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Red blood cell oleic acid levels reflect olive oil intake while omega-3 levels reflect fish intake and the use of omega-3 acid ethyl esters: The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico–Heart Failure trial

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ABSTRACT

The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico–Heart Failure (GISSI-HF) study reported benefits of n-3 fatty acid (FA) treatment on cardiovascular (CV) events, but the effects of treatment on a putative CV disease risk factor, the red blood cell (RBC) n-3 FA level (the omega-3 index), have not been examined in this context. We hypothesized that treatment with prescription omega-3 acid ethyl esters (O3AEE) would increase the omega-3 index to the proposed cardioprotective value of 8%. RBCs were collected from a subset of patients participating in the GISSI-HF study (n = 461 out of 6975 randomized), at baseline and after 3 months of treatment with either an olive oil placebo or O3AEE (1 g/d). RBC FA levels were expressed as a percentage of total FA. Patients also reported their typical olive oil and fish intakes. RBC oleic acid levels were directly correlated with reported frequency of olive oil consumption, and the omega-3 index was correlated with reported fish intake (P for trends <0.001 for both). After treatment, the omega-3 index increased from 4.8 ± 1.7% to 6.7 ± 1.9% but was unchanged in the placebo group (4.7 ± 1.7 to 4.8 ± 1.5%) (P < .0001 for changes between groups). At 3 months, more patients reached the proposed target omega-3 index level of 8%-12% in the treated vs placebo group (22.6% vs.

Abbreviations: ANOVA, analysis of variance; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DPAn-3, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; GISSI-HF, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico - Heart Failure; O3AEE, omega-3 acid ethyl esters; OA, oleic acid; Q, quartile; RBC, red blood cell; RBC EPA + DHA, the omega-3 index.

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1.3%, $P < .0001$), however, what omega-3 index levels were ultimately achieved after four years in this trial are unknown.

1. Introduction

There are multiple controversies surrounding the effects of saturated, mono-unsaturated and omega-6 polyunsaturated fatty acids (FAs) on coronary heart disease risk [1-4], and even around the omega-3 FA, which for many years have enjoyed virtually universal support as being cardioprotective [5,6]. In the Chowdhury et al meta-analysis [1], both intakes and blood levels of the long-chain, marine-derived omega-3 FAs (eicosapentaenoic [EPA], docosahexaenoic [DHA], and docosapentaenoic acids [DPAn-3]) were inversely related to risk, but randomized trials have, at least in recent years, not supported this relationship.

FA intakes and blood levels vary around the world, presumably driven by differences in dietary patterns. Omega-3 FA levels are typically low in most western countries where fish consumption is low, and high in cultures like Japan or Korea where consumption is high [7]. Among the European Mediterranean countries, Spain has the highest fish intake [8], but their red blood cell (RBC) omega-3 levels, although high [8] relative to the United States [9] and Germany [10] (ie, 7.1% versus 5.6% and 4.7%, respectively), have not been compared across Europe. The other FA family most commonly associated with Mediterranean diets, particularly in Italy, is oleic acid (OA), which is a major component of olive oil. Since OA levels are determined by both endogenous synthesis and exogenous consumption, its levels in the blood have not been considered to be good markers of dietary OA [11], but cross-cultural analyses using the same laboratory methods are few, and the possibility that chronically elevated intakes might be reflected in membrane OA levels has not been tested.

The primary hypotheses tested here were (1) treatment with omega-3 acid ethyl esters (O3AEE) will increase the RBC EPA + DHA level (ie, the Omega-3 Index) to the proposed cardioprotective level of 8% or greater, (2) the Omega-3 Index at baseline is directly associated with reported fish intake, and 3) RBC OA levels at baseline are directly associated with reported olive oil intake. These hypotheses were tested by measuring the RBC FA composition at baseline and after 3 months in a sub-cohort of Italian patients who participated in the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico–Heart Failure (GISSI-HF) study and who were treated with 1 g/d of O3AEE (or placebo).

2. Methods and materials

2.1. Subjects

The GISSI-HF trial was a randomized, double-blind, placebo-controlled, multicenter study that enrolled 6975 patients with clinical evidence of chronic, stable HF, as previously described [12]. The trial investigated the effect of taking 1 g/day of O3AEE providing about 850 to 882 mg of EPA and DHA combined (in the average ratio of 1:1.2) or an olive oil placebo for about 4 years. In a prospectively planned biomarker substudy, blood samples were collected at randomization and 3 months later from a subset of 461 patients recruited in 51 clinical centers [13]. A brief food questionnaire was administered that included questions on the frequency of consumption of fish and olive oil [14]. The study was approved by local ethics committees, and informed consent was obtained from all patients before the study started. The trial was registered at Clinicaltrials.gov (NCT00336336).

