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A review and evaluation of study design considerations for omega-3 fatty acid supplementation trials in physically trained participants

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Abstract

Long-chain omega-3 polyunsaturated fatty acid (LC *n*-3 PUFA) supplements, rich in eicosapentaenoic acid and/or docosahexaenoic acid, are increasingly being recommended within athletic institutions. However, the wide range of doses, durations and study designs implemented across trials makes it difficult to provide clear recommendations. The importance of study design characteristics in LC *n*-3 PUFA trials has been detailed in cardiovascular disease research, and these considerations may guide LC *n*-3 PUFA study design in healthy cohorts. This systematic review examined the quality of studies and study design considerations used in evaluating the evidence for LC *n*-3 PUFA improving performance in physically trained adults. SCOPUS, PubMed and Web of Science electronic databases were searched to identify studies that supplemented LC *n*-3 PUFA in physically trained participants. Forty-six ($n = 46$) studies met inclusion. Most studies used a randomised control design. Risk of bias, assessed using the design-appropriate Cochrane Collaboration tool, revealed that studies had a predominant judgment of 'some concerns', 'high risk' or 'moderate risk' in randomised controlled, randomised crossover or non-randomised studies, respectively. A custom five-point quality assessment scale demonstrated that no study satisfied all recommendations for LC *n*-3 PUFA study design. This review has highlighted that the disparate range of study designs is likely contributing to the inconclusive state of outcomes pertaining to LC *n*-3 PUFA as a potential ergogenic aid. Further research must adequately account for the specific LC *n*-3 PUFA study design considerations, underpinned by a clear hypothesis, to achieve evidence-based dose, duration and composition recommendations for physically trained individuals.

Key words: **LC *n*-3 PUFA: EPA: DHA: Trained participants: Physical performance**

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Introduction

There is continued appreciation for the key role of optimised nutrition for maximising adaptation to training stimulus and therefore athletic performance. This has opened the field of 'performance nutrition' which has focused primarily on optimal macronutrient consumption and total energy intake. Notwithstanding the foundational role of broader macronutrient optimisation, considerable attention has been given to sub-groups of fatty acids such as the omega-3 class of polyunsaturated fatty acids (PUFA). Omega-3 fatty acid supplements, and specifically long-chain omega-3 PUFA (LC *n*-3 PUFA), are increasingly being recommended within athletic groups and sporting institutes as a potential ergogenic aid, being recognised in 2019 by the National Collegiate Athletic Association (NCAA) as a 'permissible' supplement for athletic departments to purchase and provide to athletes[(1)] and by Australian Institute of Sport (AIS) in 2021 as a Grade B (emerging scientific support) supplement[(2)].

The LC *n*-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are the primary LC *n*-3 PUFA responsible for exerting physiological effects that may benefit physically trained participants. Furthermore, these physiological effects of LC *n*-3 PUFA supplementation are predominantly mediated through target tissue membrane incorporation of EPA and especially DHA[(3)], and the omega-3 index (O3I) (erythrocyte membrane) is a readily accessible marker of membrane composition of other tissues, including skeletal and cardiac muscle. The proportional sum of EPA + DHA within erythrocyte membranes (termed the O3I) represents a marker of habitual LC *n*-3 PUFA consumption and has been used clinically to stratify risk for

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cardiovascular disease mortality (<4% high risk, 4–8% moderate risk, >8% low risk)[4]. Currently, mean O3I of large samples of physically trained NCAA athletes from a variety of sports are consistently below 4·5%[5,6], indicating both the potential for supplementation and the need for translation from research into practice and clear LC *n*-3 PUFA consumption guidelines for athletes. More broadly, these low values of O3I are reflected in other physically trained populations who undergo similar physiological strain as physically trained athletes, such as military personnel, and who may benefit from increased LC *n*-3 PUFA consumption[7,8]. In addition to the potential for ergogenic benefit from short-term LC *n*-3 PUFA supplementation, it is important to consider longevity of an athlete's career, which may be supported by maintaining a protective O3I level long term. It is also worth noting that recent trials highlight the potential application of LC *n*-3 PUFA supplementation to mitigate the magnitude of head trauma experienced by athletes[9–11] and improve recovery from muscle-damaging exercise[12,13]. Thus, prophylactic LC *n*-3 PUFA supplementation appears to represent a feasible strategy to positively influence the physiology of multiple tissues and, subsequently, enhance functional outcomes.

Whilst the O3I tends to be low in physically trained populations and is readily raised by supplementation[14], the question remains as to whether any performance benefit can be obtained from having a higher O3I. Several narrative review articles[15–20] and one systematic review[21] have attempted to address the question of whether omega-3 supplementation can optimise sport performance or act as an ergogenic aid in physically trained participants. Whilst some reviews suggest a broadly beneficial effect of LC *n*-3 PUFA supplementation to improve aspects of sport performance[15,16,19], others indicate either the evidence is inconclusive[17,21] or there is no benefit above dietary recommended doses[20]. The considerable variability in results across these studies is most often attributed to disparate study design characteristics. However, as none of these reviews has explicitly addressed this topic, it forms the primary outcome of the current review.

The consequence of inappropriate LC *n*-3 PUFA study design characteristics is most clearly evident in clinical trials with cardiovascular endpoints. Unlike pharmaceutical trials, trials involving LC *n*-3 PUFA are unique in their inability to entirely eliminate the test substance from dietary consumption and therefore from within tissue membranes at baseline or in any control or placebo group[22]. For example, the contradictory findings in randomised control trials of LC *n*-3 PUFA supplementation and cardiovascular mortality are largely attributable to the inherent difficulties in creating clear separation between intervention and control groups prior to and following the intervention[23]. Within the context of cardiovascular outcomes, factors such as background diet LC *n*-3 PUFA consumption, measurement of LC *n*-3 PUFA blood status, LC *n*-3 PUFA dose and duration have all been identified as key elements which influence the efficacy of trials in cardiac patients[22,24]. Drawing from evidence in this cohort, these study design elements may help guide appropriate LC *n*-3 PUFA study design creation in healthy individuals with endpoints related to physical performance, muscle recovery or mitigation of neurological consequences related to head trauma injury.

