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Research Article

Effect of fortified milk on lyso-platelet-activating factor acetyltranferase and lipoprotein-associated phospholipase A_2 in hypercholesterolemic adults

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Hypercholesterolemia is associated with subclinical inflammation, characterised by elevated proinflammatory mediators. Lyso-platelet-activating factor acetyltransferase (lyso-PAF AT) and lipoprotein-associated phospholipase A_2 (Lp-PLA_2) are two key metabolic enzymes of platelet-activating factor (PAF), a potent inflammatory lipid mediator. Little information is available concerning the efficacy of a dietary intervention on the metabolism of PAF. The objective of the study was to evaluate the effect of fortified milk on the activity of these enzymes. Forty-three adults (mean age 49.8 ± 8.1 years) with body mass index < 35 kg/m^2, and total cholesterol >200 but < 310 mg/dL were randomised to two groups; (i) intervention group received 500 mL/day (two glasses) of a low-fat milk fortified with phytosterols, linoleic and alpha linolenic acids, vitamin C, vitamin E, vitamin A, vitamin B6, vitamin B12, folic acid, magnesium and selenium ($n = 22$), and (ii) placebo group received 500 mL/day of a conventional low-fat milk ($n = 21$) for 3 months. Outcome measures were the activities of lyso-PAF AT from leukocytes and serum Lp-PLA_2 determined with established methods. None of the activities changed significantly during the study in the intervention group, lyso-PAF AT (95% confidence interval: −1.7, 2.3 nmol/min/mg; $p = 0.246$), and Lp-PLA_2 (−7.8, 5.8 nmol/min/mL, $p = 0.591$). No difference was observed between the two groups. In conclusion, daily intake of two glasses of phytosterols, antioxidants, linoleic and linolenic acids via fortified milk for three months had no effect on the activity of either lyso-PAF AT or Lp-PLA_2.

Practical applications: Platelet-activating factor (PAF) was the first intact phospholipid known to have messenger functions in which the signaling results from the molecule binding to specific receptors on the plasma membrane or other membranes of the cell. It has a number of pro-inflammatory properties, and affects several critical points of atherogenesis including thrombosis, inflammation, and oxidation. Fortification of milk with nutrients that possess anti-inflammatory properties and administration to adults with elevated blood cholesterol could provide a means to controlling inflammatory process through the synthesis and degradation of PAF in a population group at risk for cardiovascular morbidity and mortality.

Keywords: Adults / Hypercholesterolemia / Lipoprotein-associated phospholipase A_2 / Lyso-PAF acetyltransferase / Milk

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Abbreviations: apo, apolipoprotein; **BMI,** body mass index; **CRP,** C-reactive protein; **DHA,** docosahexaenoic acid; **EPA,** eicosapentaenoic acid; **HDL,** high density lipoprotein; **IL-6,** interleukin-6; **LDL,** low-density lipoprotein; **LDL-C,** LDL-cholesterol; **Lp-PLA_2,** lipoprotein-associated phospholipase A_2; **lyso-PAF AT,** lyso-platelet-activating factor acetyltransferase; **PAF,** platelet-activating factor; **PG,** placebo group; **PhG,** intervention group; **PUFA,** polyunsaturated fatty acid; **TAG,** triacylglycerol; **TC,** total cholesterol; **TNF-α,** tumour necrosis factor alpha

1 Introduction

Hypercholesterolemia, which promotes atherosclerotic lesions in large arteries, is one of the major risk factors for cardiovascular disease (CVD). Several studies have associated hypercholesterolemia with subclinical inflammation, which is indicated by increased circulating levels of inflammatory

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mediators, such as C-reactive protein (CRP), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) [1].

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a biologically active phospholipid implicated in physiological as well as pathological inflammatory processes such as atherogenesis [2]. PAF affects several critical points of atherogenesis including thrombosis, inflammation and oxidation. Common risk factors for atherosclerosis, namely diabetes, cigarette smoking, oxidised low-density lipoprotein (LDL) and systemic inflammation, share elevated PAF and PAF-like lipids as a common feature [2, 3].

PAF can be synthesised via two distinctly different enzymatic routes, the remodelling and de novo pathways. The remodelling pathway involves a structural modification of pre-existing ether-linked choline-containing phospholipids that serve as structural components of membranes. The last step in PAF formation is the acetylation of lyso-PAF catalysed by at least two isoforms of acetyl-CoA:lyso-PAF acetyltransferase (lyso-PAF AT) [4, 5]. It is believed that the remodelling route plays a crucial role in inflammatory/hypersensitivity responses of PAF in various tissues and blood [3]. Detopoulou et al. [6] have found a positive correlation of leukocyte lyso-PAF AT activity with markers of subclinical inflammation (CRP, IL-6). Despite the critical role of lyso-PAF AT in the biosynthesis of PAF, only a couple of studies have attempted to determine its activity under various clinical settings [6, 7].

PAF is hydrolysed and inactivated by PAF-acetylhydrolase, a Ca^{2+}-independent, phospholipase A_2 (PLA_2) [8]. Two major forms of PAF-acetylhydrolase have been described, the secreted (plasma), designated lipoprotein associated phospholipase A_2 or Lp-PLA_2, and the intracellular (cytosolic) forms. Lp-PLA_2 circulates in blood bound to lipoproteins, especially the small and dense LDLs and catalyses the hydrolysis of short chain acyl-groups or oxidised fatty acids connected to the glycerol backbone at the sn-2 position [8, 9]. Elevated Lp-PLA_2 concentrations in plasma increase the risk for primary and secondary cardiovascular events [10], and Lp-PLA_2 activity appears elevated among patients with dyslipidemia [11, 12], while its treatment with statins or fibrates concludes in the reduction of LDL-bound-Lp-PLA_2 activity [13, 14].

