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Controversies in omega-3 efficacy and novel concepts for application

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ABSTRACT

Interest in the cardioprotective effects of long chain omega-3 polyunsaturated fatty acids (LCn3) was largely influenced by the low rates of cardiovascular disease (CVD) amongst the Inuits of Greenland who consumed a high marine fat diet rich in LCn3s. This finding stimulated years of epidemiological and clinical studies investigating the cardioprotective effects of LCn3s, thought to be primarily mediated through anti-inflammatory (and anti-aggregatory) prostaglandins that protect the vascular wall from pro-inflammatory effects of metabolic stress precipitated by poor diet and lifestyle. Although the original hypothesis of the link between LCn3s and CVD protection was based on a high LCn3 containing diet (namely a high marine fat diet) the majority of clinical trials since have focussed on EPA and DHA supplementation, and results of repeated meta-analyses have not shown conclusive evidence in support of their beneficial health effects. In this review we focus on the controversies that surround the efficacy of LCn3s in cardiovascular and other chronic diseases and present emerging areas of research for novel applications. We will examine factors that can impact on the efficacy of LCn3s such as source (plant vs marine vs supplements (algal vs marine)), stability of product, dose, trial duration, ratio of EPA:DHA, and ratio of LCn6:LCn3 fatty acids in the diet.

Contents

Abbreviations: CVD, Cardiovascular disease; AA, Arachidonic Acid; DHA, DocosaHexaenoic Acid; EPA, EicosaPentaenoic Acid; LCn3, Long Chain omega-3 polyunsaturated fatty acids; LCn6, Long Chain omega-6 polyunsaturated fatty acids; RCT, Randomized Control Trial.

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1. Introduction

Research on the beneficial aspect of LCn3s was prompted by the remarkably low rate of cardiovascular disease (CVD) amongst the Inuits of Greenland. A series of studies conducted on Inuits showed that despite consuming a high marine fat diet, the people had extremely low levels of Coronary Heart Disease (CHD) [1]. Interestingly, diabetes was essentially unknown in the population as compared to the Danish population living in Denmark [2]. The Inuits exhibited lower serum LDL-cholesterol and triglycerides, higher HDL-cholesterol and higher levels of LCn3 eicosapentaenoic acid (EPA) to LCn6 arachidonic acid (AA), compared to their Danish counterparts. Since this observation, a large number of experimental and clinical studies have demonstrated the health benefits of long chain n-3 PUFAs, however not all studies have been consistent. Bang and colleagues [3] hypothesised that eicosapentanoic acid (EPA) plays a crucial role in lowering the incidence of atherosclerosis, coronary heart disease and diabetes. In particular, the presence of EPA in the walls of the blood vessels instead of AA was reported to create an anti-thrombotic state that lowered the incidence of coronary heart disease amongst the Inuits [2]. This work has since been supported by a substantial body of evidence. In addition to this, researchers demonstrated that prostaglandins derived from LCn6 had increased pro-inflammatory activity compared with those derived from LCn3. Furthermore, studies suggested that EPA and docosohexanoic acid (DHA) derived from LCn3 are structurally and functionally distinct from the prostaglandins that are derived from AA. Marine plants and fish are the two main sources of dietary LCn3 [4]. The most concentrated source of LCn3 comes from fatty fish such as salmon, tuna and mackerel. LCn3s are incorporated into the cell membrane, thereby modulating cellular signalling, gene expression and membrane protein function. Dietary supplementation of LCn3 is known to promote secretion of anti-inflammatory prostaglandins and decrease leukotrienes [5]. These anti-inflammatory properties are thought to be beneficial in cardiovascular diseases and metabolic syndrome disorders such as central obesity, insulin resistance, hypertension and dyslipidemia [6,7]. Over the last decade, an increasing amount of experimental evidence suggests a crucial role played by DHA on neurodegenerative conditions such as mild cognitive impairment (MCI) [8,9] and Alzheimer's Disease [10].

While most epidemiological evidence and randomized control trials (RCTs) indicate the protective role of LCn3 on general health and wellbeing, there are some RCTs that have shown no effect [11]. The aims of this review are to evaluate (a) the evidence of the beneficial effects of LCn3 on various disease conditions such as metabolic syndrome and cardiovascular disease (b) discuss why some RCTs may not have positive outcomes, (c) highlight controversies in LCn3 efficacy and (d) identify emerging areas for LCn3 application as therapeutic agents.

2. Source of LCn3s: diet versus supplementation

Since the initial interest sparked by the Inuit studies, there has been a vast amount of research into the area of dietary fish intake, LCn3 supplementation and disease prevention and treatment. Controversy exists, however, as to whether LCn3 intake is responsible for benefits seen, whether fish intake itself is beneficial, or if fish intake is merely a marker for a healthier dietary pattern. The evidence for diet versus supplementation in different disease states should be considered and will be reviewed in this article.

(i) Diet versus supplementation - CVD events

Epidemiological support for the protective effect of fish is illustrated by the concept of the blue zone diets. So-called 'blue zones' are areas of the world where populations experience longevity (>100 years of age or more) and reduced morbidity compared to other populations [12]. Notable blue zone areas include the islands of Ikaria and Sardinia in the Mediterranean and Okinawa in Japan, with these areas having a high dietary intake of LCn3 amongst other dietary patterns. Fish is the major source of LCn3 intake in Japan and there are many examples of epidemiological support for a high LCn3 intake from fish, such as the Nurse's Health Study and the Chicago Western Electric Study which demonstrated an inverse relationship between fish consumption and mortality, particularly from CVD events [13–15]. Paradoxically, some epidemiological studies, show no relationship between LCn3 intake from fish and mortality [16] or that fish consumption is only protective in high risk groups [17].

Following on from the 'blue zone' diet concept there was interest in the use of supplemental LCn3 in cardiovascular disease, with significant reductions reported for secondary prevention of myocardial infarction [18]. Additionally, the Diet and Reinfarction Trial (DART) is an example of an RCT demonstrating that both fish consumption and dietary LCn3 supplementation could reduce mortality in the 2 years post-myocardial infarction [19]. Another study by Brazionis and colleagues [20], however, showed that fish consumption rather than fish oil improved risk factors of CVD such as blood pressure and waist to hip ratio. Controversy also exists in regards to how different CVD risk factors are impacted by fish oils. There is evidence supporting dietary supplementation with LCn3 fish oil in management of high triglycerides with meta-analyses showing a positive benefit on triglycerides with dietary supplementation [21], with certain populations receiving the most benefit including those with chronic renal disease or HIV positive populations receiving Highly Active Antiretroviral Therapy (HAART) [22]. A recent meta-analysis by Wei and Jacobson [23] compared the effects of supplementation of DHA and EHA concluding that both DHA and EPA lower triglycerides, with divergent effects on LDL and HDL cholesterol. Cardioprotective dietary patterns such as a Mediterranean style diet or a 'blue zones' type eating pattern include a wide variety of plant foods consumed with high levels of antioxidants and other bioactive nutrients that may act in a protective and synergistic way, increasing the quality of fatty acids incorporated into tissues.

