

Background diet influences the anti-inflammatory effect of α -linolenic acid in dyslipidaemic subjects

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(Received 15 December 2003 – Revised 18 May 2004 – Accepted 2 June 2004)

Long-chain *n*-3 PUFA from fish oils are known to have anti-inflammatory effects. We evaluated the effect of α -linolenic acid (ALA), precursor of *n*-3 fatty acids, on serum inflammatory markers and soluble cellular adhesion molecules (sCAM) of dyslipidaemic males, relative to their background diet. Participants were assigned to two groups, based upon food intake patterns: (a) twenty-one dyslipidaemic subjects who habitually ate a Mediterranean–Cretan-type diet; (b) nineteen dyslipidaemic subjects who normally ate a Westernised Greek diet. All were supplemented with 8.1 g ALA/d for 12 weeks. We determined serum amyloid A (SAA), C-reactive protein (CRP), macrophage colony-stimulating factor (MCSF), IL-6, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 and soluble E-selectin concentrations at the beginning and the end of the ALA supplementation period. Serum baseline concentrations of inflammatory markers and sCAM were similar across the diet groups. Type of diet had a significant impact on the response of inflammatory markers to ALA supplementation. The Westernised Greek diet group showed a reduction in SAA ($P < 0.001$), CRP ($P = 0.002$), MCSF ($P = 0.005$) and IL-6 ($P = 0.04$) concentrations. The Mediterranean–Cretan-type background diet group showed a significant reduction only in MCSF concentrations ($P = 0.003$). The sVCAM-1 concentrations were significantly reduced in both the Westernised Greek diet group ($P = 0.001$) and the Mediterranean–Cretan-type diet group ($P < 0.001$). The present study demonstrated that ALA supplementation lowered the serum concentrations of inflammatory markers more profoundly when the background diet was rich in saturated fatty acids and poor in MUFA.

α -Linolenic acid: Mediterranean diet: Inflammatory markers: Cellular adhesion molecules

Consumption of diets rich in *n*-3 fatty acids has been linked with low prevalence of atherosclerosis (Ascherio *et al.* 1995). Furthermore, *n*-3 fatty acids have been used to treat various chronic inflammatory conditions and have been shown to have anti-inflammatory properties (Calder, 2001). Immune system cells are inherent parts of inflammatory events involved in the development and progression of atherosclerosis. The adhesion of circulating blood monocytes to the endothelial cells and their subsequent transmigration across the vascular endothelium are prerequisite steps in the pathogenesis of the disease (Ross, 1999). It has been proposed that the anti-inflammatory properties of *n*-3 fatty acids can explain the correlation with the low prevalence of atherosclerosis (Calder, 2001). However, other human studies do not confirm the anti-inflammatory effect of *n*-3 PUFA.

Previous studies have shown variable results when reporting effects of *n*-3 PUFA on cytokine production by

mononuclear cells. Dietary supplementation studies with EPA and docosahexaenoic acid (DHA) have shown a decrease in monocyte synthesis of TNF- α , IL-1 β and IL-6 in healthy subjects (Endres *et al.* 1989; Meydani *et al.* 1991; Caughey *et al.* 1996). In contrast, other studies documented a lack of effect of *n*-3 PUFA on the synthesis of the same cytokines (Schmidt *et al.* 1996; Blok *et al.* 1997; Yaqoob *et al.* 2000). The effects of α -linolenic acid (ALA), the precursor of EPA and DHA, on inflammatory markers are also controversial. In particular, on the one hand, it has been reported that the production of TNF- α and IL-1 β was suppressed by lipopolysaccharide-stimulated monocytes of healthy volunteers after a high dose of dietary ALA (Caughey *et al.* 1996). In addition, in our previous report (Rallidis *et al.* 2003), supplementation with ALA reduced serum IL-6 and C-reactive protein (CRP) concentrations. On the other hand, other research has demonstrated that ALA did not affect the

Abbreviations: ALA, α -linolenic acid; CRP, C-reactive protein; DHA, docosahexaenoic acid; MCSF, macrophage colony-stimulating factor; SAA, serum amyloid A; sCAM, soluble cellular adhesion molecules.

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production of TNF- α , IL-1 β , IL-6, IL-2 or interferon- γ by mononuclear cells (Thies *et al.* 2001). Recent evidence has documented that intake levels of ≤ 9.5 g ALA/d or ≤ 1.7 g EPA+DHA/d did not alter the production of cytokines by monocytes and lymphocytes although the fatty acid composition of mononuclear cells was significantly altered (Kew *et al.* 2003).

