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Real-world omega-3 supplementation patterns and whole-blood fatty-acid biomarkers: a cross-sectional analysis of 762,150 dried blood spot samples

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Abstract

Background Long-chain omega-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as compositional measures such as the arachidonic acid-to-EPA ratio (AA/EPA), have been extensively examined in fatty-acid biomarker research and in relation to cardiometabolic risk. However, large-scale characterisation of how these biomarkers and broader fatty-acid profiles vary across real-world omega-3 supplementation-exposure categories remains limited. This study aimed to characterise whole-blood fatty-acid profiles, with particular emphasis on EPA+DHA, AA/EPA, and their distributions across three pre-existing real-world supplementation-exposure groups in a large direct-to-consumer DBS dataset.

Methods A retrospective cross-sectional analysis was performed using 762,150 whole-blood DBS samples submitted to a commercial fatty-acid profiling service between 2012 and 2023. Based on self-reported supplementation exposure, samples were classified as non-user, self-supplemented (≥120 days), or program-supplemented (≥120 days within a structured program including a standardized fish-oil/extra-virgin olive-oil formulation, weight-based dosing guidance, and biomarker testing/feedback). Whole-blood fatty-acid profiles, EPA+DHA, and ln(AA/EPA) were compared across groups after adjustment for age, sex, BMI, and geographic region. Additional analyses examined AA/EPA across comparable EPA+DHA intervals and regional variation in AA/EPA.

Results Supplementation group was associated with differences in the measured relative whole-blood fatty-acid profile, with the largest group-related effect sizes observed for long-chain n-3-related measures and AA/EPA. In primary adjusted models, supplementation group had partial η^2 values of 0.274 for EPA+DHA and 0.352 for ln(AA/EPA). Adjusted mean EPA+DHA was progressively higher across the non-user, self-supplemented, and program-supplemented groups (3.30%, 4.16%, and 5.61%, respectively), while adjusted geometric mean AA/EPA was progressively lower (16.6, 10.9, and 5.2, respectively). The self-supplemented group showed intermediate but broadly dispersed biomarker values. By contrast, the program-supplemented group showed a more compact AA/EPA distribution, lower AA/EPA at comparable EPA+DHA levels below approximately 10%, and less regional heterogeneity in AA/EPA.

Conclusions In this large cross-sectional DBS dataset, pre-existing real-world supplementation-exposure groups were associated with differences in covariate-adjusted EPA+DHA and AA/EPA, and biomarker dispersion.

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Simple self-reported supplement-use categories captured group-level biomarker differences but masked substantial within-category heterogeneity. AA/EPA provided complementary information on fatty-acid profiling not summarized by measured EPA+DHA alone and may help characterize biomarker patterning across real-world omega-3 supplementation contexts. These observational findings should be interpreted as associations rather than evidence of causal effects of specific supplementation strategies.

Keywords Omega-3 status · EPA+DHA · AA/EPA ratio · Whole blood fatty acids · Dried blood spot · Omega-3 supplementation · Fatty acid biomarkers · Observational study · Real-world data

Background

Long-chain omega-3 polyunsaturated fatty acids (n-3 LCPUFA), including eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), have been widely studied in relation to cardiovascular, neurocognitive, and systemic health [1–4]. Robust prospective studies have associated higher circulating EPA and DHA levels with lower cardiovascular risk and more favourable physiological outcomes, while mechanistic evidence supports biologically plausible roles in membrane fatty-acid composition and lipid-mediator biology [5–7]. Blood fatty-acid biomarkers are therefore commonly used to characterize omega-3 status in population research and have been proposed as modifiable omega-3 status markers relevant to population health [8–10].

Despite these associations, global assessments consistently show low habitual intake of EPA and DHA and low blood omega-3 status across many regions [11–14]. In Western populations, omega-3 biomarker levels often cluster in ranges considered low, and only a minority of individuals achieve levels associated with lower cardiovascular and all-cause mortality risk in large prospective cohorts [6, 8, 15]. Observational and intervention studies further suggest that current intake recommendations, such as two seafood meals per week or low-dose supplements of ~250 to 400 mg/day EPA+DHA, may be insufficient to shift many adults into higher omega-3 status ranges [16, 17].

A persistent challenge in nutritional practice is the gap between biomarker changes demonstrated under controlled supplementation trials and the more modest, variable biomarker differences observed among free-living supplement users [18]. In real-world settings, omega-3 supplement use is highly heterogeneous: commercial products differ in EPA+DHA content, EPA/DHA composition, chemical form, and recommended dose, while use patterns vary in duration, frequency, and adherence [17, 18]. Population studies of self-directed supplement users therefore tend to report only modest average improvements in blood omega-3 biomarkers relative to non-users [17, 19, 20], despite larger biomarker changes observed under controlled, adequately dosed regimens [16, 21]. Broad self-reported supplement-use categories are useful for population-level comparisons but may obscure substantial heterogeneity in actual blood status associated with differences in formulation, dose, frequency, adherence, baseline status, and other unmeasured characteristics of real-world supplementation practices [18, 22]. Characterizing the distributions and broader fatty-acid profiles within such categories may therefore help illustrate the range of biomarker values observed within broadly defined supplementation groups and the extent to which these broadly defined groups are homogeneous at the biomarker level.

Blood-based omega-3 status can be assessed in several biological matrices, including plasma, erythrocytes, and whole blood, which reflect partly different lipid pools and therefore yield matrix-specific fatty-acid values [23–25]. The Omega-3 Index (O3I) is an established biomarker defined as the sum of EPA and DHA in erythrocyte membranes, expressed as a percentage of total erythrocyte fatty acids, and has been associated with coronary heart disease, incident cardiovascular disease, and all-cause mortality in prospective studies [5, 6, 15]. Mechanistically, these associations are thought to reflect broader differences in membrane fatty-acid composition and downstream lipid-mediator biology [2, 26]. In this context, the AA/EPA ratio may provide complementary information because AA and EPA constitute a metabolically related fatty-acid pair that participates in shared enzymatic pathways [27–29], and AA/EPA-related measures have been extensively examined in cardiovascular,

cerebrovascular, and inflammatory-context clinical outcomes [9, 10, 30, 31]. Accordingly, EPA+DHA and AA/EPA can be understood as related but distinct biomarkers within fatty acid profiling: EPA+DHA as a measure of long-chain omega-3 status in the measured blood matrix, and AA/EPA as a compositional marker describing the relative AA–EPA profile. AA/EPA should therefore be interpreted alongside its constituent fatty acids and EPA+DHA.

Dried blood spot (DBS) fatty-acid profiling provides a practical whole-blood biomarker platform for population-level assessment of EPA+DHA status and broader fatty-acid patterns [23, 24, 32]. In whole blood DBS analysis, fatty acids are reported as relative proportions within the measured fatty-acid profile, which reflects contributions from both circulating and cellular lipid fractions rather than a single isolated lipid pool [23, 25]. DBS-derived whole-blood values show strong correspondence with erythrocyte- and plasma/serum-based measures for both omega-3 metrics [24, 25, 33] and AA/EPA-related ratios [34–36], although values from different blood compartments are not directly interchangeable. This correspondence, combined with the recent expansion of commercially available DBS-based omega-3 status testing and at-home sampling, has further increased the feasibility of assembling large real-world biomarker datasets outside traditional clinical research settings [37, 38].

However, few large-scale studies have examined how broad self-reported omega-3 supplementation categories relate simultaneously to EPA+DHA status, AA/EPA, and biomarker distributions. Existing large-scale studies have largely focused on fish intake, supplement use, and achieved Omega-3 Index (O3I) or plasma n-3 LCPUFA levels [17, 18, 39, 40]; although some have also reported AA/EPA, these measures are rarely analysed in relation to one another, and whether AA/EPA carries information beyond EPA+DHA status remains largely unexamined. Moreover, many observational studies of omega-3 supplementation necessarily classify exposure using self-reported use versus non-use, without access to detailed information on dose, formulation, frequency, or adherence [41–43]. Large direct-to-consumer DBS datasets therefore provide an opportunity to examine what these real-world exposure categories represent at the biomarker level, using complementary fatty-acid measures.

Accordingly, the aim of the present study was to characterize and compare whole-blood fatty-acid profiles across three pre-existing real-world omega-3 supplementation-exposure groups in a large global direct-to-consumer DBS dataset, with particular emphasis on EPA+DHA, AA/EPA, and their distributions. The study analysed whole-blood fatty-acid profiles in 762,150 DBS samples from a global direct-to-consumer testing service. Building on a previous analysis of this global DBS database [19], the present study extends that work by incorporating samples from an additional supplementation-exposure group and by focusing specifically on EPA+DHA, AA/EPA, distributional variability, and AA/EPA levels at comparable EPA+DHA levels. Supplement use and demographic characteristics were self-reported at sample registration, enabling comparisons across three pre-existing real-world supplementation groups: samples from non-users reporting no long-term omega-3 supplement use, samples from self-supplemented test users reporting long-term omega-3 supplement use without information on product formulation or dose, and samples from program-supplemented users reporting use of a structured commercial omega-3 program that included a standardized fish oil/olive oil product, weight-based dosing guidance, and periodic biomarker feedback.