(ii) Diet versus supplementation - Cognitive function

The role of LCn3 have also been examined in relation to cognitive function. Once again the current evidence suggest that certain populations may benefit more than others and different LCn3 may be important. A prospective cohort study by Morris and colleagues [24] demonstrated an inverse relationship between DHA and total LCn3 intake in older people, with no protective effect seen with EPA. Intervention trials have so far failed to support a role for supplementation to improve cognitive function in those with Alzheimer's Dementia [25] and with mixed results reported in healthy individuals [26,27]. A possible theory behind the conflicting and lack of positive results of supplement trials is the oxidisable nature of fish oil supplements which may reduce the bioavailability.

(iii) Diet versus supplementation - Fatty liver disease

An example illustrating the complexity of this research area is in fatty liver disease studies. A recent meta-analysis by Parker et al. [28] examined the role of LCn3 supplementation in reducing liver size. This was stimulated by previous research that showed patients with Non-Alcoholic Fatty Liver Disease (NAFLD) had habitually low consumption of fish compared to individuals without NAFLD

[29,30]. The results of the meta-analysis suggest that supplementation with LCn3 may be helpful in reducing liver fat, however it was noted by the authors that some studies included in the meta-analysis included concurrent dietary modification which may have potentially confounded the results [28]. Challenges in study design and assessment of habitual LCn3 consumption are also echoed by Issa and colleagues [31] in their recent meta-analysis examining the effect of LCn3 intake on cognitive function.

The question remains, is it more than the LCn3 content of fish that has a role to play in both the secondary and primary prevention of disease? Are other nutrients present in fish important, or are dietary patterns associated with a high fish intake higher in other protective nutrients as well?

(iv) Variety is key

An extension of this idea is that a wide variety of LCn3 sources may be an important factor in disease prevention. Simopoulos [32] asserts that some Mediterranean diets, namely the traditional Greek diet, have high omega-3 intake from both animal sources (fish, seafood and eggs) as well as from plants (namely leafy greens such as purslane and legumes). Fish contains other potentially important nutrients such as taurine, selenium and astaxanthin which may displace less healthy components of the diet. Other traditional diets high in LCn3 have also been shown to be effective in both the primary and secondary prevention of CVD. The Mediterranean diet is high in LCn3 content and traditional dietary patterns in Japan are also associated with a lower rate of CVD, with average n-3 PUFA intake of 1–2 g/day, which is 3–4 times the intake of most Western populations [33]. Notably, both dietary patterns have a high intake of plant based food including fruits, vegetables and fish and lower intakes of red or processed meat and refined grains, and have LCn3 from a variety of sources.

A study examining the Seventh Day Adventist Population in North America, notably another blue zone and long-living population, concluded that nut intake may be protective against mortality [16]. Nuts, such as walnuts, are a high dietary source of LCn3s namely alpha linolenic acid. A more recent European study, the PREDIMED trial, showed that a Mediterranean diet supplemented with nuts (walnuts, almonds and hazelnuts) increased protection against metabolic syndrome [34]. This study examined the effects in older adults, a high risk population, once again suggesting that different interventions may be suitable or more effective in different populations. Another meta-analysis by Pan et al. suggested high intakes of ALA were associated with lower CVD events [35]. The dietary strategy of increased ALA consumption is appealing, as plant sources of LCn3 are more accessible and affordable for the majority of the population. However, the conversion from ALA to EPA is limited (approximately 8% in men and 9% in women) and conversion to DHA can be very low (reports of <0·1% in men and 9% in women) [36] and so the main sources of long chain are marine-based.

(v) Evolving dietary habits

A further area of complexity and controversy relates to the potential decline of dietary LCn3 that coincides with an increase in urbanisation and affluence in a population. Red meat consumption generally increases with affluence and can contribute significantly to the ratio of LCn3:LCn6 in the human diet. A recent study by Daley and colleagues [37] showed grass fed beef contained up to five times higher levels of LCn3 than grain fed beef. Furthermore, game meats such as deer and kangaroo, although low in total fat, have proportionally high levels of LCn3 derived from their diet of wild edible plants rich in ALA [38]. Therefore, as suggested by Howe and colleagues [35], the source of red meat and production methods are important considerations, as these factors impact the fatty acid composition of the meat.

With a shift towards a Western dietary pattern that occurs with increased affluence there is a corresponding decrease in protein sources that are high in LCn3 such as offal and brain. Consumption of higher fat protein sources would improve the LCn3 to LCn6 ratio and it is this that may be key when it comes to disease prevention and treatment as discussed elsewhere in this article. Similarly, when fish intake is examined, some studies demonstrate lower levels of LCn3 in farmed fish [39] whereas others claim that the total fat content of farmed fish is higher. For example, Cahu and colleagues [40] suggest that while the proportion of EPA and DHA in farmed fish is low compared to wild fish, when expressed as a proportion of total fat, the total amount in grams is higher. These authors also noted that fish feed has an influence on the fatty acid profile. The cooking method utilised can also alter the fatty acid composition with methods such as deep frying changing the fatty acid profile [41]. Some studies show reduced cardiac benefits with fried fish and fish sandwiches compared to grilled or baked fish [42]. In a recent meta-analysis, Li [43] suggests that the variability in the type of fish consumed, total LCn3 intake and cooking method may a possible reason for conflicting results in epidemiological studies.

Lifestyle and dietary changes are notoriously hard to achieve and maintain, and may potentially be environmentally unsustainable, whereas compliance with dietary supplements may be easier and more accessible for some individuals. Furthermore, the sustainability of fish oil supplements, which deplete our reserves of oily fish, needs to be considered. The emerging use of algal oils in LCn3 supplementation offers a lot of promise. Although the findings of trials examining singular nutrients have yielded mixed results, it is apparent that some population groups benefit more than others and ongoing research is justified [44].

