PAPER

BLOOD N-3 FATTY ACIDS INCREASE AFTER CONSUMPTION OF AN ENRICHED YOGHURT

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ABSTRACT

The effects of a n-3 fatty acid (FA) enriched yoghurt on blood FA were investigated in 23 females and 23 males, who consumed 2 yoghurts/day for 6 weeks, providing 64 mg EPA and 74 mg DHA. Blood samples were collected before treatment, at the beginning, end, and after wash out. Yoghurt intakes increased blood EPA (35%) and DHA (11%) with different trends of changes in women versus men. In conclusion, the daily consumption of a yoghurt containing small amounts of n-3 FA significantly increases blood n-3 FA, providing a useful approach to reach the recommended intakes in populations.

- Key words: FA incorporated in yoghurt, gender differences, omega-3 -

INTRODUCTION

There is a vast amount of literature supporting the favourable impact of n-3 long chain polyunsaturated fatty acids (LC PUFA) in the prevention and treatment of chronic diseases, especially of the cardiovascular (CV) system (YASHODHARA *et al.,* 2009; GALLI and RISE', 2009). Studies on the effects of the administration of n-3 fatty acid (FA) preparations, mainly in the form of capsules, are generally based on doses in the order of at least 1 g/day, and the low doses mentioned in some investigations refer to at least 600 mg/day (BAYS, 2007). The nutritional features of these compounds, in relation to human physiology at different ages, the patho-physiological processes at the basis of chronic diseases, and the changes in dietary habits (individual and age-related), clearly indicate that the intake of essential and bioactive nutrients, such as n-3 LC PUFA, should be maintained throughout one's entire life (FLEITH and CLANDININ, 2005; CALDER, 2007). Since these FA are rather precious and are present in very small amounts in most diets (HIBBELN *et al.,* 2006), formulations that optimize their bioavailability and facilitate strategies aimed at maintaining optimal intakes should be developed. The ingestion of n-3 FA as components of foods rather than formulated preparations allows greater bioavailability (VISIOLI *et al.,* 2003; ELVEVOLL *et al.,* 2006; GALLI and MARANGONI, 2006). This type of approach has been used, e.g. through the intake of milk enriched with n-3 FA (VISIOLI *et al.,* 2000), giving significant increments of the circulating levels of these FA, even with relatively low intake. Milk-derived products such as yoghurt, a rather popular food item in most countries, can also represent a potential form of administration of n-3 LC PUFA, contributing to optimizing the intake of these compounds, especially in the case of subjects who do not generally consume fish or may not appreciate this type of food.

HLAVATY *et al.* (2008) reported increments of circulating n-3 FA after 3 weeks of consumption of a yoghurt providing about 600 mg/day of n-3 LC PUFA; these amounts were lower than those used by DAWCZYNSKI *et al.* (2009), i.e. 1.2 g/day of n-3 LC PUFA, a dose with favourable effects on the cardiovascular system and on rheumatoid arthritis. However, the consumption of the yoghurt containing 600 mg/day of n-3 LC PUFA (HLAVATY *et al.,* 2008), in addition to increasing the proportion of n-3 FA in serum lipids, prevented unfavourable changes in FA composition of serum lipid classes in obese females under a low caloric diet.

On the basis of the above considerations we planned to specifically investigate the effects of a newly designed yoghurt preparation with low amounts of n-3 FA, i.e. 184 mg/day, lower than those used by HLAVATY *et al.* (2008), on the FA profile in whole blood of volunteers. This study was conducted for six weeks, a time period that is followed in most nutritional interventions.

MATERIALS AND METHODS

Subjects and study design

Subjects (46) were recruited by the Department of Experimental Medicine, University of Parma, from people living in the Province of Parma. The informed consent was obtained from all volunteers participating in the study.

At the beginning of the study, serum total cholesterol and triglycerides were assessed by enzymatic methods using commercially available kits.