Specifically, the objectives were (i) to characterize and compare whole-blood fatty-acid profiles, and the distribution of EPA+DHA and AA/EPA across three pre-existing real-world omega-3 supplementation-exposure groups and (ii) to estimate covariate-adjusted between-group differences in EPA+DHA and AA/EPA, after accounting for age, sex, BMI, and geographic region, and (iii) to examine whether AA/EPA differed between supplementation-exposure groups within comparable EPA+DHA intervals, thereby assessing whether these two biomarkers provide complementary information for characterizing whole-blood fatty-acid profiles.

Materials and methods

Study design

The present study was a retrospective cross-sectional analysis of an overlapping global whole-blood DBS fatty-acid database previously reported in a large-scale analysis of overall PUFA status measures and their associated determinants among commercial test users [19]. The previous paper examined broad differences in whole-blood omega-3 status according to supplement use versus non-use, including variation by sex, age, BMI, and geographic region. The present analysis addressed a distinct research question and analytic framework by additionally including test users who reported participation in a structured omega-3 supplementation program and by characterizing covariate-adjusted between-group differences in individual fatty acids, prespecified summary measures, EPA+DHA, AA/EPA, and their distributions across three pre-existing real-world supplementation-exposure groups.

This cross-sectional observational study included 762,150 DBS samples collected between August 2012 and September 2023 from test users of a commercial fatty-acid profiling service provided by Zinzino Operations, Sweden. Samples were obtained via self-administered finger-prick DBS, either at home or during consultations with health practitioners. Test users registered anonymous sample IDs online and completed a questionnaire covering demographic characteristics and self-reported omega-3 supplement use. Fatty-acid analyses were performed at Vitas Laboratory, Oslo, Norway, as part of routine commercial testing throughout the 2012–2023 study period, using the established whole blood DBS fatty-acid method described previously [19]. Ethical approval for this cross-sectional analysis of anonymous commercial test and questionnaire data was obtained from the Joint Research Ethics Committee at the University of Central Lancashire, United Kingdom (HEALTH 01195).

Study population and sample selection

The analytic dataset comprised DBS samples collected between August 2012 and September 2023. This period was selected because the pre-existing online questionnaire consistently captured all variables required for the present analysis, including age, sex, body weight, height, country of residence, and self-reported omega-3 supplementation information. Additional information on for example pregnancy and lactation was not collected in the questionnaire during this period and was therefore not available for analysis.

Samples were eligible if they included valid DBS fatty-acid measurements, complete demographic information, and self-reported omega-3 supplement status. Samples with incomplete data, implausible anthropometric values, or inconsistent supplementation information were excluded. No additional sampling or selection was performed on the basis of age, geographic region, or measured fatty-acid values.

Because the database was anonymized by design, DBS samples could not be linked to individual test users. Some individuals may therefore be represented by more than one sample across different exposure groups over time; however, as the present analysis is cross-sectional and does not assess within-person change, each sample was treated as an independent observation of the exposure group to which it belonged at the time of testing.

Demographic and geographic classification

Demographic variables included age, sex, country of residence, weight, and height. BMI was calculated from self-reported weight and height and included as a continuous covariate. For descriptive tabulations in Table 1, BMI categories were calculated using conventional adult cut-offs among test users with reported age $\geq$20 years; younger test users were excluded from BMI-category tabulations. Age-specific BMI cut-offs for older adults were not applied because BMI categories were not used in the primary inferential models.

Because most samples originated from Europe, European countries were subdivided into Central & Eastern, Northern, Southern, and Western Europe according to the EuroVoc classification. The geographic variable used in

Table 1 Demographic characteristics by supplement-use group

	Total (n = 762,150)	Non-user (n = 519,382)	Self-supplemented (n = 76,187)	Program-supplemented (n = 166,581)	P value	Effect size	
Age, years	48.0 ± 16.5	46.8[a] ± 16.5	50.1 [b] ± 15.8	50.9 [c] ± 16.3	< 0.001	0.012	●
BMI, kg/m²	25.4 ± 4.5	25.5 [a] ± 4.5	25.2 [b] ± 4.3	25.7 [a] ± 4.3	< 0.001	0.001	
Sex					< 0.001	0.02	
Female	63.8%	63.3% [a]	66.2% [b]	64.2%[c]			
Male	36.2%	36.7% [a]	33.8% [b]	35.8% [c]			
Age group					< 0.001	0.08	
Children 1–9 years	1.5%	1.5% [a]	1.0% [b]	1.5%$_{a}$			
Adolescents 10–19 years	3.9%	4.4% [a]	2.0% [b]	3.0% [c]			
Young adults 20–39 years	23.7%	25.7% [a]	22.0% [b]	18.1% [c]			
Middle aged 40–59 years	45.5%	45.5% [a]	45.4% [a]	45.4% [a]			
Older adults 60–79 years	23.8%	21.3% [a]	27.6% [b]	29.8% [c]			
Elderly 80 + years	1.7%	1.5% [a]	1.9% [b]	2.1% [c]			
BMI category, adults aged ≥ 20 years only					< 0.001	0.02	
Underweight	1.9%	2.1% [a]	1.9% [a]	1.6%[b]			
Normal weight	48.6%	48.1% [a]	51.5% [b]	48.7% [c]			
Overweight	33.8%	33.7% [a]	32.8% [b]	34.8% [c]			
Obese	15.7%	16.2% [a]	13.7% [b]	14.9% [c]			
Geographic region					< 0.001	0.11	●
Africa	0.4%	0.5% [a]	0.6% [b]	0.3% [c]			
Asia	1.9%	2.3% [a]	1.2% [b]	1.1% [b]			
Central & Eastern Europe	22.7%	24.8% [a]	15.2% [b]	19.5%$_{c}$			
Latin America & Caribbean	0.2%	0.2% [a]	0.2% [a]	0.1% [b]			
North America	2.7%	2.5% [a]	3.4% [b]	2.9% [c]			
Northern Europe	41.4%	36.9% [a]	55.5% [b]	49.1% [c]			
Oceania	1.3%	1.4% [a]	1.4% [a]	0.9% [b]			
Southern Europe	2.6%	3.0% [a]	1.7% [b]	1.8% [b]			
Western Europe	26.8%	28.5% [a]	20.9% [b]	24.3% [c]			

Notes: Values are mean ± SD for continuous variables and percentages for categorical variables. Mean BMI is based on the full analytic sample. BMI-category distributions are restricted to test users aged ≥ 20 years because conventional adult BMI cut-offs are not applicable to children and adolescents. Superscript letters indicate statistically significant pairwise differences between supplement-use groups within each characteristic; groups sharing the same letter do not differ significantly. Effect sizes are reported as partial η^2 for continuous variables and Cramér's V for categorical variables. P values are from one-way ANOVA for continuous variables and χ^2 tests for categorical variables. Dots indicate approximate effect-size magnitude based on conventional thresholds

● = small (η^2: 0.010–0.059; V: 0.10–0.29)

●● = medium (η^2: 0.06–0.139; V: 0.30–0.49)

●●● = large (η^2: ≥ 0.14; V: ≥ 0.50)

adjusted models therefore combined non-European global regions with European subregions. For country-level visualisations and descriptive country-specific summaries, only countries with at least 35 samples per supplementation group were included, following recommendations used in comparable omega-3 status mapping studies [12].

Supplementation-exposure categories

The supplementation categories were not assigned by the investigators and did not represent intervention groups. Instead, they reflected self-reported omega-3 supplementation behaviours recorded at the time of sample registration through a pre-existing commercial questionnaire, which asked about omega-3 supplement use during the preceding 120 days and whether the reported supplement was the standardized program product or another omega-3 supplement. This 120-day threshold was used to distinguish long-term supplementation from more

recent or intermittent use, as it approximately corresponds to erythrocyte turnover and represents the integration period relevant to steady-state blood fatty-acid status. Information on product type, formulation, dose, use frequency, and adherence was not collected as part of this questionnaire.

Based on these responses, samples were classified into three mutually exclusive supplementation-exposure categories. **Non-users** comprised respondents who reported either no omega-3 supplement use or having used omega-3 supplements only for a short period; these response categories had been grouped together in the pre-existing dataset. **Self-supplemented users** reported omega-3 supplement use for ≥120 days without further product-level detail, representing a heterogeneous real-world exposure category. **Program-supplemented users** reported use for ≥120 days of a structured commercial omega-3 program comprising a standardized triglyceride-form fish oil and extra-virgin olive oil formulation (60:40), containing approximately 164 mg labelled EPA+DHA per mL of oil and an EPA/DHA ratio of approximately 1.9:1. The program recommended a weight-based volume of 0.15 mL oil/kg body weight, corresponding to approximately 25 mg labelled EPA+DHA/kg body weight, and included periodic DBS-based whole-blood fatty-acid testing, feedback on the measured fatty-acid profile, and optional results-based dietary guidance.