A frequently overlooked consideration in LC *n*-3 PUFA study design is the prior dietary intake of EPA + DHA through fatty fish, fish oil and other marine sources, which dictate baseline LC *n*-3 PUFA membrane composition[25]. This, along with dose, duration and delivery method of EPA + DHA, influences the incorporation of supplemental LC *n*-3 PUFA within target tissues and may therefore contribute to the mixed conclusions found across review articles. It is currently not possible to ascertain the true benefit, or mechanism by which LC *n*-3 PUFA are acting within physically trained participants until control of variables known to influence outcome measures is addressed. Therefore, the present study aims to review the methods and methodology of studies supplementing LC *n*-3 PUFA in physically trained participants to address and correct the issues that might be contributing to the variability in reported outcomes.

Methods

Study overview and inclusion criteria

The primary objective of this systematic literature review was to evaluate the methodological practices in LC *n*-3 PUFA trials aiming to determine whether LC *n*-3 PUFA improves physiological performance or recovery metrics in physically trained individuals.

Inclusion criteria were as follows: (a) supplementation with LC *n*-3 PUFA, regardless of delivery method, dose or duration, (b) inclusion of physically trained participants and (c) measurement of a performance or recovery outcome. Studies were excluded if they met the following criteria: (a) participants were described as physically untrained, (b) study did not provide a description of participant training status to determine if participants were physically trained, (c) study did not include a physical performance outcome or physiological marker related to performance or recovery, (d) study assessed musculoskeletal injury or trauma as a primary outcome and (e) study was a conference paper, book chapter or conference proceeding. Further, studies which met inclusion criteria and (a) shared participant data and (b) included co-ingestion of other nutrients with LC *n*-3 PUFA were included in the review.

Search parameters and study selection

A systematic search of the literature was conducted in SCOPUS, PubMed and Web of Science electronic databases (from earliest record to April 2022). The search strategy used various combinations of the following keywords: fish* OR krill* OR omega* OR EPA OR eicosapentaenoic* OR DHA OR docosahexaenoic* OR PUFA* AND elite OR professional OR trained OR compet* AND athlet*. Where possible, database results were limited to include only studies involving humans.

The screening process began with the removal of duplicate studies using Endnote, version X8 (Thomson Reuters, Philadelphia, PA, USA). Following this, screening of the title and abstract was performed to remove irrelevant or off-topic studies. The full text of the remaining studies was then retrieved and screened using the inclusion and exclusion criteria. Two authors (G.E.P.: medical and exercise physiologist; R.A.:

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nutritionist) screened each article, and any discrepancies between the two authors were resolved by consulting a third author (M.J.M.: medical and exercise physiologist).

Assessment of study quality

The Cochrane Collaboration's Risk of Bias tool for randomised trials version 2 (RoB 2)[26] was used to assess the internal validity for each randomised control trial (RoB 2 for RCT) and randomised crossover trial (RoB 2 for crossover trials). Each study is classified as having either low risk, some concerns or high risk for each domain. The internal validity for each non-randomised study was determined by the Risk of Bias in Non-randomised Studies of Interventions (ROBINS-I) tool[27]. With this tool, each study is classified as having either low risk, moderate risk, serious risk or critical risk for each of seven domains. Two authors (G.E.P.; R.A.) judged the risk of bias, and any discrepancies between the two authors were resolved by consulting a third author (M.J.M.).

To assess the study design, specifically for LC *n*-3 PUFA interventions, a previously used[12] custom five-point quality assessment scale was used on the basis of recommendations from James *et al.*[22]. Criteria included the following: (i) exclusion of participants with a baseline erythrocyte membrane EPA and/or DHA level above a certain threshold, (ii) whether supplementation resulted in a change in erythrocyte membrane EPA and/or DHA, (iii) exclusion of participants that consumed LC *n*-3 PUFA supplements, (iv) exclusion of participants consuming more than one fish meal per week, and (v) minimum supplementation duration of 4 weeks. The scale scored each criterion as either satisfied (1 point) or not satisfied (0 points), resulting in a score ranging from 0 to 5.

Results

Studies selected

A total of 369 publications were identified from the literature search of databases, and an additional 3 publications were identified through review of reference lists of identified studies and review articles (Fig. 1). After removal of duplicates, 251 publications were removed on the basis of title and abstract screening. Of the remaining fifty-seven publications, a further eleven were excluded on the basis of full-text screening. Figure 1 outlines the selection process, with reasons for exclusion. A total of forty-six publications met the inclusion criteria and were included in the review (Table 1).

Assessment of study quality

The majority ($n = 31$) of studies included in this review were randomised control trials (RCTs), and their internal validity was assessed using the Cochrane Collaboration's RoB 2 tool for RCTs (Figs. 2a and 3a). Five trials were randomised crossover trials and were assessed with the Cochrane Collaboration's RoB 2 tool for randomised crossover trials (Figs. 2b and 3b). The internal validity of the remaining non-randomised studies ($n = 10$) was assessed with the Cochrane Collaboration's ROBINS-I tool (Figs. 2c and 3c).

Randomised control trials. Most RCTs received an overall risk of bias judgement of 'some concerns'. This was largely a result of only three studies[28,54,60] providing sufficient detail regarding the randomisation process to warrant a low risk of bias. All studies had some concerns regarding selection of the reported result as no study met the requirements of providing detail of analysis intentions prior to unblinding of outcome data. Those studies which did register details regarding analysis intentions (i.e. through clinicaltrials.gov) often did so after the study was already published. Two studies[44,45] received an overall high risk of bias due to providing insufficient information regarding deviations from the intended intervention.

Randomised crossover trials. The five studies included in this review which utilised a randomised crossover design all received an overall high risk of bias. The bias predominantly arose from period and carryover effects. High-risk classification was determined by the lack of confirmation that LC *n*-3 PUFA blood levels returned to baseline values with a measurement of erythrocyte membrane EPA and/or DHA levels prior to beginning placebo and intervention supplementation. Only one study[69] provided sufficient detail regarding participant randomisation to receive low risk for the randomisation process. Mickleborough *et al.*[31] and Bunn *et al.*[62] were the only studies which satisfied criteria required for a low risk classification in deviations from the intended intervention.

Non-randomised studies. Of the ten non-randomised studies, there were some concerns in five of those studies, whereas four studies were assessed as having an overall high risk of bias. One study[67] was assessed as having an overall critical risk of bias. The primary concern in the studies that were assessed as having an overall high risk and critical risk was bias due to confounding. Buonocore *et al.*[67] received a critical risk for this domain as there was no control over baseline measures, nor was there a direct control group.