A number of *in vitro* studies suggest a suppressive effect of *n-3* fatty acids on adhesion molecule expression by vascular endothelial cells (De Caterina *et al.* 1994; Weber *et al.* 1995; Khalfoun *et al.* 1996; Collie-Duguid & Wahle, 1996). Results from human supplementation studies, however, are inconsistent. Some have shown that supplementation with EPA+DHA reduced the plasma concentration of soluble forms of adhesion molecules (Abe *et al.* 1998; Thies *et al.* 2001), while other studies report the opposite (Seljeflot *et al.* 1998; Johansen *et al.* 1999).

Results of fish oil experiments on plasma CRP concentrations also are inconsistent. Although a significant inverse correlation between serum CRP concentrations and content of DHA in granulocytes has been demonstrated (Madsen *et al.* 2001), a recent study by the same research group failed to reproduce this finding (Madsen *et al.* 2003). Similarly, the administration of *n-3* PUFA to healthy individuals did not alter plasma CRP levels (Vega-Lopez *et al.* 2004).

Evidence since the 1950s has demonstrated that Mediterranean countries display rates of chronic diseases that are among the lowest in the world with corresponding life expectancies that are among the highest (Nestle, 1995). In a recent study conducted in the Greek population, the adoption of the Mediterranean diet by subjects with the metabolic syndrome was associated with a significant reduction of the risk of developing acute coronary events (Pitsavos *et al.* 2003).

Our objective was to examine the effect of dietary ALA on inflammatory markers and soluble cellular adhesion molecules (sCAM) related to atherosclerosis with regard to a dietary variable. For this purpose, volunteers were recruited who followed either a Mediterranean–Cretan-type diet or a Westernised Greek diet.

Subjects and methods

Subjects

Subjects were recruited from the treadmill test unit in the Department of Cardiology of Laiko Hospital after screening by medical history, physical examination, electrocardiograph, and laboratory analysis. Fifty male volunteers aged 35 to 67 years, first diagnosed for dyslipidaemia, without evidence of CHD, were recruited. Entry criteria for recruitment included a total cholesterol concentration higher than 5.20 mmol/l, and/or an HDL-cholesterol concentration lower than 1.03 mmol/l. Subjects with evidence of infection or coexistent diabetes mellitus, hypertension, renal, liver or inflammatory disease were excluded. In addition, subjects taking lipid-lowering drugs, habitually consuming more than thirty units alcohol per week, smoking ten or more cigarettes per d, or habitually vigorously exercising at the level of >6 h/week were excluded. Of the fifty men enrolled, one withdrew before completion of the study because of gastrointestinal discomfort.

An additional nine subjects either withdrew or were excluded for non-compliance during the 12-week intervention. Thus, forty subjects completed the entire study.

Study design

A single intervention of parallel design was carried out. Dietary assessment was performed before the intervention to allocate volunteers according to their background diet into two groups. The first group was a group of subjects habitually following a diet characteristic of the so-called Mediterranean–Cretan diet as described in the Seven Countries Study (Kafatos *et al.* 2000) (n 21); the second group of subjects habitually followed a Westernised Greek diet (n 19).

All subjects were supplemented with 15 ml flaxseed oil/d for 12 weeks. Supplements contained approximately 8 g ALA (*n-3*) and were taken three times daily, one 5 ml teaspoon/meal. The subjects were asked to maintain their dietary habits and usual lifestyle and were instructed to avoid the intake of anti-inflammatory drugs, vitamins or other dietary supplements throughout the intervention period. The subjects were supervised in respect of their dietary habits, alcohol consumption and physical activity habits by phone calls once per week and by visits to the hospital. The subjects were weighed monthly. At the time of the patient visits, they were provided with the oil supplements. Heights and weights were measured with the subjects in light clothing and shoes removed. Flaxseed-oil supplements were provided by Savant International (Savant Distribution Ltd, Leeds, UK). Flaxseed oil was extracted by cold pressing under N₂. It was stored in brown bottles at 4°C. No additional antioxidants were added. Fatty acid composition of the oil is given in Table 1. All subjects provided written informed consent, and the study protocol was approved by the ethical committee of Harokopio University.