For both supplemented groups, actual intake, dose, use frequency, and adherence were not recorded, and neither group should therefore be interpreted as a confirmed dose-exposure category. The self-supplemented group reflects reported long-term use with no available product-level information, whereas the program-supplemented group reflects reported participation in a standardized program with defined product and dosing guidance, without verified individual consumption or adherence.

DBS fatty-acid analysis

The samples were analysed at Vitas Laboratory using the established whole-blood DBS fatty-acid method described in detail elsewhere [19]. Briefly, DBS samples were air-dried after collection and transported to the laboratory in sealed aluminium-laminate pouches containing desiccant. Upon arrival at Vitas Laboratory, samples were stored at 4 °C and analysed within one week. Whole-blood total fatty acids were transesterified to fatty-acid methyl esters (FAMEs), extracted into hexane, and analysed by gas chromatography with flame-ionization detection (GC-FID). Individual FAMEs were identified according to chromatographic retention times relative to an external FAME reference standard. No internal standard was used in this relative-composition assay. Whole-blood DBS samples were analysed for the 11 fatty acids included in the predefined analytical panel: palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1n-9), linoleic acid (LA, C18:2n-6), γ-linolenic acid (GLA, C18:3n-6), α-linolenic acid (ALA, C18:3n-3), dihomo-γ-linolenic acid (DGLA, C20:3n-6), arachidonic acid (AA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), and docosahexaenoic acid (DHA, C22:6n-3). The predefined panel includes major whole-blood fatty acids together with selected n-3 and n-6 fatty acids relevant to nutritional fatty-acid status. Results were expressed as percentage of the total peak area of the 11 quantified FAMEs. EPA+DHA and AA/EPA were calculated directly from relative DBS fatty-acid measurements. Analytical consistency across batches and over time was monitored using matrix-matched DBS QC samples evaluated against predefined control limits; replacement QC materials were bridged against the preceding QC before routine implementation.

Outcomes

Fatty-acid outcomes included the 11 individual fatty acids and grouped fatty-acid sums, which were used to characterise broader fatty-acid profile differences across supplementation-exposure groups. The primary outcomes were whole-blood EPA+DHA percentage, representing measured long-chain omega-3 status in the DBS matrix, and the AA/EPA ratio, used as a compositional marker of the relative AA–EPA profile. A secondary analysis examined between-group differences in AA/EPA across comparable EPA+DHA intervals. Additional analyses characterised geographic patterning of AA/EPA across supplementation-exposure groups. Because AA/

EPA was right-skewed, it was analysed on the natural log scale; back-transformed estimates are presented for interpretability.

Statistical analysis

Continuous variables are presented as means and standard deviations or medians and interquartile ranges where appropriate. Categorical variables are presented as percentages. Given the large sample size, statistical significance was defined as $p < 0.001$, and 99.9% confidence intervals (CIs) were reported. Effect sizes are presented alongside *P* values. Analyses were performed using IBM SPSS Statistics, version 28.

Demographic comparisons

Between-group differences in age and BMI were assessed using one-way ANOVA, with Welch's ANOVA reported when homogeneity of variance was violated. Categorical variables were compared using Pearson's χ^2 test. Effect sizes were expressed as partial η^2 for continuous variables and Cramér's V for categorical variables.

Fatty-acid profile models

Overall differences in fatty-acid profiles between supplementation exposure groups were assessed using multivariate analysis of covariance (MANCOVA), with the 11 individual fatty acids as dependent variables, supplementation exposure group as the fixed factor, and age, sex, BMI, and geographic region as covariates. Pillai's trace was the primary multivariate statistic given unequal group sizes and covariance heterogeneity.

Separate ANCOVA models were then fitted for individual fatty acids, fatty-acid sums, EPA + DHA, and log-transformed AA/EPA, using the same covariates. Bonferroni-corrected pairwise comparisons were conducted for significant group effects. Adjusted means, 99.9% CIs, and partial η^2 are reported; for AA/EPA, back-transformed geometric means are presented.

For descriptive comparison across fatty acids and indices, standardised adjusted mean differences were calculated for the self-supplemented and program-supplemented group versus the non-user group as the adjusted mean differences divided by the ANCOVA root mean square error (RMSE). Effect sizes were summarised in a clustered bar plot.

Sensitivity analyses for AA/EPA were performed using winsorized values and, where variance heterogeneity was present, Welch's ANOVA with Games-Howell post-hoc tests. Additional sensitivity analyses for both EPA + DHA and ln(AA/EPA) repeated the primary models using categorical rather than continuous specifications of age and BMI, with supplementation-group interactions examined to assess potential effect modification. Potential calendar-time and seasonal variation was assessed by additionally including calendar year and month of sample registration as categorical factors. Conclusions were materially unchanged across these analyses.

EPA + DHA-stratified analyses of AA/EPA

To examine whether AA/EPA differed between supplementation exposure groups at comparable EPA + DHA levels, samples were stratified into 1-percentage-point EPA + DHA percentage intervals defined according to the nearest whole percentage (e.g., 2.50–3.49% was classified as 3%). All intervals below 13% met the prespecified minimum requirement of at least 35 observations per group; therefore, only the upper-tail intervals were combined into a single ≥ 13% category to avoid sparse strata, giving 13 final categories from ≤ 1% to ≥ 13%. A general linear model was fitted with ln(AA/EPA) as the dependent variable and supplementation group, EPA + DHA interval, and their interaction included as fixed factors, with sex and geographic region as additional categorical factors and age and BMI as continuous covariates. Adjusted geometric mean AA/EPA values were subsequently estimated for each supplementation group within each EPA + DHA interval.

Geographic and distributional analyses

To assess regional patterning of AA/EPA within each supplementation exposure group, stratified general linear models of ln(AA/EPA) were fitted separately by group and adjusted for age, sex, and BMI. Partial η^2 for geographic region was used to quantify the partial association between geographic region and ln(AA/EPA) within each group.

Geographic and distributional visualisations were used to describe regional and country-level variation in AA/EPA across supplementation exposure groups. Decile cut-points were defined from the pooled sample distribution of observed AA/EPA values and then applied uniformly across groups, regions, and countries. Regional heatmaps show the percentage of samples within each geographic region falling into each pooled AA/EPA decile category. Country-level choropleth maps show the median AA/EPA ratio for each country, classified according to the same pooled decile cut-points. Geographic visualisations were restricted to regions or countries with at least 35 samples in the supplementation groups.

As an observational, non-randomised study, all analyses are subject to residual confounding despite adjustment for available demographic and regional variables.

Results

Demographic characteristics

Of the 762,150 DBS samples analysed between August 2012 and September 2023, mean reported age was 48.0 years (SD 16.5), and sex was recorded as female for 63.8% of samples. Overall, 68% were classified as non-user, 10% as self-supplemented, and 22% as program-supplemented. As shown in Table 1, the three supplementation groups were broadly similar in demographic and anthropometric characteristics, with several small but statistically detectable differences, as expected given the very large sample size.

Mean age was slightly higher in the two supplemented groups than among the non-user group, at approximately 51 vs. 47 years, respectively (partial $\eta^2 \approx 0.012$), which was reflected in a greater proportion of older adults in both supplemented groups. Sex distribution was comparable, with women comprising roughly two-thirds of the sample and only a small association between sex and supplementation status (Cramér's $V \approx 0.02$). Mean BMI was nearly identical across supplementation groups (partial $\eta^2 \approx 0.001$). BMI-category distributions among adults aged ≥ 20 years were also very similar (Cramér's $V \approx 0.02$), despite highly significant P values attributable to the large sample size. All groups were predominantly European, but geographic composition differed across supplementation groups (Cramér's $V = 0.106$), mainly reflecting a higher proportion of Northern European samples and lower proportions from Central & Eastern and Western Europe in the two supplemented groups than in the non-user group.

Given these small but detectable differences, and the larger difference in geographic composition, all subsequent analyses were adjusted for age, sex, BMI, and geographic region.

Covariate-adjusted fatty-acid biomarkers by supplementation group

An omnibus MANCOVA including the 11 measured fatty acids as dependent variables showed a large multivariate association between supplementation group and the whole-blood fatty-acid profile after adjustment for age, sex, BMI, and geographic region (Pillai's trace = 0.459, F(22, 1,524,254) = 20,644.4, $P < 0.001$, partial $\eta^2 = 0.230$). Follow-up ANCOVA models for individual fatty acids, predefined aggregates, and key indices showed significant group differences for all markers ($P < 0.001$; Table 2).