At T0 the subjects began to consume 2 yoghurts/day, providing 184 mg/day of total n-3 FA, of which 64 mg were eicosapentaenoic acid (20:5 n-3, EPA) and 74 mg were docosahexaenoic acid (22:6 n-3, DHA). This regimen was maintained for 6 weeks (T6) and then followed by 4 weeks of washout, for a total duration of 10 weeks. Since the study was aimed exclusively at assessing the changes in blood FA profiles during the consumption of a LC n-3 FA-enriched yoghurt, a control group consuming a preparation without these FA was not included.

Blood samples for FA analysis were collected at the following times: 2 weeks before initiation of treatment (run-in period, i.e. T -2), at the beginning of treatment (T0), at the end of 6 weeks of treatment (T6) and after 4 weeks of wash-out (T10). During the run-in period, recommendations were given to the subjects to avoid foods rich in linoleic acid (LA, 18:2 n-6), namely seed oils with high LA levels, e.g. corn, soy bean, sunflower, and especially foods rich in n-3 FA, such as walnuts and spinach containing ALA (alpha linolenic acid, 18:3 n-3), and fish providing EPA and DHA.

The yoghurt used in the study (yoghurt Omega 3 Plus® produced by Parmalat, Parma, Italy) consisted of a 115 g preparation/dose, containing (g/100 g) 1.6 g fat, 60 mg EPA + DHA, 0.1 g dietary fiber, 4 mg Vitamin E, 0.4 μg Vitamin B12 and 80 μg folate. The detailed FA composition of the yoghurt is presented in Table 1. The enriched yoghurt was obtained by adding 0.25% of fish oil (ROPUFA ® '30') that contained n-3 FA as triglycerides.

Whole blood FA analysis

Whole blood drops, obtained from a fingertip, were collected on a special adsorbent paper embedded with the antioxidant butyl hydroxyl toluene both contained in a specific kit (Sigma-Aldrich, St. Louis, MO). The FA analysis of whole blood was then carried out as previously described (MARANGONI *et al.,* 2004). FA methyl esters (FAME) were directly prepared by transes-

Table 1 - Percentage levels of major fatty acids in the yoghurt enriched with omega 3.

FA	% *(w/w)*
C4:0	3.38
C6:0	2.25
C8:0	1.20
C10:0	2.75
C12:0	3.23
C14:0	11.35
C14:1	0.94
C15:0	1.08
C16:0	32.20
C16:1	2.29
C17:1	0.19
C18:0	8.70
C18:1 n-9	16.48
C18:1 n-7	0.96
C18:1 n-5	0.26
C18:2 n-6	1.21
C18:2 *trans*	0.20
CLA	0.29
C18:3 n-3	0.59
C18:4 n-3	0.32
C20:0	0.15
C20:1 n-9	0.50
C20:4 n-6	0.14
C20:4 n-3	0.26
C20:5 n-3	1.91
C22:5 n-3	0.32
C22:6 n-3	2.22

Values are the average of data obtained on several samples. FA, fatty acids; CLA, conjugated linoleic acid.

terification with 600 μL of 3N HCl/methanol and maintained in a dry bath at 90°C for 1 h. Then, after extraction with a KCl solution (2 mL) and hexane (400 μL), FAME were analysed by gas liquid chromatography (Shimadzu FAST GC 2010), equipped with a PTV injector (220°C) and a FID detector (270°C). An Agilent DB-FFAB capillary column (15 m length, 0.10 mm I.D., 0.10 mm film thickness) was used with helium as carrier gas. Oven temperature was programmed from 150° to 248°C. FA from 16 to 24 carbon atoms were identified by the use of pure reference compounds, and were quantified as relative percentages of total FA.

Statistical analysis

It was calculated that the sample size, established also on the basis of previous data (VISIOLI *et al.*, 2003), and allowing a type I error (α) level of 0.05 and a power of the test of 90% with the expected SD for EPA and DHA (30% of mean levels), would allow a change of 10% in variation with the same power to be detected. Data analysis was performed with the statistical software SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Results are expressed as mean±SD; one-way ANOVA univariate between groups and Tukey post-hoc for multiple comparisons were applied and a value of $p<0.05$ was considered significant. The significance of differences for FA analyses between males and females, at different times, was assessed using the unpaired Student T-test.