Only a few studies have investigated the impact of dietary interventions on PAF metabolism. Hatoum et al. [15] have found that the replacement of 5% of energy from carbohydrates with energy from protein was associated with 2.2 nmol/min/mL lower levels of Lp-PLA_2 (95% CI: −3.1, −0.4) activity, and every 15-g/day increase in alcohol consumption was associated with 4.4 nmol/min/mL lower levels of Lp-PLA_2 activity (95% CI: −6.4, −2.4). Furthermore, Nelson et al. [16] did not find any significant changes in Lp-PLA_2 mass or activity after supplementing the diet of older adults with olive oil, safflower oil or fish oil capsules for 8 weeks. As far as we are aware of, the activity of AT has not yet been investigated in the context of a dietary intervention program in humans.

In the current dietary intervention study we tested the hypothesis that administration of a low-fat milk fortified with phytosterols, linoleic and alpha linolenic acids, antioxidant vitamins and minerals during a lifestyle modification program could attenuate the activity of the enzymes lyso-PAF AT and Lp-PLA_2 in leukocytes and serum, respectively, in adults with elevated blood cholesterol.

Several of the aforementioned compounds or similar ones can modulate PAF metabolism in cellular and animal experiments. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) suppress PAF synthesis by human monocytes and endothelial cells [17, 18]. Yeo et al. [19] have shown that renal microsomal lyso-PAF AT activity in rats is reduced after a long-term consumption of an EPA and DHA-enriched diet. Oleic and Arachidonic acids have been found to reduce the activity of lyso-PAF acetyltransferase in neutrophils [20]. PAF biosynthesis by lyso-PAF AT is strongly inhibited by lycopene alone or in combination with alpha-tocopherol or tomato lipophilic extracts under oxidative stress in endothelial cells [21]. Regarding Lp-PLA_2, an inverse association with serum retinol levels was found in the Bruneck study, but no correlation was observed between serum levels of tocopherol and the activity of Lp-PLA_2 [22]. Finally, selenium deficiency has been reported to increase the production of PAF in bovine aortic endothelial cells (BAEC), through activation of PLA2 and lyso-PAF-AT, while PAF-acetylhydrolase activity was not affected by selenium status [23].

On the other hand, nutrients, such as phytosterols, that have not been studied as to their direct effect on PAF metabolism may act indirectly on it by attenuating cardiovascular disease risk factors, such as hyperlipidemia, oxidative stress and inflammation. It is known that those factors can trigger PAF synthesis [2, 3] and a possible modulation of PAF's enzyme activities after the consumption of the fortified milk concurrently with markers of dyslipidemia and inflammation could support PAF's role in cardiovascular diseases.

2 Materials and methods

2.1 Materials and instrumentation

All reagents were of analytical grade and were supplied by Sigma (St. Louis, MO, USA). 2-thio-PAF was purchased from Cayman Chemical (Michigan, USA). Analytical grade solvents, liquid-chromatography grade solvents and silica gel G used for thin-layer chromatography, were supplied from Merck (Darmstadt, Germany). PAF-induced platelet aggregation was measured in a Chrono-Log (Havertown, PA, USA) aggregometer (model 400-VS) coupled to a Chrono-Log recorder (Havertown, PA, USA) at 37°C with constant stirring at 1200 rpm. Centrifugations of blood samples were performed in a refrigerated Eppendorf Centrifuge 5810R while the centrifugations for the isolation of washed rabbit platelets were conducted in a Sorvall RC-5B refrigerated Superspeed centrifuge (Sigma) Homogenizations were conducted with an

ultrasonic Bandelin sonopuls HD 2070, Bandelin electronic UW 2070 apparatus (Berlin, Germany). To determine the protein concentration with the Bradford method, we used the spectrophotometer Pharmacia Biotech Novaspec II. For the determination of Lp-PLA_2 activity, a BioTek PowerWave XS microplate reader (Vermont, USA) was used.

2.2 Participants and study design

This was a simple randomisation, double-blind, placebo-controlled, parallel-group study with a 1:1 allocation ratio that took place at the Department of Nutrition and Dietetics of Harokopio University in Athens, Greece. Eligible participants were 43 adults aged 40–60 years old, with a body mass index (BMI) <35 kg/m^2, and total cholesterol (TC) >200 but <310 mg/dL. Exclusion criteria were lipid-lowering medication use, and metabolic disorders other than hypercholesterolemia (i.e. diabetes, thyroid, renal, and hepatic disease). All subjects were recruited via advertisement. The duration of the intervention was three months. All subjects came to the laboratory for anthropometric, nutritional, and clinical evaluation, and blood sample collection the week before the intervention, and after one and three months. Anthropometric assessment included body weight, standing height, BMI, waist and hip circumferences, and body composition assessed with bioelectrical impedance analysis. Nutritional information was collected via a 3-day 24-h recall (two consecutive weekdays and one weekend day). Early morning venous blood samples were obtained from each subject after a 12-h overnight fast and biochemical measurements were performed within a reasonable time. The experimental protocol was approved by the Ethics Committee of Harokopio University, Athens, and all subjects gave their signed informed consent.

