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Symposium on 'The challenge of translating nutrition research into public health nutrition'

Session 3: Joint Nutrition Society and Irish Nutrition and Dietetic Institute Symposium on 'Nutrition and autoimmune disease' PUFA, inflammatory processes and rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease manifested by swollen and painful joints, bone erosion and functional impairment. The joint lesions are characterised by infiltration of T lymphocytes, macrophages and B lymphocytes into the synovium and by synovial inflammation involving eicosanoids, cytokines and matrix metalloproteinases. In relation to inflammatory processes, the main fatty acids of interest are the *n*-6 PUFA arachidonic acid, which is the precursor of inflammatory eicosanoids such as PGE_2 and leukotriene B_4, and the *n*-3 PUFA EPA and DHA, which are found in oily fish and fish oils. Eicosanoids derived from the *n*-6 PUFA arachidonic acid play a role in RA, and the efficacy of non-steroidal anti-inflammatory drugs in RA indicates the importance of pro-inflammatory cyclooxygenase pathway products of arachidonic acid in the pathophysiology of the disease. EPA and DHA inhibit arachidonic acid metabolism to inflammatory eicosanoids. EPA also gives rise to eicosanoid mediators that are less inflammatory than those produced from arachidonic acid and both EPA and DHA give rise to resolvins that are anti-inflammatory and inflammation resolving. In addition to modifying the lipid mediator profile, *n*-3 PUFA exert effects on other aspects of immunity relevant to RA such as antigen presentation, T-cell reactivity and inflammatory cytokine production. Fish oil has been shown to slow the development of arthritis in an animal model and to reduce disease severity. Randomised clinical trials have demonstrated a range of clinical benefits in patients with RA that include reducing pain, duration of morning stiffness and use of non-steroidal anti-inflammatory drugs.

Cytokine: Eicosanoid: Fatty acid: Fish oil: Inflammation

Immune system overview

The immune system is responsible for the host's response to the presence of bacteria, viruses, fungi and parasites; it is also involved in protection against growth of certain tumours and in the response to injury and trauma. The immune system acts to distinguish between 'self' and 'non-self', permitting tolerance to self antigens and to non-threatening environmental agents such as food proteins and commensal gut bacteria. The system has two functional divisions: the innate (or natural) immune system and the acquired (also termed specific or adaptive) immune system. Both components involve various blood-borne factors and cells. Immune cells originate in bone marrow and are found circulating in the bloodstream, organised into lymphoid organs such as the thymus, spleen, lymph nodes and gut-associated lymphoid tissue or dispersed in other locations. The immune response is typified by cellular

Abbreviations: HLA, human leucocyte antigen; IFN, interferon; LT, leukotriene; NSAID, non-steroidal anti-inflammatory drug; RA, rheumatoid arthritis.

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interactions and by the movement of cells to sites of infection or other immune activity. The four key functional activities of the immune response are:

to act as an exclusion barrier;
to distinguish self from non-self;
to develop tolerance to, or to eliminate the source of, non-self antigens;
to retain memory of immunological encounters.

In order to allow effective functioning of the immune system many different cell types, each specialised in a limited range of functions, are involved. These cells work in a coordinated integrated manner in order to assure a successful immune response.

Loss of tolerance can lead to disease and to adverse patient outcome. For example, autoimmune diseases result from loss of tolerance to self antigens, allergic diseases result from loss of tolerance to normally benign environmental or food components and inflammatory bowel diseases result from loss of tolerance to commensal gut bacteria. The loss of tolerance leads to an immunological response that is damaging to the host.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects about 1% of the adult population and is more common in women than in men[1]. RA is characterised by symmetric polyarthritis[1]. Joint inflammation is manifested by swelling, pain, functional impairment, morning stiffness, osteoporosis and muscle wasting. Erosion of bone occurs commonly in the joints of the hands and feet. The joint lesions are characterised by infiltration of activated T lymphocytes, macrophages and antibody-secreting B lymphocytes into the synovium (the tissue lining the joints) and by proliferation of fibroblast-like synovial cells called synoviocytes[1,2]. These cells and new blood vessels form a tissue termed pannus that leads to progressive destruction of cartilage and bone, which is most likely to be a result of cytokine- and eicosanoid-mediated induction of destructive enzymes such as matrix metalloproteinases. RA is also characterised by signs of systemic inflammation, such as elevated plasma concentrations of some cytokines (e.g. IL-6), acute-phase proteins and rheumatoid factors.