RESULTS

The characteristics of the subjects are shown in Table 2. The average ages of men and women were rather similar and ranged from 40 to 50 years. Anthropometric data reflected average values of the population in the area, also including a certain percentage of smokers (> 5 cigarettes/day). A rather small percentage of subjects were moderately hypercholesterolemics (average value of total cholesterol 250±22 mg/dL) and the proportion of hypertriglyceridemics (average value of triglycerides 360±170 mg/dL) was even lower. A certain percentage of subjects occasionally took some drugs (antibiotics, anti-inflammatory), which however do not affect lipid metabolism.

The FA profiles of whole blood lipids, expressed as percentage values of total FA, at the different time periods are reported in Table 3. At the various time points, there was a significant difference in the trend of changes, especially for EPA, docosapentaenoic acid (DPA) and DHA. This is reflected in changes of SFA, PUFA and of the unsaturation index (U.I., it is the sum of the percentage of each FA x its number of double bonds) over time. Also total n-6 and n-3 PUFA, n-6 and n-3 HUFA were significantly different at the different time points.

In particular, there were significant increments in the n-3 LC PUFA, while the precursor ALA, present in very small amounts in the yoghurt, decreased at T6 versus T0. In fact, there was an increase of EPA from 0.40 to 0.54% (35%) between T0 and T6, followed by a decline from T6 and T10 (13%), although the final value (0.47%) remained higher than that at T0. As for DHA, the increments followed a different

Table 2 - Characteristics of the subjects.

	All n=46	Females n=23	Males n=23
Age (y)	43.2±7.0	42,2±6.7	44.3±7.3
Weight (kg)	73.3±14.8	64.7±11.4	82.0±12.8
Height (cm)	170.8±8.7	164.7±5.3	177.0±6.8
BMI	25.0±4.1	24.0±4.7	26.1±3.2
% Smokers	13	13	13
% Hyperchol	15	17	13
% Hypertrigl	6	9	4
% Drugs	24	30	17

BMI, body mass index (the ratio between the weight and the squared height in meters).

Table 3 - Whole blood fatty acid profiles of healthy subjects (n=46), consuming a yoghurt rich in n-3 fatty acids.

% FA *(w/w)*	T-2	TO	T6	T10
16:0	25.72±1.51	25.49±1.60	25.91±1.86	24.95±1.61§
18:0	11.46±0.88	11.25±0.85	11.28±0.98	10.91±0.75§
16:1	1.38±0.66	1.44±0.65	1.48±0.67	1.39±0.56
18:1 n-9	20.32±2.45	20.61±2.98	19.88±3.95	20.29±2.66
20:3 n-9	0.13±0.03	0.13±0.04	0.13±0.04	0.13±0.04
18:2 n-6	18.24±1.63	18.55±2.07	18.26±2.17	18.96±2.00
18:3 n-6	0.20±0.07	0.23±0.08	0.26±0.11	0.24±0.09
20:3 n-6	1.38±0.26	1.38±0.30	1.46±0.30	1.45±0.27
20:4 n-6	7.46±1.19	7.67±1.25	7.91±1.36	8.24±1.05
22:4 n-6	0.96±0.23	0.96±0.22	1.00±0.25	1.02±0.20
22:5 n-6	0.21±0.07	0.21±0.06	0.22±0.06	0.23±0.06
18:3 n-3	0.29±0.19	0.29±0.09	0.24±0.06	0.28±0.11
20:5 n-3	0.43±0.14	0.40±0.11	0.54±0.20	0.47±0.17#
22:5 n-3	0.62±0.13	0.63±0.13	0.70±0.15	0.71±0.14#
22:6 n-3	1.78±0.45	1.76±0.40	1.95±0.50	2.03±0.59§
SFA	41.60±1.53	40.96±1.71	41.21±1.90	39.77±1.75#
MUFA	26.68±2.44	26.83±3.01	26.05±3.75	26.40±2.65
PUPA	31.57±2.21	31.99±2.68	32.57±3.28	33.67±2.31#
U.I.	119.90±5.84	121.29±5.93	123.48±7.11	126.87±5.57#
n-6	28.46±2.05	29.00±2.69	29.10±2.85	30.15±1.93§
n-3	3.12±0.62	3.07±0.57	3.43±0.75	3.48±0.80#
n-3 HUFA	2.84±0.65	2.79±0.57	3.19± 10.76	3.20±0.81#
n-6 HUFA	10.02±1.45	10.23±1.55	10.59±1.70	10.94±1.26§