2.2. Laboratory methods

Blood samples were collected in EDTA tubes in the fasting state. The tubes were centrifuged, an aliquot of the packed RBC fraction was transferred to a cryovial and placed in a –70°C freezer, all within 30 minutes of collection. The samples remained at temperature until thawed at room temperature for analysis. RBC FA composition was analyzed according to the HS-Omega-3 Index® methodology as modified from Harris et al [10]. FA methyl esters were generated from erythrocytes by transesterification with boron trifluoride and analyzed by gas chromatography. FAs were identified by comparison with a standard mixture of fatty acids characteristic of RBCs. Omega-3 index results are given as EPA plus DHA expressed as a percentage of 20 total FAs (Table) after calibration-curve derived response factor correction was applied to each fatty acid [15]. The average coefficient of variation for FAs of <1% prevalence was 11.3%; for those between 1% and 5% prevalence, 3.4%; and for those with >5% prevalence, 1.6%.

2.3. Statistical analyses

Categorical variables are presented as proportions and continuous variables as means (±SDs) or medians (Q1-Q3). The effect of the study drugs (O3AEE vs placebo) on 3-month changes in circulating FA in RBC was tested by analysis of variance (ANOVA). Baseline associations between reported olive oil intake and RBC OA levels, and between fish intake and RBC EPA + DHA, were evaluated by ANOVA after checking normality distribution assumptions and log-transforming any data that were not normally distributed. Although multiple hypothesis tests were carried out, a nominal 2-sided significance level of 0.05 was used, with no formal adjustment for multiple testing given the exploratory nature of the present investigation. All analyses were performed with SAS software, version 9.3 (SAS Institute, Cary, NC).

3. Results

The mean (SD) age of the 461 patients comprising the GISSI-HF subcohort was 67.1 (11.5) years, 77% were male, the mean (SD) body mass index was 26.8 (4.6) and omega-3 index was 4.7% (1.7%). The effects of treatment with O3AEE (1 g/d) or

Table – RBC Fatty acid content at baseline and 3-month follow-up.

	Placebo (n = 231)		Omega-3 FA (n = 230)		P [a]
	Baseline	3 months	Baseline	3 months	
Myristic (MA) 14:0	0.43 ± 0.17	0.45 ± 0.23	0.44 ± 0.17	0.44 ± 0.16	.26
Palmitic (PA) 16:0	23.6 ± 2.3	23.1 ± 2.4	23.4 ± 2.0	23.1 ± 2.1	.32
Trans palmitoleic 16:1n7t	0.13 ± 0.04	0.12 ± 0.03	0.13 ± 0.03	0.12 ± 0.03	.72
Palmitoleic (POA) 16:1n7	0.75 ± 0.38	0.71 ± 0.34	0.77 ± 0.40	0.67 ± 0.33	.03
Stearic (SA) 18:0	16.3 ± 2.1	16.4 ± 1.9	16.2 ± 2.0	16.4 ± 1.8	.90
Trans oleic C18:1 t	0.40 ± 0.11	0.37 ± 0.10	0.40 ± 0.11	0.38 ± 0.11	.98
Oleic (OA) 18:1n9	18.9 ± 2.7	18.9 ± 2.8	18.9 ± 2.5	18.4 ± 2.3	.05
Trans linoleic 18:2n6t	0.13 ± 0.04	0.13 ± 0.04	0.13 ± 0.04	0.13 ± 0.04	.25
Linoleic (LA) 18:2n6	11.8 ± 2.7	11.5 ± 2.7	11.8 ± 2.7	11.1 ± 2.4	.40
Gamma-linolenic (GLA) 18:3n6	0.14 ± 0.06	0.14 ± 0.06	0.15 ± 0.06	0.12 ± 0.04	.0005
Eicosenoic (EEA) 20:1n9	0.30 ± 0.07	0.30 ± 0.07	0.29 ± 0.06	0.28 ± 0.06	.005
Alpha-linolenic (ALA) 18:3n3	0.10 ± 0.07	0.09 ± 0.06	0.10 ± 0.07	0.09 ± 0.05	.87
Eicosadienoic (EDA) 20:2n6	0.28 ± 0.06	0.27 ± 0.05	0.28 ± 0.06	0.26 ± 0.06	.06
Dihomo-γ-linolenic (DGLA) 20:3n6	1.74 ± 0.39	1.71 ± 0.37	1.83 ± 0.39	1.60 ± 0.31	<.0001
Arachidonic (ARA) 20:4n6	13.8 ± 2.7	14.3 ± 2.6	13.9 ± 2.7	13.5 ± 2.1	.02
Eicosapentaenoic (EPA) 20:5n3	0.54 ± 0.56	0.51 ± 0.38	0.55 ± 0.38	1.24 ± 0.58	<.0001
Docosatetraenoic (DTA) 22:4n6	2.59 ± 0.87	2.72 ± 0.79	2.60 ± 0.91	2.24 ± 0.66	<.0001
Docosapentaenoic 22:5n6	0.58 ± 0.20	0.62 ± 0.19	0.59 ± 0.19	0.49 ± 0.14	<.0001
Docosapentaenoic (DPAn3) 22:5n3	1.59 ± 0.54	1.63 ± 0.51	1.61 ± 0.46	2.10 ± 0.54	<.0001
Docosahexaenoic (DHA) 22:6n3	4.19 ± 1.36	4.30 ± 1.23	4.20 ± 1.41	5.49 ± 1.46	<.0001
Omega-3 Index (EPA + DHA)	4.73 ± 1.70	4.81 ± 1.49	4.75 ± 1.68	6.73 ± 1.93	<.0001

