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# Evaluation of cardiovascular risk and oxidative stress parameters in hypercholesterolemic subjects on a standard healthy diet including low-fat milk enriched with plant sterols☆

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**Abstract**

A healthy diet and plant sterols (PS) are recommended for reducing low-density lipoprotein (LDL) cholesterol and, subsequently, the risk of premature cardiovascular disease. PS mediate a decrease in fat-soluble vitamin concentration, which can lead to a general impairment of antioxidative defenses and an increase in oxidative stress. Thus, we evaluated the effects of a healthy diet, including PS-enriched low-fat milk, on cardiovascular risk and oxidative stress parameters in hypercholesterolemic subjects.

This was a randomized parallel trial employing 40 subjects and consisting of two 3-month intervention phases. After 3 months on a standard healthy diet, subjects were divided into two intervention groups: a diet group and a diet+PS group (2 g/day). Lipid profile, apolipoproteins, high-sensitivity C-reactive protein and oxidative stress parameters were analyzed. Diet significantly reduced total and LDL cholesterol (4.0% and 4.7%, respectively), produced an increase in the level of β-carotene (23%) and improved the antioxidant capacity of LDL cholesterol particles (4.6%). PS induced a significant decrease in total cholesterol (6.4%), LDL (9.9%) and the apolipoprotein B100/apolipoprotein A1 ratio (4.9%), but led to a decrease in cryptoxanthin level (29%) without any change being observed in the antioxidant capacity of LDL cholesterol particles, total antioxidant status or lipid peroxidation. After 3 months, we observed the positive effect of including a PS supplement in dietary measures, as the lipoprotein-mediated risk of cardiovascular disease was reduced. Despite a decrease in the concentration of cryptoxanthin, no evidence of a global impairment of antioxidative defenses or an enhancement of oxidative stress parameters was found.
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*Keywords:* Plant sterols; Carotenoids; Lipid peroxidation; Antioxidant status; LDL oxidation

## 1. Introduction

Elevated concentrations of plasma low-density lipoprotein (LDL) cholesterol are a major risk for the development of premature cardiovascular disease. The first step towards reducing LDL cholesterol levels in individuals with mild to moderate hypercholesterolemia is to modify lifestyle and diet, in particular by reducing the intake of total and saturated fats [1]. The effects of such a measure can vary, with a mean decrease in LDL cholesterol of between 5% and 10% [2,3]. Among other recommendations, daily consumption of foods rich in plant sterols (PS) has been shown to reduce the plasma concentration of LDL cholesterol by 10–15% [4,5] without changing high-density lipoprotein (HDL) cholesterol or triglyceride (TG) levels [6,7]. The primary dietary sources of PS are fat-rich vegetables including vegetable oils and nuts — foods that are usually consumed in the Mediterranean region at maximum concentrations of 375 mg/day [8]. PS lower blood cholesterol levels principally through competitive replacement of cholesterol in bile salt micelles, resulting in a reduced absorption of unesterified cholesterol from the small intestine [5]. Thus, hypercholesterolemic patients are recommended a daily intake of PS in the range of 1–2 g/day [1], which should be accompanied by other dietary measures [9].

In most previous studies, it has been suggested that the dispersion of PS in different food forms substantially affects the degree of LDL cholesterol decrease achieved [4]. However, few studies have examined the hypocholesterolemic effect of PS

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supplementation in low-fat dairy beverages in the absence of lipid-lowering medication [6,7,10,11].

Chronic inflammation and oxidative stress are thought to promote the development and progression of atherosclerosis. A risk factor recently related to the inflammatory hypothesis of cardiovascular heart disease (CHD) is high-sensitivity C-reactive protein (hsCRP) [12,13]. However, there is a lack of data regarding the potential impact of PS supplementation on this marker [14–17]. Oxidative stress is produced by an imbalance between prooxidants (free radicals or reactive oxygen species) and antioxidants (enzymes and vitamins), which occurs as a result of an increase in prooxidants. Oxidation of LDL cholesterol has been implicated at both early and late stages of the pathogenesis of atherosclerosis, during which plaque rupture leads to further clinical events. Attack of reactive oxygen species against biomembranes or lipoproteins leads to oxidative destruction of polyunsaturated fatty acids by a process called lipid peroxidation, of which malondialdehyde (MDA) is a product and, therefore, an indicator of oxidative stress in cells and tissues.