Five fatty acid measures showed large partial effect sizes for the supplementation-group factor (partial $\eta^2 \geq 0.140$): EPA (0.361), AA/EPA ratio (0.352), total n-3 PUFA (0.278), EPA + DHA (0.274), and DPA (0.214). EPA

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Table 2 Covariate-adjusted whole-blood fatty acid profiles by supplement-use group

Fatty acid variable	Non-user		Self-supplemented		Program-supplemented			
	Adjusted mean	(99.9% CI)	Adjusted mean	(99.9% CI)	Adjusted mean	(99.9% CI)	*P* value	Effect size
C16:0, PA	25.29	(25.27–25.31)	25.00	(24.97–25.03)	25.07	(25.04–25.1)	<0.001	0.003
C18:0, SA	13.68	(13.66–13.69)	13.68	(13.66–13.71)	13.78	(13.76–13.80)	<0.001	0.001
C18:1n-9, OA	23.43	(23.40–23.46)	22.89	(22.85–22.94)	22.67	(22.63–22.71)	<0.001	0.011 ●
C18:2n-6, LA	21.38	(21.36–21.41)	21.80	(21.75–21.84)	20.89	(20.86–20.93)	<0.001	0.008
C18:3n-3, ALA	0.36	(0.35–0.36)	0.38	(0.38–0.39)	0.35	(0.34–0.35)	<0.001	0.002
C18:3n-6, GLA	0.21	(0.21–0.22)	0.20	(0.20–0.20)	0.17	(0.17–0.18)	<0.001	0.028 ●
C20:3n-6, DGLA	1.46	(1.46–1.47)	1.37	(1.37–1.38)	1.17	(1.17–1.18)	<0.001	0.105 ●●
C20:4n-6, AA	9.72	(9.70–9.74)	9.25	(9.22–9.28)	8.63	(8.60–8.65)	<0.001	0.048 ●
C20:5n-3, EPA	0.71	(0.70–0.72)	1.07	(1.06–1.08)	2.18	(2.17–2.19)	<0.001	0.361 ●●●
C22:5n-3, DPA	1.17	(1.16–1.17)	1.26	(1.26–1.27)	1.66	(1.66–1.67)	<0.001	0.214 ●●●
C22:6n-3, DHA	2.59	(2.58–2.60)	3.09	(3.07–3.1)	3.43	(3.42–3.44)	<0.001	0.127 ●●
Total SFA	38.96	(38.94–38.99)	38.69	(38.65–38.72)	38.85	(38.82–38.88)	<0.001	0.001
Total PUFA	37.60	(37.56–37.64)	38.42	(38.36–38.48)	38.48	(38.43–38.53)	<0.001	0.010
Total n-3 PUFA	4.82	(4.80–4.84)	5.80	(5.78–5.83)	7.61	(7.59–7.64)	<0.001	0.278 ●●●
Total n-6 PUFA	32.78	(32.75–32.82)	32.62	(32.57–32.67)	30.86	(30.82–30.91)	<0.001	0.051 ●
EPA + DHA	3.30	(3.29–3.32)	4.16	(4.13–4.18)	5.61	(5.59–5.63)	<0.001	0.274 ●●●
AA/EPA	16.61	(16.44–16.78)	10.90	(10.80–11.02)	5.16	(5.16–5.21)	<0.001	0.352 ●●●

Notes: Values are adjusted means and 99.9% confidence intervals expressed as percentage of total measured fatty acids, unless otherwise indicated. Fatty-acid measures are expressed as percentages of the summed peak area of the 11 quantified FAMEs. Estimates were adjusted for age, sex, BMI, and geographic region. The AA/EPA ratio is presented as a geometric mean with 99.9% confidence interval, back-transformed from models fitted to ln(AA/EPA). Effect sizes are reported as partial η^2. P values are from covariate-adjusted general linear models. Dots indicate approximate effect-size magnitude based on conventional thresholds

● = small (partial η^2: 0.010–0.059)

●● = medium (partial η^2: 0.060–0.139)

●●● = large (partial η^2: ≥ 0.140)

and AA/EPA exhibited particularly large partial effect sizes, both exceeding 0.30, indicating strong group-related separation for these measures compared with the remaining fatty-acid measures. DHA approached the large-effect threshold ($\eta^2 = 0.127$), whereas omega-6 related measures (AA, DGLA, total n-6 PUFA, GLA) showed small-to-moderate partial effect sizes (partial $\eta^2 < 0.12$).

Adjusted group means indicated a clear gradient across supplementation categories (Table 2). Long-chain n-3-related measures showed progressively higher adjusted values from the non-user group to the self-supplemented group and the program-supplemented group, whereas AA/EPA and several n-6-related measures showed progressively lower adjusted values. Notably, the effect size associated with supplementation group was larger for AA/EPA than for EPA+DHA (partial $\eta^2 = 0.352$ vs. 0.274).

Pairwise Bonferroni-adjusted contrasts were used to compare each supplemented group with the non-user group and to quantify the direction and magnitude of these adjusted differences. Standardized mean differences are shown in Fig. 1 and Supplementary Table S1.

Figure 1 presents standardized adjusted mean differences describing the magnitude and direction of between-group differences relative to the non-user group. For long chain *n*-3-related measures, including EPA, total n-3 PUFA, DPA, DHA, and EPA+DHA, both supplemented groups showed positive standardized differences relative to the non-user group, whereas ln(AA/EPA) showed negative standardized differences. These standardized

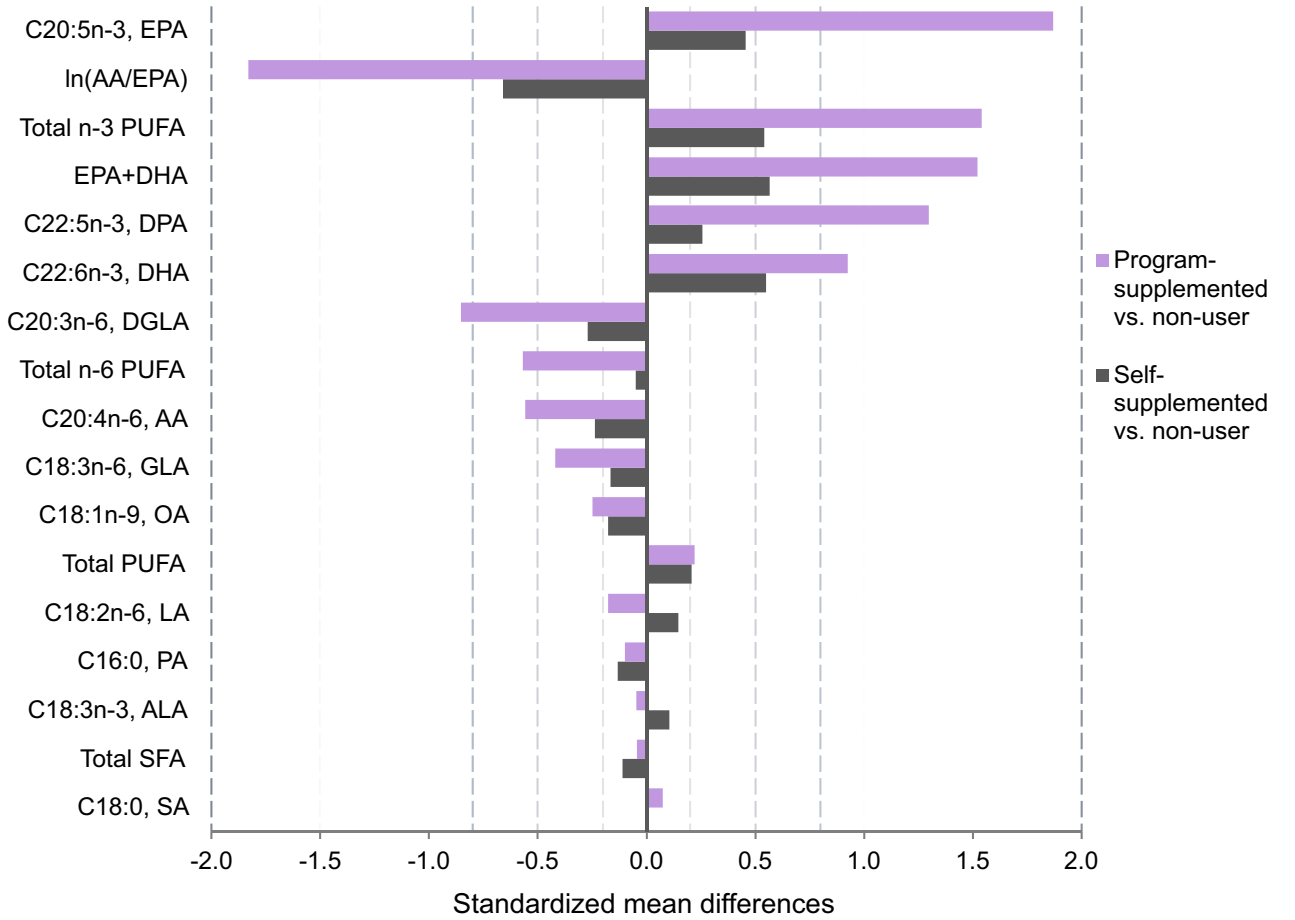


Fig. 1 Standardized mean differences for pairwise comparisons of fatty-acid variables between the self-supplemented and non-user groups and between the program-supplemented and non-user groups, after adjustment for age, sex, BMI, and geographic region. Positive values indicate higher adjusted values in the supplemented group than in the non-user group, whereas negative values indicate lower adjusted values. Vertical dashed lines indicate conventional thresholds for small ($|d| \approx 0.2$), medium ($|d| \approx 0.5$), and large ($|d| \approx 0.8$) standardized mean differences

differences were generally larger in the program-supplemented vs. non-user comparison than in the self-supplemented vs. non-user comparison. This contrast was most evident for EPA, DPA, and DGLA, where the program-supplemented comparison crossed at least one additional conventional standardized-difference threshold compared with the self-supplemented comparison. By contrast, ALA, LA, PA, SA, total SFA, and total PUFA showed negligible-to-small standardized differences relative to the non-user group in both pairwise comparisons. Among n-6-related measures, DGLA, AA, and total n-6 PUFA showed negative standardized differences, with the largest absolute differences observed in the program-supplemented vs. non-user comparison.