Genetic studies have linked susceptibility to, and severity of, RA to genes in the MHC II locus; in human subjects these genes encode the human leucocyte antigen (HLA) II proteins involved in antigen presentation. RA is associated with specific alleles of the HLA-DRB1 gene, although other HLA-DR alleles may also play a role[3]. As the function of HLA-DR is antigen presentation to T lymphocytes, the genetic association indicates a role for T-cells in RA[4]. In total the HLA region contributes 30–50% of the genetic component of RA. The second largest genetic risk for RA lies with a variant in the protein tyrosine phosphatase non-receptor 22 gene, which encodes an intracellular protein tyrosine phosphatase[3]. The variant may act to reduce the ability to down regulate activated T-cells. Recently, novel risk loci have been described[3].

Synovial fluid from patients with RA contains high levels of pro-inflammatory cytokines including TNFα, IL-1β, IL-6, IL-8 and granulocyte–macrophage colony-stimulating factor[5]. Synovial cells cultured *ex vivo* spontaneously produce TNFα, IL-1β, IL-6, IL-8 and granulocyte–macrophage colony-stimulating factor for extended periods of time[5]. Synovial fluid from patients with RA also contains high levels of anti-inflammatory cytokines such as transforming growth factor β, IL-10, IL-1 receptor antagonist and soluble TNF receptors[5]. Thus, the inflamed synovial joint contains excessive amounts of both pro- and anti-inflammatory cytokines, but given the ongoing state of inflammation there must be an imbalance in favour of the former.

Arachidonic acid, eicosanoids and the link with inflammation

Eicosanoids are key mediators and regulators of inflammation[6,7] and are generated from C_{20} PUFA. As inflammatory cells typically contain a high proportion of the *n*-6 PUFA arachidonic acid (20:4*n*-6) and low proportions of other C_{20} PUFA, arachidonic acid is usually the major substrate for eicosanoid synthesis. Eicosanoids, which include PG, thromboxanes, leukotrienes (LT) and other oxidised derivatives, are generated from arachidonic acid by the metabolic processes summarised in Fig. 1. They are involved in modulating the intensity and duration of inflammatory responses[6,7], have cell- and stimulus-specific sources and frequently have opposing effects. Expression of both isoforms of cyclooxygenase is increased in the synovium of patients with RA[5,8] and in joint tissues in rat models of arthritis[8]. PGE_2, LTB_4 and 5-hydroxyeicosatetraenoic acid are found in the synovial fluid of patients with active RA[9]. Infiltrating leucocytes such as neutrophils, monocytes and synoviocytes are important sources of eicosanoids in RA[9]. PGE_2 has a number of pro-inflammatory effects, including increasing vascular permeability, vasodilation, blood flow and local pyrexia and potentiation of pain caused by other agents. It also promotes the production of some matrix metalloproteinases and stimulates bone resorption. The efficacy of non-steroidal anti-inflammatory drugs (NSAID), which act to inhibit cyclooxygenase activity, in RA indicates the importance of this pathway in the pathophysiology of the disease. However, although these drugs provide rapid relief of pain and stiffness by inhibiting joint inflammation, they do not influence the course of the disease. LTB_4 increases vascular permeability, enhances local blood flow, is a potent chemotactic agent for leucocytes, induces release of lysosomal enzymes and enhances release of reactive oxygen species and inflammatory cytokines such as TNFα, IL-1β and IL-6.