The potential effect of a daily consumption of PS on the level of oxidative stress is yet to be established. It is possible that the decrease in the serum concentration of fat-soluble vitamins produced by a PS dietary supplement [6,11,18,19] undermines antioxidative defenses and, therefore, increases oxidative stress. In contrast, due to the decrease in the serum concentration of LDL cholesterol that is also produced by PS consumption (caused by a drop in the number of circulating LDL cholesterol particles susceptible to oxidation), atherosclerotic lesions are less likely to form. Most related studies have been performed over relatively short periods of time (a few weeks) and without introducing dietary measures prior to or during administration of PS [6,7,19,20]. Therefore, the objectives of the present study were to analyze, in a long-term period, (a) the additive effect of dietary measures and daily consumption of 2 g of PS on cardiovascular risk parameters in moderate hypercholesterolemic subjects and (b) the effect of daily intake of PS plus a healthy diet on oxidative stress parameters.

## 2. Materials and methods

### 2.1. Subjects

Subjects with untreated moderate hypercholesterolemia were recruited from the Service of Endocrinology and Nutrition of University Hospital Dr. Peset (Valencia, Spain). Patients between the ages of 18 and 75 years (inclusive) were eligible for inclusion in the study. Further inclusion criteria were as follows: a serum LDL cholesterol concentration of between 160 and 190 mg/dl in patients with less than two cardiovascular risk factors or a serum LDL cholesterol concentration of 130–160 mg/dl in patients with two or more cardiovascular risk factors, and a TG concentration of <400 mg/dl in both cases. Cardiovascular risk factors were defined as follows: age (≥45 years in men and ≥55 years in women), smoking habit, hypertension (≥140/90 mmHg), diabetes mellitus, HDL cholesterol concentration of <40 mg/dl, and a family history of cardiovascular disease. Exclusion criteria were pregnancy or lactation, change of oral contraceptive formulation, severe disease, history of cardiovascular disease or chronic inflammatory disease, hypersensitivity to milk proteins and use of lipid-lowering medication.

The study protocol was carefully explained to all subjects before they provided their written informed consent. The study was approved by the Ethics Committee of the hospital. On the basis of these criteria, 40 subjects were included in the study and were randomly assigned to the healthy diet group or the healthy diet plus PS group. No subject dropped out of the study prior to its conclusion.

### 2.2. Study design

The study consisted of a randomized parallel trial of two 3-month intervention phases. All subjects were submitted to a 3-month run-in period of a standard healthy diet recommended by the Adult Treatment Panel III [1] in order to stabilize dietary patterns prior to treatment. During the following 3 months of the study, two intervention groups were evaluated: an experimental group, whose members consumed 2g/day PS in 500 ml of low-fat milk, and a control group, which followed the dietetic guidelines of the Adult Treatment Panel III and whose diet included 500 ml/day standard low-fat milk.

Clinical examination was performed during the first visit (selection and inclusion), after the stabilization period (3 months) and at the end of the trial (6 months).

Participants were given dietary guidance throughout the trial by an experienced dietician and received detailed written and oral instructions about the diet, including the precise amounts of food to be eaten and the quality of food, according to the main food groups. The recommended diet included total cholesterol (<200 mg/day), saturated fat (≤7% of daily total energy), simple sugar of low content (<10%), enhanced consumption of monounsaturated fatty acids from olive oil, ω-3 fatty acids from fish, two to three pieces of fruit and unlimited vegetables.

With respect to daily energy intake, 2000 kcal were proposed for men and 1700 kcal were proposed for women (18–19% proteins, 52–53% carbohydrates and 29–30% fats, containing up to 20% monounsaturated fatty acid with respect to total energy and 20–30 g of dietary fiber).

Adherence to the diet was monitored by means of 3-day food records (compiled on weekdays) and 24-h diet recall at baseline, 3 months and 6 months (carried out during appointments with the dietician). Food intake was converted into energy and nutrients with the help of the Spanish Food Composition Table [21]. The composition database was created with AYS44 Diet Analysis software (ASDE SA, Valencia, Spain). The diet recalls and records revealed no deviations from the guidelines during the course of the study. Subjects were encouraged to maintain their normal pattern of activity.

