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## Review

## Conducting omega-3 clinical trials with cardiovascular outcomes: Proceedings of a workshop held at ISSFAL 2014

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## ABSTRACT

In contrast to earlier long-chain (LC) omega-3 (i.e. EPA and DHA) investigations, some recent studies have not demonstrated significant effects of EPA and DHA on cardiovascular disease (CVD) outcomes. The neutral findings may have been due to experimental design issues, such as: maintenance on aggressive cardiovascular drug treatment overshadowing the benefits of LC omega-3s, high background LC omega-3 intake, too few subjects in the study, treatment duration too short, insufficient LC omega-3 dosage, increase in omega-6 fatty acid intake during the study, failure to assess the LC omega-3 status of the subjects prior to and during treatment and lack of clarity concerning which mechanisms were expected to produce benefits. At the 11th ISSFAL Congress, a workshop was held on conducting LC omega-3 clinical trials with cardiovascular outcomes, with the goal of gaining a better understanding concerning aspects of experimental design that should be considered when planning clinical studies related to EPA and DHA and potential cardiovascular benefits.

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## 1. Introduction

At the 11th Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL), the Global Organization for EPA and DHA Omega-3s (GOED) sponsored a workshop on conducting long chain omega-3 (LC omega-3) clinical trials with cardiovascular outcomes. The goal of the workshop was to gain a better understanding, from scientists intimately involved in LC omega-3 fatty acid (EPA/DHA) research, about aspects of experimental design that should be considered when planning clinical studies related to EPA/DHA and potential cardiovascular benefits.

The inspiration for the workshop was frustration among GOED personnel during recent years with the publication of a number of cardiovascular studies of EPA/DHA demonstrating neutral results,

but with less than optimal experimental designs [1–7]. While any, or all, of these studies could have been neutral because there are no cardiovascular benefits associated with increased EPA/DHA intake, given the past positive findings [8–11], that is not considered to be likely. Some studies with neutral findings have been heavily publicized, gaining widespread attention, yet the findings are rarely put into proper context, thus they may be misinterpreted or the findings exaggerated. The conclusion from this becomes that LC omega-3s (EPA/DHA) are not “heart healthy.” One danger of this is that consumers become confused, understandably, and so stop eating fish and/or taking LC omega-3 supplements.

In contrast to earlier investigations [8–11], some recent studies [1–7] have not demonstrated significant effects of the LC omega-3 fatty acids EPA and DHA on cardiovascular disease (CVD) event risk. Neutral findings from these more recent studies may be attributable to a number of possible causes, including: maintenance on aggressive cardiovascular drug treatment overshadowing the benefits of LC omega-3s, high background LC

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omega-3 intake, too few subjects in the study, treatment duration too short, insufficient LC omega-3 dosage, and increase in omega-6 fatty acid intake during the study. These issues were addressed during the workshop. Five scientists with expertise in LC omega-3 fatty acids presented during the workshop and this review summarizes their presentations in the order given.

## 2. Statistical power considerations in the design of omega-3 trials with cardiovascular outcomes (Aldo Bernasconi, PhD)

There is a long history of clinical research showing that LC omega-3 fatty acids have a cardio-protective effect. This research includes both observational studies, of which there are a large number, and several large randomized control trials (RCTs). One surprising aspect of this research is that while the earlier RCTs showed that consumption of fatty fish or of LC omega-3 supplements have a clear benefit for cardiovascular health, later studies with similar design have found smaller effects, or failed to find a significant effect.

The first large RCT on this subject was the Diet and Reinfarction Trial (DART), conclusions from which were published in 1989 [8]. The researchers recruited 2033 men who had recovered from a myocardial infarction, and randomized them to receive advice on three possible dietary changes: 1) to increase consumption of fatty fish, 2) to increase the ratio of polyunsaturated to saturated fat, or 3) to increase cereal consumption. The study found that consumption of two weekly servings of fatty fish not only reduced the risk of death from ischemic heart disease (IHD), but also reduced all-cause mortality by 29% compared with the results for those not given advice about consuming fatty fish.

A large, open-label, RCT, the GISSI-Prevenzione trial [9], confirmed a protective effect of LC omega-3 fatty acids. In a group of 11,324 patients who had survived a recent myocardial infarction, LC omega-3 fatty acid ethyl ester consumption (1 g/d) reduced the risk of all-cause mortality, sudden death, and coronary death compared with usual care. The findings of these two large studies were concordant, suggesting that LC omega-3 fatty acids were protective against cardiovascular mortality in patients who had already suffered a myocardial infarction, and they supported results from several prospective cohort studies. The findings from these studies were reviewed by Harris et al. [12], who found that LC omega-3 fatty acid consumption was associated with decreased coronary heart disease mortality.

Later studies, on the other hand, have failed to find these same effects on mortality ([2–4], for example), reporting either evidence of a much smaller protective effect, or no effect at all. This development has caused confusion amongst consumers as to whether fatty fish and LC omega-3s are “heart healthy.” As a result, there is some debate about whether omega-3 supplements reduce cardiovascular risk, and even about whether the dietary recommendations to eat more fish are appropriate.

The reason for this apparent contradiction may be that advances in the treatment and prevention of CVD have radically changed the environment in which trials of EPA/DHA are conducted. As a consequence, the effect of the LC omega-3 fatty acid interventions seen in clinical trials has been reduced, and study designs that were adequate even 20 years ago, now result in studies that are underpowered for the outcomes being investigated.

To understand why this is the case, one needs to examine some aspects about the statistical framework used for hypothesis testing, known as the Neyman–Pearson paradigm [13]. Under this approach, before conducting an experiment the researcher chooses a significance value or *p*-value cutoff. This cutoff, usually denoted by the Greek letter alpha, is a statement about the acceptable risk of getting a false positive result. A researcher

choosing an alpha value of 0.01 is stating that, for this experiment, he or she is willing to accept a 1% risk of a false positive result. Along with the alpha level, the researcher will generally select a desired statistical power (defined as the probability of reaching a true positive result), and select the number of participants and other study design aspects accordingly. The choice of a particular value for statistical power is a statement of the researcher's tolerance for false negative results. For example, if a researcher plans an experiment to have 90% power, he is in fact stating that he is willing to accept a 10% risk that the experiment will miss (fail to declare statistical significance at the specified alpha level) a result that is, in fact, true.

There are several methods by which a researcher can increase the power of an experiment, but they are not always practicable, and each comes with a cost. It is important to note that these associated costs are not always in terms of money or time. In a sense, designing an experiment is a form of optimization: finding the design that maximizes statistical power, while keeping the costs at an acceptable level. Most of these methods fall into one of the following categories:

1. Increasing the number of samples/subjects;
2. Increasing the effect of the intervention;
3. Reducing the sample/subject variability that is not caused by the intervention;
4. Selecting a more appropriate statistical analysis.

### 2.1. Increasing the number of samples/subjects

Intuitively, the more samples or subjects used in a study, the better and more precise all numerical estimates will be. This makes it easier to discern an intervention effect amid the variability in the data, thereby increasing power. The obvious drawback is that it is not always possible to recruit more subjects, either because the population of interest that is willing and able to participate is too small, or because increasing recruitment would make the cost of the study prohibitive. Other potential costs may include longer times to collect the necessary samples and data, the need to develop collection, storage and analysis procedures to handle the increased volume, or longer times from sample collection to analysis, which may affect measurements of less stable analytes.