[a] Relative changes compared between randomized treatments (t test). Values are percentages of total fatty acids, means ± SDs.

placebo for 3 months on RBC FA composition are shown in Table. O3AEE increased the omega-3 index by 2 percentage points (a 42% increase), and levels of DPAn-3 by 30%. Treatment also lowered levels of all long chain omega-6 FAs downstream of linoleic acid including gamma-linolenic (–20%), docosapentaenoic n-6 (–17%), docosatetraenoic (–14%), and dihomo-gamma linolenic (–13%) acids (all $P < .0001$). The major long-chain omega-6 fatty acid, arachidonic acid, decreased by only 3% ($P = .02$). The major saturated and monounsaturated FAs were not affected. More patients reached the proposed target omega-3 index level of 8%-12% in the treated vs placebo group (22.6% vs 1.3%, $P < .0001$). The same was true for other potential cut-points, eg, 7% (48% vs 7%) or 6% (66% vs 14%, both $P < .001$).

The reported intake categories of olive oil in the GISSI-HF cohort were reflected in higher RBC OA levels (Figure top); similarly, the reported categories of intake for fish were directly associated with the omega-3 index (P for trend <.001 both; Figure bottom). RBC OA ranged from 16.1 ± 2.0% in the "never consume olive oil" group to 19.3 ± 2.5% in the "regular" consumers. Across the span of reported fish intake, the omega-3 index was 4.00 ± 1.54% for the lowest intake and 6.04 ± 2.17% in the highest.

4. Discussion

The purposes of this study were to estimate the effects of 1 capsule of O3AEE per day on RBC FA composition in the GISSI-HF study, and to determine the extent to which RBC omega-3 and OA levels correlated with reported fish and olive oil intakes, respectively. Based on our findings, we reject the hypothesis that 3 months of treatment would result in a mean omega-3 index of ≥8%, but accept the hypotheses that the omega-3 index and RBC OA levels would be directly correlated with reported intakes of fish and olive oil, respectively.

Baseline levels of the omega-3 index in this cohort were about 4.7%. As alluded to in the Introduction, omega-3 index levels appear to vary by culture. For example, the average omega-3 index (determined using the same method as here) was 4.7% in both the Women's Health Initiative Memory Study [16] and in a recent Canadian government survey [17], 5.5% in Framingham [9], and 5.3% in a clinical lab database of about 160 000 Americans [18]. At the extremes are Alaska Natives [19] at 8.9% and healthy Koreans [20] at 9.1% versus US vegans [21] and deployed Army personnel [22], both at 3.7%. Although not formally tested, there is a clear suggestion that dietary habits are important in determining the omega-3 index.