Assessment of LC n-3 PUFA study designs

The mean LC *n*-3 PUFA study design score across all forty-six studies was 1·48 ± 1·01 (± SD) (out of a potential 5) with a range of 0 to 4 (Table 1). There were only three studies that achieved a score of 4[14,59,71]. One study excluded participants with a baseline erythrocyte membrane EPA and/or DHA level above a set threshold[71]; however, no study successfully met all the criteria for a score of 5.

LC n-3 PUFA dose, duration and delivery method

A combination of EPA + DHA was predominantly used ($n = 38$) usually as a fish oil or concentrated fish oil, with one study using isolated EPA and seven studies using isolated DHA. Of the thirty-eight studies that used a combined EPA + DHA supplement, twenty-four studies used an EPA dominant capsule, nine studies used a DHA dominant capsule, four studies used an equal EPA + DHA ratio and one study did not state the EPA + DHA content. In the forty-five studies that reported the component doses of EPA and DHA separately, the average EPA dose was 1242 mg/d (±1284 SD; min, 120 mg/d; max, 6000 mg/d) and

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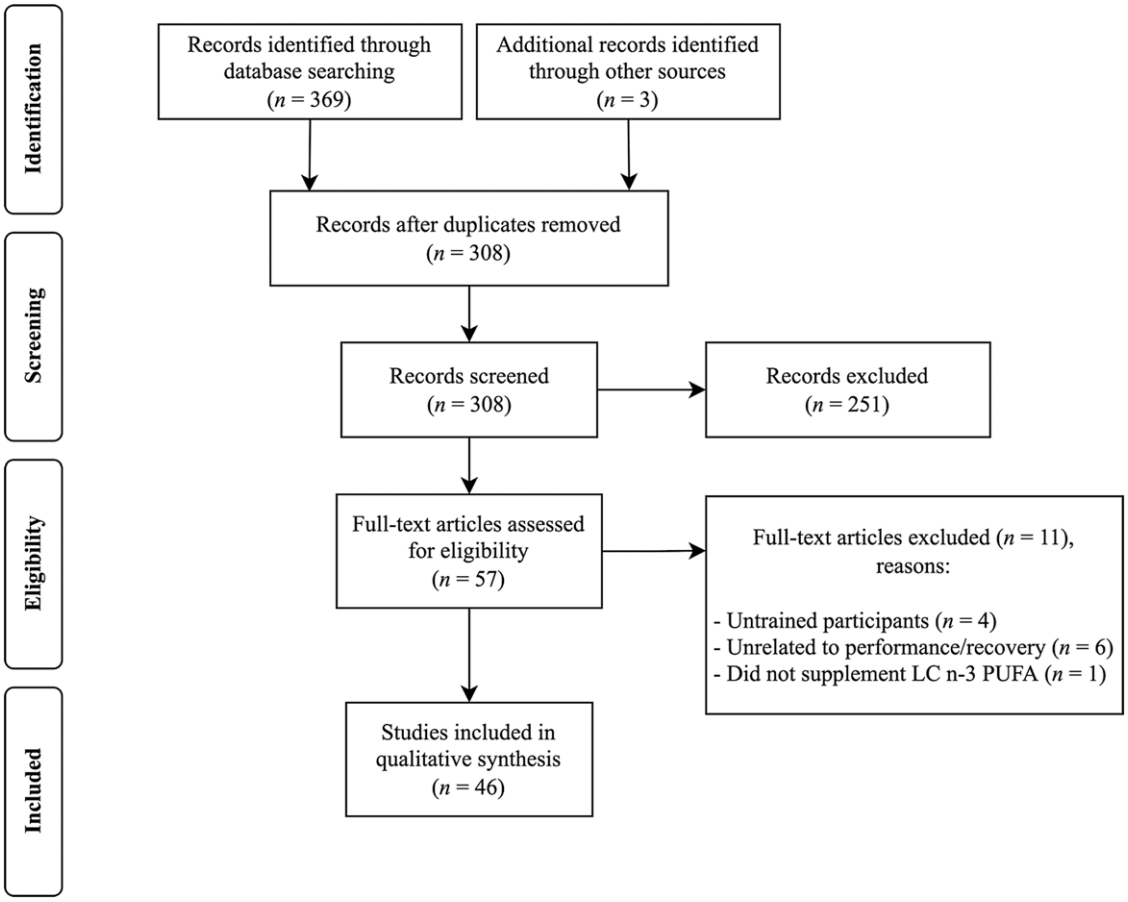

Fig. 1. Prisma flow diagram showing study selection process.

the average DHA dose was 978 mg/d (±765 SD; min, 96 mg/d; max, 3500 mg/d). Supplementation duration ranged from 1 d (acute) to 84 d, with an average of 40 d (±20 SD).

Supplemental LC *n*-3 PUFA delivery was achieved through capsules in 67% of studies ($n = 31$) and as a beverage in 22% ($n = 10$) of studies. The remaining studies used pills ($n = 1$), used chewable supplements ($n = 2$) or did not state delivery method ($n = 2$). Authors identified the source of LC *n*-3 PUFA as fish-derived in 61% of studies ($n = 28$), whereas 26% of studies ($n = 12$) did not state the origin of LC *n*-3 PUFA. The remaining studies used krill oil ($n = 4$), algal oil ($n = 1$) or seal oil ($n = 1$).

Measurement of LC n-3 PUFA blood status

Overall, 54% of studies ($n = 25$) included a biological measure of LC *n*-3 PUFA status. Erythrocyte membrane LC *n*-3 PUFA was predominantly chosen as the biological marker in those studies ($n = 12$), with LC *n*-3 PUFA of blood plasma measured in six studies ($n = 6$), whole blood in five ($n = 5$) and neutrophils in two ($n = 2$).

Dietary assessment

A form of dietary assessment was conducted in 48% of studies ($n = 22$). Most studies used food records ($n = 16$) ranging from 1 d to 7 d. Other forms of dietary assessment included food frequency questionnaire ($n = 4$), or food recall ($n = 1$), with one study omitting description of the tool used for dietary assessment. One study[65] provided all food items and fluids to participants with the intent to create an energy deficit to induce weight loss. In the twenty-two ($n = 22$) studies that measured dietary intake, sixteen ($n = 16$) studies reported no difference in major nutrients or energy intake between groups at baseline; however, only six ($n = 6$) of those studies reported no difference in baseline and post-supplement dietary assessments. The remaining six ($n = 6$) studies did not provide dietary data or sufficient detail to determine if there were differences in dietary intake between groups or after supplementation.