2.3 Study groups

Participants in the intervention group (PhG) ($n = 22$) received two portions/day (250 mL each) of a milk fortified with 0.5 g of plant sterols, 0.43 g of linoleic acid, 0.04 g of alpha linolenic acid, 40 mg of vitamin C, 7.88 mg of vitamin E, 111 μg of vitamin A, 2.5 mg of vitamin B6, 2.5 μg of vitamin B12, 100 μg of folic acid, 22 mg of magnesium, and 25 μg of selenium per 100 mL. Participants in the placebo group (PG) ($n = 21$) received the same labelled low-fat milk that was not fortified (PG). The milk consumed by both groups contained (per 100 mL) 3.5 g of protein; 4.6 g carbohydrates; 1.8 g of fat for a total energy of 49 kcal. The study products were all packaged in 1000 mL bottles (Friesland Campina Hellas). Additionally, subjects in PhG and PG attended seven 1-h dietary and lifestyle counselling sessions, held biweekly in order to increase awareness regarding health issues and motivate subjects to set targets and change certain dietary and physical activity habits for improving cardiovascular risk and health status in general.

2.4 Determination of lyso-PAF AT activity in leukocyte homogenates

Determination of lyso-PAF AT activity in leukocyte homogenates was based on the measurement of the produced PAF, after thin-layer chromatography separation, by the washed rabbit platelet aggregation assay according to previously published methods [24]. All assays were performed in duplicate. Enzymatic activities were expressed as specific activity in nmol/min/mg protein.

2.5 Determination of Lp-PLA_2 activity

We determined Lp-PLA_2 activity using a photometric assay based on the hydrolysis of the acetyl-thioester bond of a thio-analogue of PAF (2-thio-PAF) by Lp-PLA_2 and the subsequent determination of the lyso-thio-PAF free thiol by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Specifically, serum samples are removed from -80°C and left to thaw at constant temperature. 90 μL Tris–HCl (100 mM)–EGTA (1 mM), 40 μL DTNB (1 mM) and 10 μL serum are placed in each well of a microplate, and the reaction starts with the addition of 60 μL 2-thio-PAF (500 μM). Sixty microliter Tris–HCl–EGTA instead of 60 μL 2-thio-PAF (500 μM) is added in the case of the non-enzyme controls. The plate containing the samples is introduced into the microplate reader and the absorbance at 414 nm is recorded continually for 5 min at 37°C. The reaction is linear for incubation times up to 5 min. We determined the rate of ΔA/min for the no-enzyme controls and subtracted it from that of the sample wells. To calculate the activity, we used the following formulae:

$$\text{Activity of Lp-PLA}_2\ \mu\text{mol/min/mL} = \frac{(\Delta A/\text{min}) \times 0.200\,\text{mL}}{10\,\text{mM}^{-1} \times 0.01\,\text{mL}}$$

All assays were performed in duplicate. Enzymatic activities were expressed as specific activity in nmol/min/mL.

2.6 Other biochemical analyses

Fasting TC [25], high density lipoprotein (HDL)-cholesterol [26], triglyceride [27] and glucose [28] were determined by photometric method. Apolipoprotein A_1 (apo-A_1) and apolipoprotein B (apo-B) concentrations were determined by an immunoturbiometric method [29] and insulin by chemiluminescence [30]. All analyses were carried out by an automated centrifugal analyzer with commercially available assays (Roche/Hitachi modular system P800 module). LDL-cholesterol (LDL-C) was calculated with the Friedewald equation [31]. Total fasting plasma homocysteine concentration was measured by immunoturbiometric method with an automated centrifugal analyzer with commercially available assays (ABBOTT/AXSYM) [32]. Plasma folic acid

and vitamin B_{12} concentrations were measured by electrochemiluminescence (Roche/E170) [33]. Total antioxidant capacity (TAC) was measured using Trolox as standard [34]. CRP was assayed by immunonephelometry (Date-Behring Marburg, Marburg, Germany) [35]. IL-6 was measured with a high-sensitivity enzyme-linked immunoassay (R&D Systems Europe, Abingdon, United Kingdom). TNF-α was also measured by an ELISA method (Quantikine HS/human TNF-alpha immunoassay kit, R&D Systems, Minneapolis, Minnesota).

2.7 Statistical analysis

Continuous variables with normal distribution are summarised for each group by mean and SD. Quantitative data with an asymmetrical distribution are reported as median (25th, 75th quartiles). Normal distribution of variables was tested with histograms and cumulative distribution graphs at baseline, 1st and 3rd months of the study and was verified with the Kolmogorov–Smirnov test. The two groups were compared with the Student's *t*-test for independent samples. We used the non-parametric Mann–Whitney test to compare the two groups regarding the number of cigarettes smoked per day. We calculated the bivariate linear zero-order correlations at baseline using Pearson's coefficient for pairs of quantitative variables. Sex and smoking status are reported as numbers and proportions. Associations between categorical variables (gender and group, smoking and group) were tested with the chi-squared test. Multiple linear regressions controlled for confounding factors of lyso-PAF AT or Lp-PLA_2 activity at baseline. We used the ANOVA for repeated measures in order to check for any change in the activity of lyso-PAF AT or Lp-PLA_2 at the end of the 3-month period of the study in the intervention group. We consulted the results of Levene's test for homogeneity of variance and Mauchly's test to check for homoschedacity and sphericity, respectively. Post hoc analysis was carried out using paired samples *t* tests (for ANOVA) with the Bonferroni correction to control for type I error. All 95% confidence intervals and probability values (p) are based on two-tailed tests, and the level of significance used is 5%. All statistical calculations were made with SPSS version 13.0 (Statistical Package for Social Sciences, SPSS, Chicago, Illinois, USA).

3 Results

This is the first study that investigates the influence of a functional food consumed by a group of hypercholesterolemic, but otherwise healthy individuals, on the activity of two key metabolic enzymes of PAF – lyso-PAF AT and Lp-PLA_2. Baseline demographic, anthropometric, clinical and dietary characteristics of the intervention and placebo groups are shown in Tables 1 and 2. There were no significant differences between the two groups at baseline, except for consumption of DHA which is probably caused by chance.