In the adjusted ANCOVA models, the two primary biomarkers showed parallel but unequal between-group separation. Adjusted whole-blood EPA+DHA was 3.30% in the non-user group, 4.16% in the self-supplemented group, and 5.61% in the program-supplemented group, corresponding to 26% and 70% higher adjusted levels, respectively, relative to the non-user group. For AA/EPA, back-transformed adjusted geometric means from models of ln(AA/EPA) were 16.6, 10.9, and 5.2 in the non-user, self-supplemented, and program-supplemented groups, corresponding to 34% and 69% lower values in the two supplemented groups (Table 2; Fig. 1).

AA/EPA by supplementation group at comparable EPA + DHA levels

Given that AA/EPA showed large between-group differences in the primary adjusted models, AA/EPA was compared across supplementation groups within EPA+DHA strata to describe whether group differences were also present at comparable measured EPA+DHA levels. Samples were grouped into 1-percentage-point EPA+DHA intervals (≤1% to ≥13%). Adjusted geometric mean AA/EPA values for each supplementation group within each EPA+DHA interval were estimated using a general linear model of ln(AA/EPA) that included supplementation group, EPA+DHA interval as a categorical factor, and their interaction, with age, sex, BMI, and geographical region included as additional covariates (Supplementary Table S2; Fig. 2).

In this interval-based analysis, adjusted geometric mean AA/EPA declined across increasing EPA+DHA intervals in all three supplementation groups. The decline was steepest across the lower EPA+DHA intervals, and became progressively smaller at higher EPA+DHA intervals, with estimates converging at approximately ≥10–11% EPA+DHA. Between-group differences in adjusted geometric mean AA/EPA remained evident

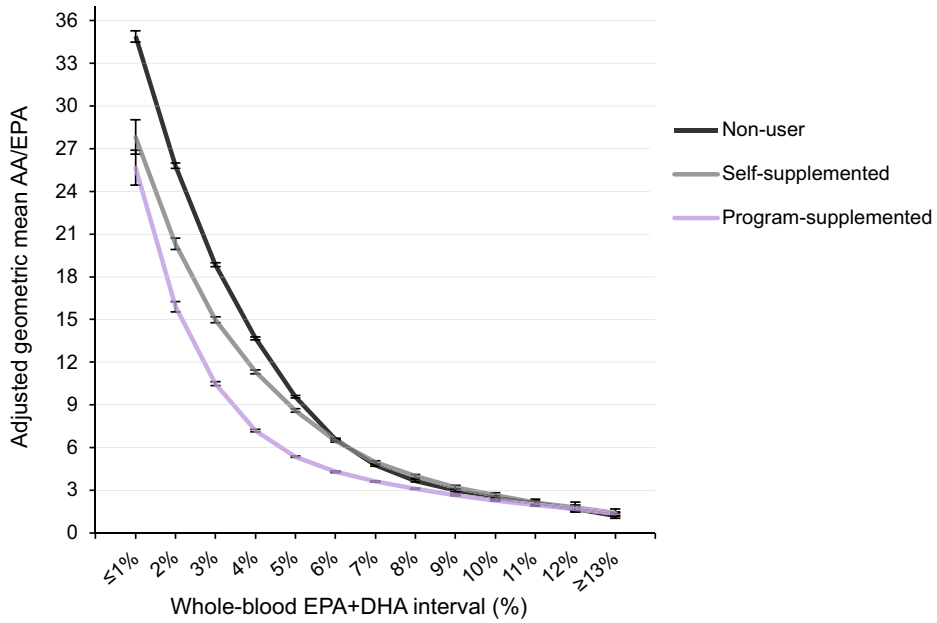


Fig. 2 Relationship between whole-blood EPA+DHA interval and adjusted geometric mean AA/EPA by supplementation group. EPA+DHA intervals are based on percentages of the summed peak area of the 11 quantified FAMEs. Values are back-transformed adjusted geometric means estimated from a general linear model of ln(AA/EPA), adjusted for age, sex, BMI, and geographic region. Error bars represent 99.9% confidence intervals

Table 3 Partial η^2 and adjusted R^2 from covariate-adjusted general linear models of EPA + DHA and ln(AA/EPA)

Model term / statistic	EPA + DHA model	ln(AA/EPA) model	ln(AA/EPA) model with EPA + DHA interval
Sex	0.002	0.000	0.001
Geographic region	0.079	0.166	0.100
Age	0.023	0.065	0.045
BMI	0.014	0.004	0.001
Supplementation group	0.274	0.352	0.123
EPA + DHA interval	—	—	0.493
Model R^2 (adjusted)	0.362	0.485	0.739

Note: Values are partial η^2 for individual predictors unless otherwise indicated. Model R^2 values are adjusted R^2. AA/EPA models were fitted using ln(AA/EPA). All models included age, sex, BMI, geographic region, and supplementation group; the expanded AA/EPA model included EPA + DHA interval as a categorical factor

Table 4 Observed EPA + DHA and AA/EPA distributions by supplementation group

Supplementation group	EPA + DHA (%), median (Q1–Q3)	AA/EPA, median (Q1–Q3)
Non-user	3.3 (2.5–4.2)	14.4 (9.0–23.0)
Self-supplemented	4.4 (3.3–5.6)	8.0 (5.1–12.7)
Program-supplemented	5.9 (4.6–7.3)	3.6 (2.6–5.5)
Total sample	**3.8 (2.8–5.2)**	**10.9 (5.4–19.2)**

Note: EPA + DHA is expressed as a percentage of the summed peak area of the 11 quantified FAMEs

within several EPA + DHA intervals, particularly below 10% EPA + DHA (Fig. 2, Supplementary Table S2). At EPA + DHA levels < 5%, both supplemented groups had substantially lower AA/EPA than the non-user group. For example, at 4% EPA + DHA, adjusted geometric mean AA/EPA was 13.7 in the non-user, 11.4 in the self-supplemented, and 7.2 in the program-supplemented group, corresponding to approximately 17% and 47% lower AA/EPA, respectively, relative to the non-user group. Around the mid-range, for example at 6% EPA + DHA, differences between the non-user and self-supplemented groups were small, whereas AA/EPA remained lower in the program-supplemented group. At ≥ 10–11% EPA + DHA, adjusted geometric mean AA/EPA estimates converged and between-group differences were small (Supplementary Table S2; Fig. 2).

Model summary statistics and partial η^2 values are summarized in Table 3. The primary EPA + DHA and ln(AA/EPA) models explained 36.2% and 48.5% of the variance, respectively. In both primary models, supplementation group had the largest partial η^2 among the included model terms. Geographic region had a larger partial η^2 in the ln(AA/EPA) model than in the EPA + DHA model, whereas sex and BMI had very small partial η^2 values. When EPA + DHA interval was added to the ln(AA/EPA) model, adjusted R^2 increased from 0.485 to 0.739. EPA + DHA interval had the largest partial η^2 in this expanded model, while supplementation group and geographic region remained associated with AA/EPA after accounting for EPA + DHA interval. These findings indicate that AA/EPA was strongly related to measured EPA + DHA level, while group- and region-related variation in AA/EPA was not fully described by EPA + DHA interval alone.

Sensitivity analyses using alternative age and BMI specifications and additional adjustment for calendar year and month of sample registration did not materially alter the supplementation-group findings; supplementation-group interactions with sex and with age and BMI categories were negligible in magnitude (partial $\eta^2 \leq 0.001$).

Observed EPA + DHA and AA/EPA distributions

Observed EPA + DHA and AA/EPA distributions by supplementation group are shown in Table 4. Median EPA + DHA was 3.3% (Q1–Q3: 2.5–4.2) in the non-user group, 4.4% (3.3–5.6) in the self-supplemented group, and 5.9% (4.6–7.3) in the program-supplemented group. Median AA/EPA was 14.4 (9.0–23.0), 8.0 (5.1–12.7), and 3.6 (2.6–5.5), respectively. The AA/EPA interquartile ranges showed limited overlap between groups, indicating broad distributional separation rather than differences confined to the upper tail of the distribution. EPA + DHA

also showed an ordered increase across supplementation groups, although its interquartile ranges showed greater overlap than those observed for AA/EPA. Full percentile distributions for EPA+DHA and AA/EPA are provided in Supplementary Table S3.