Discussion

This review has identified a shortfall in design considerations within studies that have investigated the effects of LC *n*-3 PUFA in physically trained populations. Firstly, the risk of overall study bias, as classified via the Cochrane Collaboration's and ROBINS-I tools, was extraordinarily high, with the majority of studies (65%) identified as 'some concerns', the remaining studies (35%) categorised as 'high risk' and no studies achieving 'low risk' (Fig. 2). Across studies, the risk of bias was often attributed to critical flaws in the design, conduct, analysis and reporting of outcomes. Most importantly, serious concern should be drawn

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Table 1. Study characteristics and omega-3 study design score

Study	LC *n*-3 PUFA score	Study design	Sport	Population (*n*), age	Dose (EPA/DHA, mg/d)	Duration (d)	LC *n*-3 PUFA tissue measure
Leaf & Rauch[28]	1	RCT	Runners, crew racers, cyclists, weightlifters	Male (24), 19–35 years	4200/1800 (high), 2100/900 (low)	42	Unmeasured
Oostenbrug *et al.*[29]	1	NRS	Cyclists	Male (24), 19–42 years	1050/750	21	Erythrocyte
Raastad *et al.*[30]	2	RCT	Soccer players	Male (28), 24 years	1600/1040	74	Plasma
Mickleborough *et al.*[31]	0	Randomised crossover trial	Triathletes, cross-country and track runners	Male (10)/female (10), 22–23 years	3200/2200	21	Neutrophil
Andrade *et al.*[32]	1	RCT	Swimmers	Male (20), 20–35 years	950/500	45	Plasma
Malaguti *et al.*[33]	2	NRS	Volleyball players	Male (11), 29–32 years	3000 (combined)	60	Erythrocyte
Peoples *et al.*[34]	2	RCT	Cyclists	Male (16), 23–27	800/2400	56	Erythrocyte
Buckley *et al.*[35]	3	Matched control trial	Australian rules footballers	Not stated (25), 22–23 years	360/1560	35	Erythrocyte
Fontani *et al.*[36]	0	RCT	Karate	Male (12)/female (6), 20–53 years	1200/600	21	Unmeasured
Nieman *et al.*[37]	1	RCT	Cyclists	Male (32)/female (7), 26–28 years	220/180	24	Unmeasured
Nieman *et al.*[38]	0	RCT	Cyclists	Male (19)/female (4), 24–27 years	2000/400	42	Plasma
Reza *et al.*[39]	1	RCT	Basketball players	Male (34), 17–35 years	2000/0	42	Unmeasured
Filaire *et al.*[40]	2	RCT	Judo	Male (20), 22–23 years	600/400	42	Unmeasured
McAnulty *et al.*[41]	1	RCT	Cyclists	Male/female (48), 22–27 years	2000/400	42	Plasma
Skarpanska-Stejnborn *et al.*[42]	2	RCT	Rowers	Male (17), 21 years	170/110	42	Unmeasured
Tartibian *at al.*[43]	1	RCT	Wrestlers	Male (40), 19 years	180/120	84	Neutrophil
Filaire *et al.*[44]	2	RCT	Judo	Male (28), 22–23 years	600/400	42	Unmeasured
Guzman *et al.*[45]	2	RCT	Soccer players	Female (24), not stated	0/3500	28	Unmeasured
McAnulty *et al.*[46]	0	RCT	Cyclists	Male (32)/female (7), 26–28 years	220/180	17	Unmeasured
Atashak *et al.*[47]	1	RCT	Handball players	Male (20), 20–22 years	720/480	7	Unmeasured
Santos *et al.*[48]	1	RCT	Marathon athletes	Male (21), 37 years	300/1500	60	Unmeasured
Capo *et al.*[49]	2	RCT	Soccer players	Male (15), 19–20 years	0/1140	56	Erythrocyte
Macartney *et al.*[14]	4	Matched control trial	Cyclists, runners	Male (26), 23–24 years	140/560	56	Erythrocyte
Martorell *et al.*[50]	1	RCT	Soccer players	Male (15), 20 years	0/1140	56	Plasma
Capo *et al.*[51]	2	RCT	Soccer players	Male (15), 20 years	0/1140	56	Erythrocyte
Capo *et al.*[52]	1	RCT	Soccer players	Male (15), 20 years	0/1140	56	Erythrocyte
Delfan *et al.*[53]	2	RCT	Paddlers	Male (22), 24–26 years	2400/1200	28	Unmeasured
Lewis *et al.*[54]	1	RCT	Rowers, sailors, triathletes, runners	Male (30), 24–26 years	375/510	21	Plasma
Martorell *et al.*[55]	1	RCT	Soccer players	Male (15), 20 years	0/1140	56	Erythrocyte
Price *et al.*[56]	0	Non-randomised cross-over trial	Runners, cyclists, triathletes	Male (9)/female (1), 35 years	3000/3000	21	Unmeasured
Zebrowska *et al.*[57]	1	Randomised crossover trial	Cyclists	Male (13), 23 years	660/440	21	Unmeasured
Gravina *et al.*[58]	2	Matched control trial	Soccer players	Male (19)/female (7), 21–24 years	~700/~200*	28	Whole blood
Hingley *et al.*[59]	4	Matched control trial	Cyclists, runners	Male (26), 23–24 years	140/560	56	Erythrocyte
Jakeman *et al.*[60]	0	RCT	Not stated	Male (27), 26 years	~6000/~400 (high), ~1200/~800 (low)*	1	Unmeasured

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Table 1. (*Continued*)