O3AEE treatment predictably raised RBC EPA and DHA (and DPAn-3) levels, but the dose used in the GISSI-HF study (and in several other large omega-3 randomized controlled trials [23-26]) only increased the mean omega-3 index by 1.9 percentage points (from 4.8% to 6.7%) after 3 months of supplementation. Less than 25% of those randomized to O3AEE achieved the proposed target omega-3 index value of 8% [15,27]. Since a new RBC EPA + DHA steady state requires at least 4 months of supplementation [28,29], the final levels achieved in the full 4-year GISSI-HF trial can be assumed to have been higher than that observed here, but how many patients would have achieved an omega-3 index in the 8%-12% range is unknown.

We are aware of only four other studies in which the approximate dose of EPA + DHA given here was used, and the effects on the omega-3 index were reported. Two lasted for five months; in one (n = 9, 1000 mg/d), the index increased by 5.2 units (from 4.7% to 9.9%) [15], whereas in the other (n = 24, 900 mg/d), it increased by 3.2 units (4.3% to 7.5%) [30]. Another

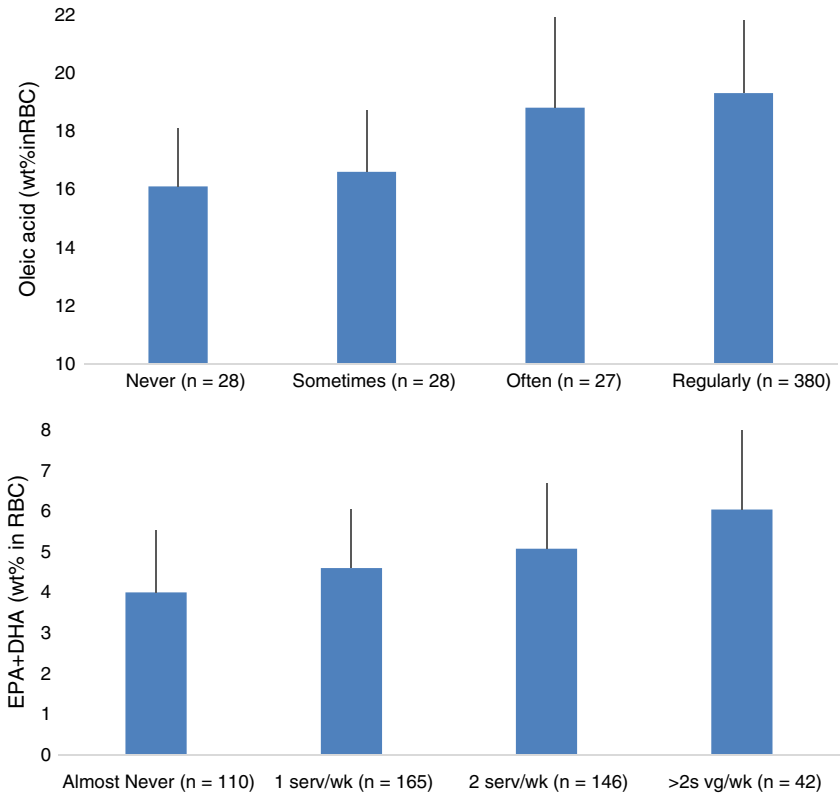

Figure – Association between the reported frequency of olive oil intake and RBC oleic acid levels (top), and fish intake and RBC EPA + DHA (bottom) (means and SDs). Values are means + SD. *P* values from ANOVA (overall comparison); *P* < .0001 for both. Top, *P* < .002 for comparisons of regularly versus never and sometimes. Bottom, *P* < .008 for comparisons of >2 servings per week vs all other categories.

lasted for 4 months and included 157 subjects (all already taking some kind of omega-3 supplement); each was given an additional 984 mg of EPA + DHA per day [31]. Here, the omega-3 index only increased by 1.2 units (6.1% to 7.3%). The fourth study lasted only 2 months but used the same dose and product as was used in the GISSI-HF study [32]. In the 26 patients tested, the index rose only 1.5 units (5% to 6.5%). Hence, the 1.9-unit rise observed in the current study is roughly in line with previous experience, with the obvious exception of the 5.2-unit rise noted above. The oil used in that study and in two of the others [30,31] was a triglyceride-based product, whereas in Skulas-Ray et al [32] and the GISSI-HF study ethyl ester-based products were used. Unfortunately, it was the 2004 study in which 1 g/d produced an omega-3 index of 9.9% [15] that was used in the original estimation of the cardioprotective target for the omega-3 index. Had data been available from these 3 additional studies and included in the estimate of what omega-3 index likely characterized the GISSI-Prevenzione [24] and the Diet And Reinfarction Trial [33] patients, then the proposed target omega-3 index would have been lower, approximately 7%. A target omega-3 index of 7–8% would have been consistent with subsequent findings linking similar levels with reduced risk for acute coronary syndromes [34], slowed rates of telomere attrition [35], and (using plasma phospholipid EPA + DHA to predict the index) a lower risk for total mortality [36]. Further data are needed to better define optimal omega-3 index values for reducing risk for cardiovascular disease (and possibly for neurocognitive/behavioral disorders [37]).