According to the answers provided at the end of the 1st month, 85 and 73% of the participants in the placebo and intervention groups, respectively, complied perfectly with the recommended quantity of milk (two glasses of milk/day, either conventional or enriched). However, 70% of the participants in the PG and 82% of their PhG counterparts consumed two glasses of either conventional or enriched milk per day at the end of the 3rd month. To assess compliance to the dietary and lifestyle counseling sessions we use the criterion of attending >4 sessions (compliant). We found that 57% of the participants in the PG and 64% of those in the enriched milk group attended at least four counselling sessions.

Significant unadjusted linear correlations at baseline were found for the activity of lyso-PAF AT with white blood cell count ($r = 0.336$, $p = 0.045$, $N = 36$), and systolic blood pressure ($r = -0.325$, $p = 0.036$, $N = 42$). Total antioxidant capacity ($r = 0.276$, $p = 0.085$, $N = 40$) tended to be linearly associated with the activity of lyso-PAF AT. White blood cell count remains significantly associated with the activity of lyso-PAF AT even after controlling for gender, age and systolic blood pressure (Table 3).

Activity of Lp-PLA_2 was linearly associated with age ($r = -0.376$, $p = 0.014$, $N = 42$), total antioxidant capacity ($r = -0.334$, $p = 0.035$, $N = 40$), fat free mass ($r = 0.326$, $p = 0.040$, $N = 40$), body fat percentage ($r = -0.479$, $p = 0.002$, $N = 40$), apo-B ($r = 0.322$, $p = 0.038$, $N = 42$) and LDL-C ($r = 0.304$, $p = 0.050$, $N = 42$). Multiple linear regression models 1 and 2 suggest that men in the sample have higher specific activity of Lp-PLA_2 compared to women (Table 3). Furthermore, participants who smoked during the study period have higher specific activity of Lp-PLA_2 than those who refrained from smoking after controlling for gender, age and LDL-C (Table 3, model 3).

Table 4 summarises the differences observed in anthropometric and clinical characteristics between the placebo and intervention group at the end of the 3rd month of the study period. Total cholesterol, LDL-cholesterol, apolipoprotein-B and homocysteine concentrations were significantly lower in the PhG than in the PG. On the other hand, folic acid concentration was higher in the PhG compared to PG after the completion of the 3rd month. Regarding dietary intake, the only significant differences found were for vitamins B_6 (95% CI: −9.3 to −5.2 mg/day), C (−194.9 to −85.8 mg/day), E (−32.5 to −18.1 mg/day) and folate (−415.1 to −218.3 μg/day). There were no significant differences in lyso-PAF AT and Lp-PLA_2 activity between the intervention and placebo groups at three months.

Finally, we checked for probable changes in the activity of lyso-PAF AT or Lp-PLA_2 over time for each study group (Fig. 1A and B). Neither activity has significantly changed during the three months of the study ($p_{\text{for effect of time}} > 0.05$ for both lyso-PAF AT and Lp-PLA_2. Although non-significant, the average activity of lyso-PAF AT is higher in the PhG compared to PG at baseline, and it remains higher at the end of the 1st and 3rd months. On the contrary, the average

Table 1. Baseline demographic, anthropometric, and clinical characteristics*

	PG ($n = 21$)	PhG ($n = 22$)	Significance level
Age (years)	49.0 (8.1)	50.7 (8.1)	0.519
Sex (female)	11 (52%)	11 (50%)	0.876
Current smoking status			
Smokers	6 (30%)	9 (45%)	0.327
Non-smokers	14 (70%)	11 (55%)	
Cigarettes/day	0 (0, 8)	0 (0, 16)	0.624
Weight (kg)	74.7 (11.7)	80.1 (14.9)	0.192
BMI (kg/m^2)	27.6 (3.9)	28.4 (3.7)	0.502
Waist circumference (cm)	90.2 (10.6)	91.9 (11.9)	0.633
Waist to hip ratio	0.87 (0.08)	0.88 (0.09)	0.875
Body fat (% body weight)	39.3 (7.2)	40.1 (5.8)	0.675
Fat free mass (kg)	44.5 (8.1)	48.5 (11.8)	0.209
Systolic blood pressure (mmHg)	132.1 (14.0)	126.6 (14.1)	0.207
Diastolic blood pressure (mmHg)	83.3 (8.7)	81.4 (13.3)	0.580
Glucose (mg/dL)	98.5 (9.0)	97.4 (12.4)	0.738
Total cholesterol (mg/dL)	254.4 (35.4)	242.8 (33.8)	0.278
LDL cholesterol (mg/dL)	172.9 (33.7)	167.0 (30.7)	0.554
HDL cholesterol (mg/dL)	56.0 (15.9)	51.9 (15.8)	0.397
TAGs (mg/dL)	127.7 (37.4)	118.4 (34.8)	0.403
Apolipoprotein-A$_1$ (mg/dL)	171.0 (31.3)	162.2 (30.8)	0.357
Apolipoprotein-B (mg/dL)	118.5 (21.8)	115.9 (20.6)	0.699
Insulin (μUI/mL)	10.4 (4.8)	10.8 (4.1)	0.625
White blood cell count (cells $\times 10^6$/μL)	6.6 (1.7)	6.4 (1.7)	0.782
Total antioxidant capacity (nmol/μL)	50.7 (3.7)	51.0 (3.5)	0.792
IL-6 (pg/mL)	1.3 (2.4)	0.4 (0.5)	0.187
TNF-α (pg/mL)	11.9 (5.7)	11.0 (2.3)	0.765
CRP (mg/dL)	2.7 (2.4)	3.3 (2.9)	0.525
Homocysteine (μmo/L)	11.5 (2.6)	12.3 (3.8)	0.434
Vitamin B$_{12}$ (pg/mL)	455.7 (137.1)	415.0 (101.4)	0.274
Folic acid (ng/mL)	8.8 (3.0)	8.6 (3.6)	0.856
Lyso-PAF AT (nmol/min/mg)	4.5 (3.9)	5.1 (3.4)	0.540
Lp-PLA$_2$ (nmol/min/mL)	27.0 (7.9)	28.9 (8.1)	0.454