Study	LC *n*-3 PUFA score	Study design	Sport	Population (*n*), age	Dose (EPA/DHA, mg/d)	Duration (d)	LC *n*-3 PUFA tissue measure
Black *et al.*[61]	1	NRS	Rugby Union players	Not stated (20), 23 years	1102/1102	35	Whole blood
Bunn *et al.*[62]	0	Randomised crossover trial	Resistance trained	Male (20), 21 years	0/1500	1	Unmeasured
Georges *et al.*[63]	2	RCT	Resistance trained	Male (18), 22 years	393/240	56	Unmeasured
Philpott *et al.*[64]	2	NRS	Soccer players	Male (30), 23 years	1100/1100	42	Whole blood
Philpott *et al.*[65]	2	RCT	Resistance trained	Male (20), 23 years	1000/1000	42	Whole blood
Avila-Gandia *et al.*[66]	2	RCT	Cyclists	Male (38), 35–36 years	120/975	30	Unmeasured
Buonocore *et al.*[67]	2	Intervention trial	Middle- and long-distance runners	Male/female (39), 24 years	1600/800	56	Unmeasured
James *et al.*[68]	2	Randomised crossover trial	Cyclists	Male (10), 38 years	3606/1518	28	Whole blood
Ramos-Campo *et al.*[69]	1	Randomised crossover trial	Not stated	Male (15), 36 years	240/2100	70	Unmeasured
Storsve *et al.*[70]	1	RCT	Triathletes	Male (35)/female (12), 40–41 years	193/96	35	Unmeasured
Drobnic *et al.*[71]	4	RCT	Power athletes	Male (27)/female (8), 32–34 years	400/150	84	Erythrocyte
Zebrowska *et al.*[72]	2	RCT	Marathon athletes	Male (24), 33–35 years	852/1602	21	Erythrocyte

* Estimates based on body mass.
NRS, non-randomised study.

from the lack of appreciation for designing and implementing studies that adhere to principles for interrogating the specific effects of LC *n*-3 PUFA including (i) measurement of LC *n*-3 PUFA tissue status, (ii) assessment of background diet, (iii) baseline LC *n*-3 PUFA biomarker informed exclusion criteria and (iv) the interaction of LC *n*-3 PUFA dose and duration. Such widespread lack of adherence to fundamental study design considerations explain why it has been difficult to either draw conclusions relating to the benefits or detriments of LC *n*-3 PUFA supplementation in physically trained populations, or make recommendations for applied practice.

Measurement of LC n-3 PUFA tissue status

The physiological effects of LC *n*-3 PUFA are predominately governed by their relative contribution to and, following supplementation, their alteration of cell membrane fatty acid proportional composition within the target tissue[3,73–75]. Many studies examined in this review sought to determine the effects of LC *n*-3 PUFA on outcome measures related to improved cardiovascular endurance and aerobic capacity, physical strength and power, and the attenuation of exercise-induced inflammatory and muscle damage processes. Often the emphasis is placed on cardiac and skeletal muscle as the primary tissues where improvement is likely to be actualised in outcome measures. Measurement of LC *n*-3 PUFA at these target tissues would provide evidence that the LC *n*-3 PUFA intervention was successful in altering target tissue cell membrane fatty acids and therefore give confidence of a causal relationship between LC *n*-3 PUFA supplementation and outcome measures. In the case of LC *n*-3 PUFA status in skeletal muscle, a muscle biopsy would represent the gold standard for establishing LC *n*-3 PUFA incorporation within the target tissue. Whilst this has been undertaken previously[76–78], there remain limitations around participant burden, cost and researcher skill. Furthermore, animal studies reveal systematic variations according to muscle fibre type analysed[79], therefore raising questions of choice or access to muscles that correlate with human physiological or functional outcomes.

If a skeletal muscle biopsy is not practical, or if cardiac muscle is the target tissue, an appropriate blood biomarker that is reflective of LC *n*-3 PUFA incorporation in cardiac and skeletal muscle cell membranes should be used as a surrogate to indicate target tissue cell membrane LC *n*-3 PUFA status. The selection of an appropriate blood biomarker requires evaluation of temporal changes occurring within the target tissue cell membrane, in parallel with the chosen blood biomarker, following LC *n*-3 PUFA supplementation. Erythrocyte membranes appear to be the blood biomarker that most appropriately reflects LC *n*-3 PUFA incorporation into most major organs[80]. Changes within erythrocyte membranes follow a delayed incorporation and desaturation curve and have lower biological variability[81] than plasma phospholipids[82]. This is mirrored in skeletal and cardiac muscle, which follow similar incorporation rates to that of erythrocyte membranes[80,83,84]. Relationships between cardiac muscle and blood markers are best demonstrated by simultaneously sampling blood and atrial biopsy during cardiac surgery. Such studies reveal that erythrocyte membrane fatty acid

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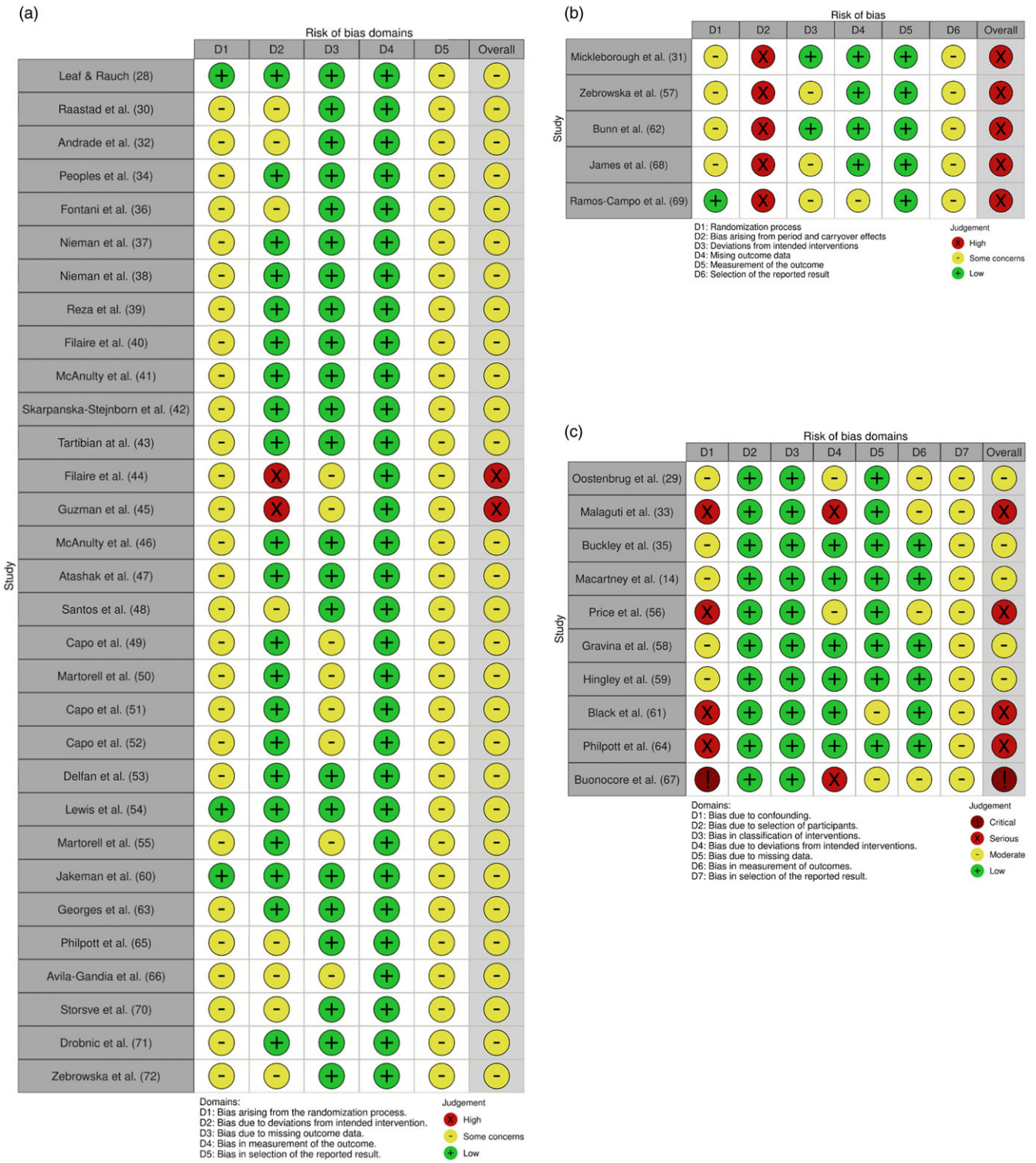