Diets

Dietary assessments were based on a validated food-frequency questionnaire that included food items from the Mediterranean diet pyramid (Trichopoulou, 2000). Frequencies of milk and dairy products, bread, fruits, vegetables, legumes, olive oil, fish, poultry, red meat and meat products, and wine consumption were recorded and a monthly food consumption score calculated. Dietary compositions of the two groups are depicted in Table 2. Dietary compliance was assessed by a total of three 4 d dietary records (one every 4 weeks). The recorded days

Table 1. Fatty acid, total sterol and total tocopherol composition of the flaxseed oil (per 100 g)

Constituents	
Palmitic acid (g)	5.9
Stearic acid (g)	3.6
Oleic acid (g)	18.2
Linoleic acid (g)	13.9
α -Linolenic acid (g)	54.2
Total sterols (g)	0.4
Total tocopherols (mg)	54.27

Table 2. Nutrient composition of the two diets (Mean values and standard deviations)

Diet...	Mediterranean–Cretan-type diet		Westernised Greek diet		P value
	Mean	SD	Mean	SD	
Energy (J)	9169.86	1118.46	9154.32	959.28	0.348
Proteins (%)	13.2	3.1	16.0	1.8	0.009
Carbohydrates (%)	47.8	3.8	44.4	4.4	0.007
Fats (%)	37.7	4.8	38.2	4.2	0.467
SFA (g)	22.3	2.5	38.6	3.2	0.018
MUFA (g)	49.5	5.8	32.6	4.9	0.034
PUFA (g)	13.7	2.0	12.1	1.7	0.171
α -Linolenic acid (g)	0.9	0.3	1.1	0.2	0.215
EPA (g)	0.3	0.05	0.3	0.08	0.249
DHA (g)	0.4	0.04	0.3	0.05	0.311
Cholesterol (mg)	214.8	24.4	370.1	31.0	0.004
Alcohol %	1.3	0.3	1.4	0.4	0.741
Vitamin C (mg)	122.4	13.0	117.8	18.4	0.002
Dietary fibre (g)	26.7	3.3	22.4	4.1	0.021

SFA, saturated fatty acids; DHA, docosahexaenoic acid.

included three weekdays and one weekend day. The diet diaries were analysed using the Nutritionist V Diet Analysis software (version 2.1, First Data Bank Inc., San Bruno, CA, USA) as modified for the Greek population (Yannakoulia *et al.* 2003).

Analytical procedures

The volunteers attended the Department of Cardiology at Laiko Hospital on two occasions for blood collection; at the beginning and end of the experimental period. At each visit blood samples were collected at 08.00 hours after the subjects had fasted overnight (12 h). Blood was collected into a glass tube without preservative (vacutainer tube; Becton Dickinson Labware, Franklin Lakes, NJ, USA) for the determination of serum inflammatory indices. All samples were collected without occlusion. The vacutainer tubes were kept on ice until centrifuged at 3000 rpm for 10 min at 4°C within 2 h of blood collection. Serum samples were stored at –80°C for further analysis. CRP and serum amyloid A (SAA) were assayed by particle-enhanced immunonephelometry (N Latex; Date-Behring Marburg GmbH, Marburg, Germany) with a range from 0.175 to 1100 mg/l and 0.75 to 1000 mg/l, respectively. IL-6 and macrophage colony-stimulating factor (MCSF) were measured by high-sensitivity ELISA (R & D Systems Europe Ltd, Abingdon, Oxfordshire, UK). The intra-assay and inter-assay CV were <5% for CRP, SAA and MCSF and <10% for IL-6. Soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1 and soluble E-selectin were assayed by quantitative sandwich ELISA (R & D Systems Europe Ltd). The intra-assay and inter-assay CV for sCAM was <10%. All samples were assayed twice by a single operator blinded to sample timing and diet category. To minimise variability, samples before and after the ALA supplementation for each subject were run in one assay. All measurements were performed at the biochemistry laboratory of the General Hospital of Nikea.