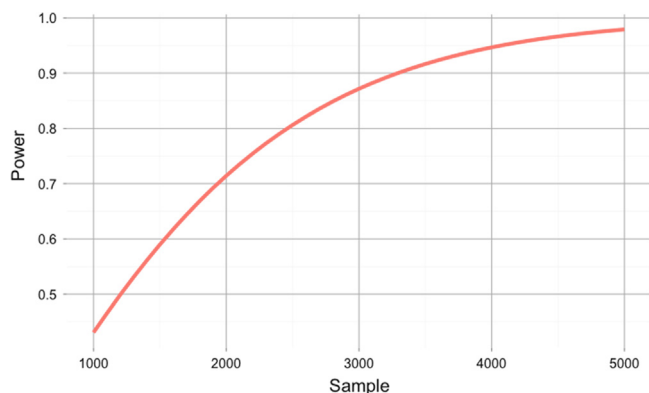
Researchers must then strive to find the right balance between study size and cost. By relying on prior knowledge or assumptions about a desirable intervention effect size, they can use statistical power computations to estimate the number of samples necessary to reach a pre-specified statistical power. Several common paradigms for measuring the quality of reporting of clinical trials require that the method used to decide the number of samples or participants is described, but adherence to this requirement is low. In a more specialized application, Guo et al. [14] examined articles on cancer nursing RCTs published between 1984 and 2010 for compliance to CONSORT [15] quality criteria, and estimated that only 45% of these publications explicitly describe the method by which sample size was selected. Turner et al. [16] estimated that only 48% of articles based on RCTs published in medical journals comply with this requirement.

One often overlooked aspect of increasing the number of subjects is that the increased benefits of adding participants diminish as the number of subjects increases. This is best explained with an example. Enns et al. [17] reviewed existing evidence and conducted a meta-analysis to determine whether LC omega-3 fatty acid supplementation reduces the risk of cardiovascular events in patients with existing peripheral arterial disease (PAD) (Table 1). The meta-analysis identifies that LC omega-3 supplementation confers a risk reduction from 5.6% to 3.9% (relative risk=0.69), but

**Table 1**

Incidence of cardiovascular events in patients with existing peripheral arterial disease receiving LC omega-3 fatty acids or placebo, as reported by the systematic review and meta-analysis of [17]. The events for myocardial infarction and cardiovascular death were obtained by combining the results of [18,19]. Results for stroke and angina were obtained from [18,19], respectively.

Outcome	LC Omega-3 fatty acids	Control
Myocardial infarction	6/156	8/132
Cardiovascular death	5/156	7/132
Stroke	3/60	1/60
Angina	5/177	8/106
<b>Total:</b>	<b>19/489</b>	<b>24/430</b>



**Fig. 1.** Relationship between sample size and statistical power in a two-sample proportion test to detect a reduction in incidence in the risk of cardiovascular events from 5.6% to 3.9% in a high-risk population with Peripheral Arterial Disease [17].

that the difference is not statistically significant. Fig. 1 shows the relationship between the number of samples in each group (LC omega-3 treated and control) and the statistical power in a study of an outcome with these risks. If this protective effect is real, a study with 2000 participants in each group would have a 71.4% chance of detecting it with significance  $< 0.05$ . Increasing the number of participants to 3000 would increase this power to 87.2%. However, increasing the number of participants from 4000 to 5000 only increases the power from 94.7% to 97.9%. A researcher must decide at what point the costs of adding additional subjects outweigh the benefits of increased power.

One method used to achieve a higher number of participants is to combine the results of previous trials by means of a meta-analysis. Enns et al. [17] combined the results of two prior trials [18,19]. The two studies were conducted in different populations, with different demographic and socioeconomic profiles, genetic makeup, lifestyles, diets and standards of medical diagnosis and care. Each one of these differences introduces some noise into the final data. In a way, a meta-analysis is an attempt to achieve more power by increasing sample size, but it comes at the cost of introducing variability into the final data set and often some difficulty in interpreting the results. Because of this extra variability, a meta-analysis will not have higher power than an RCT with an equal number of samples. In this case, both the control and treated groups contain fewer than 500 participants, so the power is well below 50%. In other words, even if supplementation with LC omega-3 fatty acids confers a cardioprotective effect on patients with PAD, a meta-analysis this size would have less than a 50% chance of detecting it at a significance level of  $< 0.05$ . The correct interpretation of the results of this meta-analysis is that existing evidence is insufficient to determine whether or not intake of LC omega-3 fatty acids prevents cardiovascular outcomes in this population.

## 2.2. Increasing the effect of the intervention

An alternative approach to increase power is to modify the intervention in order to magnify its effect. One extreme form of this approach is seen in toxicology or drug safety experiments performed on animal models. The laboratory animals are exposed to much larger effective doses of the compound of interest than any reasonably expected potential exposure in humans. For obvious reasons, this is not always a practical or ethical approach to use in trials involving human subjects, but some approaches can still be used, including:

1. Increasing dosage where reasonable;
2. Extending the length of the intervention and follow-up;
3. Selecting study participants from higher-risk populations. Each one of these approaches comes at a price. Extending the length of a study, for example, increases its cost, and restricting it to a higher-risk population may reduce the number of participants or affect the generalizability of the results.

Increasing the dosage of LC omega-3 fatty acids used in clinical studies is both a low-cost and safe method to potentially increase study power. EPA and DHA have been established to be safe and well-tolerated at levels well above those used in clinical studies. The EFSA panel on Dietetic Products, Nutrition and Allergy has determined that intake levels of 5 g/d are safe [20], and the Norwegian Scientific Committee for Food Safety considers 6.9 g/d [21] to be safe. Neither of these two levels is seen as a maximum safe intake, but rather an intake level that has been determined to be acceptable – a potential maximum level would be higher than both, but has never been determined. However, both of these intakes are many times higher than habitual intakes and the levels of supplementation used in clinical trials (typically in the 500 mg to 1 g/d range).

It is important to note that, depending on the outcome of interest, the effect of an intervention is not necessarily proportional to dosage, and that the effect of supplementation will often depend on the baseline characteristics of the population being studied. According to Papanikolaou et al. [22], the average intake of EPA+DHA among US adults is 113 mg/d, of which 83 mg/d came from food, and the rest from dietary supplements. There are no reliable estimates of EPA+DHA intakes in the late 1980s, at the time in which DART was being conducted, but fish intake estimates compiled by the U.S. Environmental Protection Agency indicate that U.S. per capita fish consumption has been steadily, albeit slowly, increasing since 1982 [23]. During DART [8], when the use of LC omega-3 supplements was low, only a minimal amount of EPA+DHA would have come from supplementation in the general population, so it is reasonable to assume that EPA+DHA intake would have been somewhat lower than 83 mg/d. Fig. 2, adapted from Mozaffarian and Rimm [24], shows the relationship between EPA+DHA intake and the relative risk of death caused by coronary heart disease (CHD).

Some observations are important:

1. There appears to be little benefit in supplementing beyond 250 mg/d. This is an outcome for which increasing dosage would not result in an increased treatment effect and more power.
2. The effect of 1000 mg/d EPA+DHA supplementation on a population with baseline intake of 83 mg/d (a reasonable estimate of the U.S. adult intake at the time of DART, as described above) would have a much larger protective effect than that observed in the ORIGIN trial [4]. In other words, because of the shifting baseline, studies with identical designs would have had more statistical power 30 years ago. Newer studies require more participants, longer follow-up times, or the use of other approaches to increase their power.

3. Because of the cardioprotective reputation of LC omega-3 fatty acids, people with a history of heart disease may eat more fish and be more likely to take supplements, their habitual intake of EPA+DHA may be higher than that of the general population. A study using supplementation as the intervention would have a small expected treatment effect, low power, and be unlikely to detect a significant protective effect. This may be part of the reason why newer secondary prevention trials for cardiovascular outcomes have failed to detect a significant effect.