Fig. 2. Risk of bias graph: review authors' judgements about each risk of bias item for each included study. *Legend*. (a) Risk of bias graph generated using Cochrane Risk of Bias 2 tool for RCT studies. (b) Risk of bias graph generated using Cochrane Risk of Bias 2 tool for randomised crossover studies. (c) Risk of bias graph generated using ROBINS-I tool for non-randomised studies.

composition provides the best correlate of any blood component with cardiac incorporation[85]. Furthermore, slower fluctuations in incorporation and washout, plus a nearer to 1:1 relationship between erythrocyte and atria show erythrocytes to be a better long-term marker of muscle incorporation, compared with plasma. In the present review, an appropriate blood biomarker, namely erythrocyte EPA, DHA or EPA + DHA, was collected in only 28% of studies, and within the studies that did measure

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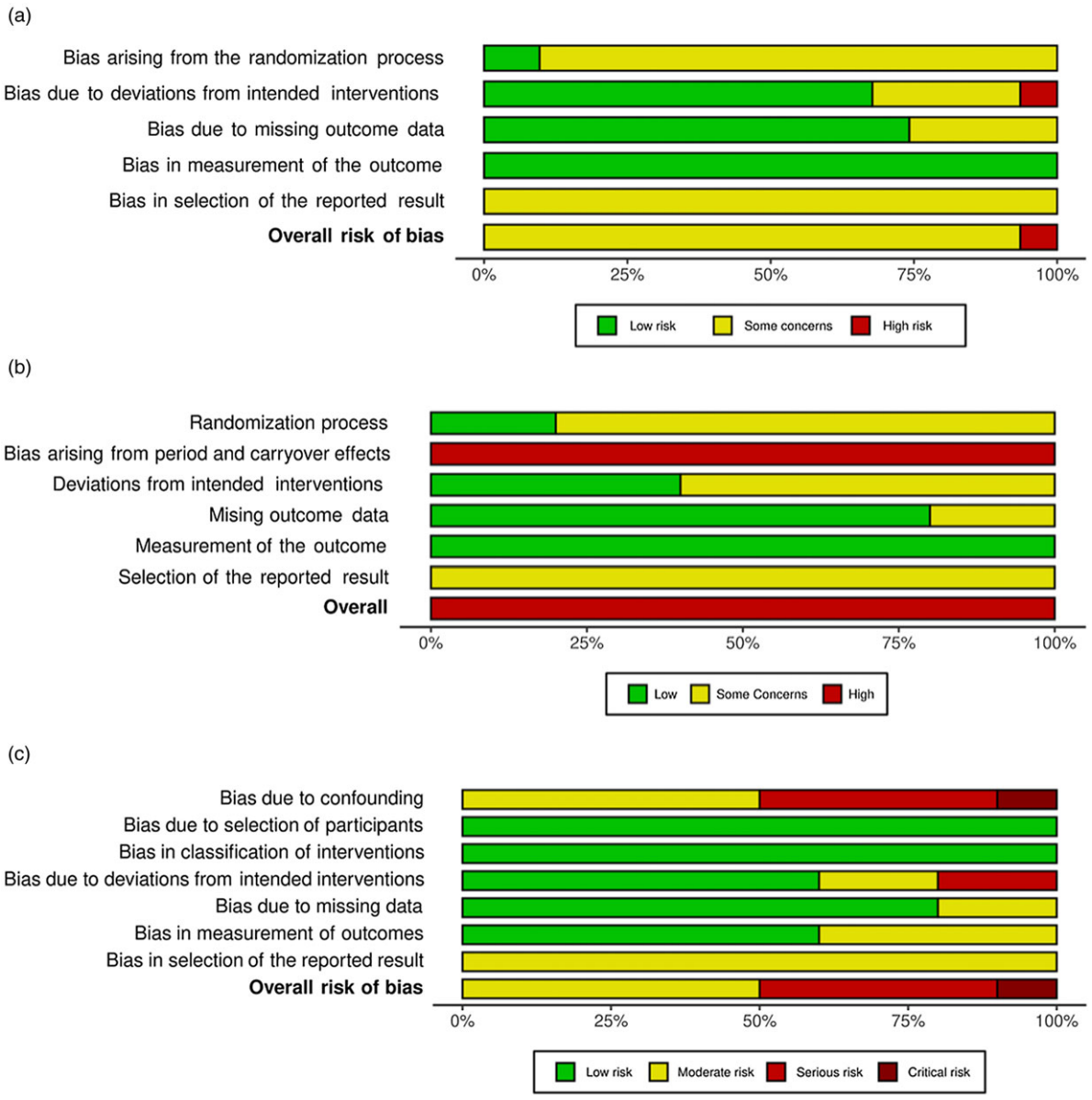

Fig. 3. Risk of bias summary: review authors' judgements about each risk of bias item presented as percentages across all included studies. *Legend.* (a) Risk of bias summary generated using Cochrane Risk of Bias 2 tool for RCT studies. (b) Risk of bias summary generated using Cochrane Risk of Bias 2 tool for randomised crossover studies. (c) Risk of bias summary generated using ROBINS-I tool for non-randomised studies.

erythrocyte membrane fatty acids, few established marked changes from baseline.