Regional heterogeneity in AA/EPA

Because geographic region remained associated with AA/EPA after adjustment for EPA+DHA interval in the overall model, regional heterogeneity in AA/EPA was examined in greater detail. To assess whether regional variation differed by supplementation group, group-stratified models of ln(AA/EPA) were fitted with geographic region as the main factor and age, sex, and BMI as covariates. Geographic region showed a large association with ln(AA/EPA) in the non-user and self-supplemented groups (partial $\eta^2 = 0.192$ and 0.179, respectively), and medium association in the program-supplemented group (partial $\eta^2 = 0.094$). When the models were additionally adjusted for EPA+DHA interval, the regional association was attenuated to the medium range in non-user and self-supplemented groups (partial $\eta^2 = 0.128$ and 0.096, respectively), and to the small range (partial $\eta^2 = 0.038$) in the program-supplemented group (Supplementary Table S4). Thus, adjustment for EPA+DHA interval attenuated regional AA/EPA heterogeneity in all groups; however, the residual regional association remained medium in non-user and self-supplemented groups but was small in program-supplemented group.

Figure 3 provides a descriptive visualization of regional AA/EPA distribution patterns using pooled AA/EPA decile cut points applied uniformly across supplementation groups. Regions were ordered according to the proportion of non-user samples in the highest AA/EPA decile (D10), and the same order was retained for the self-supplemented and program-supplemented groups to facilitate visual comparison. Across most regions, the non-user group was more frequently represented in the higher AA/EPA deciles, whereas the program-supplemented group was more frequently represented in the lower deciles and less represented in the upper deciles. The self-supplemented group showed an intermediate, more diffuse pattern across the decile distribution. For the corresponding EPA+DHA decile distribution, see Supplementary Figure S1.

Country-level median AA/EPA patterns were broadly consistent with the regional heatmaps and are shown in Supplementary Figure S2, with country-specific medians and sample sizes provided in Supplementary Table S5.

Discussion

Principal findings

In this large real-world DBS dataset, supplementation-exposure categories were associated with differences in the measured relative whole-blood fatty-acid profile, based on the multivariate model including the 11 individual fatty acids. Follow-up models of individual fatty acids and derived biomarkers indicated that the largest group-related effect sizes were observed for long-chain n-3-related measures, particularly EPA, DPA, total n-3 PUFA, and EPA+DHA, together with marked differences in AA/EPA. Thus, the group differences were not confined to a single fatty acid or ratio, but reflected broader patterning within the relative whole-blood fatty-acid profile.

Within this broader profile pattern, samples in both supplemented groups had higher whole-blood EPA+DHA and lower AA/EPA than samples in the non-user group, with the self-supplemented group occupying an intermediate position and the program-supplemented group showing the most pronounced omega-3-enriched, lower-AA/EPA biomarker pattern. AA/EPA showed stronger between-group separation than EPA+DHA in this dataset, and observed AA/EPA distributions showed limited overlap of interquartile ranges between groups, indicating broad distributional separation rather than differences confined to the mean. Importantly, AA/EPA remained lower in the program-supplemented group at comparable EPA+DHA levels, particularly below approximately 10% EPA+DHA, showing that the between-group biomarker differences were not limited to total EPA+DHA

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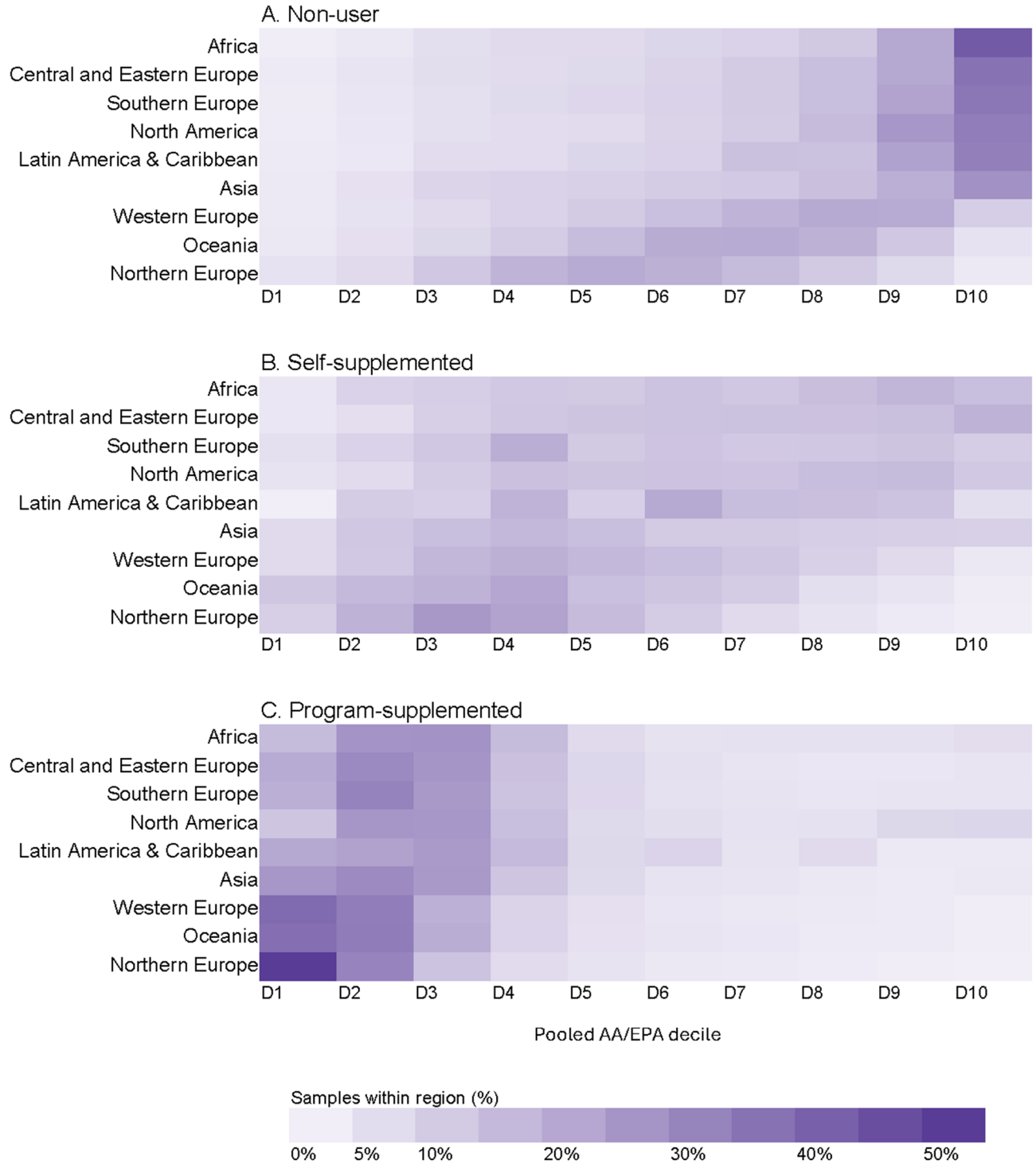


Fig. 3 Regional distributions of observed whole-blood AA/EPA by supplementation group. Heatmaps show the percentage of samples in each pooled AA/EPA decile within each region and supplementation group. Panels represent (**A**) non-user, (**B**) self-supplemented, and (**C**) program-supplemented groups. Decile cut-points were defined from the pooled sample-level AA/EPA distribution and applied uniformly across groups and regions: D1 ≤ 2.97, D2 2.98–4.44, D3 4.45–6.36, D4 6.37–8.51, D5 8.52–10.85, D6 10.86–13.55, D7 13.56–16.97, D8 16.98–21.88, D9 21.89–31.25, and D10 ≥ 31.26. Darker shading indicates a higher proportion of samples within the corresponding decile. Regions are ordered according to the proportion of non-user samples in D10

enrichment. Regional AA/EPA heterogeneity was also lowest in the program-supplemented group, a pattern explored further below.

Whole-blood biomarker interpretation

When interpreting whole-blood EPA+DHA in this dataset, it is important to recognise that these values are not directly interchangeable with erythrocyte O3I percentages [25]. In the O3I literature, erythrocyte EPA+DHA values of ≤4% and ≥8% are often considered indicative of relatively low and high status, respectively [5]. Published conversion work suggests that whole-blood EPA+DHA values are generally lower than corresponding erythrocyte percentages, such that an erythrocyte O3I of 8% may broadly correspond to whole-blood EPA+DHA values of approximately 5.7–6.7% [(25)]. Because these conversions were derived using broader fatty-acid panels than the present assay, they should not be interpreted as directly applicable thresholds. Using these published conversion relationships as an approximate external comparison, only the upper tail of the non-user group and above the upper quartile of the self-supplemented group approached this corresponding whole-blood range, whereas the program-supplemented group had a distribution extending toward and beyond it. This pattern is consistent with prior reports that relatively high omega-3 status is uncommon in free-living Western populations [6, 12].