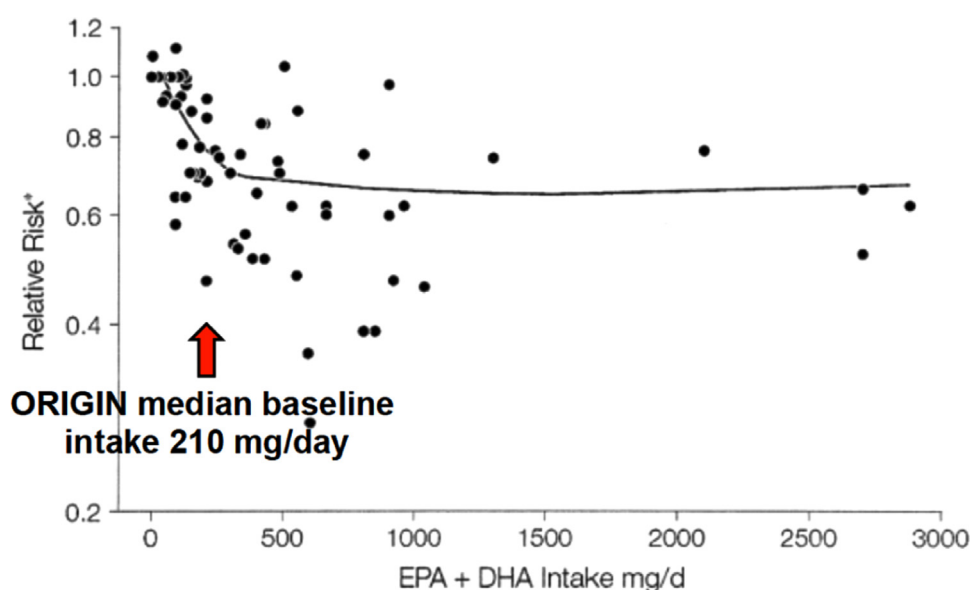
There are some outcomes for which increasing the intervention dose would result in an increased effect size. Fig. 3, taken from Mozaffarian and Rimm [24] shows the relationship between treatment dosage of EPA+DHA and the protective effect strength for multiple cardiovascular outcomes. While an increase in dosage

would be unlikely to increase statistical power for a trial concerning arrhythmia, or, as discussed above, CHD death, it would have an effect for multiple other indications.

While increasing dosage does not always increase the treatment effect, requiring participants to refrain from taking supplements that are not part of the study, or selecting participants from groups with lower baseline intake (as proposed by James et al. [25]) would decrease EPA+DHA levels in the control group, and may increase the treatment effect.

### 2.3. Reducing the sample/subject variability that is not caused by the intervention

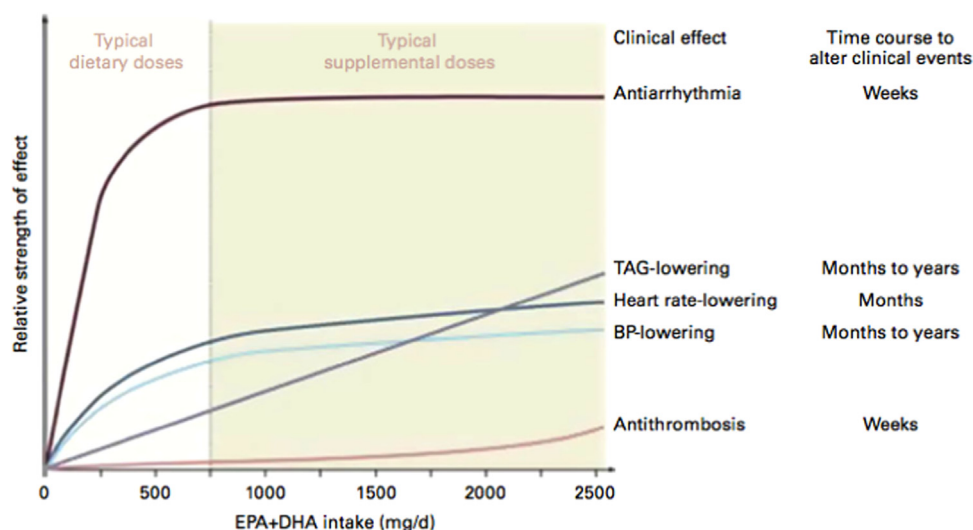
A third method to increase power is to reduce the variability among samples that is not directly caused by the intervention. In



Abbreviations: CHD = coronary heart disease, DHA = docosahexaenoic acid, EPA =

eicosapentaenoic acid, ORIGIN = Outcome Reduction with an Initial Glargine Intervention

**Fig. 2.** Pooled analysis of prospective cohort studies and randomized clinical trials of the relationship between EPA+DHA intake and relative risk for CHD death. Copyright © (2006) American Medical Association. All rights reserved. Adapted with permission from [24] with data marked from the ORIGIN trial [4].



**Fig. 3.** Relative protective effect for several cardiovascular outcomes, depending on intervention dosage. Copyright © (2006) American Medical Association. All rights reserved. Reprinted with permission from [24].

the case of studies involving laboratory animals, this is accomplished by using animals of the same sex and strain, and by making sure that the environments and diets of all groups are as close to identical as possible. This cannot be done in studies involving human subjects, but some control over unwanted variability can be accomplished by selecting study participants of the same sex, age group, genetic makeup or disease predisposition, among other variables. While selecting relatively similar participants can increase the statistical power of the study, it often comes at the cost of fewer participants or a loss of generalizability.

Other methods to reduce unwanted data variability are to control the conditions under which samples are taken (after fasting, for example), the length and condition of sample storage, and the methods used for sample preparation and analysis.

#### 2.4. Selecting a more appropriate statistical analysis

A final way to maximize the power of a study is to select statistical methods that take the fullest possible advantage of the data collected. One common analytical technique is to separate the participants into groups (quintiles, for example) depending on their risk, the strength of their response, or the level of a biomarker of interest, and then to compare the two most extreme groups. Doing so frequently increases the measured intervention effect, but it comes at the cost of only using a smaller number of participants. In most cases it is more effective to use all the data collected and draw conclusions from a linear model, logistic regression to estimate risk of an outcome, or survival analysis to study time-to-event.

In many cases it is impossible to select participants or to control the experimental conditions to completely remove the most important confounding variables, but it is possible to explicitly incorporate some of these variables in the data modeling, as a way to remove (or at least reduce) their effect on the analysis of the parameters of interest. Some of the most important confounding variables in cardiovascular research involving LC omega-3 fatty acids include:

1. Socioeconomic variables: For many populations, habitual fish intake and LC omega-3 supplement use are associated with higher socioeconomic status, which is connected with a better standard of care and faster diagnostics.
2. Baseline EPA+DHA levels: As described previously, the effect of an intervention frequently depends on baseline levels, so it makes sense to consider this variable during final analysis.
3. Use of medication: The standard of care for both primary and secondary cardiovascular prevention has changed dramatically in recent years, with more patients at risk for cardiovascular events taking lipid lowering and other cardio-protective medications, and use of these drugs may overwhelm the true effect of LC omega-3 fatty acids. A subgroup analysis of the meta-analysis in Kwak et al. [26] showed LC omega-3 supplementation to have a stronger cardio-protective effect in trials where most subjects were not already using lipid-lowering drugs. It would be difficult to conduct a trial involving only participants not already taking any of these agents, but including drug usage in the data analysis may reduce their confounding effect.