T-2 is the start of run in period; after 2 weeks the start of treatment is T0; T6 (after 6 weeks) the end of treatment and T10 the end of wash out (a 4 week period). During the treatment, volunteers consumed 2 yoghurt/day that provided 202 mg/day of n-3 FA. Values are mean ±SD; one-way ANOVA univariate between groups (different time periods) is applied and the significant differences are §: $p<0.05$; #: $p<0.01$.
FA, fatty acids, SPA, saturated FA; MUFA, monounsaturated FA, PUPA, polyunsaturated FA; U.I. unsaturation index; HUFA, highly unsaturated FA.

trend and were of lower magnitude: from T0 to T6 there was an 11% increment, but the value was further increased after wash-out (15% versus T0). As for the changes in DPA, the intermediate compound in the conversion of EPA to DHA, there was an increment especially during consumption of the yoghurt, of the same magnitude as that of DHA (11%), and the increment was substantially maintained after wash-out (12.6%).

A further type of evaluation concerns differences between females and males in the response to the consumption of the yoghurt. At T0 the EPA and DHA levels were similar in the 2 groups with a tendency to be higher in females than in males. By comparing the changes of EPA and DHA levels from T0 to T10 it appears that increments of both DHA and EPA were greater in women than in men (Fig. 1). In fact, EPA increments in women, over values at T0 were 49% at T6 and 24% at T10, and those of DHA were 16% at both T6 and T10, while in men the changes for EPA were 21 and 13% and for DHA were 5 and 17%, respectively. In addition, increments of DHA in women took place mainly in the T0 to T6 time period, and remained constant during wash out, while in men increments occurred mainly during wash-out.

DISCUSSION

Increments in the consumption of n-3 LC PUFA are presently widely accepted as part of an effective strategy for optimizing physiological processes and preventing major diseases (HOOPER *et al.,* 2004). The relationship between intake of fish or fish oil and relative risk of death from cardiac heart disease shows that the modest consumption of 250 mg/day EPA and DHA appears sufficient for primary prevention (MOZAFFARIAN and RIMM, 2006). A contribution to this strategy may be provided by the use of food items containing these compounds or food items enriched with n-3 FA, and generally consumed by large population groups.

Among natural foods, milk is characterized by highly dispersed features such as fat globules (ranging from 0.1 to 10 μm in diameter), a condition that favours the digestion and intestinal absorption of fats and FA. These characteristics are retained in yoghurt, a type of milk-derived product that is widely consumed.

The intake of 2x115 g of partially defatted yoghurt (1.6% fat *versus* 3.2% in a standard yoghurt) fortified with omega 3 and providing only 64 mg EPA and 74 mg DHA/day resulted in significant increments of both FA in blood lipids

after 6 weeks. The increments were statistically significant and especially concerned EPA, as predictable on the basis of lower starting values of this FA, that is also mainly incorporated into metabolically less stable lipid pools (RISE' *et al.,* 2007). This last consideration partly explains the decline of EPA during the wash-out, in agreement with the trend towards a reduction of the FA during the run-in period. Reduction of blood EPA levels during the wash-out period was also observed in a previous study (GALLI and MARANGONI, 2006). Conversion of the supplemented EPA to its derivatives, supported also by the increments of DPA and especially of DHA even during wash-out, may contribute to the reduction of EPA after its intake is interrupted. We observed a similar trend, i.e. increments of blood DHA levels at the end of wash-out following a 3 weeks intake of LC n-3 PUFA through either fish consumption or ingestion of encapsulated preparations in male subjects and this increment was greater when the FA were ingested through food (GALLI and MARANGONI, 2006).