Few studies consider the washout characteristics of LC *n*-3 PUFA from cardiac muscle or skeletal muscle cell membranes following cessation of LC *n*-3 PUFA supplementation. However, their slower incorporation rate would indicate that clearance of LC *n*-3 PUFA would follow a delayed, rather than expedient time course. Fatty acid washout is much slower from erythrocyte membranes than plasma[82]. Indeed, LC *n*-3 PUFA, especially DHA, can remain elevated in erythrocytes beyond 18 weeks after cessation of fish or fish oil supplementation for 6 weeks[86] and for as long as 6 months[87] after 12 months supplementation. This raises concern in the four studies that used a crossover design with a washout period ranging from 2 weeks to a maximum of 4 weeks. Cao *et al.*[82] has demonstrated that 4 weeks after cessation of LC *n*-3 PUFA supplementation, erythrocyte membrane EPA + DHA remained statistically higher than baseline and did not entirely return to baseline levels until 16 weeks after supplement cessation. If a crossover study design is to be used, a measurement of erythrocyte membrane EPA + DHA should be collected before and after each supplement block to confirm LC *n*-3 PUFA levels have returned to baseline during the washout period, prior to crossing over to the alternate supplement.

Background diet LC n-3 PUFA

As has been explored by James *et al.*[22], intervention trials involving LC *n*-3 PUFA are unique when compared with pharmacological drug trials. In drug trials it is common, possible

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and indeed essential to eliminate consumers of the intervention drug entirely from the control group. LC *n*-3 PUFA are found commonly within foods consumed on a semi-regular basis, and whilst population intakes are below desirable targets[88,89], their presence in food and within the body should be acknowledged. The identification and therefore exclusion of participants who consumed one or more fish meals per week was conducted in only four of the forty-six studies in this review. More studies ($n = 19$) considered supplementation and established criteria for exclusion for those taking fish oil supplements, yet only five of those studies measured a biomarker of habitual LC *n*-3 PUFA consumption (erythrocyte membrane EPA + DHA). Without exclusion parameters, the often wide ranges in LC *n*−3 PUFA levels at baseline and subsequent failure to avoid overlap between treatment and control groups after treatment were identified as a common flaw in randomised control trials of cardiovascular outcomes, preventing valid assessments of outcomes and conclusions[23].

Broad dietary intakes involving total energy and macronutrients have a profound effect on sport performance, particularly in athletes undergoing physiologically stressful exercise[90]. Standardisation and monitoring of dietary intake is recognised as an important component in trials assessing performance related outcomes in physically trained participants[91]. Whilst standardisation of dietary intake between trials would not be possible due to the variance of dietary requirements between individuals and sports, it should be considered and accounted for within individual trials. As a minimum, measuring dietary intake at baseline and post-intervention limits the confounding of key variables known to influence performance. The selection of an appropriate dietary assessment tool for use within physically trained populations is outside the scope of this review[92,93]; however, regardless of the dietary assessment tool used, only six studies in the current review reported no change in dietary intake at baseline and post-intervention periods. Considering these trials sought to manipulate diet through LC *n*-3 PUFA supplementation, the general lack of dietary assessment data and the heterogeneous approaches taken to assess and report on dietary intake is surprising. Nevertheless, with respect to LC *n*-3 PUFA, dietary assessment cannot match the accuracy and reliability of a blood biomarker such as O3I, due to the difficulty in capturing the infrequent consumption pattern of fatty fish[94].

Baseline exclusion

The LC *n*-3 PUFA enrichment of erythrocyte fatty acid membranes through dietary feeding of LC *n*-3 PUFA is largely a function of current erythrocyte levels, whereby a higher erythrocyte EPA + DHA level is less responsive to further increases[82]. It is therefore paramount to start all participants at an equal and low erythrocyte membrane EPA + DHA level if LC *n*-3 PUFA dosage and duration are fixed. This is particularly important considering that some physiological effects may be derived from achievable dietary intakes[14,59] rather than pharmacological doses as described for cardiovascular effects[3]. None of the studies in this review established predefined criteria for exclusion based on erythrocyte membrane EPA and/or DHA. The precise value of erythrocyte membrane EPA + DHA to be used as a criterion for the purpose of athletic benefit has not been established. The O3I (erythrocyte EPA + DHA expressed as a percentage of total fatty acids) has predominantly been used to categorise risk associated with cardiovascular mortality[4], whereby those with a value below 4% are associated with the highest risk, those between 4% and 8% associated with intermediate risk and those above 8% associated with the lowest risk. An O3I below 6·5% was arbitrarily chosen as a criterion for baseline participant exclusion in trials investigating incorporation of EPA and DHA within blood in both short[95] and long-term studies[96], and James *et al.*[22] demonstrated in a cardiovascular RCT that this would likely give satisfactory separation of treated and control groups. However, it may be appropriate to use statistical methods, such as exclusion of the 90th percentile of O3I, until further research clarifies the optimal ranges for athletic benefit.

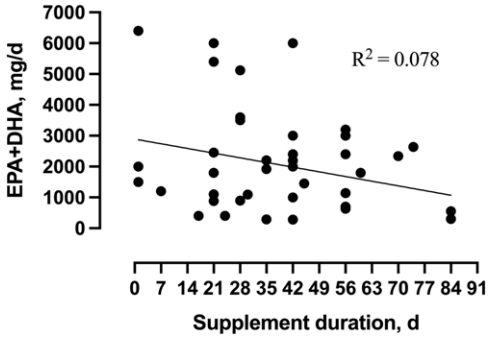

Fig. 4. Correlation between supplement dose and supplement duration for included studies ($n = 46$).

Separation of groups and the interaction of LC n-3 PUFA dose and duration

Upon establishment of homogeneous and low LC *n*-3 PUFA tissue status at baseline, the proposed LC *n*-3 PUFA intervention must be assessed for its ability to create clear group separation post-supplementation. This is a function of both the dosage of EPA and DHA, in addition to supplement duration. Browning *et al.*[25] explored this in their paper on incorporation of varying doses of EPA + DHA over a lengthy duration of 12 months. Whilst moderate doses (approximately two portions of fish per week) were able to establish a similar maximal erythrocyte membrane EPA and DHA incorporation compared with higher doses (approximately four portions of fish per week), the time to maximal incorporation was more than double that of the higher dose. Furthermore, the objectives of the study and primary mechanism of LC *n*-3 PUFA should also be considered when assessing optimal dose and duration. Higher doses (>2 g/d EPA + DHA) which favour EPA over DHA are often chosen in studies in which LC *n*-3 PUFA supplementation is found to influence muscle mass[97], strength[97,98] and muscle protein metabolism[99,100] in older adults. The implementation of higher doses should be taken with caution as the mechanism by which LC *n*-3 PUFA exert effects on muscle protein metabolism is unclear and therefore dose and duration require further investigation.