To conclude, baseline levels of EPA+DHA intake and status, the incidence of cardiac events, and the medical standard of care have changed dramatically since the publication of DART [8] and GISSI Prevenzione [9], the first large RCTs on the effect of LC omega-3 fatty acids on cardiovascular mortality, and this has profound implications for the design of newer trials. Study designs that were effective 35 years ago may lack sufficient power today, leading to inconclusive results and confusion about the role of LC omega-3

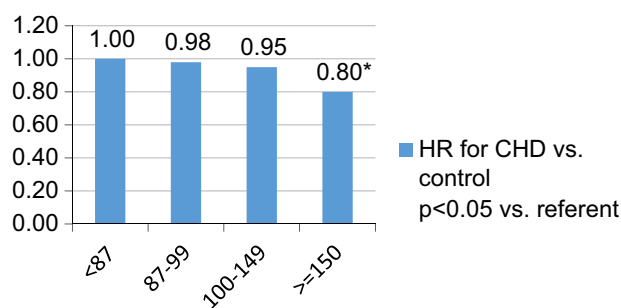
fatty acids in cardiovascular prevention. Researchers need to consider these changes when planning future trials.

### 3. Design issues for clinical trials of omega-3 products assessing cardiovascular outcomes (Kevin C. Maki, PhD)

The first observational evidence, nearly four decades ago, for a cardioprotective effect of LC omega-3 fatty acids came from the discovery that Greenland Inuits, who consumed a diet high in EPA+DHA, had lower mortality from heart disease than Americans and Europeans, who consumed less of these fatty acids [27–30]. A substantial body of epidemiological evidence for the association between reduced cardiac events/mortality and higher intakes (mostly from fish) and blood levels of LC omega-3 fatty acids have accumulated since that time [31–33]. In 1999, findings from a large-scale, open-label, RCT were published that aligned with the observational evidence [9,34,35]. The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione trial of 11,323 patients with recent (< 3 months) myocardial infarction results suggested that 1 g/d of LC omega-3-acid ethyl esters significantly reduced the risk of total mortality (relative risk [RR] 0.59, 95% confidence interval [CI] 0.36–0.97,  $p=0.037$ ) as early as three months after starting the omega-3 fatty acids, as well as sudden cardiac death (RR 0.47, 95% CI 0.219–0.995,  $p=0.048$ ) after 4 months, and cardiovascular, cardiac, and coronary deaths after six to eight months of treatment [34,35].

In the years since the GISSI-Prevenzione trial, several RCTs have failed to detect the same significant cardioprotective effects, prompting controversy and debate about the utility of LC omega-3 fatty acids for the reduction of cardiovascular risk [6,36]. Findings from recent meta-analyses of RCT data from trials with LC omega-3 fatty acid interventions have provided evidence for a modest benefit on risk for cardiac death, but have generally failed to show significant reductions in other cardiovascular disease (CVD) outcomes [36–39].

Careful examination of the RCTs indicates, however, that there have been several study design issues that may have biased the results toward the null, reducing the likelihood that beneficial effects of LC omega-3 fatty acids on CVD outcomes would be detected. Among these are 1) the use of low dosages of EPA and/or DHA, 2) a failure to assess the LC omega-3 status of the subjects prior to and during treatment, 3) the concurrent use of cardiovascular and lipid-altering medication(s) that might have overwhelmed the effects of the omega-3 fatty acids, and 4) a lack of clarity as to which mechanisms were expected to produce benefits. It is important to consider these points so that future clinical trials can be designed in a manner that optimizes the potential for demonstrating benefits, if present, of LC omega-3 fatty acid



Abbreviations: CHD = coronary heart disease, EPA = eicosapentaenoic acid, HR = hazard ratio,

JELIS = Japan EPA Lipid Intervention Study

**Fig. 4.** Relationship between on-treatment plasma EPA concentration (μg/mL) and adjusted risk of major coronary events among participants in JELIS [40].

interventions on CVD outcomes, while better defining the subsets of the population that are most likely to benefit from such interventions.

To date, clinical trials investigating cardiovascular effects of LC omega-3 fatty acids have generally used relatively low dosages (median [interquartile range] 1.0 [0.5–1.8] g/d EPA and/or DHA ethyl esters) [36]. Evidence from a subgroup analysis of the Japan EPA Lipid Intervention Study (JELIS), which administered 1.8 g/d EPA as ethyl esters, indicated that the greatest cardiovascular event risk reduction occurred in subjects who achieved plasma levels of EPA in the highest quartile ( $\geq 150 \mu\text{g/mL}$ ), whereas no statistically significant benefit was observed in subgroups that achieved lower levels of plasma EPA [10,40] (Fig. 4). A cohort study reported by Mozaffarian et al. [41] showed that higher circulating individual and total omega-3 levels were associated with lower total mortality, especially coronary heart disease (CHD) death, and that subjects in the highest quintile of plasma total omega-3 fatty acids (median=6.04%), compared to the lowest quintile (median=3.17%), had a 27% relative reduction in total mortality. Based on extrapolation of results from dose–response studies (e.g., Browning et al. [42]), it is unlikely that consumption of LC omega-3 products providing 1 g/d EPA+DHA ethyl esters would be sufficient to raise mean levels of omega-3 fatty acids to the range where the greatest benefit has been observed ( $\geq 150 \mu\text{g/dL}$  plasma EPA and  $\geq 6\%$  for plasma phospholipid total omega-3 fatty acids).

In many recent clinical trials examining LC omega-3 treatments in high-risk individuals, the majority of subjects were concurrently taking cardiovascular and/or lipid-altering medications as standard of care, and this may have overwhelmed the LC omega-3 effect. In the Outcome Reduction with an Initial Glargine Intervention (ORIGIN) trial, an examination of LC omega-3 fatty acids and cardiovascular disease outcomes in patients with dysglycemia, for example, approximately 54% of subjects were taking a statin, 69% were taking an angiotensin converting enzyme inhibitor or angiotensin II receptor blocker, 53% were taking a beta-blocker, and 69% were taking aspirin [4]. Notably, a subgroup analysis of a meta-analysis of randomized controlled trials by Kwak et al. [26] showed a trend toward cardiovascular event risk reduction with LC omega-3 fatty acid supplementation in the trials where the majority of subjects were not using lipid-lowering agents (RR 0.74, 95% CI 0.54–1.03), compared to the trials in which a large percentage of subjects were using lipid-lowering agents (RR 1.02, 95% CI 0.92–1.12). In future clinical trials enrolling high-risk subjects, the potential confounding effects of concomitant use of cardiovascular and lipid-altering medications should be considered.

LC omega-3 fatty acids are hypothesized to produce cardiovascular benefits through a variety of mechanisms including: antiarrhythmic effects, lipoprotein lipid modifications, hemodynamic changes (reduction of blood pressure and heart rate), and antithrombotic and anti-inflammatory effects. The various proposed mechanisms have different dose–response characteristics that need to be considered at the trial design stage (Fig. 3).

As previously mentioned, the strongest evidence from randomized trials is for reducing the risk of cardiac death, which may be attributable to antiarrhythmic effects, particularly with regard to reducing the propensity toward potentially fatal ventricular arrhythmias triggered by ischemia [24,39,43,44]. Data from observational studies suggest a possible threshold effect for the relationship between LC omega-3 fatty acid intake and CHD death. In a pooled analysis of prospective cohort studies and RCTs, a 36% lower risk of CHD death was observed between 0 and 250 mg/d of EPA+DHA consumption (RR 0.64, 95% CI 0.50–0.80,  $p=0.94$ ), but little further benefit was observed at intakes above 250 mg/d [24]. In recent years, LC omega-3 fatty acid intakes in the populations of developed countries have increased due to public awareness of

potential health benefits and the availability of foods fortified with LC omega-3 fatty acids [45]. It is notable that the estimated median baseline intake of EPA+DHA in the ORIGIN trial, designed to investigate the prevention of cardiovascular events in patients with recent myocardial infarction or heart failure, was 210 mg/d, which is close to the threshold of 250 mg/d suggested by the analysis above (Fig. 2), thus, potentially reducing the ability to detect an effect on risk for CHD death.