3. Importance of the dietary LCn6:LCn3 fatty acid ratio and controversies surrounding the role of fatty acids in CVD

Modulating dietary fat continues to be the mainstay of treatment for cardiovascular disease and non-communicable chronic diseases generally [45]. However, recent meta-analyses have challenged our understanding of the role of fatty acids in CVD risk, concluding that apart from trans fats that are positively associated with CVD risk, the evidence for an association between all other fatty acids (selected saturated fats, monounsaturated fats and polyunsaturated fats (LCn6 and LCn3)) and CVD risk is not strong or is inconclusive [46]. It has been argued however, that the lack of conclusive evidence linking fatty acids to CVD risk may be due to the poor quality of published trials including self-reported measurement of fatty acid intakes, variations in trial length and measurement of biomarkers of fatty acids and genetic variation in trial participants affecting response to interventions [42,46]. Another issue is trials including LCn6 and LCn3s together yet they have distinctively different and opposing properties [47,48]. Although there is general consensus for the need to replace trans fats and saturated fats with healthier fats such as monounsaturated and polyunsaturated fats, there is emerging evidence that excess consumption of n-6 polyunsaturated fatty acids (beyond 10% of energy intake) may increase CVD risk by increasing oxidation of LDL cholesterol, and increased vasospasm, vasoconstriction and increased platelet aggregation [49]. Recent studies have highlighted the importance of the LCn6/LCn3 ratio in CVD risk, rather than the amount of individual fatty acids [49].

The two essential fatty acids in the diet that cannot be synthesised internally are linoleic acid (or omega-6; C18:2n-6; LNA)

and alpha-linolenic acid (or omega-3; C18:3n-3; ALA). LNA is a precursor of arachidonic acid, which in turn is a precursor for a variety of vasoconstrictive, pro-inflammatory and pro-aggregatory molecules that are important in the body's response to inflammation. ALA is a precursor of both eicosapentanoic acid (C20:5n-3; EPA) and docosahexanoic acid (C22:6n-3; DHA), which have important anti-inflammatory [50] vasodilator and anti-aggregatory functions [51]. The LCn6 and LCn3 fatty acids therefore have opposing effects which highlights the importance of an appropriate n-6 to n-3 fatty acid ratio. As argued by Simopoulos [32], the ratio of LCn6:LCn3 fatty acids in the diet has rapidly increased to about 20:1 with industrialisation and westernization of society and a move away from traditional diets such as the traditional archetypal Mediterranean diet of the 1950s. The diets of paleolithic man, which were rich in plant foods and wild animals, were reported to have an LCn6 to LCn3 ratio of close to unity [32]. The traditional Greek-style Mediterranean diet is reported to have a LCn6:LCn3 ratio of 2:1 due to high plant sources of ALA, high EPA and DHA from fish and other seafoods, and consumption of free range eggs, chickens and meat (goat and lamb) that graze on a high ALA diet from wild edible plants and herbs, snails and worms [32].

In our experience it is difficult to achieve a low LCn6:LCn3 ratio with the current Westernised food supply with its abundance of processed foods, intensively reared animal foods and vegetable cultivars that are low in LCn3s [52]. Careful planning is required to select free range produce, oils and nuts rich in LCn3s, oily fish and seafoods rich in LCn3, and breads and cereals that are supplemented with LCn3s.

4. LCn3s action in biological systems

(i) Metabolic syndrome

Metabolic syndrome is a constellation of risk factors for cardiovascular disease and diabetes which includes obesity, insulin resistance, hypertension and dyslipidaemia [44]. It is commonly suggested that metabolic abnormalities, including metabolic syndrome, increase with the level of obesity. When nutrient intake exceeds the metabolic demands of the body, the excess energy is stored as triglyceride in various tissues of the body including skeletal muscle, adipocytes and liver. The adipocytokines released by adipose tissue regulate various functions including inflammation and also components of the metabolic syndrome [53]. Inflammation appears to play a central role in the pathophysiology of metabolic syndrome and it is known to act mainly through an initiation phase. During the initiation phase, the LCn6 arachidonic acid (AA) is released, which is converted to pro-inflammatory eicosanoids such as prostaglandin (PGE2), thromboxane, 5-lipoxygenase and leukotriene [50]. These pro-inflammatory leukotrienes and eicosanoids activate NF-Kβ, which is a crucial mediator of inflammation and regulates more than 200 genes involved in pro-inflammatory cascades and apoptosis. The resolution of inflammation in metabolic syndrome is achieved by a transcellular processes that use the LCn3s EPA and DHA to synthesize specialized pro-resolving lipid mediators [6]. These mediators terminate neutrophil infiltration into sites of injury and control neutrophil apoptosis and the clearance of apoptotic neutrophils by macrophages, thereby returning inflamed tissue to a state of homeostasis [6]. While it is a concern that metabolic syndrome is a growing pandemic worldwide, dietary modifications and physical activity have been suggested to tackle this issue to reduce one or more risk factors for metabolic syndrome [19].

(ii) Cardiovascular disease

Cardiovascular disease is major cause of mortality worldwide due to the underlying multifactorial causes. Several key studies have examined diet-derived LCn3 and health interactions. The Diet and Reinfarction study (DART) study included men recovering from myocardial infarction who were given dietary advice including consumption of at least two weekly portions of fatty fish. Men who complied with this dietary advice had a 29% reduction in mortality compared with men who did not receive this dietary advice [19,54]. In addition, the Oslo study, which was a 5 year observational study, also showed that dietary changes and smoking cessation resulted in a lower incidence of coronary events [55]. While there are numerous data suggesting the cardioprotective effects of LCn3s, emerging reports call for a careful consideration on the age, gender and pathophysiology of the CVD factors which could potentially affect the metabolism of LCn3s. One study showed that after acute EPA supplementation, males exhibited a reduction in both platelet aggregation and microparticle activity, whereas females showed significantly reduced platelet aggregation but not microparticle activity after DHA supplementation only [56].