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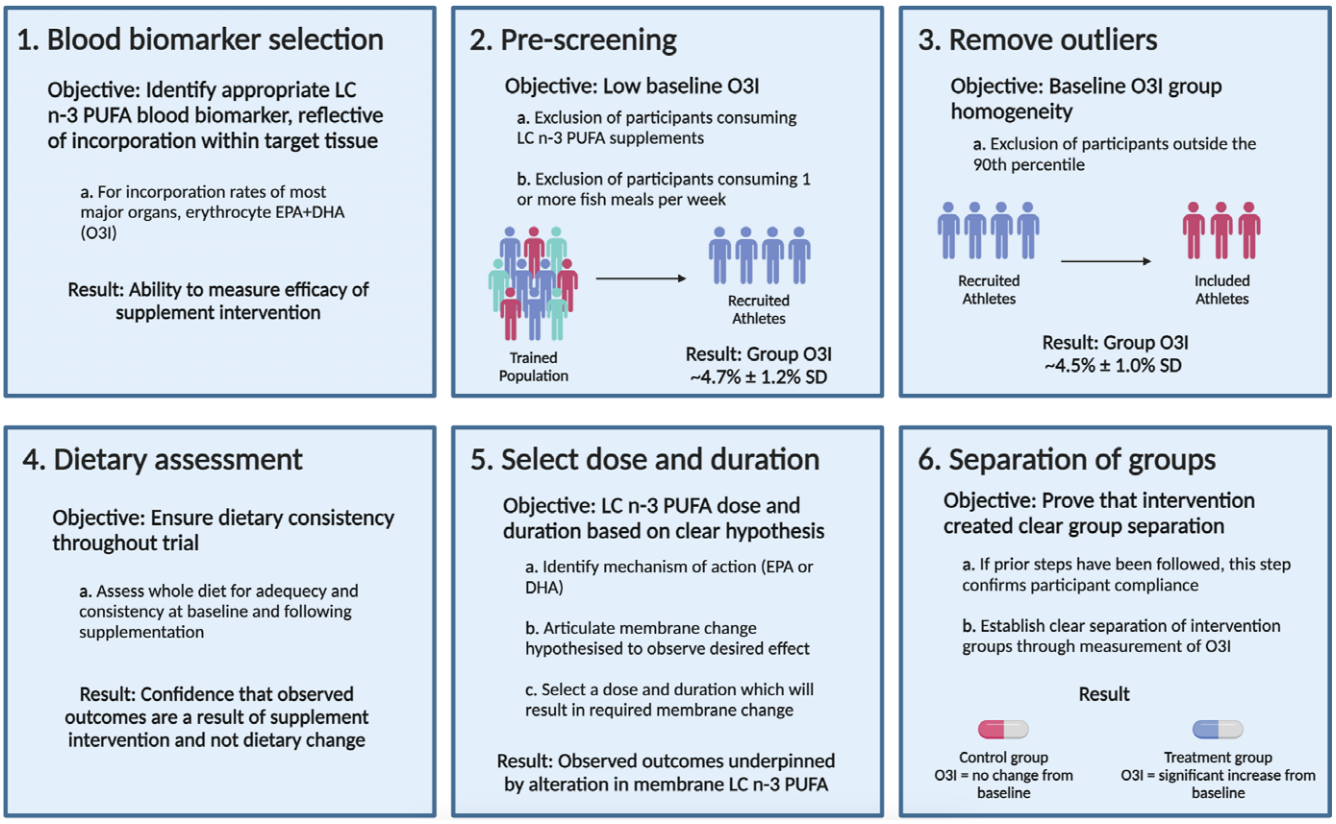

Fig. 5. Study design considerations for LC *n*-3 PUFA trials in physically trained individuals.

The present review has highlighted a broad range of EPA + DHA doses (min: 300 mg/d; max: 6400 mg/d), with supplement duration ranging from 1 d to 84 d. With supplement dose and duration being linked to incorporation within various lipid pools and therefore physiological effects, it was expected that a negative association between dose and duration would be present across studies in this review. In fact, there was no association between selection of EPA + DHA dose and supplement duration (Fig. 4), nor substantial reasoning for either. Whilst a statistically significant increase was reported in all studies that measured erythrocyte membrane EPA and/or DHA, there were clear differences in the magnitude of group separation. Zebrowska *et al.*[72] reported a significant increase in O3I following LC *n*-3 PUFA intervention, both within group and between groups post-supplement; however, O3I was increased by only 0·9%, reaching a final mean value of 4·8% (versus 3·9% in the control group). Conversely, group separation was more clearly defined by others[29,33,35], with increases in O3I ranging from 2% to >4%, with no change from baseline in the relatively low O3I of the control groups.

Conclusion and future recommendations

LC *n*-3 PUFA has recently gained substantial interest from sport scientists and researchers aiming to improve athletic outcomes. This is evident from more than half of the research papers in this review being conducted within the past 10 years. Nevertheless, despite this research intensity, the potential benefits of LC *n*-3 PUFA remain inconclusive. The current review has revealed many contributary design and measurement inconsistencies. First, a clear mechanistic hypothesis for LC *n*-3 PUFA was rarely articulated, resulting in substantial variation of dose and duration across individual studies. Second, inadequate assessment of broader background dietary intake and background dietary LC *n*-3 PUFA consumption. Finally, the paucity of appropriate LC *n*-3 PUFA blood biomarker assessment, leading to uncertainty in intervention efficacy. This highlights the need for widespread implementation of the LC *n*-3 PUFA study design considerations outlined throughout this review (shown graphically in Fig. 5) as a priority for future research in physically trained individuals.

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Conflict of interest

None.

Authorship

All the authors have contributed to the manuscript and approved the final submission. Study design, data collection and data

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analysis were carried out by R.A., G.E.P. and M.J.M. Data interpretation and manuscript preparation were undertaken by all authors.

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