These results also illustrate the importance of measuring LC omega-3 fatty acid status at baseline and during treatment in order to exclude subjects with high baseline LC omega-3 status who may have less potential to benefit from supplemental EPA+DHA. Another reason for measuring LC omega-3 fatty acid status during and after treatment is to provide a physiological measurement for confirmation of compliance with the LC omega-3 fatty acid intervention.

The low dosages used in most LC omega-3 intervention studies are below the range recommended for management of hypertriglyceridemia of 2–4 g/d of EPA+DHA [46,47]. In addition, the trials completed to date were not designed to assess whether the lipid-altering effects of LC omega-3 fatty acids reduce CVD morbidity and mortality, both because of the low dosages employed, and because they did not select participants based on the presence of hypertriglyceridemia. In hypertriglyceridemic subjects, therapeutic dosages of EPA+DHA typically reduce triglyceride (TG) and very low-density lipoprotein cholesterol levels, and modestly increase high-density lipoprotein cholesterol (HDL-C) [48]. These levels of intake generally have a neutral effect on low-density lipoprotein cholesterol (LDL-C), but may raise LDL-C in some, primarily those with severe hypertriglyceridemia [49,50]. In the JELIS trial, a particularly strong reduction in cumulative incidence of major coronary events was observed in the subgroup with TG  $\geq 150 \text{ mg/dL}$  and HDL-C  $< 40 \text{ mg/dL}$  (hazard ratio 0.47, 95% CI 0.23–0.98,  $p=0.043$ ) [51]. Omega-3 fatty acids alter lipoprotein lipids in a manner similar to the effects of fibric acid medications [49,50,52]. Subgroup analyses from trials of fibric acids have also shown that the subjects with high TG plus low HDL-C had significant reductions in CHD risk, which was generally larger than observed in participants without the high TG/low HDL-C phenotype [53–55]. Thus, a trial to test the efficacy of LC omega-3 fatty acids as lipid-altering agents would ideally employ a dosage of EPA or EPA+DHA in the 2–4 g/d range, and enroll participants with the high TG/low HDL-C phenotype.

In conclusion, investigators designing outcomes trials should carefully consider several study design issues in order to effectively investigate the efficacy of LC omega-3 fatty acids for CVD risk reduction.

1. An adequate LC omega-3 fatty acid dose should be used to produce the mechanistic change(s) through which a cardiovascular benefit might be anticipated.
2. An appropriate study population should be enrolled to test the hypothesized effect; e.g., high-CVD risk subjects with low LC omega-3 status at baseline. To test the efficacy of the lipid-altering effects of omega-3 fatty acids, subjects should be enrolled with high TG and low HDL-C.
3. Consideration is needed of the possible effects of cardiovascular medications in higher risk subjects, and the potential of these agents to confound the effect of the LC omega-3 intervention.
4. Measurement of LC omega-3 status should be performed at baseline and during treatment to ensure that subjects studied have low baseline LC omega-3 fatty acid status, and to allow assessment of compliance with the intervention.

#### 4. Measuring omega-3 fatty acid status (William S. Harris, PhD)

To increase the odds of detecting an effect in a clinical study of LC omega-3 fatty acids with cardiovascular outcomes, low (normal) EPA+DHA status should be an inclusion criterion. Fatty acid status can be measured in a variety of tissues, most commonly from the blood. Fatty acids in the blood are found in the plasma (~50%), red blood cells (~50%) and white blood cells and platelets (<1%). In plasma, the fatty acids are found in 4 main lipid classes: phospholipids (~45%), cholesteryl esters (~11%), triglycerides (~42%) and non-esterified fatty acids (~4%). Red blood cell (RBC), white blood cell and platelet fatty acids are virtually all carried in membrane phospholipids.

In general, a circulating (e.g. plasma, platelets, monocytes, and RBC) fatty acid measurement of LC omega-3 fatty acids is going to provide a reasonably good indication of LC omega-3 fatty acid status, as demonstrated in a recent study [42].

There are advantages and disadvantages associated with each sample type used for the analysis of LC omega-3 fatty acid status (Table 2). With respect to ease of use in the clinical laboratory, whole plasma (or serum), whole blood and red blood cells rank the highest, followed by plasma phospholipids (or PL subclasses) and plasma cholesteryl esters. The most logistically/analytically challenging sample types include: platelets, leukocytes, adipose tissue and cheek swabs.

Based on our work over the last 10 years, we have found that the RBC fraction has several advantages over other blood-based metrics for tracking LC omega-3 fatty acid status. As originally proposed in 2004, the “Omega-3 Index” was defined as the EPA+DHA content of RBCs expressed as a percent of total identified fatty acids, as described elsewhere [57–60]. Several reviews have discussed the clinical utility [61] and the research utility [62] of the Omega-3 Index, and the extent to which it fulfills the criteria for a risk factor for CVD [63].

In addition to there being multiple sample types in which LC omega-3 fatty acid status can be estimated, there are multiple ways of expressing the LC omega-3 fatty acid content of the same sample type. Using either whole blood or RBCs as examples, where the Omega-3 Index is calculable, Lands has proposed using the ratio of n-3 HUFA (highly unsaturated fatty acids) to total HUFA [64]. Whether in whole blood or in RBCs, these two metrics are very highly correlated. Using data from the Heart and Soul Study [65], and from the Framingham Offspring Study [66], we have shown correlation coefficients of 0.93 and 0.95, respectively [56]. Consequently, one could use either metric to get an idea of omega-3 status. An 8% Omega-3 Index, which is the target cardioprotective level [57], corresponds to a 40% n-3 HUFA to total HUFA ratio.

Red blood EPA+DHA content has a low biological (or intra-individual) variability, as well as being a long-term marker of LC omega-3 fatty acid status. To explore this, LC omega-3 fatty acid status from whole blood, whole plasma, plasma phospholipids and RBCs was measured in 20 healthy, free living volunteers for six consecutive weeks [67]. Intra-individual variability of EPA+DHA for the different sample types was as follows: RBCs 4.1%, whole blood 6.7%, plasma 16% and plasma phospholipids 15%. Thus, the RBC biological variability was ¼th of that of plasma.

In addition to being associated with low biological variability, RBCs are also impervious to acute intakes of LC omega-3 fatty acids. In clinical practice in particular, it is important to use a marker that is not acutely altered from normal by a single large intake of fatty fish or fish oil because it can be misleading. To examine this question, we recruited another 20 healthy volunteers and tested them five times over 24 hours after giving them a breakfast including 3.4 g of EPA+DHA [68]. The percent change from baseline was calculated at each time point for RBC EPA+DHA (% of total), plasma EPA+DHA (% of total), and plasma EPA+DHA concentration (µg/mL). While both plasma EPA+DHA (% of total) and plasma EPA+DHA concentration (µg/mL) increased by 20–40% within six hours, Omega-3 Index was unaffected. This also demonstrates the value of the Omega-3 Index to reflect usual intake as opposed to acute deviations from usual. In this regard, the Omega-3 Index may be compared with hemoglobin A1c testing in diabetes. Compared to plasma glucose levels which can vary markedly within an individual during the day, the hemoglobin A1c test (which, like the Omega-3 Index, is measured in RBCs and expressed as a percent) is a far more stable reflection of the patient's usual glycemic status. The same may be said for the Omega-3 Index with respect to omega-3 fatty acid status.