LCn3 supplementation has also been shown to have cardioprotective effects in patients with coronary heart disease. A meta-analysis which included fourteen RCTs showing a reduction on LCn3 supplementation from 3 months to more than 1 year duration, indicated that supplementation decreased the risk of death due to all causes and also from sudden cardiac death [57]. In addition to these RCTs showing a reduction in CVD events and death due to all causes, there are reported benefits among patients without documented CVD as well [58,59]. One of the potential causes of finding no effect in other studies could be attributed to a low dose of LCn3 (<1 g), since the therapeutic dosage for hypertriglyceridemia is ~4 g/day and significant variations in study design, including differences in baseline levels of LCn3, duration of supplementation and inadequate study power [60]. Also, whilst positive benefits for LCn3 supplementation in addition to standard therapy in heart failure have been reported [22], medications used in CVD treatment may also compromise potential benefits of LCn3 supplementation, as shown in the instance of statin prescribed patients [61]. Further studies with higher doses of LCn3 are required in CVD patients, as well patients without documented CVD, with the monitoring of co-prescriptions so that this therapy can be uniformly recommended in primary prevention patients.

5. Controversies in LCn3 efficacy

(i) Stability of LCn3 supplements

There are LCn3 supplement intervention studies that, despite very similar intervention design and patient population, have quite different outcomes [62,63]. One of the possible reasons for inconsistencies in the reported benefits of LCn3 supplementation may be due to the instability of fats contained within LCn3 supplements. A recent study by Albert and colleagues [64] investigating the fatty acid quality and content of fish oil supplements, measured oxidation markers and the EPA and DHA concentrations of 32 supplements available in New Zealand. The majority of the products analysed exceeded the recommended indices of oxidative markers, with only 8% meeting the recommendations. Peroxide values (PV) and anisidine values (AV) were measured, allowing for calculation of total oxidation (Totox). However, at a recent meeting of experts in Geelong, Australia (December 2015), the validity of the analyses in the Albert et al. [64] paper were questioned and considered flawed as similar analyses conducted by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) of a similar range of fish oil capsules demonstrated good compliance with the label claims and oxidation of these lipids was not

identified [65]. The experts noted that the Albert et al. paper used non-standard techniques in their analyses.

Furthermore, another study investigating 11 LCn3 products for persistent organic pollutant profiles reported that products adhered closely to manufacturer specifications and met chemical guideline thresholds [66]. The variation in findings, when independently analysing products, raises another issue of consistent methodologies used in analyses of LCn3 stability. Whilst measures of oxidation should be reported for baseline and post-intervention supplements in future fish-oil studies to identify quality of fatty acids throughout intervention, validated methods should be used to avoid inconsistencies of results.

The quality of fats contained within an LCn3 products can be impacted by heat and light conditions of the environment in which they are stored in, factors that cannot be controlled for in a free living intervention study. Moreover, even in a controlled environment, foods enriched with LCn3 show an increase in peroxide values over time [67]. Most LCn3 intervention studies that have been published do not report oxidation analytics of supplemental products used within the intervention. Analytical data should be reported on products used within clinical trials at baseline and at end point, to identify any compromise of fatty acid quality which may have occurred throughout the study. These analytical assessments of the supplement quality at end point may be conducted on excess capsules returned at the cessation of the intervention. Analytical reports should include concentrations of EPA and DHA fatty acids and oxidation indices including PV, AV and Totox using validated analytical methodologies. The addition of antioxidants to LCn3 enriched products can improve oxidative stability of fatty acids [67] and studies that have reported different findings despite similarities of LCn3 intervention design may have different outcomes dependent on the longevity of the product quality. An example of the possible impact of antioxidants added to LCn3 supplements on outcome, is the reported benefits in implantable cardioverter defibrillator (ICD) patients and outcomes of ventricular tachycardia or ventricular fibrillation in a study that used an LCn3 supplement containing added antioxidants [63], in comparison to a study reporting no ventricular tachycardia or ventricular fibrillation benefits in ICD patients using an LCn3 supplement not reported to contain added antioxidants [62].

Given the possibility of oxidation affecting the benefits of LCn3 supplements, considerations should be made in intervention studies to use a supplement which contains added antioxidants or even the use of LCn3 supplement sources that are naturally rich in antioxidants. Consideration for supplementation using LCn3 sources naturally containing greater amounts of antioxidants compared to shrimp, trout or salmon may include krill oil, rich in the antioxidant astaxanthin, Vitamin A and Vitamin E [68]. Additionally, krill oil may be more effective compared to fish oil as a source of LCn3s due to the phospholipid structure of krill oil. A recent study showed that krill oil intervention was more effective than fish oil in increasing LCn3, reducing LCn6:LCn3 ratio, and improving the omega-3 index [69].

(ii) Disparity in reported fatty acid concentration of supplement

Analytical data has shown disparity in the content of DHA and EPA within LCn3 products compared to the reported concentrations provided by manufacturers on product labels.

In the Albert and colleagues [64] study investigating fish oil supplements available in the New Zealand market, it was found that a number of major suppliers of LCn3 capsules were over-reporting LCn3 concentration, with supplements containing on average 68% of the reported EPA and DHA concentrations and some supplements containing as little as 32% of the EPA and DHA concentrations reported on the product label. Only three out of 32 analysed supplements had equal or higher quantities of EPA and DHA than reported on the product label [64]. However, as noted above, the findings of the Albert et al. (2015) study have been disputed by experts at a recent scientific meeting, due to non-standard analysis techniques. Alternatively, other studies investigating disparity between product claimed concentrations of fatty acids and independent analysis showed similar concentrations between the manufacturer and independent analysis [70].

Whilst analytical methodologies used should be validated and recognised, it is apparent that reliance on label reported EPA and DHA values weakens the strength of evidence from LCn3 supplement intervention studies. In the potential instance that previous studies with no effect from LCn3 intervention used a supplement that contained much lower quantities of LCn3 than indicated by manufacturer labelling, this may have affected outcomes of the study. This supports that future LCn3 supplementation studies should have independent analytical verification, using validated methods, of capsule fatty acid concentration. These analytical results should also be reported within publications arising from these intervention studies. LCn3 content and quality data should be provided in publications to aid not only quality control of intervention but also determine true intervention dose.

(iii) Omega-3 index levels - Compliance indicators and eligibility criteria

Studies on LCn3 supplementation commonly report objective compliance by analysing circulating plasma LCn3 blood levels before and after the intervention. Plasma LCn3 levels, while good indicators of acute increase or decrease in fatty acid intake, are not indicative of sustained intake [71]. Plasma LCn3 levels reflect recent intakes whereas red blood cell LCn3 reflects long-term intakes [72]. Reporting erythrocyte LCn3 levels at baseline at post-intervention would be a more robust method of reporting objective compliance in LCn3 intervention studies. The EPA and DHA concentration in the erythrocyte membrane constitutes the omega-3 index, and the omega-3 index has been proposed as a predictor of CVD mortality [73,74]. The omega-3 index has been shown to increase in a dose-dependent manner with increases in EPA and DHA supplementation [75]. Reporting objective compliance by measuring the omega-3 index of study population at baseline and post intervention is likely to strengthen consistency of study findings in the area of LCn3 interventions.