Statistical analysis

Clinical and dietary traits are expressed as mean values and standard deviations, unless otherwise stated. Data on inflammatory markers and adhesion molecules were not normally distributed and therefore were expressed as the median and the 25th and 75th percentiles. Differences in the baseline concentrations of clinical and dietary traits between diet groups were assessed using Student's independent samples *t* tests (after log transformation), with the exception of smoking status where the χ^2 test was used. Since data on inflammatory markers and sCAM concentrations were not normally distributed even after the logarithmic transformation of the values, non-parametric analyses were performed to determine differences at baseline (Mann–Whitney *U* test), and before and after ALA supplementation (Wilcoxon signed ranks test). The Mann–Whitney *U* test also was used to compare percentage differences of inflammatory markers and sCAM concentrations before and after the ALA supplementation period between the two diet groups. Percentage difference was calculated as the median of the percentage differences of individual values. Statistical significance was set at $P < 0.05$. Data were analysed using SPSS statistical software (SPSS for Windows, version 10.0.5; SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics

The subjects enrolled in the two dietary groups did not differ in regard to age, BMI, blood pressure and leucocyte, lymphocyte, or monocyte–granulocyte count. The number of light smokers (less than ten cigarettes per d) was similar across the two groups (Table 3).

Body weight and leucocyte, lymphocyte, and monocyte–granulocyte counts did not change significantly during the course of the study. A review of diet diaries indicated that the subjects' background diet remained unchanged throughout the study.

Table 3. Baseline clinical data of the two different background diet groups (Mean values and standard deviations)

Diet...	Mediterranean–Cretan-type diet (n 21)		Westernised Greek diet (n 19)		P value
	Mean	SD	Mean	SD	
Age (years)	50.2	6.3	48.7	8.3	NS
BMI (kg/m ²)	28.6	3.6	27.8	3.6	NS
Leucocytes (× 10 ³)	6.4	1.6	7.3	1.9	NS
Neutrophils (× 10 ³)	3.8	1.1	4.1	1.3	
Lymphocytes (× 10 ³)	2.2	0.9	2.3	0.7	NS
Monocytes (× 10 ³)	0.32	0.12	0.37	0.14	NS
Blood pressure (mmHg)					
Systolic	126.6	16	125.6	16.7	NS
Diastolic	80.8	11	80.2	11	NS
Light smokers: yes (n)		4		4	NS
no (n)		17		15	

Blood inflammatory markers

Median values of SAA, CRP, MCSF and IL-6 of the two dietary groups before and after the ALA supplementation are presented in Table 4. The only inflammatory marker to present a statistically significant reduction in both groups after ALA supplementation was MCSF, while CRP, SAA and IL-6 were decreased significantly, but only in the Westernised Greek diet group. The difference of response of the inflammatory markers to the ALA supplementation in the two background diets was confirmed by comparison of percentage differences within each of the two diet groups (Fig. 1). Percentage changes of SAA and CRP in the Westernised Greek diet group were significantly different from changes in the group following the Mediterranean–Cretan-type diet ($P=0.005$ and $P=0.038$, respectively). No significant correlations were found between dietary cholesterol, saturated fatty acids, MUFA and PUFA, and the investigated inflammatory markers in the two groups. Furthermore, correlations between dietary vitamin C intake and inflammatory markers were not statistically significant.

Soluble cellular adhesion molecules

Levels of soluble vascular cell adhesion molecule-1 decreased significantly with ALA supplementation in both patient groups ($P<0.001$ in the Mediterranean–Cretan-type diet and $P=0.001$ in the Westernised Greek diet). The levels of soluble intercellular adhesion molecule and soluble E-selectin also remained unaltered in both groups (Table 5).

Discussion

Our data reveal that an increased dietary intake of ALA for 3 months was sufficient to reduce inflammatory markers and soluble vascular cell adhesion molecule-1 concentrations in dyslipidaemic subjects. This effect was more evident for the group following the Westernised Greek diet compared with the group following the Mediterranean–Cretan-type diet.

Nutrient intakes were different in the two diet groups. The Westernised Greek diet group showed higher intakes of protein, while carbohydrate intakes were lower

Table 4. Serum concentrations of inflammatory markers in dyslipidaemic volunteers following two different background diets before and after α -linolenic acid supplementation* (Median values and 25th and 75th percentiles)