The PS-enriched milk was produced by Unilever (Spain) and packed in white containers. In addition to 0.4 g of vegetal sterols, every 100 ml of milk provided 3.2 g of protein, 4.7 g of carbohydrate, 1.8 g of fat (0.25 g of saturated fat, 0.50 g of monounsaturated fat and 1.05 g of polyunsaturated sat) and 48 kcal of energy. The PS consisted of vegetable-oil-based sterols esterified with sunflower oil fatty acids and contained β-sitosterol (70%), campesterol (15%) and β-sitostanol (10%).

The control group received a commercially available low-fat milk with similar macronutrient composition and energy intake as that consumed by the PS group, but which did not include vegetable sterols. Subjects were recommended to consume the milk two times per day with meals.

Compliance was evaluated by interviewing the subjects and by counting the unopened and unconsumed product packages returned to the clinic, and was recorded as percentage of the scheduled servings consumed. Noncompliance was defined as consumption of <80% of the scheduled servings during the study period.

### 2.3. Blood sampling

Venous blood samples were collected from the subjects after 12 h of overnight fasting at baseline, 3 months and 6 months. To separate serum from blood cells, we immediately centrifuged samples at 2000×*g* for 15 min at 4°C. Freshly separated serum was employed to determine lipid profile and hsCRP, and the remaining aliquots of serum were stored at −80°C until assessment for susceptibility to oxidation in LDL cholesterol particles, total antioxidant status, measurement of major carotenes and MDA concentration.

#### 2.3.1. Cardiovascular risk parameters

Total cholesterol and TG were measured by means of enzymatic assays [22,23], and HDL cholesterol concentrations were recorded with a Beckman LX-20 autoanalyzer (Beckman Coulter, La Brea, CA, USA) using a direct method [24]. The intraserial variation coefficient was <3.5% for all determinations. When TG values were under 300 mg/dl, LDL cholesterol concentration was calculated using the method of Friedewald et al. [25]. Non-HDL cholesterol concentration was obtained by calculating the difference between total cholesterol and HDL cholesterol. Apolipoprotein A1 (ApoA) and apolipoprotein B100 (ApoB) were determined by immunonephelometry (Dade Behring BNII, Marburg, Germany) to have an intra-assay variation coefficient of <5.5%.

hsCRP levels were quantified by a latex-enhanced immunonephelometric assay (Behring Nephelometer II; Dade Behring, Inc., Newark, DE, USA) to have an intra-assay coefficient of variation of 8.7% and a sensitivity of 0.01 mg/L.

#### 2.3.2. Oxidative stress parameters

LDL cholesterol oxidation was measured by monitoring the formation of conjugated dienes after *in vitro* $Cu^{2+}$-catalyzed oxidation. In short, LDL cholesterol was isolated using a dextran sulfate LDL adsorption column (Liposorber® LA-15 System Facts). Salts and EDTA were removed by passing the LDL samples through a polyacrylamide column (Bio-Gel P6). Samples were diluted to 100 μg/ml LDL ApoB with a phosphate-buffered saline (pH 7.4) and incubated at 37°C with 5 μM/L $Cu^{2+}$ for 5 h. Conjugated diene formation was measured by continuous spectrophotometric monitoring at 234 nm. Lag time was estimated as the incubation time corresponding with the intersection of two lines representing changes in optical density: one through the initial slowly rising curve, which represented consumption of endogenous antioxidants, and the other through the rapidly rising curve, which represented the propagation phase of LDL oxidation [26].

Total antioxidant status was measured spectrophotometrically at 600 nm using the Trolox Equivalent Antioxidant Capacity assay (Randox, San Francisco, CA, USA), a method developed by Miller et al. [27], following the manufacturer's instructions. The intra-assay and interassay coefficients of variation were 1.7% and 3.5%, respectively.

Serum concentrations of hydrocarbon carotenoids (β-carotene and lycopene) and oxygenated carotenoids (lutein and β-cryptoxanthin) were determined at the beginning and at the end of the trial by employing an HPLC method described by

Olmedilla et al. [28], with slight modifications. In brief, 400 μl of serum samples was mixed with an equivalent quantity of ethanol and an internal standard solution (0.6 μg/ml) of β-apo-8-carotenal. Fat-soluble compounds were extracted twice with *n*-hexane (1 ml), each time stabilized with 0.01% butylated hydroxytoluene (BHT), and the extract was evaporated to dryness under nitrogen. The samples were dissolved once more in 150 μl of tetrahydrofurane stabilized with BHT 0.01% for HPLC reversed-phase analysis (Waters, Eschborn, Germany). We employed an isocratic method with dichloromethane/acetonitrile/methanol (20:70:10, vol/vol/vol) as mobile phase, with a flow of 1.3 ml/min. Detection was performed at 450 nm with AUFS 0.005.