HLAVATY *et al.* (2008) showed combined increments of EPA + DHA of about 60% from basal values, after the daily intakes of an n-3 FA (620 mg/day) enriched yoghurt. Interestingly in our study, the increment of 15% in these FA in blood was obtained with a dose of about 25% (138 mg/day) of that in the study by HLAVATY *et al.* (2008). On the other hand, the basal values of DHA, which in a previous comparative assessment in plasma versus whole blood lipids were very similar (RISE' *et al.,* 2007), were significantly higher in our study than in the Czech population, possibly reflecting the low intake of fish in this population, as mentioned in the article (HLAVATY *et al.,* 2008).

Another interesting finding concerns the different patterns of EPA and DHA changes, i.e., greater in women than in men. In addition, during washout EPA declined in women more than in men while DHA remained stable in women and increased in men, although remaining lower than in women. Higher circulating levels of DHA associated with lower levels of the precursor DPA, suggesting more efficient conversion to the end product, have been previously reported in pre-menopausal women versus men (MARANGONI *et al.,* 2007). These differences have been attributed to estrogenic effects (GILTAY *et al.,* 2004) and greater efficiency in the conversion of ALA to DHA has been described using stable isotope techniques (BURDGE and WOOTTON, 2002). In addition gender-related differences have been described in the kinetics of various "exogenous" compounds, such as drugs (bioavailability, distribution, metabolism and elimination) (GHANDI *et al.,* 2004). The gender differences in blood increments with the same doses of n-3 FA should, however, be more carefully explored, although they were recently described (METHEREL *et al.,* 2009), also in term of functional responses to the different increments, in order to optimize the supplementation of these compounds in women versus men.

Our data show that the consumption, for a relatively short duration, of a low fat yoghurt containing small amounts of n-3 FA, compared to the generally administered doses in most supplementation studies, resulted in appreciable increments in blood levels of these compounds, thus providing a useful approach to reach the recommended intake in large population groups consuming this type of food item.

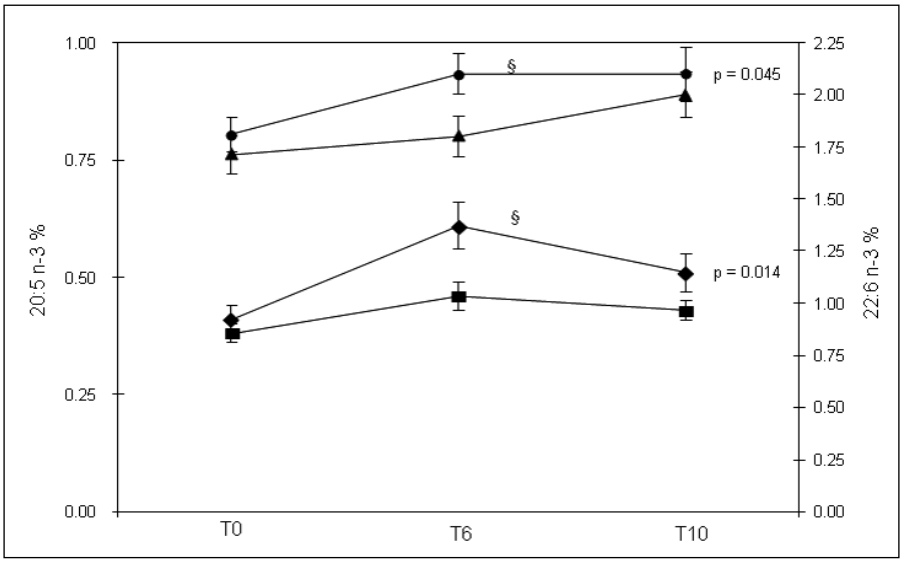

Fig. 1 - 20:5 n-3 and 22:6n-3 levels in whole blood of male and female subjects.
Values represent the mean±SEM; -■- 20:5 n-3 male; -◆- 20:5 n-3 female; -▲- 22:6 n-3 male; -●- 22:6 n-3 female.
One-way ANOVA univariate between groups (p values) was applied; differences between males and females, at different times, were assessed using the unpaired Student T-test. The differences were significant for §: $p<0.05$.

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