Variables	Before†		After		Percentage difference‡	P value
	Median	25th–75th percentile	Median	25th–75th percentile		
Mediterranean–Cretan-type diet group (n 21)						
SAA (mg/l)	2.51	2.20–4.5	2.40	1.70–3.00	–3.6	0.16
CRP (mg/l)	0.96	0.54–1.82	0.99	0.56–2.10	2.0	0.51
MCSF (pg/ml)	283	257–309	258	226–292	–15.0	0.006
IL-6 (pg/ml)	2.0	1.12–3.15	1.66	0.99–2.13	–4.5	0.08
Westernised Greek diet group (n 19)						
SAA (mg/l)	3.65	2.5–5.8	2.38	1.39–3.40	–36.4	0.0002
CRP (mg/l)	1.8	1.19–4.4	1.10	0.59–1.60	–36.1	0.002
MCSF (pg/ml)	279	237–328	232.0	195.0–284.0	–21.9	0.005
IL-6 (pg/ml)	2.28	1.34–4.80	1.70	1.26–2.40	–7.9	0.03

SAA, serum amyloid A; CRP, C-reactive protein; MCSF, macrophage colony-stimulating factor.

* For details of subjects and diets, see Tables 1–3 and pp. 650–652.

† The median values of the inflammatory markers for the Mediterranean–Cretan-type diet group were not significantly different from the corresponding values for the Westernised Greek diet group at baseline.

‡ Percentage difference was calculated as the median of the percentage differences of individual values.

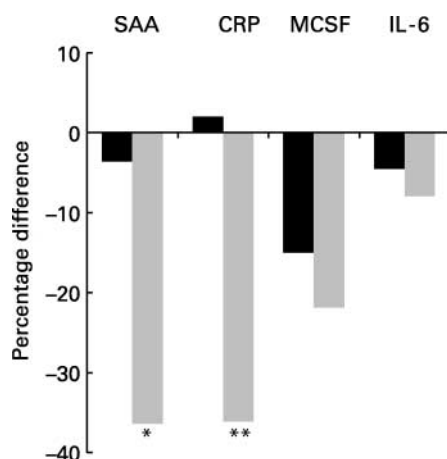


Fig. 1. Percentage differences of concentrations of inflammatory markers after the α -linolenic acid supplementation of subjects with the two different background diets, the Mediterranean–Cretan-type diet (■) and the Westernised Greek diet (▒). SAA, serum amyloid A; CRP, C-reactive protein; MCSF, macrophage colony-stimulating factor. Percentage difference was calculated as the median of the percentage differences of individual values. The comparison of percentage differences was made by the Mann–Whitney *U* test. There were significant differences between the two diet groups: * $P < 0.05$, ** $P < 0.01$.

compared with the Mediterranean–Cretan-type diet group. Intakes of saturated fatty acids and cholesterol were significantly higher in the Westernised Greek diet group than the Mediterranean–Cretan-type diet group. Approximately 16% of energy intakes in the Westernised Greek diet group came from saturated fatty acids, a pattern that would be considered high when compared with the American Heart Association recommendations (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). On the other hand, the Mediterranean–Cretan-type diet group showed a higher intake of MUFA, vitamin C and dietary fibre. These intakes are in accord with the basic definition of the Mediterranean–Cretan diet (Kafatos *et al.* 2000) and the ‘Westernised’ diet profile (Lewis *et al.* 1981), respectively.

Atherosclerosis is a chronic inflammatory disorder characterised by a dynamic interaction of inflammatory

cells, cytokines and chemokines (Ross, 1999). Leucocyte adhesion to endothelial cells and subsequent transendothelial migration, an initial event in the atherosclerotic process, is largely mediated by cell adhesion molecules (Price & Loscalzo, 1999). The sCAM originate from the shedding or proteolytic cleavage of membrane-bound molecules and may serve as markers of endothelial cell activation (Gearing & Newman, 1993). On the other hand, there is growing evidence that inflammatory markers such as CRP may also play a direct pathogenic role in the atherosclerotic process (Pasceri *et al.* 2000). Other inflammatory markers such as SAA, MCSF and IL-6 are present in atherosclerotic lesions and research has suggested that these markers play a role in the development of atherosclerotic plaques (Clinton *et al.* 1992; Meek *et al.* 1994; DeGraba, 1997). Such results indicate the importance of interventions aimed at the reduction of inflammatory markers, which may be translated in clinical benefit regarding the prevention of CHD.