Very-long-chain *n*-3 PUFA and inflammatory processes

Oily fish and fish oils contain the very-long-chain *n*-3 PUFA EPA (20:5*n*-3) and DHA (22:6*n*-3). Increased consumption of these fatty acids results in their incorporation into immune cell phospholipids[10–13], which

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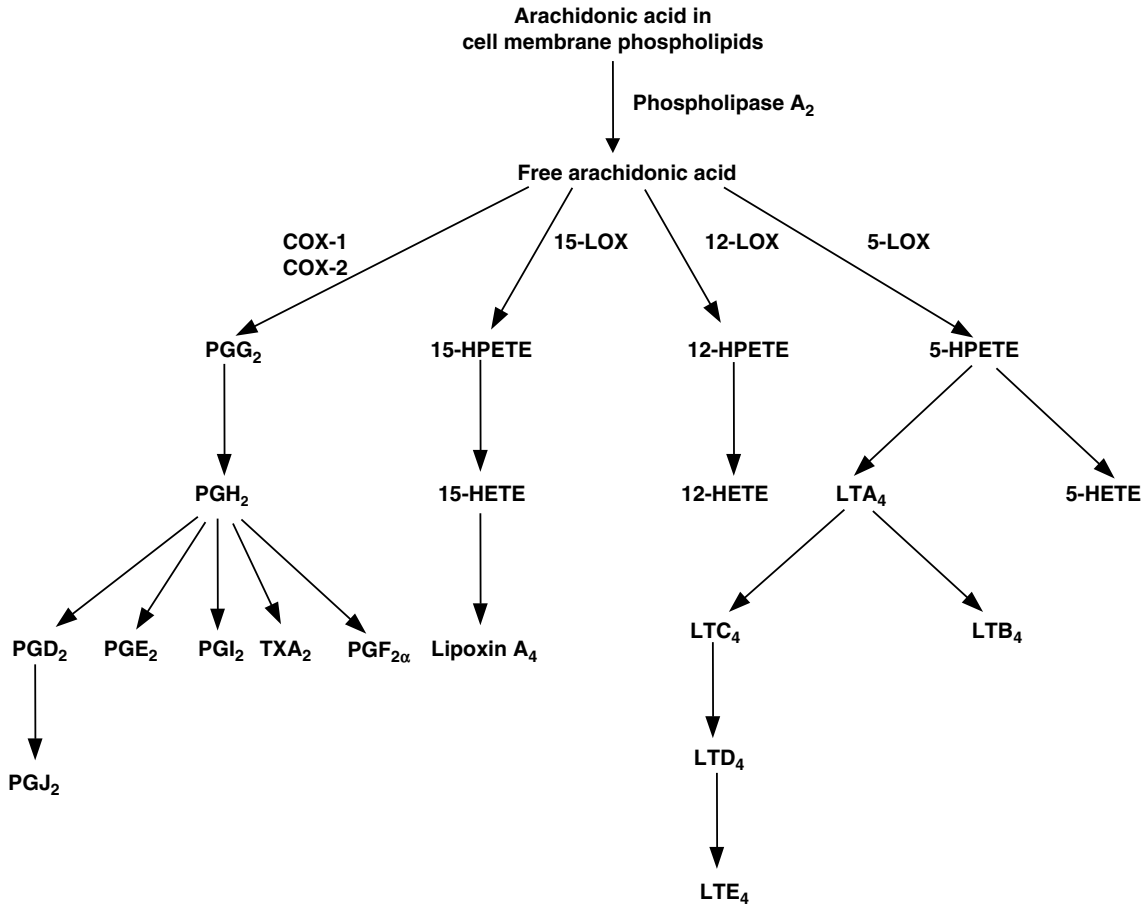

Fig. 1. Outline of the pathway of eicosanoid synthesis from arachidonic acid. COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; TX, thromboxane.

occurs in a dose–response fashion and is partly at the expense of arachidonic acid. The changed membrane fatty acid composition is believed to influence immune cell function and inflammatory processes[14] (Fig. 2). There have been numerous reviews of the influence of *n*-3 PUFA on many aspects of immune function in recent years[10–27] and the reader is referred to these articles for details beyond those provided in the following sections.