Free-radical formation through lipid peroxidation was determined using MDA thiobarbituric-acid-reactive substances, as described by Yagi [29]. In short, after the separation of proteins from MDA, a fluorescent complex (MDA-TBA) was formed, and fluorescence between the excitation wavelength and the emission wavelength (544–590 nm) was measured in a Fluoroskan Ascent FL (Thermo Electron Corporation).

### 2.4. Statistical analysis

The sample size was 20 subjects per group in order to provide 80% statistical power and thus detect differences between the two groups in values of the primary efficacy criterion (LDL cholesterol variation) equal to or greater than 15 mg/dl, assuming a common S.D. of 23 mg/dl.

Data are expressed as mean±S.D. (for tables) or S.E.M. (for figures) for parametric data, and as medians with 25th and 75th percentiles, respectively, for nonparamentric data. Statistical analyses were conducted using GraphPad Prism software version 4 (GraphPad Software, San Diego, CA, USA). In the first intervention phase, paired Student's *t* test was used to analyze differences between baseline and 3 months. In the second intervention phase, between-groups and within-group differences were analyzed using two-factor repeated-measures analyis of variance (ANOVA) followed by Student's *t* test for parametric data, and using Friedman test followed by Wilcoxon signed-rank tests for nonparametric data. $P<.05$ was considered significant.

## 3. Results

A total of 40 subjects in the age range of 24–69 years completed the study. Anthropometric parameters of the subjects before and after addition of PS to their diet are presented in Table 1. Since there were no significant differences in age, weight, body mass index (BMI), waist–hip index or systolic/diastolic blood pressure between the diet group and the diet+PS group at baseline, values are presented as the mean of the 40 subjects. Men and women were distributed similarly between the two groups: 30% of the diet group and 25% of the diet +PS group were composed of men (data not shown). During the first 3 months of the trial, subjects followed dietary measures to homogenize their serum parameters prior to the introduction of PS into their diet. Diet significantly reduced weight and BMI in the entire population (Table 1). No significant changes were observed between the anthropometric parameters of subjects receiving beverages enriched with PS and the anthropometric parameters of subjects not receiving beverages enriched with PS (Table 1).

During the first 3 months of the standard healthy diet, subjects showed a significant decrease of 4–5% in total cholesterol, LDL cholesterol and non-HDL cholesterol. Enrichment with PS induced an additional decrease of 6.4%, 9.9% and 7.1%, respectively, which represented a total drop from 10.5%, 14.6% and 14.1%, respectively, at the onset of the study. These lipid parameters were maintained in subjects undergoing dietary therapy only, therefore marking significant differences with the other group (Fig. 1A, Table 2). ApoB/ApoA ratio, considered an even better marker of CHD than lipid concentration per se [13], decreased in subjects receiving low-fat milk enriched with PS (Fig. 1B). No changes in HDL cholesterol, TG, ApoB or hsCRP were found in any of the groups (Fig. 1C, Table 2).

The serum concentrations of liposoluble antioxidants (measured as carotenoid levels) in subjects at the beginning and at the end of the trial are shown in Table 3. Lutein and lycopene concentrations were similar in the two groups at baseline and at the end of the study. However, in the group whose diet was not enriched with PS, we observed a significant increase of 23% in β-carotene after 6 months, whereas no change was observed in the other group. In subjects supplemented with PS, there was a significant decrease of 29% in the level of cryptoxanthin with respect to baseline levels, showing that the supplement of PS had reduced carotenoid levels, either directly or indirectly, by preventing the increase in antioxidants provoked as a consequence of the healthy diet. This effect was evident in LDL cholesterol particles' susceptibility to oxidation, since the diet alone improved lag time by 4.6% but did not exert the same effect on the PS group (Fig. 2A). However, the enhancement of endogenous antioxidants observed as a result of adherence to the diet did not alter the total antioxidant status of the subjects (as can be seen in Fig. 2B) or lipid peroxidation (an indicator of oxidative stress; as shown in Fig. 2C).