* Data are means (SD), median (25th, 75th quartiles) or absolute numbers (%).

activity of Lp-PLA$_2$ is higher in the PhG in comparison with PG at baseline, but it is lower at the end of the 1st and 3rd months.

4 Discussion and conclusions

PAF is a crucial mediator of endothelial dysfunction and vascular diseases [2, 3] and modulation of PAF metabolism can be proven a good alternative for the regulation of PAF actions in the vasculature. Lyso-PAF AT and Lp-PLA$_2$ are involved in PAF biosynthesis and hydrolysis, respectively, and their relative activities can determine its extracellular and intracellular levels. Lp-PLA$_2$ is a well characterised enzyme and many cellular and clinical studies have shown that it is a promising biomarker of cardiovascular implications [7, 8]. The mass and activity of Lp-PLA$_2$ can be pharmacologically manipulated by drugs targeting cholesterol and triacylglycerol (TAG) metabolism [13, 14] but the ability of dietary interventions and supplementations to affect the activity of the enzyme is much less studied. On the other hand, the determination of lyso-PAF AT activity under various clinical settings and manipulations is limited [36] since no molecular characterisation of the enzyme had been achieved until recently when the research team of Takao Shimizu demonstrated the existence of two acyl-CoA acyltransferases isoforms (namely LPCAT1 and LPCAT2) which also possess lyso-PAF AT activities [2, 3]. A few cellular and animal studies have also shown that lyso-PAF AT can be modulated by certain dietary micronutrients such as omega-3 fatty acids [17–19], selenium [23] and polyphenols [37, 38]. Under this perspective, the objective of the current study was to compare the effect of two low-fat milk products, a conventional and a fortified one, administered during a lifestyle modification program, on the activities of

Table 2. Baseline daily dietary intake of macronutrients, vitamins, and minerals*

	PG ($n = 21$)	PhG ($n = 22$)	Significance level
Energy (kcal)	1586 (498)	1548 (600)	0.822
Carbohydrate (g)	168.6 (59.8)	156.2 (86.1)	0.587
Protein (g)	66.6 (21.4)	67.2 (27.4)	0.931
Fat (g)	72.1 (30.8)	74.2 (30.6)	0.826
SFA (g)	22.9 (13.3)	22.0 (13.5)	0.826
PUFA (g)	9.5 (4.8)	10.5 (5.3)	0.536
Linoleic acid (g)	7.7 (4.2)	8.6 (5.2)	0.547
Alpha linolenic acid (g)	0.9 (0.8)	0.7 (0.3)	0.348
Eicosapentaenoic acid – EPA (g)	0.009 (0.011)	0.114 (0.309)	0.127
DHA – DHA (g)	0.027 (0.026)	0.178 (0.328)	0.042
MUFA (g)	34.7 (14.5)	36.7 (14.8)	0.652
Oleic acid (g)	31.9 (13.6)	33.8 (14.5)	0.652
Vitamin A (μg)	886.4 (846.3)	968.9 (722.1)	0.733
Vitamin C (mg)	134.9 (114.2)	104.7 (72.2)	0.302
Vitamin E (mg)	8.4 (4.5)	10.4 (7.9)	0.332
Vitamin B_6 (mg)	1.4 (0.5)	1.4 (0.9)	0.818
Vitamin B_{12} (μg)	2.6 (1.3)	3.7 (3.8)	0.226
Folate (μg)	272.0 (141.3)	300.4 (170.2)	0.556
Magnesium (mg)	270.8 (90.8)	263.3 (111.8)	0.810
Selenium (mg)	0.105 (0.081)	0.156 (0.278)	0.420

* Data are means (SD).

lyso-PAF AT and Lp-PLA_2 in a sample of hypercholesterolemic adults. According to literature, the added components have the potential to modulate predisposition to chronic oxidative and inflammatory conditions and may have a role in their therapy. These components act through a variety of mechanisms including a decrease in inflammatory mediator production through effects on cell signalling and gene expression (EFA, especially their derivatives, ω-3 fatty acids), a reduction in the production of damaging oxidants (vitamin E and other antioxidants) [39]. Since hypercholesterolemia is associated with both low-grade systemic inflammation and increased oxidation [40] we believed that a pleiotropic dietary attenuation of several biochemical mechanisms leading to atherosclerosis is much better than monotherapy. Under this perspective a combination of hypolipidemic, antioxidant and antinflammatory compounds were added in the fortified milk.