Even using the same sample type (e.g., RBCs), omega-3 testing methods need to be standardized because of differences in methods (reagents, temperatures, timing, sequence of solvent addition, which fatty acids are included in the total, etc.) To demonstrate at least that different labs give different results, five U.S. commercial labs were sent the same blood sample and asked to measure the level of EPA+DHA in RBCs. The results demonstrated a difference of +32% to –63%, i.e. results differed by a factor of 3.5 from what is called the “HS-Omega-3 Index<sup>®</sup>” technology (i.e., the specific method described in Harris et al. [69]). It is safe to assume that the same applies to other non-commercial laboratories. The reasons for these differences are unknown (since the other laboratories do not publish their methods), but the point remains that major differences between laboratories exist. In the absence of standardization, it is important that the same laboratory and the same method are used to measure the pre- and post-intervention samples from within any trial.

**Table 2**

Sample types used to assess LC omega-3 fatty acid status. Adapted with permission of Nova Science Publishers, Inc. from [56].

Tissue	Comment
Whole plasma (serum)	Includes all lipid classes (CE, PL, triglycerides, free fatty acids) and thus variations in plasma lipoprotein levels may affect composition. High biological variability but no lipid class separation required
Plasma phospholipids (PL)	More enriched in LC omega-3 fatty acids than whole plasma and reasonably reflective of tissue PL fatty acid composition, but relatively high biological variability, and requires lipid class separation
Plasma cholesteryl esters (CE)	Carries EPA, with very little DHA, and requires lipid class separation
Whole blood	Includes plasma (with all lipid classes) and cells, hence more enriched in PL than plasma; lower biological variability than plasma or plasma PL; and requires no lipid class separation
Red blood cells	Easily recovered and no lipid class separation necessary; essentially pure PL. Low biovariability and reflects tissue composition. Longest ½ life of any circulating sample type
Platelets	More steps in recovery than plasma or RBC, but no lipid class separation necessary; essentially pure PL. Shorter ½ life than RBCs
Leukocytes	Special methods needed to isolate, but no lipid class separation necessary; essentially pure PL. Shorter ½ life than RBCs
Adipose tissue	Challenging to collect, and not available in the clinical setting. Essentially pure TG, low content of EPA and DHA
Cheek swabs	Non-invasively collected but time-consuming preparation and isolation. No lipid class separation necessary; essentially pure PL

In conclusion, to increase the odds of detecting an effect of LC omega-3 fatty acids in a clinical study with cardiovascular outcomes, low EPA+DHA status should be an inclusion criterion. While LC omega-3 fatty acid status can be determined by several different approaches, in our view the Omega-3 Index (i.e., RBCs analyzed for EPA+DHA by the HS-Omega-3 Index<sup>®</sup> technology) has many advantages.

## 5. Omega-3 status and study design (Clemens von Schacky, MD)

Epidemiologic studies demonstrated an inverse relation of occurrence of severe events like death, sudden cardiac death, fatal and non-fatal myocardial infarctions and intake of EPA+DHA, be it in the form of fish or dietary supplements [70,71]. In contrast, meta-analyses of large RCTs focusing on the same endpoints demonstrated no effect of intake of EPA+DHA, with most trials performed with dietary supplements [38]. As a result, some, but not all guidelines of cardiac societies recommend EPA and DHA for prevention of cardiac events, either in the form of fatty fish or as dietary supplements [72,73]. The following paragraphs focus on the discrepancies between epidemiologic findings and results of intervention trials, try to explain them, and, based on these explanations, suggest novel approaches in designing intervention trials.

### 5.1. Epidemiology: intake vs. levels

A recent systematic review and meta-analysis of observational and intervention studies found in a total of 422,786 participants with 9089 coronary events (like fatal and non-fatal myocardial infarction, coronary death, angina and others) that the highest tertile of intake of EPA+DHA was associated with a 13% lower risk for coronary events (relative risk, RR 0.87, 95% confidence interval, CI, 0.78–0.97), as compared to the lowest tertile [38]. However, when levels were assessed in 20,809 participants with 4073 events, the highest tertile of levels of EPA+DHA (measured in whole blood, serum, plasma, erythrocytes or adipose tissue) was associated with a 25% lower risk for coronary events (RR 0.75, 95% CI 0.62–0.89), as compared to the lowest tertile [38]. Similar findings have been reported elsewhere, as reviewed in [70,71]. Why is it that the relative risk associated with LC omega-3 fatty acid intake is different from the relative risk associated with LC omega-3 fatty acid levels?

Some 50% of participants in observational studies on diet do not report plausible data [74]. In contrast to estimates of dietary intake, blood levels of LC omega-3 fatty acids are not subject to recollection bias, and other subjective influences, and therefore, a clearer picture is likely to emerge. However, assessing blood levels of LC omega-3 fatty acids is subject to biological and analytical variabilities: pre-analytical, analytical, and post-analytical problems, such as biological variability, storage conditions and issues of analytical quality management. Few studies have formally compared biologic variabilities of blood compartments, but in one study, the biological variability of erythrocytes was found to be 1.3%, substantially lower than the biological variabilities of whole blood (5.7%), whole plasma (13.6%) or plasma phospholipids (13.9%) [67]. The low biological variability of erythrocyte fatty acid composition could only be discerned with a method with a low analytical variability. A specific analytical method (HS-Omega-3 Index<sup>®</sup>), standardizing every step of sample preparation, gas-chromatographic analysis and evaluation of the chromatogram brought analytical variability down to 3.9% [67,70,71]. Since it correlates strongly with the EPA+DHA contents of phospholipids in many tissues, the HS-Omega-3 Index<sup>®</sup> assesses an individual's

status of EPA+DHA [70,71]. With the use of this method, it was found that overall mortality was 7% in the lowest tertile, and 26% in the highest tertile of erythrocyte EPA in the first two years in 1144 patients after a myocardial infarction (unadjusted for conventional risk factors, with adjustment for conventional and unconventional cardiovascular risk factors [75]. Studies in other populations had similar results [70,71]. As discussed in more detail elsewhere, a low HS-Omega-3 Index<sup>®</sup> fulfills almost all criteria of a cardiovascular risk factor, according to current criteria of the American Heart Association, except that there is not a HS-Omega-3 Index<sup>®</sup>-based intervention trial with clinical endpoints [70,71].

### 5.2. Levels and neutral results of previous intervention trials

For previous intervention trials with clinical endpoints, participants were recruited irrespective of their baseline status in EPA and DHA, both in the cardiovascular area and in other fields. Moreover, in most large cardiovascular trials, no material was stored to make later analyses of status of EPA and DHA possible, neither at baseline nor during the trial, with two exceptions [2,11]. This was like conducting a trial with a blood pressure lowering agent without measuring blood pressure, or with a lipid lowering agent without measuring blood lipids, neither at baseline nor during the trial. In all populations studied so far, erythrocyte EPA+DHA had a statistically normal distribution, with a varying proportion of individuals in the target range suggested for the HS-Omega-3 Index<sup>®</sup> of 8–11% [70,71]. Demonstrating an effect of EPA+DHA in individuals with a high status in EPA+DHA at baseline will be difficult, if not impossible. Since cardiovascular disease is multifactorial, and has a high prevalence, it is not expected that individuals with cardiovascular disease will have a vastly lower status in EPA+DHA than the population at large. Indeed, this is what we found with the HS-Omega-3 Index<sup>®</sup> [70,71]. This reasoning indicates that recruiting trial participants for LC omega-3 intervention trials, irrespective of baseline omega-3 status is likely to bias the results toward the null for cardiovascular outcomes, as well as for other multi-factorial diseases.

What was just discussed is compounded by the inter-individual variability in response in terms of status to a given dose of EPA+DHA: When 40 individuals with a HS-Omega-3 Index<sup>®</sup> < 5% were given 0.5 g EPA+DHA/day, their mean HS-Omega-3 Index<sup>®</sup> increased significantly, but the increase varied by a factor of 13 inter-individually [76], which could be due to genetic variants [77]. Translated to clinical trials, this indicates that levels of EPA+DHA will overlap between the intervention and the control or placebo groups. Indeed, this has been observed, when investigated, with omega-3 levels of the intervention group and the placebo group overlapped in up to 80% of study participants [25,78]. One of the most important issues in an intervention trial is the distinction between intervention and control or placebo groups in terms of the intervention. With a large proportion of study participants being undistinguishable in terms of EPA+DHA levels, it is a small wonder that an effect of an intervention cannot be discerned.