## 4. Discussion

Two of the strengths of this randomized parallel study are its duration and the fact that participants were given dietary guidance by an experienced dietician throughout the trial. Our results show that the diet followed by subjects reduced total cholesterol, LDL cholesterol and non-HDL cholesterol, as well as increased levels of liposoluble antioxidants and improved the antioxidant capacity of LDL cholesterol particles. In the second phase of intervention, enrichment with PS induced an additional decrease in total cholesterol, LDL cholesterol, non-HDL cholesterol and ApoB/ApoA ratio. Furthermore, it produced a decrease in the levels of liposoluble

Table 1
Anthropometric parameters of subjects before and after addition of PS to the diet

| | | Baseline | 3-month diet | Postrandomized | 6 months |
|---|---|---|---|---|---|
| Age (years) | | 50.0±10.2 | –– | A: – | A: – |
| | | | | B: – | B: – |
| Weight (kg) | | 74.6±16.1 | 73.7±15.7* | A: 73.2±11.6 | A: 74.0±12.0 |
| | | | | B: 73.8±20.3 | B: 73.8±21.7 |
| BMI (kg/m$^2$) | | 28.3±4.7 | 27.7±4.5* | A: 27.8±4.6 | A: 28.0±4.8 |
| | | | | B: 27.6±4.9 | B: 27.6±5.2 |
| Waist–hip index | Male | 0.96±0.09 | 0.96±0.07 | A: 0.95±0.07 | A: 0.96±0.09 |
| | | | | B: 0.98±0.07 | B: 0.97±0.04 |
| | Female | 0.85±0.12 | 0.83±0.05 | A: 0.85±.04 | A: 0.86±0.04 |
| | | | | B: 0.81±0.04 | B: 0.84±0.09 |
| Systolic blood pressure (mmHg) | | 131±20 | 126±15 | A: 129±16 | A: 131±15 |
| | | | | B: 130±13 | B: 130±19 |
| Diastolic blood pressure (mmHg) | | 82±11 | 81±14 | A: 86±10 | A: 85±10 |
| | | | | B: 85±10 | B: 86±14 |

Data are expressed as mean±S.D.
After the first 3 months of dietary therapy, 40 subjects were randomized into two groups (20 subjects each group): one group followed dietary therapy (A) whereas the diet of the other group was enriched with 2g/day PS (B).
* $P<.05$, when compared using paired Student's *t* test in the first 3 months of the study, or two-factor repeated-measures ANOVA followed by paired Student's *t* test after the addition of PS.