Some studies which report baseline and post-intervention circulating LCn3 levels show large variation between baseline levels within intervention and control groups. As it has been reported that an LCn3 index of ~8% is associated with cardioprotection and an index of ~4% associated with the least protection [73] outcomes of LCn3 intervention are likely to be more pronounced in the instance of participants beginning with low omega-3 index levels compared to those beginning with moderate to high, highlighting the importance for researchers to take into account baseline LCn3 levels – particularly baseline erythrocyte LCn3 levels, as this will be a stronger indicator of dietary LCn3 intake 3 months prior to participation and may reduce confounders to effect size. Within the study design of future LCn3 intervention trials, including a cut off for baseline LCn3 index levels in the inclusion/exclusion criteria may further strengthen the consistency of research outcomes. Alternatively, participants with higher omega-3 index baseline levels should be removed from data analysis or results categorised based on these values.

(iv) Variability in trial methodology – LCn3 dose

A recent review on LCn3 supplementation in patients with mild cognitive impairment [76] compared evidence from five RCTs; four studies reported consistent improvement in cognition measures whereas one study, which used a low dose intervention in comparison to the other interventions, found no improvement in cognition [76] (Table 1). These findings highlight the importance of dose and for independent assessment of DHA and EPA concentration of supplement in order to reach an effective dose. Cognitive functions were evaluated by Kotani et al. [77] before and after 90 days supplementation of 240 mg/day of arachidonic acid and DHA or 240 mg/day of olive oil (placebo), in both patients with mild cognitive impairment (MCI) and Alzheimer's disease. There were no significant improvements in either Alzheimer's disease patients or a placebo group, however patients treated with arachidonic acid and DHA with MCI showed a significant improvement of the immediate and memory score.

Similarly, a 24 week double blinded RCT conducted by Chiu et al. (2008) detected a significant improvement in the cognitive portion of the Alzheimer' s Disease Assessment Scale (ADAS-cog) in the group of participants with MCI who received LCn3 1.8 g/day compared to the placebo group ($p = 0.03$) [78]. These results were not observed in the participants with Alzheimer's disease. As Sinn et al. (2010) suggest, these results could be indicative that the early stages of cognitive decline may be optimal for intervention with LCn3 [79].

As illustrated in Fig. 1, there appears a clear delineation in benefits of omega-3 for cognition according to dose. Jackson et al. [80] suggest studies which administer less than 2 g LCn3s per day are unlikely to produce clinically relevant cognitive performance enhancements in healthy populations. Whilst larger doses may appear to illicit stronger outcomes, particularly in cognition, omega-3 research is complex, with variability in individual baseline intake and variability in trial methodology (study duration, supplements used, dosage, populations etc.). There may also be a need for caution in some populations, as indicated by the conflicting results of the DART and DART-2 trials in patients with ischaemic heart disease. Whilst the DART trial demonstrated a reduction in all-cause mortality among those taking fish oil, the DART-2 trial reported that participants taking fish oil had a higher risk of cardiac death [81].

Recently conducted RCTs investigating DHA supplementation in healthy populations detected little or no evidence of cognitive enhancing effects of 400 mg of DHA supplemented for 25 and 50 days [82] nor with supplementation with 252 mg of DHA, 60 mg of EPA and 10 mg of Vitamin E was for 90 days [83]. Plasma DHA levels and compliance were not reported by Benton et al. [82]; additionally, a lack of significant change in these interventions may be due to insufficient dose and supplementation period. Stough et al. [83] reported significantly higher plasma DHA levels in the intervention compared to the placebo group post-treatment; this increase did not translate to enhancement of cognitive function. Investigators speculated that the negative findings may reflect that the existing diet of participants did not provide sufficiently low levels on LCn3s to permit a benefit from supplementation [82].

(v) Duration of intervention

Results of a recent trial suggest EPA-rich supplementation may be more beneficial in improving behavioural cognitive outcomes compared to DHA-rich supplementation in healthy populations. Bauer et al. [87] compared a high EPA intervention group (400 mg fish oil; 3:1 ratio EPA:DHA) and a high DHA intervention group (365.7 mg fish oil; 4:1 ratio DHA:EPA) supplemented for 30 days. Behavioural outcomes were significant in the EPA-rich group; reaction times were decreased compared with supplementation rich in DHA ($p = 0.04$) whereas DHA-rich supplementation did not induce any behavioural improvement. Brain activation changes were detected in both groups; EPA-rich supplementation produced a decrease in the functional activation in the anterior cingulate cortex compared with prior to supplementation. DHA-rich supplementation increased functional activation in the precentral gyrus. It was concluded that following the 30-day intervention period EPA-rich supplementation was more successful than DHA-rich supplementation in improving neural efficiency during higher order cognitive tasks [87].

A Cochrane review [88] of three high quality RCTs found no benefit to cognitive function from LCn3 supplementation in cognitively healthy adults over 60 years of age. These authors identified length of supplementation as a limitation of the trials; the shortest trial duration was 26 weeks and the longest was 40

Table 1
Summary of DHA/EPA dietary intervention trials in patients with mild cognitive impairment (MCI) in the last 10 years [76].

Ref	Clinical trials with MCI patients (n, mean age)	Dosage of DHA/EPA per day	Trial duration and design	Measures	Outcome
[78]	Patients with MCI (23, 74 yrs)	0.72 g DHA + 1.08 g EPA or placebo	6 months Randomized double blinded placebo-controlled trials	ADAS-cog; CIBIC - plus	Significant improvement in ADAS-cog; in patients with MCI after omega-3 supplementation
[77]	Patients with MCI (23, 68 yrs)	240 mg DHA + 240 mg AA or placeboo	3 months, Placebo controlled trial	Japanese version of RBANS (5 cognitive domains)	Improvement of immediate memory and attention in omega-3 supplemented group
[84]	Elderly persons with MCI (36, 66 yrs)	1.3 g DHA + 0.45 mg of EPA or placebo	12 months, Randomized double blinded placebo controlled trial	RAVLT, MMSE, CDT, WAIS-R	Significant improvement in cognitive function in omega-3 supplemented group
[85]	Elderly patients suffering from MCI. (11, 85 yrs)	1.4 g DHA + 572 g EPA or placebo	3 months, Randomized double blinded placebo controlled trial	MMSE	Significant improvement in MMSE, semantic verbal fluency and olfactory sensitivity assessment in omega-3 supplemented group
[86]	Older people with MCI (100, 74 yrs)	180 mg DHA + 120 mg EPA or placebo	6 months, Randomized double blinded placebo controlled trial	MMSE, AMT	Low prescription dose had no effect on cognitive function in omega-3 supplemented group