Antigen-presenting cell function

There have been several studies of the effects of *n*-3 PUFA on MHC II or HLA expression or antigen presentation via class II molecules[28]. These studies have typically found that class II expression and antigen presentation via class II molecules are decreased by *n*-3 PUFA. An *in vitro* study in which spleen cells were incubated with EPA has reported decreased ability of those cells to present antigen[29]; this study did not report class II expression. Incubating murine macrophages with DHA decreases expression of the class II molecules (termed Ia in mice)[30]. Likewise, incubating mouse macrophages with EPA or DHA decreases interferon (IFN)-γ-induced up-regulation of class II molecules[31] and incubating mouse dendritic cells with DHA decreases endotoxin-induced class II molecule up-regulation[32] EPA and DHA treatment has been reported to diminish the up-regulation of HLA-DR and HLA-DP associated with IFN-γ stimulation of human monocytes[33]. It has subsequently been demonstrated that these fatty acids decrease the ability of human monocytes to present antigen[34]. Three studies, one in mice[35], one in rats[36] and one in human subjects[37] have reported effects of dietary *n*-3 PUFA on class II expression. Feeding mice fish oil, which contains EPA and DHA, results in a reduction in MHC II expression on peritoneal cells (mainly B lymphocytes and macrophages)[35]. A human supplementation study with fish oil has reported decreased expression of HLA-DR, -DP and -DQ on IFN-γ-stimulated blood monocytes[37], with similar effects to those seen with *n*-3 PUFA *in vitro*[33]. These studies did not examine antigen presentation activity. However, a study that involved feeding an EPA-rich oil to mice has shown decreased antigen (keyhole limpet (*Megathura crenulata*) haemocyanin) presentation by spleen cells to T-cell clones[29]. Perhaps the most thorough study of this type to date is that in which feeding a fish oil-rich diet to rats was found to result in decreased expression of MHC II on dendritic cells[36]. These cells were found to have a much reduced

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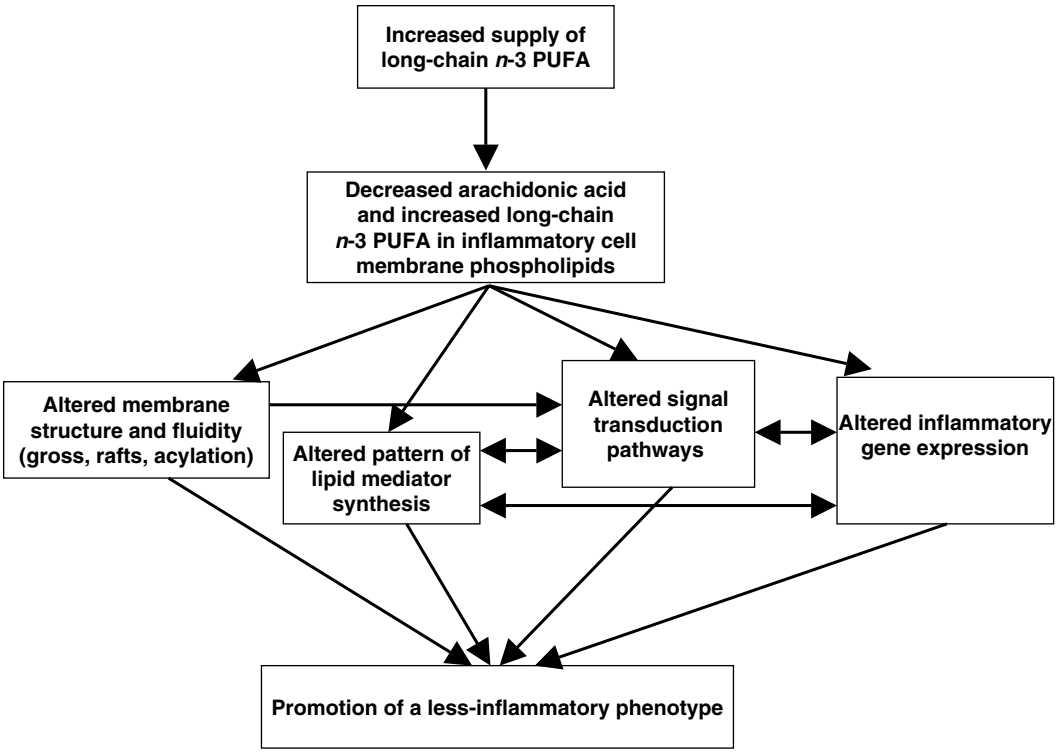

Fig. 2. Mechanisms by which *n*-3 PUFA can affect inflammatory cell activity.

capacity to present antigen (keyhole limpet haemocyanin) to antigen-sensitised spleen T-cells. The reduction in antigen presentation is probably much greater than could be explained by the reduction in class II expression, suggesting that other interactions between antigen-presenting cells and T lymphocytes are affected by dietary *n*-3 PUFA. It was reported that levels of the co-stimulatory molecules CD2, CD11a and CD18 are also decreased on dendritic cells from fish oil-fed rats[36].