The multivariable linear regression models for lyso-PAF AT activity (Table 3) at baseline revealed a linear positive correlation with white blood cell count (WBC) and a marginally significant inverse correlation with systolic blood pressure. Elevated white blood cell count implies a worse subclinical inflammatory profile which in turn may upregulate lyso-PAF AT. Indeed a previous study has shown strong positive correlations of lyso-PAF AT with CRP and IL-6 in heart failure patients [5]. The fact that this study did not show such correlations may be due to subtle variations of cytokine levels from our volunteers which may render such correlations difficult to obtain statistically. As far as we are aware of, there is no other study that has found any association between specific activity of lyso-PAF AT and blood pressure. Previous hemodynamic studies emphasised that synthetic PAF, in microgram doses, lowered arterial pressure in guinea pigs [41], rats [42] and rabbits [43] in normal and hypertensive states after intravenous or oral administrations. Although the mechanisms of PAF-induced hypotension are not completely understood, some data indicate that the action of PAF on the heart, peripheral vasculature, and microcirculation may account, at least in part, for the reduction of systemic blood pressure [44].

The multivariable linear regression models for Lp-PLA_2 activity at baseline (Table 3) confirmed the well known and previously demonstrated associations of Lp-PLA_2 with gender (men have higher activity of Lp-PLA_2 than women), smoking (smokers have higher activity of Lp-PLA_2 than non-smokers) and LDL-C (positive correlations) [8–10]. The results with reference to the association of Lp-PLA_2 with body fat percentage and fat free mass obtained from our study do not agree with those of other recently published works. For example, patients with the metabolic syndrome, who are characterised by central adiposity, have been found to display higher levels of Lp-PLA_2 activity [45]. Moreover, the main findings from another study on healthy adults indicated that measures of upper adiposity, evaluated by dual X-ray absorptiometry (i.e. fat in the DXA ROI and arms fat) had the highest explanatory ability of Lp-PLA_2 activity among other indices of adiposity in men in multi-adjusted models [46].

Table 3. Multiple linear regression models predicting specific activity of lyso-PAF AT (nmol/min/mg) and Lp-PLA_2 (nmol/min/mL) at baseline ($p < 0.05$) (bold values have a significance level of <0.05)

	R^2	Adjusted R^2	$B \pm$ standard error	β	t	p	95% confidence interval for B
Dependent variable: Lyso-PAF AT activity							
Model 1	0.247	0.166					
Constant			5.490 ± 5.476	–	1.002	0.323	−5.606, 16.586
Men versus women			−0.222 ± 1.071	−0.037	−0.207	0.837	−2.392, 1.948
Age (years)			0.060 ± 0.055	0.163	1.089	0.283	−0.051, 0.171
White blood cell count (cells × 10^6/μL)			0.663 ± 0.273	0.358	2.430	**0.020**	**0.110, 1.217**
Systolic blood pressure (mmHg)			−0.064 ± 0.036	−0.302	−1.777	0.084	−0.136, 0.009
Dependent variable: Lp-PLA_2 activity							
Model 1	0.350	0.279					
Constant			49.774 ± 18.129	–	2.746	0.009	13.041, 86.507
Men versus women			4.827 ± 2.334	0.307	2.069	**0.046**	**0.099, 9.556**
Age (years)			−0.236 ± 0.138	−0.241	−1.705	0.097	−0.515, 0.044
Total antioxidant capacity (nmol/μL)			−0.403 ± 0.315	−0.178	−1.281	0.208	−1.042, 0.235
Apolipoprotein-B (mg/dL)			0.069 ± 0.054	0.183	1.284	0.207	−0.040, 0.177
Model 2	0.368	0.280					
Constant			56.756 ± 19.389	–	2.927	0.006	17.434, 96.078
Men versus women			7.352 ± 3.414	0.467	2.154	**0.038**	**0.429, 14.276**
Age (years)			−0.280 ± 0.145	−0.286	−1.932	0.061	−0.574, 0.014
Apolipoprotein-B (mg/dL)			0.067 ± 0.054	0.178	1.250	0.219	−0.042, 0.175
Fat free mass (kg)			−0.173 ± 0.171	−0.219	−1.013	0.318	−0.520, 0.173
Total antioxidant capacity (nmol/μL)			−0.359 ± 0.318	−0.159	−1.129	0.266	−1.004, 0.286
Model 3	0.450	0.392					
Constant			23.829 ± 7.959	–	2.994	0.005	7.716, 39.941
Men versus women			4.098 ± 2.077	0.262	1.973	0.056	−0.107, 8.303
Age (years)			−0.251 ± 0.125	−0.256	−2.007	0.052	−0.503, 0.002
Smokers versus non-smokers			5.666 ± 2.110	0.339	2.686	**0.011**	1.396, 9.937
LDL-cholesterol (mg/dL)			0.073 ± 0.031	0.297	2.374	**0.023**	0.011, 0.136

In our study, the low-fat milk contained 2.5 g of phytosterols and reduced TC and LDL-C by 7.6 and 12% while the unfortified milk had no effect on the respective values. Previous studies have shown that a daily dose of at least 1.5–3 g/day has been calculated as being the amount needed to achieve a 10–15% reduction in LDL-C. The cholesterol lowering effect appears to level off at higher doses and no significant benefits are obtained [47]. A meta-analysis of 41 trials with different enriched food products showed that the most recommendable daily intake of phytosterols is 2 g and that this dose reduces LDL-cholesterol by 10% [48].