AA = Arachadonic Acid; DHA = Docosahexaenoic acid; EPA = Eicosapentaenoic acid; MMSE = Mini-Mental State Examination; ADAS-cog = cognitive portion of the Alzheimer's Disease Assessment Scale; CIBIC plus = Clinician's Interview-Based Impression of Change Scale; RBANS = Repeatable Battery for the Assessment of Neuropsychological Status; RAVLT: Rey's Auditory Verbal Learning Test; CDT = Clock Drawing Test; WAIS-R = Wechsler Adult Intelligence Scale; AMT = Abbreviated Mental Test.

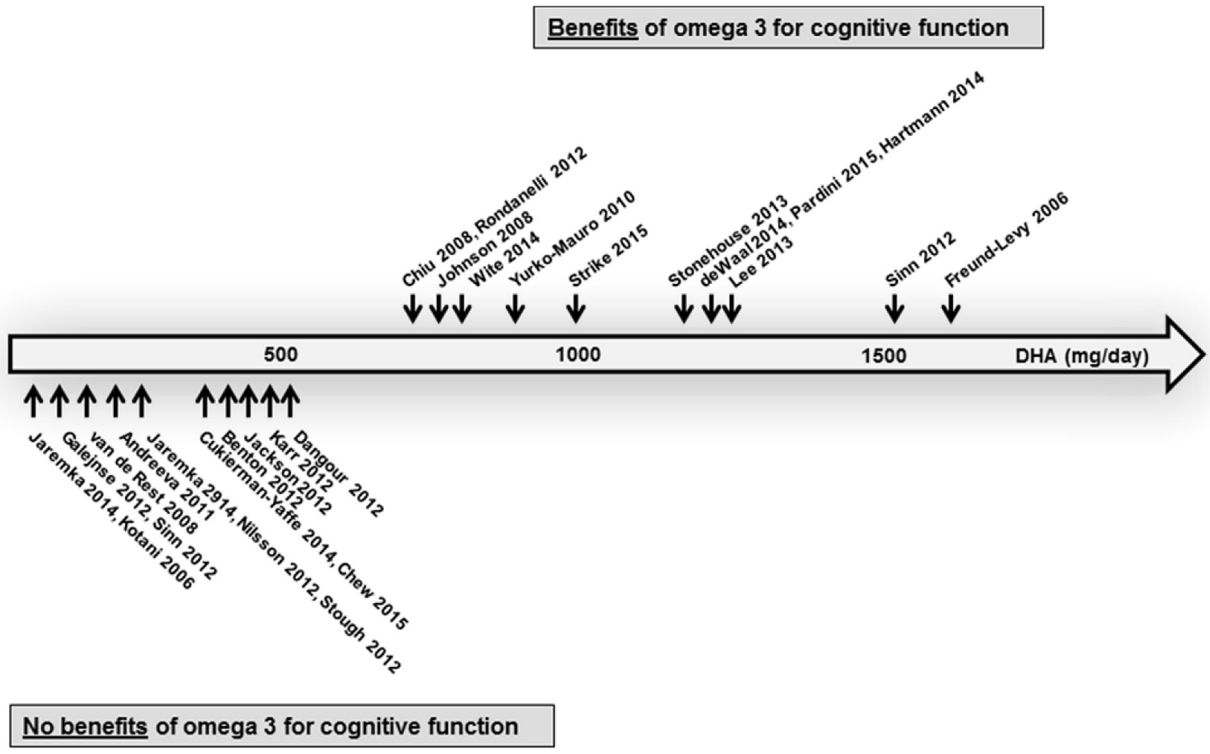

Fig. 1. Effect of LCn3s on cognitive function in cohort and RCT studies by dosage category of LCn3 consumption. Of interest, a number of the studies showing benefits of omega-3 for cognition (high dose studies) are in elderly populations; whereas a number of the other studies showing no benefits have primarily been conducted in younger participants. Presumably the elderly cohorts are also at increased risk and therefore more likely to see positive benefit. RCT, randomized control trial; DHA, docosohexanoic acid. Adapted from http://www.goedomega3.com/index.php/files/thumb/333/0 [9,77,78,84,91–112].

months. One included trial [89] of 2 years duration concluded cognitive function did not decline in either the placebo or intervention group, indicative that the relatively short intervention period may have limited capacity to detect potential delays to cognitive decline. Dosage and supplement formulation were not discussed as potential limitations of the studies. This is an important issue to consider since, as illustrated in Fig. 1, there appears a clear delineation in benefits of omega-3 for cognition according to dose.

(vi) Intervention population

In cognitively impaired, but non-demented people, both DHA and total LCn3s are lower. Low serum DHA level is considered a significant risk factor for the development of Alzheimer's dementia [90], and thus the population's baseline serum DHA levels may impact on efficacy of intervention on outcomes. Whilst efficacy of DHA intervention appears to be stronger in people with low baseline serum DHA levels, interventions in younger healthy populations may help identify the role of LCn3s in longer-term preventative treatment and progression to a cognitively impaired phenotype.

Currently, a consensus is yet to be reached as to optimal variables required to produce cognitive enhancing effects. RCTs have produced inconsistent results; discrepancies between trials could partially be attributed to these dose and duration variations of LCn3 and additionally the health status in the populations studied. Additionally, increasing duration of studies, whilst improving our understanding of LCn3 supplementation in health outcomes, may increase participant burden and result in reduced compliance and increased attrition, hence reducing study quality.

(vii) False positive and negative outcomes

Another area of controversy includes the variability of outcome measure tools used across studies. As an example, contrasting findings regarding the effects of omega-3 on cognitive function may be due to more sensitive outcome tools developed in recent years. The emergence of computerised battery testing, compared with traditional paper-based assessments of cognitive function are generally agreed to reduce the risk of false positive and negative findings (Type 1 and Type 2 statistical errors), which may have been more prevalent in earlier studies.