As we have previously reported (Rallidis *et al.* 2003), dietary ALA supplementation reduces the concentrations of inflammatory markers. The mechanisms responsible for the suppression of the concentrations of inflammatory markers by ALA, however, remain unknown, although suppression of eicosanoid production may be involved (James *et al.* 2000). We have proposed elsewhere that the reduction of prostaglandin E_2 and leucotriene B_4 production and the subsequent reduction of pro-inflammatory cytokines IL-1 and TNF- α decreases IL-6 release which in turn reduces the hepatic production of CRP and SAA (Rallidis *et al.* 2003). IL-1 and TNF- α are known to induce a large increase in MCSF secretion from both vascular endothelial and smooth muscle cells in cultures (Clinton *et al.* 1992), and thus control MCSF expression. Moreover, cellular adhesion molecules are expressed on the endothelial membrane in response to several inflammatory stimuli, such as CRP and pro-inflammatory cytokines (Price & Loscalzo, 1999).

The present study was designed to investigate associations between the anti-inflammatory effect of ALA and background dietary patterns. We report here a greater responsiveness of the inflammatory markers in the

Table 5. Soluble cellular adhesion molecule concentrations in dyslipidaemic volunteers following two different background diets before and after α -linolenic acid supplementation*

(Median values and 25th and 75th percentiles)

Variables	Before†		After		Percentage difference‡	P value
	Median	25th–75th percentile	Median	25th–75th percentile		
Mediterranean–Cretan-type diet group (<i>n</i> 21)						
sVCAM-1 (ng/ml)	610	534–787	543	487–601	–10.1	<0.001
sICAM-1 (ng/ml)	201	173–232	204	173–254	1.7	0.19
sE-selectin (ng/ml)	44.2	35.0–55.1	44.0	37.5–55.1	–3.9	0.99
Westernised Greek diet group (<i>n</i> 19)						
sVCAM-1 (ng/ml)	592	506–625	495	430–570	–18.3	0.001
sICAM-1 (ng/ml)	219	196–264	222	192–279	0.1	0.91
sE-selectin (ng/ml)	47.8	31.6–65.9	47.8	31.6–67.1	–2.9	0.31

sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; SE-selectin, soluble E-selectin.

* For details of subjects and diets, see Tables 1–3 and pp. 650–652.

† The median values of the soluble cellular adhesion molecules for the Mediterranean–Cretan-type diet group were not significantly different from the corresponding values for the Westernised Greek diet group at baseline.

‡ Percentage difference was calculated as the median of the percentage differences of individual values.

Westernised Greek diet group. Still, we found no correlations between the dietary intake of specific nutrients and concentration of inflammatory markers. Animal studies, however, have demonstrated suppressive effects of individual nutrients, such as oleic acid on *in vivo* immune responses (Yaquob, 2002), while a high-MUFA diet resulted in a reduction in the expression of adhesion molecules by human peripheral blood mononuclear cells (Yaquob *et al.* 1998). A limited number of studies have suggested an anti-inflammatory effect of vitamin C. An association between vitamin C deficiency and immune dysfunction has been reported (Schoenherr & Jewell, 1997). Serum CRP concentrations have been correlated negatively with serum vitamin C concentration in peripheral arterial disease (Langlois *et al.* 2001), and critically ill patients (Schorah *et al.* 1996). Although intakes of MUFA and vitamin C were different in the two diet groups we investigated, our data do not reveal a direct correlation between the dietary intake of these nutrients and the anti-inflammatory effect of ALA.

One limitation of the present study is the lack of a control group. A second limitation is that plasma phospholipid fatty acid composition data were not collected; therefore, associations between fatty acids and inflammatory markers could not be drawn. Still, our data on dietary fatty acid composition, based upon participants' dietary diaries, revealed no association. Furthermore, no associations were observed between vitamin C and inflammatory markers, although these nutrients were consumed in different quantities by the two dietary groups. Although our data indicated that some inflammatory markers were decreased more when ALA was supplemented in Westernised diets compared with a Mediterranean diet, our data could not confirm that the Mediterranean diet possessed an intrinsic anti-inflammatory effect.

In conclusion, we speculate that ALA supplementation would be more effective in the reduction of some inflammatory markers if patients were consuming a Westernised diet, rather than a Greek–Mediterranean type of diet. The mechanisms for these effects remain to be elucidated.

Acknowledgements

The authors thank Dr E. Karpodini, Dr E. Theriou, and the staff of the Department of Cardiology, Laiko Hospital, for assistance with the management of the subjects. We also wish to thank Dr N. Yiannakouris (Harokopio University) for his review of the manuscript. This project was supported by the Greek Ministry of Development, General Secretariat for Research and Technology (grant 97EL-55) and in part by Becel Institute/Unilever Bestfoods Greece.

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