Whether the mean achieved omega-3 index of 6.7% after 3 months of supplementation in this study afforded the best *milieu* for cardiovascular risk reduction remains unclear. It if was maintained throughout the trial, it clearly provided some benefit since risk for death from any cause after 4 years was reduced by 9% [12]. More globally, whether one of the potential reasons [38-40] for the null outcomes of some of the more recent omega-3 cardiovascular disease (CVD) trials using this dose of EPA + DHA could be their failure to achieve truly optimal omega-3 levels cannot be determined at this point. Two current RCTs testing the effects of 4 g of EPA with or without DHA - The Reduction of Cardiovascular Events with EPA Intervention Trial (REDUCE-IT, NCT01492361) and the Statin Residual Risk Reduction with Epanova in High Cardiovascular Risk Patients with Hypertriglyceridemia (STRENGTH, NCT02104817), will address the dose question more directly. Both studies include hyperlipidemic patients on statin therapy, and CVD events are the primary endpoints. The former is testing the effects of 4 g/d of EPA ethyl esters in about 8000 patients, whereas the latter is testing the effects of the same dose of EPA + DHA given as free fatty acids in about 13 000 patients. Both are event-driven studies with results expected between 2017 and 2019. The omega-3 index has not been reported either at baseline or after treatment with omega-3 fatty acids in any of the major prospective

randomized clinical trials (RCTs) with CVD endpoints, hence it is difficult to know what levels were finally achieved, and moreover the extent to which the achieved omega-3 index values correlated with rates of CVD events. Again, further research on this point is sorely needed (and may be provided by the STRENGTH trial since the omega-3 index is being tested at baseline and one year).

The categories of reported fish intake were directly related to the omega-3 index, as has been observed in several other studies [8,9,41-43]. A correlation between the reported intake of olive oil and RBC OA has rarely been examined, however. Another study using a similar RBC FA methodology to the one used here reported that OA levels were 17.6% in a Spanish cohort (also a high-olive-oil-consuming culture), a value closer to the 18.9% observed here than the 13.9% in the Framingham Offspring study [8]. In the GISSI-HF cohort, 82% reported "regular" (as opposed to "often," "sometimes," or "never") use of olive oil. While a similar survey was not taken by the US cohort, according to an olive oil trade group, the annual per capita consumption of olive oil in Italy is 12.5 L compared to 1 L in the US [44]. At about 70% OA, this would translate into olive oil-provided OA intakes of about 23 g vs 2 g per person per day. Within Europe, the Italians are second only to the Spanish in per capita OA consumption (14% vs 18% energy) [45,46] and both are higher than US intakes (12%) [47]. Thus, the observed difference in RBC OA between the Italian and US cohorts is consistent with the differences in population olive oil intakes, recognizing, of course, that OA also comes from many other foods and from endogenous synthesis.

This study was limited by relatively short duration of exposure to O3AAE before blood sampling, the lack of compliance data at 3 months, and the lack of detail regarding fish and olive oil intakes from in the simple diet questionnaires. Its strengths include being the only study to date to determine the effects of 1 g of O3AEEs on the omega-3 index in the context of a large, prospective RCT with fish oils. The collection of such data (both baseline and end of treatment) has recently been recommended by a workshop on how to improve the design of future RCTs with fish oil [48].

In conclusion, we have shown that the intakes of OA and of omega-3 fatty acids (using the surrogates of olive oil and fish consumption, respectively) were directly related to RBC OA and EPA + DHA levels. In addition, 3 months of supplementation with 1 g of O3AEE increased the omega-3 index by about 2 units in this cohort. Since such treatment (over 4 years) reduced total mortality [12], clearly a degree of protection was afforded, but whether higher doses (and thus higher blood omega-3 levels) might have provided further benefit cannot be known. The ongoing, higher-dose studies will provide important further data on that question.

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