6. Emerging novel concepts for application

(i) LCn3s and mental health

There is an increasing body of evidence suggesting that LCn3s are important in mental well-being. DHA is a major structural component of neuronal membranes, and changing the fatty acid composition of neuronal membranes leads to functional changes in the activity of receptors and other proteins embedded in the membrane phospholipid [113]. LCn3s are known to have important physiological functions that directly acts on neuronal activity. Studies indicate a strong association between depression and low dietary intake of LCn3s [114] as well as correlation between rates of homicide and mental disorders with LCn3 levels [115]. Interestingly, studies show reduced levels of LCn3s in red blood cell membranes of depressive and schizophrenic patients [114]. Double-blind, placebo-controlled trials in schizophrenia, and trials in depression, have reported therapeutic benefit from LCn3s, when given in addition to antidepressant therapy [114,116,117].

At a cellular level, LCn3s are incorporated into all cell

membranes, but those of the brain, myocardium and retina are particularly enriched. Furthermore, these fatty acids perform a plethora of actions, including facilitating the conformational changes of rhodopsin, assisting in nerve cell signalling and neurodevelopment, modulating the activities of cardiac ion-channel proteins, and modifying gene expression [113,114,116,117]. At the molecular level, LCn3s can affect gene and protein expression, modulate membrane protein activity, and serve as a reservoir for bioactive molecules. Several mechanisms have been proposed by which LCn3 acts as an antidepressant, these include (i) regulating serotonergic and dopaminergic neurotransmitters in signal transduction [118,119], (ii) EPA may have a beneficial effect on hypothalamic-pituitary-adrenal axis dysfunction, treatment-resistant depression, and multidrug resistance through the action of P-glycoprotein, which transports many substrates, including steroids [115,120], and (iii) LCn3 beneficial effects on depression and mood could be by interfering with parts of the arachidonic acid cascade such as inhibitory effect of LCn3 on phospholipase A2 (PLA2) [121,122]. Neuroimaging studies are also being used to help to evaluate the neurobiological effects of omega-3 in mental health. For example, a current RCT is assessing the effect of LCn3 treatment in an older age cohort on depressive symptoms and correlating these with brain changes [123].

(ii) LCn3s and cognitive function - therapeutic/preventative

Studies in children with attention deficit hyperactivity disorder (ADHD) or other developmental disorders supplemented with fish oil supplements have shown improvements in their learning and performance at school [124–126]. In older more vulnerable groups, it also appears that LCn3 may help to improve cognitive function [76] and to slow conversion from MCI to Alzheimer's disease [127] In healthy young and older individuals evidence from randomized clinical trial research had been mixed with regard to seeing a benefit with cognition. For example, a large trial in 70–80 year olds in the UK (OPAL; n = 687 participants), supplemented for 2 years with 700 mg per day of EPA and DHA and powered for cognitive effects, did not show improvements in cognition [89]. Yurko-Mauro et al. [9] did show improvements in paired-associates learning using a sensitive computerised cognitive battery (CANTAB) in 485 individuals supplementing with 900 mg of DHA for 24 weeks. The choice of cognitive endpoints has also been raised as an issue, with earlier studies using very course measures of cognition. In more recent years, computerised cognitive tasks have proven useful for assessing potential improvements in cognitive faculties that decline most with age [9,128].

Positive studies have also highlighted the importance of using an optimal experimental design that includes selection of participants with low levels of LCn3. Stonehouse et al. [27] selected 176 younger healthy individuals (18–45) with low levels of LCn3 and found significant improvements in cognition following 6 month supplementation. Similarly, Witte et al. [108] showed in healthy older individuals (50–75) significant improvements in cognition and brain structure following omega-supplementation. Moreover, improvements in executive function were correlated with increases in the omega-3 index. Studies which have not selected on the basis of lower omega-3 status have found negligible cognitive effects in healthy adults supplemented for 6 months with DHA in an older cohort aged 50–70 years [129] and in younger adults supplemented with EPA or DHA (1 g) for 12 weeks [80]. Interestingly in the younger adults there was evidence of increases in cerebral blood flow [80,99].

Other studies using functional neuroimaging found more efficient brain activity in healthy young participants following supplementation with EPA rich supplements as compared to DHA rich supplements [87,130]. Another recent study showing improvements in cardiovascular function and cognition [131] may have been facilitated by the concurrent supplementation with a multivitamin [132]. The authors suggested that co-factors in the supplement and the diet may be important for the uptake of omega-3 into red cells and therefore important for health endpoints such as cognition.

(iii) LCn3s and the microbiome

The gut microbiome, its metabolites and the integrity of the gut wall (intestinal permeability) are emerging as important contributors to human health and the development of chronic diseases. Over the last 10 years, there is evidence that chronic diseases may in fact have infective contributors which are influenced by the food we eat [133]. This represents a paradigm shift in our thinking regarding the cause and treatment of many chronic diseases. On the one hand, adverse changes to gut microbes have been implicated in the development of diet-induced obesity [134], chronic inflammation [135,136] and insulin resistance [137]. On the other hand, beneficial changes to gut microbes and their metabolites may explain the health benefits of the plant-based Mediterranean diet [138,139].

The discovery that gut dysfunction and elevated serum lipopolysaccharides (LPS) from bacterial cell walls were associated with obesity or insulin resistance in mice [135,136,140] and humans [141,142] provided new insights into a potential linkage between gut microbes, gut barrier function, white adipose tissue inflammation and diet. The human gastrointestinal tract is host to a complex microbial ecosystem of hundreds of bacterial species also known as the gut microbiome. A diverse microbiome appears to play a crucial role in the development of a healthy gut and immune system, while disturbances (or reduction in diversity) have been associated with systemic inflammation and chronic diseases. Animal, and a limited number of human, studies have shown that diets high in animal protein, animal fat and sugar and low in fibre and unrefined carbohydrates are associated with reduced microbiota diversity, increased relative abundance of undesirable bacteria and their toxic metabolites, including the cardiotoxin trimethylamine-N-oxide (TMAO) [143].

Gut microbes are not only influenced by fibre, resistant starch and carbohydrates but also by the type and amount of dietary fat, which, in turn, may directly or indirectly impact the host. Recent rat studies found that dietary lipids, mainly saturated fats, adversely affect specific populations of gut microbes and their metabolic end products, for example by secreting pro-inflammatory products that impair gut barrier function, leading to systemic endotoxemia and inflammation as well as insulin resistance [144]. These pro-inflammatory products may also enhance energy harvest, leading to a positive energy balance and obesity. This in turn may further adversely influence gut microbes creating self-perpetuating cycles of gut and systemic dysfunction.

