to be 20:5n-3 (EPA), 22:5n-3 and 22:6n-3 (DHA). Product to precursor ratios were used to estimate the activity of the  $\Delta 5$  (20:4n-6/20:3n-6) and  $\Delta 6$  (18:3n-6/18:2n-6) desaturases.

Plasma fatty acids	Correlation of plasma fatty acids with change in DAS28 (p value)		
	Baseline	Week 12	Mean
18:2n-6	0.408* (0.03)	-0.117 (0.30)	0.125 (0.29)
18:3n-6	0.031 (0.45)	0.211 (0.17)	0.162 (0.24)
18:3n-3	0.138 (0.27)	0.216 (0.17)	0.162 (0.24)
20:2n-6	0.181 (0.21)	-0.158 (0.24)	0.013 (0.48)
20:3n-6	0.072 (0.38)	0.034 (0.44)	0.067 (0.38)
20:4n-6	0.029 (0.45)	0.062 (0.39)	-0.001 (0.50)
20:4n-3	0.072 (0.38)	-0.303 (0.09)	-0.186 (0.20)
20:5n-3	-0.324 (0.07)	-0.394* (0.04)	-0.513** (0.007)
22:4n-6	0.132 (0.28)	-0.068 (0.38)	0.050 (0.41)
22:5n-3	0.043 (0.42)	-0.368* (0.046)	-0.269 (0.11)
22:6n-3	-0.137 (0.27)	-0.113 (0.31)	-0.167 (0.23)
Total n-3 PUFA	-0.187 (0.20)	-0.360 (0.05)	-0.366* (0.047)
Total long-chain n-3 PUFA	-0.190 (0.20)	-0.373* (0.04)	-0.425* (0.02)
Total n-6 PUFA	0.301 (0.09)	-0.066 (0.39)	0.144 (0.26)
n-3/n-6 PUFA	-0.435* (0.02)	-0.178 (0.21)	-0.363* (0.048)

EPA/AA	-0.234 (0.15)	-0.535** (0.005)	-0.478* (0.01)
$\Delta$ 5 desaturase activity	0.001 (0.50)	0.128 (0.29)	0.058 (0.40)
$\Delta$ 6 desaturase activity	-0.241 (0.14)	0.047 (0.42)	-0.125 (0.29)