Bioavailability of EPA+DHA depends on the amount of fat of the concurrent meal (if any), whether or not, and how, the EPA+DHA were emulsified, other matrix effects, and to a much lesser degree on the molecule in which they are bound (in the following order: phospholipids > recombined triglycerides > triglycerides > free fatty acids > ethyl ester; this issue is discussed in more detail elsewhere [70,71,79]). All recent large trials with EPA+DHA in the cardiovascular field advised their participants to ingest the LC omega-3 fatty acid supplement with breakfast [70,71]. In many countries, breakfast is a low-fat meal [70,71]. In almost all large trials, EPA+DHA were used in capsules, i.e. in an unemulsified form [70,71]. In most cases, the omega-3 ethyl ester was used [70,71]. Taken together, unintentionally,

bioavailability of EPA+DHA in previous large trials was minimized. As mentioned, blood levels were not measured in most large trials, and, in most cases, no material was stored for that purpose, so it will remain impossible to prove this point by measuring levels.

Moreover, there appear to be experts in large clinical trials, distinct from experts in the field that the trials are conducted in: When the OMEGA trial was designed [3], this author was given the study protocol, but no opportunity to criticize it. On several occasions, but to no avail, this author actively sought to support the design of other large cardiovascular trials, designed by individuals not conversant with the field. Taken together, trial designers did not appear to be conversant with the field of LC omega-3 fatty acids, and did not appear to be willing to seek, let alone heed, the advice of an expert. The resulting neutral results of the large cardiovascular trials indicate that it is not possible to design and conduct a successful trial without expertise in the difficult field of LC omega-3 fatty acids.

### 5.3. Levels and design of future intervention trials

Future intervention trials can be expected to provide clearer results if the issues just mentioned are taken into consideration, and the following design features are incorporated:

- Recruitment of participants with low baseline LC omega-3 fatty acid levels,
- Treatment to a pre-defined target level. Since the HS-Omega-3 Index<sup>®</sup> has the largest database of all methods to measure levels to support its use, use of its target range of 8–11% is a distinct possibility. Since levels in erythrocytes reach a new equilibrium after approximately three months, this calls for measuring levels some three months into the study, and adjusting the dose of LC omega-3 fatty acids individually. To maintain blinding, doses of placebo will need to be adjusted accordingly. This approach will generate a separation of intervention and control or placebo group in terms of LC omega-3 fatty acid levels.
- Taking bioavailability issues into account, e.g. by advising participants to take capsules with the main meal containing sufficient fat to enhance LC omega-3 bioavailability, and/or use of an emulsion.
- All this reasoning, however, will be futile, if clinical trials continue to be designed by experts in clinical trials, not drawing on expertise from the field of LC omega-3 fatty acids.

### 5.4. Positive results of previous studies

In some health issues, like major depression or impaired cognitive functions, intervention trials with EPA+DHA produced positive results [80–82]. Of note, in individuals with these health issues, a low status in EPA and DHA has been found [70,71,83]. Therefore, the odds were increased for positive findings from intervention with EPA+DHA. However, the other points just discussed (in “Levels and design of future intervention trials”) were not part of the design of the previous intervention trials in major depression or impaired cognitive function. Thus, these trials might have substantially underestimated the potential effects of EPA+DHA.

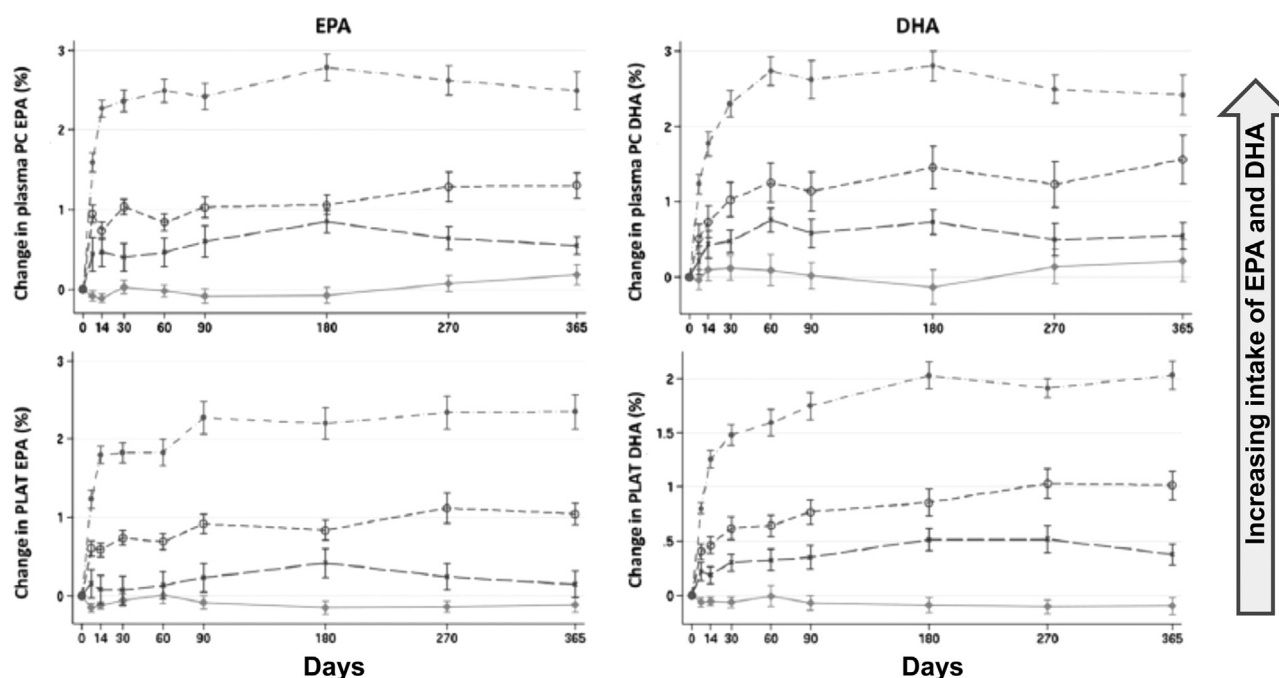
### 5.5. Conclusions

Measuring levels of LC omega-3 fatty acids has unraveled substantial deficits in the design of previous large intervention trials with cardiovascular clinical endpoints, as well as for trials in other therapeutic areas. In the cardiovascular field, these deficits

are thought to be the reason for the overall neutral results. Learning from these deficits, and incorporating the lessons learned into the design of future trials can substantially increase the impact and the chance of success for future trials.