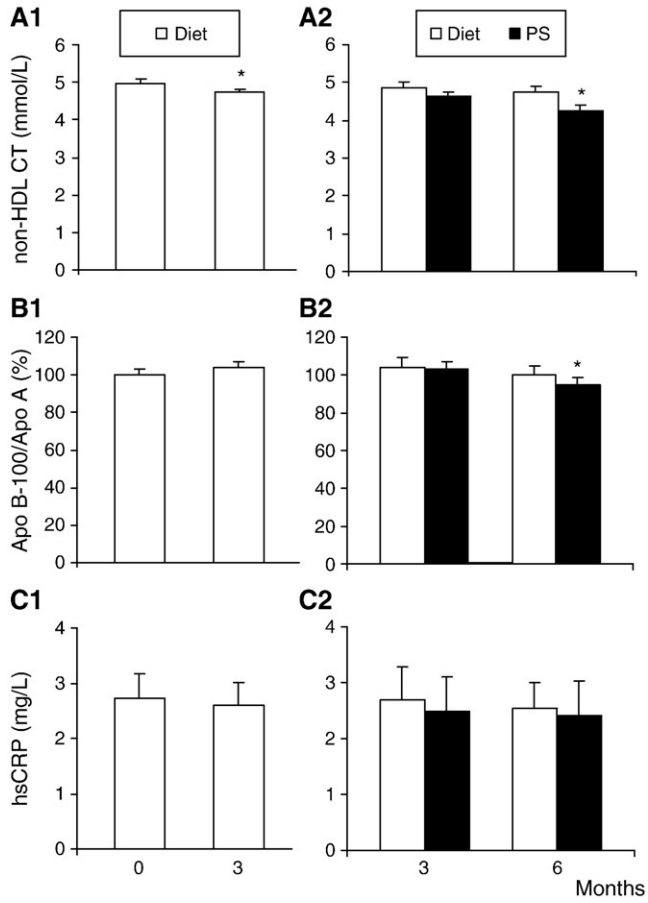


Fig. 1. Cardiovascular risk parameters in subjects before and after the addition of PS to the diet. (A1) Non-HDL cholesterol concentration during the first 3 months, corresponding with the diet standardization period. (A2) Non-HDL cholesterol during the following 3 months, corresponding with the addition of PS to the diet. (B1) The ApoB/ApoA ratio during the first 3 months, corresponding with the diet standardization period. (B2) The ApoB/ApoA ratio during the following 3 months, corresponding with the addition of PS to the diet. (C1) hsCRP concentration during the first 3 months, corresponding with the diet standardization period. (C2) hsCRP concentration during the following 3 months, corresponding with the addition of PS to the diet. Bars represent the mean±S.E.M. of 40 subjects in the first phase and of 20 subjects in the second phase after addition of PS to the healthy diet. *$P<.05$ when compared using paired Student's $t$ test in the first 3 months of the study, or two-factor repeated-measures ANOVA followed by paired Student's $t$ test after the addition of PS. Non-HDL CT: non-HDL cholesterol.

Table 3
Serum concentration of carotenoids before and after the addition of PS to the diet

| | | Lutein (μmol/L) | Cryptoxanthin (μmol/L) | Lycopene (μmol/L) | β-Carotene (μmol/L) |
|---|---|---|---|---|---|
| Diet ($n$=20) | Initial | 0.440±0.202 | 0.628±0.581 | 0.903±0.363 | 0.443±0.231 |
| | Final | 0.482±0.172 | 0.447±0.217 | 1.045±0.412 | 0.549±0.261* |
| Diet+PS | Initial | 0.450±0.216 | 0.783±0.421 | 0.788±0.397 | 0.659±0.436 |
| ($n$=20) | Final | 0.429±0.181 | 0.554±0.284* | 0.816±0.358 | 0.712±0.393 |

Data are expressed as the mean±S.D. of 40 subjects at the beginning of the study and after 6 months of diet or a combination of diet+PS.

* $P<.05$ when compared using a two-factor repeated-measures ANOVA followed by paired Student's $t$ test.

antioxidants without changing the antioxidant capacity of LDL cholesterol particles, total antioxidant status or lipid peroxidation.

Patients with hypercholesterolemia are advised, first and foremost, to limit their intake of total and saturated fats, in particular fats obtained from dairy products [1]. In the present study, the greatest change in total and LDL cholesterol occurred after the first 3 months. No additional decrease in serum total cholesterol or LDL cholesterol concentration was recorded after this period. The approximate 5% decrease in LDL cholesterol observed is in accordance with that estimated by Hunninghake et al. [2]. In fact, Bae et al. [30] also reported a similar pattern of response after 6 weeks of diet intervention, but observed no further significant decrease in the following 12 weeks.

Importantly, and in line with the results of several studies [4,18], we have demonstrated that the ingestion of approximately 2 g/day PS is associated with a 7.0–10.0% decrease in LDL cholesterol levels. PS are included in the National Cholesterol Education Program (NCEP) [1] and the American Heart Association/American College of Cardiology [31] guidelines for secondary prevention of CHD. For each percentage of LDL cholesterol reduction, the risk of CHD disease is reduced by 2–3%. In the second intervention phase of our trial, ingestion of low-fat milk enriched with PS reduced total cholesterol concentration and LDL cholesterol by 6.4% and 9.9%, respectively, whereas dietary measures alone reduced these values by only 2.1% and 1.8%, respectively. Therefore, it would appear that, in moderately hypercholesterolemic subjects, the combination of dietary therapy and PS-enriched low-fat milk may reduce the need for cholesterol-lowering drug therapy.

Recent data suggest that an increase in ApoB and hsCRP, and a decrease in ApoA are important risk factors for CHD [12,13]. To our knowledge, only two studies of PS have evaluated all these parameters [14,16]. Their findings are controversial; for instance, a daily intake of ~2 g of PS had no effect on ApoA or hsCRP, but significantly affected ApoB levels. In the present study, serum levels of