T lymphocyte reactivity

In vitro studies have demonstrated that EPA and DHA decrease T-cell proliferation[38–41] and the production of helper T-cell 1-type cytokines such as IL-2[38,39,42]. Feeding studies in rodents and supplementation studies in human subjects have also shown that fish oil decreases T-cell proliferation[43–48] and production of helper T-cell 1-type cytokines such as IL-2[42,45,47,48] and IFN-γ[42,48], although it is important to note that not all human studies report such an effect[11]. The reason for these discrepancies in the literature is not entirely clear, but dose of *n*-3 PUFA used, technical factors and differences among subjects studied are likely to be contributing factors.

The mechanism by which long-chain *n*-3 PUFA affect T-cell reactivity was initially thought to relate to altered patterns of eicosanoid synthesis; however, through the use of eicosanoid synthesis inhibitors and pure eicosanoids *in vitro* this mechanism has been shown to be unlikely[40]. Studies over the last few years have demonstrated that the inhibitory effects of *n*-3 PUFA in general, and of EPA in particular, relate to membrane-mediated effects that impact on the early stages of cell signalling[49–52].

Inflammatory mediator production

Eicosanoids. Increased consumption of very-long-chain *n*-3 PUFA such as EPA and DHA, results in decreased amounts of arachidonic acid present in immune cell membranes and available for synthesis of eicosanoids[10–13]. Thus, feeding fish oil to laboratory rodents or supplementing the diet of human subjects with fish oil has been reported to result in decreased production of a range of eicosanoids including PGE_2, thromboxane B_2, LTB_4, 5-hydroxyeicosatetraenoic acid and LTE_4 by inflammatory cells[10–13]. A recent study has demonstrated the dose–response effect to dietary EPA of PGE_2 production by endotoxin-stimulated human mononuclear cells and suggests that an EPA intake of >2 g/d is required in order to be effective[53].

EPA is also able to act as a substrate for both cyclooxygenase and 5-lipoxygenase, giving rise to eicosanoids with a slightly different structure from those formed from arachidonic acid. Thus, fish oil supplementation of the human diet has been shown to result in increased production of LTB_5, LTE_5 and 5-hydroxyeicosapentaenoic acid by inflammatory cells[10–13]. The functional importance of this outcome is that the mediators formed from EPA are frequently less potent than those formed from arachidonic acid; for example, LTB_5 is less potent as a neutrophil chemotactic agent than LTB_4[54,55].

Resolvins and related compounds: novel EPA- and DHA-derived anti-inflammatory mediators. Recent studies have identified a novel group of trihydroxyeicosapentaenoic acid mediators, termed E-series resolvins, formed from EPA by a series of reactions involving cyclooxygenase-2 (acting in the presence of aspirin) and