Whole-blood AA/EPA has similarly been proposed as a practical blood-based marker and has shown strong correspondence with both serum and erythrocyte membrane AA/EPA across published comparisons [34–36, 44]. Most published values relevant to the present comparison originate from Japanese serum-based cardiovascular and community cohorts, where results have commonly been reported as EPA/AA [9, 10, 30, 45–49]. Reported EPA/AA values of approximately 0.32–0.40 in community cohorts [30, 44, 46, 47] correspond to reciprocal serum AA/EPA values of approximately 2.5–3.1; however, based on the cross-matrix relationship reported by Nagai et al. [36], corresponding whole-blood AA/EPA values would be higher than the serum values. Using this relationship as an approximate external comparison, serum AA/EPA values in this range would broadly correspond to whole-blood AA/EPA values of approximately 3–5. In the present cross-sectional dataset, this approximate whole-blood AA/EPA range of 3–5 was observed mainly in the lower tail of the non-user distribution and the lower part of the self-supplemented distribution, whereas the central part of the program-supplemented distribution lay within or near this range. Together, these literature-based comparisons provide approximate context for interpreting how the observed EPA+DHA and AA/EPA distributions relate to values reported using other blood matrices and analytical approaches; they should not be interpreted as assay-specific reference ranges or targets.

Supplement-use categories and biomarker variability

Both supplemented groups should be interpreted as real-world exposure categories rather than intervention arms. However, they differ in exposure resolution. The self-supplemented group represents a broad self-directed supplement-use category, whereas the program-supplemented group represents a more structured supplementation context with greater standardization of product composition, dosing guidance, reassessment, and biomarker feedback. The self-supplemented category nevertheless captured a clear group-level biomarker shift, with higher EPA+DHA and lower AA/EPA than the non-user group, consistent with previous findings that omega-3 supplement use is generally associated with higher blood n-3 PUFA status [17, 18, 39].

At the same time, the broad and overlapping EPA+DHA distributions observed among non-user and self-supplemented samples were expected. Self-reported supplement use over the previous 120 days does not specify product type, EPA+DHA dose and EPA/DHA composition, chemical formulation, duration, adherence, baseline fatty-acid status, body size, background diet, or genetic factors influencing fatty-acid metabolism. These factors are all known to influence achieved omega-3 biomarker status [16, 21, 22, 50]. For example, intervention studies and pooled modelling have identified dose, body size or dose per body weight, baseline Omega-3 Index, and chemical formulation as important determinants of biomarker response [16, 22], while reviews indicate that both dose and formulation influence the likelihood of reaching higher Omega-3 Index categories [21]. Population-based

analyses similarly show that supplement use and fish intake provide useful but incomplete information about measured omega-3 status [17, 18, 39]. The heterogeneity observed in the self-supplemented group should therefore not be interpreted as unexpected or unfavourable, but as an inherent feature of a low-resolution real-world exposure category. The present analysis adds to this literature by visualizing the distributional structure behind such a category, showing that average group differences can coexist with substantial within-group biomarker heterogeneity. This distinction may help contextualize observational studies relating regular fish-oil supplement use to health outcomes, particularly when exposure classification relies on self-reported supplement-use categories rather than measured omega-3 status [51].

The program-supplemented group provides a contrasting, but still observational, real-world exposure context. Its narrower AA/EPA distribution and lower regional heterogeneity are consistent with what would be expected from a more standardized model, including defined product composition, weight-based dosing guidance, planned reassessment, and biomarker interpretation. Evidence from adult and paediatric supplementation studies supports the relevance of body-size-adjusted dosing for reducing inter-individual variability in achieved omega-3 status [16, 52]. A standardized product and weight-based dosing guidance can reduce variation in the intended exposure, but achieved blood status ultimately depends on whether the recommended intake is sustained over time. Program features such as planned reassessment, biomarker interpretation, reminders, and social support may therefore reduce behavioural variability by helping test users maintain the intended supplementation routine over the supplementation period, even in a non-clinical setting. Evidence from self-monitoring and feedback-based interventions suggests that such support can improve adherence or biomarker outcomes during active intervention periods, although effects are context-dependent and may attenuate when reinforcement stops [53, 54]. Omega-3-specific evidence is limited but directionally consistent: a web-based 120-day supplementation program increased Omega-3 Index and the proportion of participants above 8% [55], whereas a personalized-dose study in Naval Academy athletes showed that poor compliance substantially blunted the response despite a personalised dose and reminders [56]. These external findings provide a plausible rationale for why structured supplementation models may narrow biomarker distributions not only by standardizing dose recommendations, but also by improving the consistency with which the intended exposure is actually delivered.

Chemical formulation is a plausible and potentially important component of this more standardized exposure context. Formulation has been identified as a contributor to achieved omega-3 biomarker response in pooled intervention modelling and supplementation reviews [21, 22]. The standardized fish-oil and extra-virgin olive-oil lipid matrix used in the program-supplemented group may therefore represent one potential contributor, but was embedded within a broader structured supplementation context that also included weight-based dosing guidance, reassessment, biomarker feedback, and self-selection. Olive-derived phenolics such as hydroxytyrosol have been shown to reduce oxidative degradation of fish lipids in experimental systems [57]. In addition, combinations of fish oil and extra virgin olive oil have been reported to influence n-3 PUFA incorporation, lipid metabolism, and oxidative stability compared with standard fish oils [58–62]. Because actual dose consumed, adherence, baseline biomarker values, and concurrent dietary changes were not captured, the study cannot separate the relative contributions of weight-based dosing, standardized formulation, biomarker feedback, peer support, and self-selection into the program. In particular, the lower variability observed in the program-supplemented group cannot be interpreted as evidence of a more uniform biomarker response to supplementation, since it may also reflect differences in baseline fatty-acid profiles or other test-user characteristics among those entering the program.

Importantly, the distinction between the self-supplemented and program-supplemented groups was not limited to EPA+DHA enrichment. Differences in AA/EPA persisted across EPA+DHA intervals and across geographic regions, suggesting that total EPA+DHA alone did not fully summarize the group-related biomarker differences observed in this dataset. Thus, the main implication is not simply that one supplementation category had higher omega-3 status than another, but that the different real-world supplementation categories were associated with different biomarker distributions.

Complementary information from EPA + DHA and AA/EPA

In the present data, whole-blood EPA + DHA showed a strong inverse relationship with AA/EPA across all supplementation groups, such that higher EPA + DHA levels were associated with lower ln(AA/EPA). However, group differences in AA/EPA persisted at comparable EPA + DHA levels: below approximately 10%, the program-supplemented group tended to have lower AA/EPA than the non-user and self-supplemented groups. The overall adjusted fatty-acid profiles provide some context for this finding. Relative to non-users, the program-supplemented group showed a substantially larger difference in EPA than in DHA, while AA was also lower, although its overall group effect was smaller. Thus, lower AA/EPA at a given EPA + DHA level could plausibly reflect a greater relative contribution of EPA, lower AA representation, or both, consistent with differences in exposure profile and factors influencing the relative representation of AA within the measured fatty-acid profile [46, 63, 64]. In a recent systematic review and meta-analysis of 96 clinical trials, the EPA/DHA dosing ratio in the supplement was strongly associated with the blood EPA/DHA ratio, and the blood EPA/DHA ratio was identified as the strongest predictor of reduced AA representation in blood fatty-acid profiles [64]. The present interval analysis, however, was not designed to quantify the relative contributions of EPA and AA to these between-group differences in AA/EPA.

Taken together, these findings support the interpretation that EPA + DHA and AA/EPA are complementary rather than redundant markers: Whole-blood EPA + DHA reflects long-chain n-3 status arising from dietary and supplemental EPA and DHA exposure, modified by factors such as absorption, incorporation into blood lipid pools, and metabolism [24, 65]. AA/EPA describes the relative AA–EPA profile and may vary with EPA exposure, EPA/DHA composition, and factors influencing AA availability and metabolism [64, 65]. Because AA is itself a physiologically important n-6 LC-PUFA and AA/EPA is determined by both constituent fatty acids, the ratio should be interpreted alongside EPA, AA, and EPA + DHA. A lower AA/EPA should therefore not be interpreted as favourable in isolation, as it may reflect higher EPA, lower AA, or both.

Covariate patterns also supported marker-specific interpretation. Age showed a larger partial association with AA/EPA than with EPA + DHA (partial $\eta^2 = 0.065$ vs. 0.023), which may reflect age-related differences in dietary habits, supplement use, or fatty-acid metabolism, including desaturase activity [35, 66]. By contrast, sex and BMI showed small or minimal independent associations with either marker after covariate adjustment, consistent with previous reports suggesting that sex- and body-size-related differences in omega-3 status may be partly mediated by dietary intake, body composition, dose relative to body size, and life-stage-related factors such as reproductive status and ageing [8, 21, 52].

In this dataset, non-user samples remained concentrated at lower EPA + DHA and higher AA/EPA values, whereas self-supplemented samples occupied an intermediate but still broadly dispersed position. In contrast, the program-supplemented group showed both a higher median EPA + DHA and a narrower lower-AA/EPA distribution. Together with the large supplementation-group effect size observed for AA/EPA and the persistence of AA/EPA differences at comparable EPA + DHA levels, these findings highlight the potential value of considering more than total EPA + DHA enrichment when characterizing real-world omega-3 supplementation patterns. Because higher EPA + DHA and lower AA/EPA have both been associated with more favourable cardiometabolic profiles in previous cohorts [48, 67], the combined biomarker pattern observed here may warrant further prospective investigation at the population level. More broadly, this pattern supports the hypothesis that population-level omega-3 strategies may warrant evaluation not only by increases in mean EPA + DHA, but also by their ability to shift the broader distribution of fatty-acid biomarkers, including central values and distributional tails [68].