In animal studies, long chain saturated fatty acids may decrease the abundance of *A. muciniphila*, a specific type of mucin-degrading bacteria that plays a preventative role in the development of diet-induced obesity. Short chain saturated fatty acids such as those found in milk fat (and potentially coconut oil) may also preferentially select for mucosal sulfate/sulfite-reducing bacteria (i.e., *B. wadsworthia*) that diminish epithelial integrity and increase intestinal permeability through their production of the pro-inflammatory and genotoxic gas hydrogen sulfide [144]. Surprisingly little has been reported on the effects of LCn3s on gut microbes. However, consuming diets rich in LCn3 has been reported to protect intestinal cells from pro-inflammatory insults that contribute to inflammatory bowel disease, or activation of immune

cells by (a) decreasing inflammatory eicosanoid production; (b) decreasing activation of pro-inflammatory MAPKs, NF-κB, activator protein-1, and (c) increasing PPARγ [144].

Dietary LCn3s have been reported to protect intestinal epithelial cells from pro-inflammatory insults and accelerate recovery from inflammation which may reduce intestinal permeability. For example, EPA and DHA maintained the integrity of human intestinal epithelial cells exposed *in-vitro* to interleukin-4 (IL-4) by enhancing epithelial resistance and membrane integrity [145]. Consumption of fish oil-rich diets containing 25%–30% (w/w) EPA and DHA or perilla oil containing 55%–60% (w/w) α-linolenic acid (C18:3n-3) relieved chronic ileitis in senescence-accelerated P1/Yit mice by inhibiting monocyte recruitment in inflamed intestinal tissue [146]. Supplementation of LCn3s has also been shown to reduce plasma endotoxin levels in dextran sodium sulfate (DSS)-induced colitis in rats [147].

In summary, potential mechanisms by which LCn3s protect gut health include [144]:

(1) reduction of NF-κB-mediated inflammation in immune and intestinal cells. NF-κB signalling is reduced by activation of peroxisome proliferator–activated receptor (PPAR)-γ, which in turn suppresses inflammatory gene expression by directly interfering with transcriptional activation of NF-κB and activator protein-1.
(2) inhibition of inducible nitric oxide synthase and nitric oxide production.
(3) anti-inflammatory actions through incorporation into the phospholipid membranes of plasma or immune cells or gut mucosal tissue as demonstrated in human and rodent models.
(4) reduced intestinal cytokine production via reduced synthesis of phospholipids derived from LCn6s, resulting in decreased production of arachidonic acid and its pro-inflammatory, cyclooxygenase- or lipoxygenase-derived prostaglandins, thromboxanes and leukotrienes.

Rat studies have also shown that reducing the LCn6:LCn3 ratio to at least 4.5:1 significantly attenuated the abundance of pro-inflammatory thromboxanes and prostaglandins in the colon in Wistar rats [148]. Having an LCn6 to LCn3 ratio of 3:1 or lower prevented damage of intestinal mucosal layer and resolved inflammatory colitis manifestations in both Sprague–Dawley and Wistar rats [147,148].

Therefore, limiting both saturated and LCn6 fat intake and increasing sources of LCn3s, fibre and complex carbohydrate-rich foods as sources of prebiotics should help promote healthy populations of gut microbes, thereby improving intestinal health and reducing the risk of gut and systemic inflammation and related diseases.

(iv) LCn3s in parenteral nutrition and liver disease

There is emerging evidence for the use of LCn3s in specific disease states requiring specialized nutrition intervention. Areas of current research include the use of fish oils as part of nutrition support regimens in patients who are admitted to intensive care units, who require nutrition support following gastrointestinal surgery and in those who are unable to meet their nutritional needs through oral diet alone. Liver disease in patients receiving parenteral nutrition (PN), nutritional formula infused directly into the blood stream is well known, particularly in those receiving long term PN support. Other complications associated with PN include increased risk of infection, increased ventilator days in critically ill patients and increased length of stay. The proposed physiological mechanisms behind these complications are multifactorial and beyond the scope of this article, however a key theory behind these complications is increased inflammation driven by the fatty acid profile. Traditionally, soy bean oil has been the major component of parenteral lipid solution as it contained sufficient amounts of essential fatty acids, linoleic acid and α-linolenic acid to prevent deficiency. The predominant fatty acid in soybean oil is however linoleic acid, resulting in a high and unfavourable LCn6 to LCn3 ratio of 5:1 [149]. Furthermore there is emerging evidence that intravenous lipid emulsions that include mixed oil sources such as an olive oil/soy oil blend supplementing PN with fish oil may have a positive impact on PN related liver disease [150] and may have favourable outcomes such as reduced length of stay [23,151,152]. This remains an emerging area of research with more studies required to determine the optimal fatty acid profile and which groups of patients will benefit most.

7. Conclusions

The original focus on LCn3 fatty acids and cardiovascular disease was prompted by the remarkably low rates of CVD in the native Intuits of Greenland. These native peoples of Greenland consumed a high marine fat diet from seals and walruses with a modest amount of plant foods, yet remained healthy. A plethora of epidemiological studies have supported the protective effects of a high fish diet and reduced CVD and this has been supported by well controlled clinical trials such as the GISSI-Prevenzione trial. LCn3 supplementation trials have not experienced the same consistency of findings for CVD protection, nor in emerging areas of potential benefits such as reversal of metabolic syndrome and NAFLD, reversal of early cognitive decline, reversal of depression and the health of the microbiome. Recent reviews of the efficacy of supplementation trials have highlighted issues with study design such as inadequate intervention periods, poor sample size of studies, inadequate dose of supplements, variations in the ratio of EPA to DHA and issues of supplement quality due to oxidative degradation. Furthermore, the focus on LCn3 fatty acid dose may not be hitting the target, as emerging evidence supports a balance of n6:n3 towards unity in the diet rather than individual dose of LCn3. It appears that LCn3 supplementation at the appropriate dose, in the appropriate high risk population group, and for an adequate duration will ultimately have a beneficial effect on health especially in inflammatory conditions. Additionally, whilst it appears to be people with low baseline serum DHA levels, and those at an older age, that benefit the most, long term interventions in younger healthy populations will help identify the role of LCn3s in preventative treatment. However, without a focus on improving the LCn3 content of the diet and the n6:n3 ratio we are employing a short term solution for a long term chronic problem.

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