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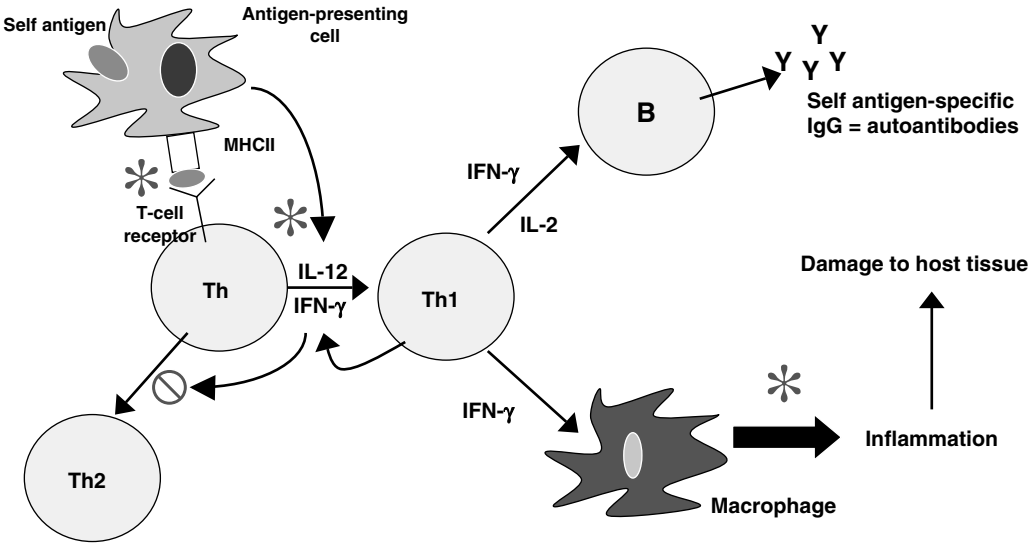

Fig. 3. Cellular sites of anti-inflammatory actions of long-chain *n*-3 PUFA. IFN, interferon; Th, helper T-cell; Y, IgG. ⁎, Sites of action of *n*-3 PUFA; ⊘, inhibits.

5-lipoxygenase. These mediators appear to exert potent anti-inflammatory actions[56–58]. In addition, DHA-derived trihydroxydocosahexanoic acid mediators termed D-series resolvins are produced by a similar series of reactions and these resolvins are also anti-inflammatory[59,60]. Metabolism of DHA via a series of steps, several involving 5-lipoxygenase, generates a dihydroxydocosatriene termed neuroprotectin D1, again a potent anti-inflammatory molecule[61]. The identification of these novel EPA- and DHA-derived mediators is an exciting new area of *n*-3 fatty acids and inflammatory mediators and the implications to a variety of conditions may be of great importance[62,63].

Inflammatory cytokines. Cell-culture studies have demonstrated that EPA and DHA can inhibit the production of IL-1β and TNFα by monocytes[64] and the production of IL-6 and IL-8 by venous endothelial cells[65,66]. Fish oil feeding decreases *ex vivo* production of TNFα, IL-1β and IL-6 by rodent macrophages[67–69]. Supplementation of the diet of healthy human volunteers with fish oil decreases production of TNF or IL-1 or IL-6 by mononuclear cells in some studies[10–13], although a number of other studies have shown little effect of *n*-3 PUFA on production of inflammatory cytokines in human subjects[11]. The reason for these discrepancies in the literature is not entirely clear, but dose of *n*-3 PUFA used, technical factors and differences among subjects studied, including genetic differences[70,71], are likely to be contributing factors.

n-3 PUFA and animal models of rheumatoid arthritis

The effects of *n*-3 PUFA from fish oil on antigen presentation, T-cell reactivity and inflammatory lipid and peptide mediator production (Fig. 3) suggest that these fatty acids might have a role both in decreasing the risk of development of RA and in decreasing severity in those patients with the disease. Indeed, dietary fish oil has been shown to have beneficial effects in animal models of arthritis. For example, compared with vegetable oil, feeding mice fish oil delays the onset (mean 34 d *v.* 25 d) and reduces the incidence (69 % *v.* 93 %) and severity (mean peak severity score 6·7 *v.* 9·8) of type II collagen-induced arthritis[72]. In another study both EPA and DHA were found to suppress streptococcal cell wall-induced arthritis in rats, with EPA being more effective[73].