The activity of Lp-PLA_2 did not change significantly over time in either placebo or intervention group, despite the fact that LDL-cholesterol and apo-B concentrations decreased in the PhG. Previous work has shown that Lp-PLA_2 is mainly bound to apo-B – containing lipoproteins and primarily with LDL, whereas a small proportion of circulating enzyme activity is also associated with HDL [8, 49] in human plasma. Moreover, the majority of the LDL-associated Lp-PLA_2 activity is bound to the atherogenic small-dense LDL (sdLDL) particles [11, 49, 50]. In addition, there was no significant change in the activity of lyso-PAF AT over time in either study group despite the lowering of TC, LDL-C and homocysteine levels. In the present study, daily consumption of two glasses of fortified milk (500 mL) offers 2.5 g phytosterols, 2.35 g polyunsaturated fatty acid (PUFA) of which 2.15 g are linoleic acid and only 0.2 g are alpha linolenic acid, 550 μg vitamin A, 200 mg vitamin C, 39.4 mg vitamin E, 12.5 mg vitamin B_6, 12.5 μg vitamin B_{12}, 500 μg folic acid, 110 mg magnesium and 125 μg selenium. Carrero et al. administered 500 mL/day of a semi-skimmed milk (1.9 g/100 mL) enriched in omega-3 PUFA, oleic acid, folic acid and vitamins E and B_6 for 8 weeks to thirty subjects aged between 45 and 65 years old with mild hyperlipidemia. Malondialdehyde, total antioxidant capacity, vitamin E and levels of oxidised LDL were measured in plasma, but no significant differences were observed at the end of the study period [51]. Moreover, linoleic and alpha linolenic acids added in the milk belong to omega-6 and omega-3 families of fatty acids, and are both called essential because human body lacks the enzymes needed to synthesize them. Alpha linolenic acid can produce EPA and DHA in humans through endogenous conversion, although not entirely efficient [52]. Furthermore, long carbon chain fatty acids, like EPA, DHA and arachidonic

Table 4. Comparison of PG with PhG at three months (bold values have a significance level of <0.05)

	PG ($n = 21$)	PhG ($n = 22$)	
	3 months mean (SD)	3 months mean (SD)	Difference (95% CI) at 3 months
Weight (kg)	73.6 (12.1)	78.5 (15.1)	−4.85 (−13.32 to 3.61)
BMI (kg/m^2)	27.2 (4.0)	27.8 (3.7)	−0.60 (−2.99 to 1.79)
Waist circumference (cm)	87.8 (10.5)	89.5 (11.6)	−1.67 (−8.48 to 5.14)
Waist to hip ratio	0.86 (0.07)	0.88 (0.08)	−0.02 (−0.06 to 0.03)
Body fat (% body weight)	35.9 (6.7)	34.9 (5.7)	1.06 (−2.79 to 4.91)
Fat free mass (kg)	47.0 (8.7)	51.3 (12.2)	−4.26 (−10.78 to 2.26)
Systolic blood pressure (mmHg)	119.5 (10.4)	122.9 (17.5)	−3.43 (−12.34 to 5.48)
Diastolic blood pressure (mmHg)	81.4 (8.2)	80.9 (10.9)	0.52 (−5.48 to 6.52)
Glucose (mg/dL)	91.6 (10.1)	93.2 (11.1)	−1.66 (−8.18 to 4.87)
Total cholesterol (mg/dL)	249.9 (37.0)	224.3 (29.0)	**25.68 (5.26** to **46.10)**
LDL cholesterol (mg/dL)	170.8 (36.7)	149.1 (24.8)	**21.67 (2.48** to **40.86)**
HDL cholesterol (mg/dL)	51.7 (14.3)	51.3 (12.9)	0.44 (−7.95 to 8.84)
TAGs (mg/dL)	136.4 (52.6)	119.4 (46.1)	17.02 (−13.40 to 47.43)
Apolipoprotein-A$_1$ (mg/dL)	157.0 (28.2)	158.0 (26.1)	−0.99 (−17.74 to 15.74)
Apolipoprotein-B (mg/dL)	118.2 (28.1)	103.3 (17.8)	**14.87 (0.44** to **29.31)**
Insulin (μUI/mL)	7.4 (5.1)	7.3 (4.4)	0.16 (−2.78 to 3.09)
White blood cell count (cells $\times 10^6$/μL)	6.4 (1.7)	6.5 (1.7)	−0.03 (−1.18 to 1.13)
Total antioxidant capacity (nmol/μL)	50.2 (4.3)	51.1 (3.0)	−0.90 (−3.16 to 1.36)
IL-6 (pg/mL)	0.8 (0.9)	0.4 (0.5)	0.36 (−0.26 to 0.98)
TNF-α (pg/mL)	10.5 (3.6)	12.4 (3.4)	−1.89 (−6.64 to 2.86)
CRP (mg/dL)	1.9 (1.5)	2.1 (1.6)	−0.17 (−1.13 to 0.79)
Homocysteine (μmo/L)	11.2 ((2.3)	9.6 (1.1)	**1.55 (0.43** to **2.68)**
Vitamin B$_{12}$ (pg/mL)	432.0 (145.1)	503.4 (157.3)	−71.36 (−161.9 to 19.27)
Folic acid (ng/mL)	9.8 (2.9)	14.5 (4.2)	**−4.72 (−6.97** to **−2.48)**
Lyso-PAF AT (nmol/min/mg)	4.6 (3.4)	5.2 (3.7)	−0.55 (−2.78 to 1.67)
Lp-PLA$_2$ (nmol/min/mL)	30.7 (8.4)	28.8 (6.5)	1.95 (−2.85 to 6.74)

acid, are more biologically potent than linoleic or alpha linolenic acids [53]. Data suggest that consumption of alpha linolenic acid should exceed 10 g/day in order to discern their anti-inflammatory properties, but even in this case, effects are weaker in comparison with those achieved after consumption of long chain omega-3 PUFAs [54]. In the study by Ravn-Haren et al. [55], 20 healthy men aged 18–40 years supplemented their usual diet with 480 μg Se contained in 1 L of enriched milk consumed daily for one week. After one week of Se supplementation, significant increases in serum Se levels were observed, but there was no increase in thrombocyte GPX activity was found when supplementing with Se-enriched milk. Furthermore, Se-enriched milk did not affect activities of plasma GR or GPX, erythrocyte GR, GPX or GST, or GR or GST measured in thrombocytes. Additionally, no treatment effect was found on plasma lipid resistance to oxidation, plasma TAG, CRP, and total, LDL- and HDL-cholesterol ($p > 0.05$, for all) [55].