Regional AA/EPA patterning beyond EPA + DHA

The adjusted models showed that both supplementation group and broad geographic region were associated with variation in EPA + DHA and AA/EPA, with a stronger regional association observed for AA/EPA. This pattern is consistent with previous reports of geographic variation in omega-3 status [12, 19] and AA/EPA-related measures

[69, 70], and may reflect regional differences in dietary fatty-acid exposure and other determinants of the relative AA–EPA profile. In the expanded AA/EPA model, adjustment for EPA+DHA interval attenuated the regional association, indicating that part of the geographic variation in AA/EPA was aligned with differences in measured EPA+DHA. However, regional differences in AA/EPA remained evident at comparable EPA+DHA levels.

The group-stratified analyses further showed that this residual regional patterning differed across supplementation groups. After adjustment for EPA+DHA interval, the regional association remained in the medium range in the non-user and self-supplemented groups but was small in the program-supplemented group (partial $\eta^2 = 0.128$, 0.096, and 0.038, respectively). The regional decile distributions visualized in the heatmap showed a corresponding qualitative pattern, with less pronounced geographic differentiation in AA/EPA within the program-supplemented group. Together with the overall distributional results, this indicates that the program-supplemented group was characterized not only by lower AA/EPA, but also by a more compact overall AA/EPA distribution and less regional heterogeneity at comparable EPA+DHA levels. This pattern may be consistent with features of the more standardized supplementation context, but the observational design cannot determine whether it reflects supplementation characteristics, adherence, self-selection, background diet, or other unmeasured factors.

Strengths and limitations

Key strengths of this work include the very large real-world sample, broad geographic coverage, and the use of a standardized whole-blood DBS fatty-acid platform across three pre-existing supplementation-exposure groups. The sample size enabled characterization of entire biomarker distributions and stable stratified analyses across supplementation groups, geographic regions, and EPA+DHA intervals, rather than reliance on mean comparisons alone. The predefined 11-fatty-acid panel, comprising major whole-blood fatty acids together with selected nutritionally relevant n-3 and n-6 fatty acids, allowed group differences in EPA+DHA and AA/EPA to be interpreted within a broader relative whole-blood fatty-acid context. A further analytical strength was the EPA+DHA-stratified analysis of AA/EPA, which allowed assessment of whether between-group differences in AA/EPA persisted at comparable measured EPA+DHA levels. Emphasis on effect sizes and 99.9% confidence intervals reduced reliance on statistical significance in this very large dataset, while distributional visualizations provided complementary information on within-group and regional heterogeneity. Robustness and sensitivity analyses addressing AA/EPA distributional properties, alternative age and BMI specifications, and broad calendar-time variation did not materially alter the principal findings.

Important limitations must be acknowledged. The cross-sectional, non-randomised design precludes causal inference, and residual confounding, particularly by diet, socio-economic status, comorbidities, and health-seeking behaviour, is likely. The dataset represents self-selected users of a commercial direct-to-consumer testing service and should therefore not be considered population-representative. Information on pregnancy, lactation, menopausal status, and other life-stage characteristics was not available. Consequently, sex-related differences associated with pregnancy, lactation, menopausal status, and other life-stage characteristics could not be examined in the present analysis. Supplementation behaviour was self-reported, and exact data on dose, non-program formulation, adherence, and detailed duration of use were unavailable. Because the database was anonymous by design, repeated samples from the same test user could not be identified, and potential within-person clustering could therefore not be accounted for. The extent of any resulting non-independence is therefore unknown, and the unit of analysis should be understood as the DBS sample rather than the individual test user. The predefined analytical panel did not characterize fatty acids outside the 11 measured analytes, and relative percentages were normalized to the summed peak area of these 11 FAMEs; values should therefore be interpreted within this compositional framework. Whole-blood fatty-acid values are matrix-specific and should not be directly equated with erythrocyte-based indices or cut-offs; however, the present analyses and conclusions are based on comparisons within the same standardized whole-blood DBS framework and are therefore not dependent on erythrocyte-based thresholds. Country-level summaries were descriptive and may have been influenced by uneven sample sizes across countries despite the application of a minimum sample threshold. Most importantly, self-selection into the

structured program is a major potential confounder: individuals who enrol may differ systematically from self-supplemented users and non-users in motivation, health awareness, diet, baseline status, and propensity to adhere to recommendations, and these differences cannot be fully addressed in the present design.

Future directions

Future research should link real-world fatty-acid profiles, including whole-blood AA/EPA, to clinical outcomes in cohorts where supplementation mode, dose, and formulation are prospectively documented. Future studies should also investigate whether dietary and biological factors contribute to differences in AA/EPA at comparable EPA + DHA levels, including variation in dietary omega-6 intake, EPA/DHA exposure ratios, and broader dietary patterns. Within program settings, longitudinal data could clarify dose–response relationships, adherence patterns, and the durability of biomarker changes over time. Comparative studies of different structured delivery approaches, varying, for example, in dosing algorithms, formulation, and feedback intensity, could test whether the distributional shifts in AA/EPA and related markers observed here are reproducible and whether they translate into measurable reductions in cardiometabolic or inflammatory risk.

Conclusion

In this large real-world direct-to-consumer dataset, self-reported long-term omega-3 supplementation was associated with differences in the measured relative whole-blood fatty-acid profile, most clearly reflected in higher EPA + DHA and lower AA/EPA compared with the non-user category. The self-supplemented group showed intermediate but broadly dispersed biomarker values, highlighting substantial within-category heterogeneity, whereas the program-supplemented group showed the highest EPA + DHA and lowest AA/EPA. Beyond differences in adjusted average biomarker levels, the program-supplemented group also showed a more compact overall AA/EPA distribution and less pronounced regional heterogeneity in AA/EPA, indicating a more consistent fatty-acid biomarker profile across geographic regions. Differences in AA/EPA persisted between supplementation groups at comparable EPA + DHA levels, indicating that AA/EPA provided complementary information for characterizing between-group differences in the whole-blood fatty-acid profile that were not summarized by total EPA + DHA alone. These findings support further longitudinal and outcome-linked research to determine whether specific features of structured supplementation models contribute to the observed biomarker distributions and whether such biomarker patterns are associated with clinically relevant outcomes.

Abbreviations

AA	Arachidonic acid
ALA	α-Linolenic acid
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BMI	Body mass index
CI	Confidence interval
DBS	Dried blood spot
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty-acid methyl ester
GC-FID	Gas chromatography with flame ionization detection

GLA	γ-Linolenic acid
IQR	Interquartile range
LA	Linoleic acid
LCPUFA	Long-chain polyunsaturated fatty acid
MANCOVA	Multivariate analysis of covariance
n-3	Omega-3
n-6	Omega-6
O3I	Omega-3 Index
OA	Oleic acid
PA	Palmitic acid
PUFA	Polyunsaturated fatty acid
RMSE	Root mean square error
SA	Stearic acid
SD	Standard deviation
SFA	Saturated fatty acid
SMD	Standardized mean difference
QC	Quality control

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Author contributions O.E. and E.G. conceptualized the study. E.G., A.M.R., T.G. and O.E. contributed to the methodology, including the study design, DBS fatty-acid methodology, and analytical approach. E.G. performed the formal analysis, statistical programming, data processing, validation checks, and visualization. E.G. and M.T. curated the data. E.G. wrote the original draft of the manuscript. A.M.R., M.T., T.G., O.E., P.C. and C.R. reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data availability The data sets generated and/or analysed during the current study are not publicly available due to proprietary data restrictions but are available from the corresponding author upon reasonable request. Requests for data access should include a clear explanation of the purpose and scope of the intended use and may require additional ethical approval depending on the request.

Declarations

Ethics approval and consent for data use The study was conducted in accordance with the principles of the Declaration of Helsinki. Ethical approval for this cross-sectional analysis of anonymous commercial test and questionnaire data was obtained from the Joint Research Ethics Committee at the University of Central Lancashire, United Kingdom (Reference Number: HEALTH 01195). Before submitting their sample and questionnaire, test users were informed about the data-management and privacy framework. They provided active consent to the analysis of their anonymous test and questionnaire data by clicking an "I agree" button at the end of the questionnaire and were informed that they could request deletion of their record by providing the relevant anonymous test ID.

Consent for publication Not applicable.

Competing interests E.G. and C.R. are employees of Zinzino Operations AB. M.T. is employed by Bioactive Foods AS,

a company owned by Zinzino Operations AB. Zinzino Operations AB markets omega-3 supplements and fatty-acid testing services related to the subject matter of this manuscript. T.G. is CEO and co-founder of Vitas Analytical Services, which performed the fatty-acid analyses used in this study. P.C., O.E., and A.M.R. serve as consultants on Zinzino's Scientific Advisory Board. No non-author representative of the above-mentioned companies had authority to control the analyses, interpretation of results, manuscript content, or decision to submit the manuscript for publication. The authors declare no other competing interests.

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