Trials of *n*-3 PUFA in rheumatoid arthritis

Several studies have reported anti-inflammatory effects of fish oil in patients with RA, such as decreased LTB_4 production by neutrophils[74–77] and monocytes[76,78], decreased PGE_2 production by mononuclear cells[79], decreased IL-1 production by monocytes[80], decreased plasma IL-1β concentrations[81], decreased serum C-reactive protein concentrations[74,82] and normalisation of the neutrophil chemotactic response[83]. A number of randomised placebo-controlled double-blind studies of fish oil in RA have been reported. The characteristics and findings of these trials are summarised in Table 1. The dose of long-chain *n*-3 PUFA used in these trials was between 1·6 and 7·1 g/d and averaged about 3·5 g/d (see Table 1). Almost all these trials have shown some benefit of fish oil (Table 1). Such benefits include reduced duration of morning stiffness, reduced number of tender or swollen joints, reduced joint pain, reduced time to fatigue, increased grip strength and decreased use of non-steroidal anti-inflammatory drugs (Table 1). A number of reviews of these trials have been published[84–90] and each has concluded that there is benefit from fish oil. In an editorial commentary discussing the use of fish oil in RA it was concluded that 'the findings of benefit from fish oil in rheumatoid arthritis are robust', 'dietary fish oil supplements in rheumatoid arthritis have treatment efficacy' and 'dietary fish oil supplements should now be regarded as part of the standard therapy for rheumatoid arthritis'[91].

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Table 1. Summary of the results of placebo-controlled studies using dietary long-chain *n*-3 PUFA (in the form of fish oil) in patients with rheumatoid arthritis

Reference	Dose of EPA + DHA (g/d)	Duration (weeks)	Placebo	Clinical outcomes improved with long-chain *n*-3 PUFA
Kremer *et al.*[74]	1·8 + 1·2	12	Paraffin oil	No. of tender joints; duration of morning stiffness
Kremer *et al.* [75]	2·7 + 1·8	14	Olive oil	No. of tender joints; no. of swollen joints; time to fatigue; physician's global assessment
Cleland *et al.*[76]	3·2 + 2·0	12	Olive oil	No. of tender joints; grip strength
van der Tempel *et al.*[77]	2·0 + 1·3	12	Coconut oil	No. of swollen joints; duration of morning stiffness
Kremer *et al.*[80]	1·7 + 1·2	24	Olive oil	No. of tender joints; no. of swollen joints; grip strength; physician's global assessment
Kremer *et al.*[80]	3·5 + 2·4	24	Olive oil	No. of tender joints; no. of swollen joints; grip strength; physician's global assessment; duration of morning stiffness
Tullekan *et al.*[78]	2·0 + 1·3	12	Coconut oil	No. of swollen joints; joint pain
Skoldstam *et al.*[95]	1·8 + 1·2	24	Mixed oils	No. and severity of tender joints; physician's global assessment; use of NSAID
Esperson *et al.*[81]	2·0 + 1·2	12	Mixed oils	No. and severity of tender joints
Nielsen *et al.*[96]	2·0 + 1·2	12	Vegetable oil	No. of tender joints; duration of morning stiffness
Kjeldsen-Kragh *et al.*[97]	3·8 + 2·0	16	Maize oil	No. and severity of tender joints; duration of morning stiffness
Lau *et al.*[98]	1·7 + 1·1	52	Air	Use of NSAID
Geusens *et al.*[99]	1·7 + 0·4	52	Olive oil	Physician's pain assessment; patient's global assessment; use of NSAID and/or disease modifying anti-rheumatic drugs
Kremer *et al.*[100]	4·6 + 2·5	26–30	Maize oil	No. of tender joints; duration of morning stiffness; physician's assessment of pain; physician's global assessment; patient's global assessment
Volker *et al.*[101]	Total 40 mg/kg (approx 2·2–3·0)	15	Mixed oils	No. of swollen joints; duration of morning stiffness; patient's assessment of pain; patient's global assessment; physician's global assessment; health assessment by questionnaire
Adam *et al.*[102]	Approx 2·4 + 1·8	12	Maize oil	No. of swollen joints; no. of tender joints; patient's global assessment; physician's global assessment; patient's assessment of pain
Remans *et al.*[103]	1·4 + 0·2 (+ 0·5 γ-linolenic acid) in a liquid supplement	16	Liquid supplement without added PUFA	None
Berbert *et al.*[104]	Total 3·0		Soyabean oil	Duration of morning stiffness; joint pain; time to onset of fatigue; Ritchie's articular index*; grip strength, patient's global assessment
Sundrarjun *et al.*[82]	1·9 + 1·5	24	Not stated	None
Galarraga *et al.*[105]	1·5 + 0·7	36	Air	Use of NSAID; patient's assessment of pain

Approx, approximately; NSAID, non-steroidal anti-inflammatory drugs.
*Based on the summation of a number of quantitative evaluations of the pain experienced by the patient when the joints were subjected to pressures when exerted over the articular margin or in some instances on movement of the joint[106].