Phytosterols, the main constituent of the enriched milk product, have not previously been tested for their effect on PAF metabolism and PAF levels. They are well known for their hypocholesterolemic actions but they have also been shown to have anti-inflammatory properties in both humans and experimental animals. Regarding the effects of phytosterols on PAF levels and metabolism, experimental and clinical data are lacking. Phytosterols should be tested in isolated leukocytes for PAF synthesis/degradation to establish an effect on lyso-PAF-AT and Lp-PLA2 activities. Furthermore, randomised clinical studies in healthy hypercholesterolemic humans are needed in order to study the effect of functional foods enriched solely with phytosterols. We should mention that combining phytosterols with other dietary components may lead to interactions that attenuate potential negative effects of the former on inflammatory mediators as shown in human studies [56]. Additionally, these studies would be preferable to apply a cross-over design to control for inter-individual differences. Finally, it would be very informative if different types of food enriched with phytosterols were tested for their efficacy in modulating inflammatory mediators, especially PAF. Taking all the above data into consideration, we assume that the type or quantity of the nutrients added to the milk were not adequate enough to induce changes in the activities of Lp-PLA$_2$ and lyso-PAF AT, known to be implicated in states of

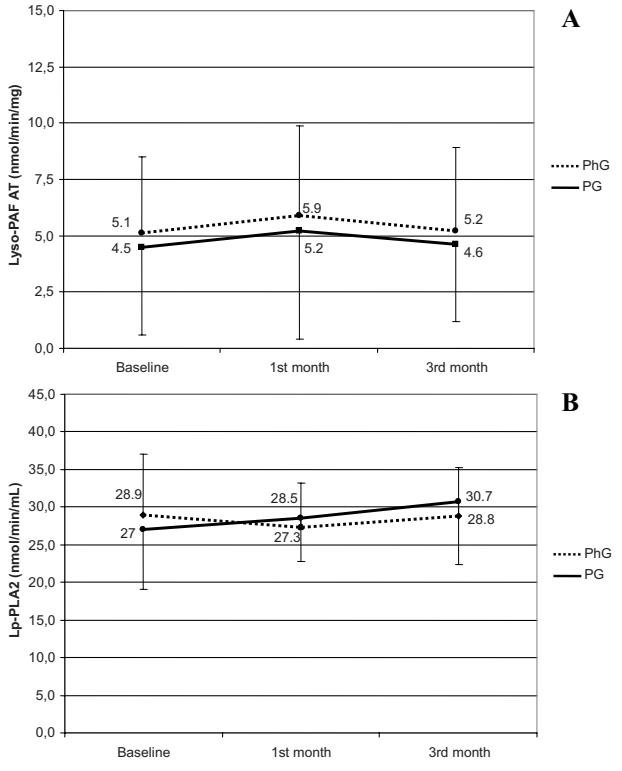

Figure 1. (A) Lyso-PAF AT activity in nmol/min/mg, at baseline, and at the end of the 1st and 3rd months of the intervention period for PG (straight line) and PhG (dot line) separately ($p_{\text{for effect of time}} > 0.05$). (B) Lp-$PLA_2$ (lipoprotein-associated phospholipase A_2) activity in nmol/min/mL at baseline, and at the end of the 1st and 3rd months of the intervention period for PG (straight line) and PhG (dot line) separately ($p_{\text{for effect of time}} > 0.05$).

inflammation and oxidative stress. Even the duration of the study was not long enough to influence markers of inflammation or oxidative stress.

There are certain limitations and strengths in our study. Activity of lyso-PAF AT is determined through a multi-step procedure during which the manipulation of samples might have influenced the results. Moreover, our lyso-PAF AT assay conditions did not discriminate between LPCAT1 and LPCAT2 acetylating activity. Furthermore, the intervention model used was parallel assignment where the whole sample was randomly allocated to one of the two study groups, allowing for inter-individual differences to interfere with the results. Another limitation could be the small sample size used which makes the effects harder to detect. Furthermore, our sample has been chosen from an urban population with hypercholesterolemia rendering our findings difficult to apply to other populations with different composition. Nonetheless, the present dietary intervention study is characterised by a randomised double-blind design that investigates the effects of two low-fat dairy products on the activity of two key metabolic enzymes of PAF, which permits to establish causal relations.

In conclusion, daily consumption of two glasses of a low-fat milk fortified with phytosterols, linoleic and alpha linolenic acids, antioxidant vitamins, magnesium and selenium for a period of 3 months by a sample of hypercholesterolemic adults has no effect on the specific activity of either lyso-PAF AT or Lp-PLA_2. This negative outcome does not necessarily mean that PAF and its metabolism is not a target of choice for the dietary attenuation of subclinical inflammation. The mild hypocholesterolemic effect of the intervention may not be capable to modify, directly or indirectly, the activities of the enzymes tested. In addition, other enzymes of the PAF's metabolism, sensitive to inflammatory stimuli, such as cytosolic phospholipase A_2 or cholinephosphotransferase may be more susceptible to such interventions. Finally, the determination of PAF, which has not been done in this study, could be helpful for the interpretation of the results and the estimation of the enriched milk ability to modulate PAF levels. Nevertheless, more studies to this direction are needed.

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