## 6. Patterns of omega-3 intake and omega-3 and -6 status (Philip C. Calder, PhD)

In common with other fatty acids, EPA and DHA are transported in the bloodstream as components of lipoproteins, in which they are esterified into triacylglycerols, phospholipids and cholesteryl esters, or non-covalently linked with albumin in the non-esterified form. EPA and DHA are stored in adipose tissue esterified into triacylglycerols and they are found in all cell membranes esterified into phospholipids and related complex lipids. Thus, EPA and DHA occur within transport pools (plasma lipoproteins and non-esterified fatty acids), functional pools (cell membranes) and the storage pool (adipose tissue). The proportional contribution of EPA or DHA to the total fatty acids present within any of the transport, functional or storage pools differs according to the pool, and in most locations there is a greater contribution from n-6 fatty acids than from LC omega-3 fatty acids, reflecting in part the greater intake from the diet of the former. For example in human peripheral blood mononuclear cells (a mixture of lymphocytes and monocytes), linoleic, dihomo-gamma-linolenic and arachidonic acids contributed an average of 10%, 1.5% and 20% of total fatty acids, respectively [84]. In contrast, EPA and DHA contributed an average of approximately 1 and 2.5%, respectively [84]. Similar differences are seen in other blood cell types like platelets, neutrophils and erythrocytes and in tissues like heart, liver, skeletal muscle and gut mucosa. An exception is brain and eye which have high DHA contents [85–87]. In most cells and tissues, DHA is present in a greater proportion than EPA; this is especially so in the brain and eye [85–87]. Increasing the intakes of EPA and DHA from fish or from LC omega-3 fatty acid supplements is reflected in increased proportions of both fatty acids in blood lipid, blood cell, and many tissue pools. Sands et al. [88] reported a significant positive relationship between habitual fish intake and erythrocyte Omega-3 Index in a cross-sectional study of 163 men and women of a wide age range; the index averaged 3% in those individuals who reported eating fish less than once per month, and increased to an average of 7% in those reporting eating fish at least twice per week. Trials with LC omega-3 fatty acid supplements have demonstrated the possibility of marked increases in EPA and DHA content of blood lipids [42,84,89–95], various blood cells types including platelets [42,89,96], white cells [42,84,93,94,97,98] and red cells [42,91,92,95], and various tissues including skeletal muscle [99], heart [100], gut mucosa [101–103] and adipose tissue [42,92] when their intake is increased. These locations all show a dose- and time-dependent incorporation of both EPA and DHA [42,84,89,92–94,97], but the precise pattern depends upon the specific location. Pools that are turning over rapidly show faster incorporation of EPA and DHA than slower turning over pools. Thus, plasma lipids incorporate EPA and DHA more quickly than blood cells do [84,92], whilst amongst blood cells, platelets and leukocytes have been usually shown to incorporate EPA and DHA more quickly than erythrocytes. A recent study examined in detail the dose- and time-dependent appearance of EPA and DHA in different transport, functional and storage pools in humans [42]. Healthy human volunteers consumed one of three doses of EPA+DHA (providing 3.27, 6.54 or 13.08 g per week; the ratio of EPA to DHA was 1:1.1 and they were provided in the triacylglycerol form) in capsules or placebo capsules daily for 12 months. Blood was collected at the start of the study and at several intervals up to 12 months; in addition adipose tissue biopsies were collected at



**Fig. 5.** Time course of changes in EPA and DHA content of human plasma phosphatidylcholine and platelets in subjects consuming placebo oil or one of three doses of fish oil. Healthy subjects supplemented their diet with capsules providing 0, 3.27, 6.54 or 13.08 g EPA+DHA per week for a period of 12 months; the ratio of EPA to DHA was 1:1.1. Plasma phosphatidylcholine and platelets were isolated at 0, 1, 2, 4, 8, 12, 24, 36 and 52 weeks and their fatty acid composition determined by gas chromatography. Data are mean  $\pm$  standard error from at least 30 subjects per group. Reproduced from [42] with permission of American Society for Nutrition.

**Table 3**

Estimated time to peak incorporation and maximum incorporation of EPA and DHA in different transport, functional and storage pools in healthy humans with low fatty fish intake. Data are median from 33 individuals who consumed 13.08 g EPA+DHA per week for 12 months. The ratio of EPA to DHA was 1:1.1 and EPA and DHA were taken over 4 days of each week. Adapted with permission of American Society for Nutrition from [42].

Pool	EPA			DHA		
	Time to peak (days)	Maximum incorporation (% of fatty acids)	Increase from base-line (% of fatty acids)	Time to peak (days)	Maximum incorporation (% of fatty acids)	Increase from base-line (% of fatty acids)
Plasma phosphatidylcholine	18	3.5	2.5	23	5.9	2.5
Plasma cholesteryl ester	24	3.2	2.2	ND	1.0	0.4
Plasma triacylglycerol	16	1.7	2.0	20	2.5	2.0
Plasma non-esterified fatty acid	38	1.1	0.5	32	3.1	2.0
Blood mononuclear cells	249	2.3	1.6	207	3.3	1.5
Erythrocytes	55	3.7	2.0	136	7.7	3.0
Platelets	25	3.1	2.2	32	3.6	2.0
Subcutaneous adipose tissue	> 365	0.3	0.1	> 365	0.35	0.15

study entry and after 6 and 12 months. It was found that all pools investigated showed time- and dose-dependent incorporation of both EPA and DHA and that, in time, pools reach a new steady state content of EPA and DHA that is determined by intake, but that the exact nature of these changes varies among pools. Fig. 5 shows the incorporation of EPA and DHA into plasma phosphatidylcholine and into platelets. EPA is incorporated more quickly than DHA into both pools, the incremental increase in both fatty acids is greater in plasma phosphatidylcholine than in platelets, and the new steady state level of EPA and DHA that is reached is precisely related to the dose of EPA or DHA being consumed. Table 3 summarises the time to reach a new steady state and the approximate increment in EPA and DHA content (as a proportion of total fatty acids) that the new steady state represents. Although some studies report greater incorporation of EPA and DHA into blood lipids in women than men [104] and greater incorporation in older than younger adults [93], this recent study saw no differences between sexes or across age groups [105].

The participants in the study of Browning et al. [42] took their LC omega-3 fatty acid supplements on one, two or four days a week, consuming placebo capsules on the other days. An

additional fifth group in the study consumed 6.54 g EPA+DHA per week but spread evenly across all days of the week [106]. This enabled the EPA and DHA incorporation pattern to be compared between 6.54 g EPA+DHA spread across two days, or across seven days of the week to be compared. The increases in EPA and DHA in plasma phosphatidylcholine, platelets and blood mononuclear cells were greater in the group consuming the EPA and DHA every day compared with the group consuming the fatty acids at a higher daily intake but on fewer days [106]. The findings suggest that regular, frequent intake of moderate amounts of EPA and DHA will achieve a higher status than less frequent intake of higher amounts.

The higher status of EPA and DHA achieved through increased intake of EPA and DHA is maintained so long as the higher intake of EPA and DHA is maintained. If, after a period of increased intake of EPA and DHA, intake returns to the earlier lower levels, then EPA and DHA status decline, eventually returning to earlier levels. This is well described for blood lipids [84,89,92], platelets [89], leukocytes [84] and erythrocytes [95]. However, just as the incorporation of EPA into different pools is faster than the incorporation of DHA, the loss of EPA is faster than the loss of DHA. Loss

of EPA and DHA from tissues has not been studied in humans. Since EPA and DHA content in lipid pools is frequently described as a percentage or proportion, the increase in content of these fatty acids is accompanied by a decrease in content (proportion) of other, usually unsaturated, fatty acids. Depending upon the pool, since once again there are differences among pools, these other fatty acids include oleic, linoleic, dihomo-gamma-linolenic and arachidonic acids. There are good demonstrations of dose- and time-dependent decreases in the proportion of arachidonic acid in leukocytes and platelets [89,93,97].

Admittedly, we do not fully understand the importance of EPA+DHA status in relation to the status of other biologically active fatty acids. While it is conceivable that EPA and DHA may interfere with beneficial effects of other fatty acids, the increased status of EPA and DHA seen with modestly increased intake has only a fairly small impact on the status of other fatty acids [107].

## Disclosure

This report summarizes the presentations from the workshop “Conducting Omega-3 Clinical Trials” held on June 30, 2014, in Stockholm, Sweden during the 11th Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL). The event was sponsored by the Global Organization for EPA and DHA Omega-3s (GOED). This report is not a consensus statement; therefore, some authors may not agree with all the opinions expressed herein.

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