A meta-analysis that included data from nine trials published between 1985 and 1992 inclusive and from one unpublished trial has concluded that 'dietary fish oil supplementation for three months significantly reduced tender joint count (mean difference −2·9; $P = 0·001$) and morning stiffness (mean difference –25·9 min; $P = 0·01$)'[92]. A more recent meta-analysis of data from trials published between 1985 and 2002 included one study of flaxseed oil, one study that did not use a control for fish oil and one study in which transdermal administration of *n*-3 PUFA by ultrasound, rather than the oral route, was used[93]. This meta-analysis has concluded that fish oil supplementation has no effect on 'patient report of pain, swollen joint count, disease activity or patient's global assessment'. However, this conclusion may be flawed, because of the inappropriate manner in which studies were combined and because of

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Table 2. Summary of the findings of the meta-analysis of Goldberg & Katz[94]

Outcome	No. of studies	No. of patients		Significance of effect of *n*-3 PUFA: *P*
		Control	*n*-3 PUFA	
Patient-assessed pain	13	247	254	0·03
Physician-assessed pain	3	61	62	0·45
Duration of morning stiffness	8	150	156	0·003
No. of painful and/or tender joints	10	210	215	0·003
Ritchie articular index*	4	68	67	0·40
NSAID consumption	3	79	77	0·01

NSAID, non-steroidal anti-inflammtory drugs.
*Based on the summation of a number of quantitative evaluations of the pain experienced by the patient when the joints were subjected to pressures when exerted over the articular margin or in some instances on movement of the joint[106].

a poor understanding of the study designs used. For example, the meta-analysis fails to recognise that patients' ability to reduce the need for using NSAID or their ability to be withdrawn from NSAID, as was done in some designs, must indicate a reduction in pain with *n*-3 PUFA use. This meta-analysis does state that 'in a qualitative analysis of seven studies that assessed the effect of *n*-3 fatty acids on anti-inflammatory drug or corticosteroid requirement, six demonstrated reduced requirement for these drugs' and concludes that '*n*-3 fatty acids may reduce requirements for corticosteroids'[93]. The effects of long-chain *n*-3 PUFA on tender joint count were not assessed by this meta-analysis, which reiterated the findings of the earlier meta-analysis that '*n*-3 fatty acids reduce tender joint counts'[92]. A recent meta-analysis of *n*-3 PUFA with data from seventeen trials included one trial of RA with flaxseed oil and two trials of fish oil not in patients with RA, but which reported joint pain[94]. Data on six outcomes were analysed and are summarised in Table 2. This meta-analysis provides further evidence of the robustness of the efficacy of *n*-3 PUFA in RA.

Several other studies have also provided information about the benefits of *n*-3 PUFA in RA. For example, in a study that has compared outcomes among patients with RA who did and did not consume fish oil supplements it was found that fish oil users are more likely to reduce use of NSAID and are more likely to be in remission[79].

Overall conclusions

Eicosanoids derived from the *n*-6 PUFA arachidonic acid play a role in RA, and the efficacy of NSAID in RA indicates the importance of pro-inflammatory cyclooxygenase pathway products in the pathophysiology of the disease. At sufficiently high intakes long-chain *n*-3 PUFA decrease the production of inflammatory eicosanoids from arachidonic acid and promote the production of less-inflammatory eicosanoids from EPA and of anti-inflammatory resolvins and similar mediators from EPA and DHA. Long-chain *n*-3 PUFA have other anti-inflammatory actions including decreasing antigen presentation via MHC II, decreasing T-cell reactivity and helper T-cell 1-type cytokine production and decreasing inflammatory cytokine production by monocytes and macrophages. Work with animal models of RA has demonstrated the efficacy of fish oil. There have been a number of clinical trials of fish oil in patients with RA. Most of these trials have reported clinical improvements (e.g. improved patient assessed pain, decreased morning stiffness, fewer painful or tender joints, decreased use of NSAID), and when the trials have been pooled in meta-analyses significant clinical benefit has emerged.

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