Table 2
Lipoprotein profile of subjects before and after the addition of PS to the diet

| | Baseline | 3-month diet | Postrandomized | 6 months |
|---|---|---|---|---|
| Total cholesterol (mmol/L) | 6.40±0.62 | 6.14±0.70* | A: 6.16±0.78 | A: 6.03±0.78 |
| | | | B: 6.11±0.62 | B: 5.72±0.75* |
| LDL cholesterol (mmol/L) | 4.43±0.57 | 4.22±0.57* | A: 4.25±0.67 | A: 4.17±0.73 |
| | | | B: 4.20±0.49 | B: 3.78±0.47* |
| HDL cholesterol (mmol/L) | 1.42±0.31 | 1.40±0.34 | A: 1.32±0.31 | A: 1.30±0.28 |
| | | | B: 1.50±0.36 | B: 1.45±0.39 |
| TG (mmol/L) | 1.08 (0.73, 1.59) | 1.05 (0.61, 1.31) | A: 1.15 (0.86, 1.57) | A: 0.94 (0.75, 1.94) |
| | | | B: 0.85 (0.59, 1.29) | B: 0.88 (0.60, 1.13) |
| ApoB (g/L) | 1.17±0.16 | 1.14±0.16 | A: 1.20±0.17 | A: 1.17±0.16 |
| | | | B: 1.08±0.11 | B: 1.05±0.14 |

Data are expressed as mean±S.D. for parametric data, and as medians (25th and 75th percentiles) for nonparametric data.
After the first 3 months of dietary therapy, 40 subjects were randomized into two groups (20 subjects each group): one group followed dietary therapy (A) whereas the diet of the other group was enriched with 2g/day PS (B).

* $P<.05$, when compared using paired Student's $t$ test in the first 3 months of the study, or two-factor repeated-measures ANOVA followed by paired Student's $t$ test after the addition of PS. No significant difference was found in nonparametric data after Wilcoxon signed-rank tests or Friedman test.

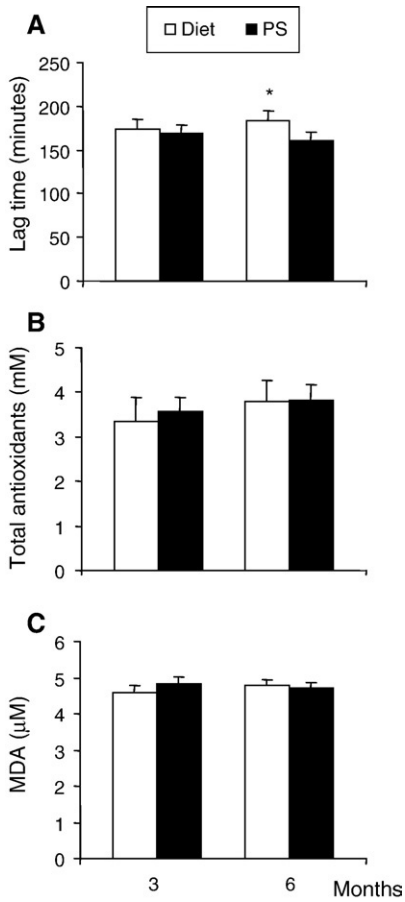


Fig. 2. Oxidative stress parameters in subjects after a 3-month supplementation of the standard healthy diet with PS. (A) Susceptibility to oxidation in LDL particles represented as lag time. (B) Global antioxidant status represented as total antioxidants. (C) Lipid peroxidation represented as MDA. Bars represent the mean±S.E.M. of 9 subjects (A), 12–16 subjects (B) and 18 subjects (C). $^{*}P<.05$ when compared using a two-factor repeated-measures ANOVA followed by paired Student's *t* test.

ApoB remained unaltered, suggesting that PS treatment affected the cholesterol content of LDL particles rather than their number. This finding is in accordance with those from previous studies [14,32], although it is true that others have reported a decrease [16,19]. We observed that the ApoB/ApoA ratio dropped by 4.9%. Interestingly, this ratio has recently been proposed as the strongest risk marker for vascular disease, even stronger than lipid concentration per se [13].

The presence of increased hsCRP has been associated with a lower decrease in total and LDL cholesterol [33]. Since the addition of PS to the standard healthy diet did not change the serum levels of hsCRP, we would suggest that PS do not mediate the decrease in total and LDL cholesterol through a reduction of inflammatory response. Indeed, previous reports have confirmed that levels of hsCRP are unaltered by addition of PS to different fat matrices [15–17]. Considered together, our findings suggest that supplementation with PS provides greater protection against CHD risk factors than does a healthy diet.

The precise mechanism by which PS lower serum carotenoid concentration and its possible long-term health implications are yet to be determined. A possible explanation for the decrease in serum carotenoid concentration after PS ingestion could be that the levels of these antioxidants were increased in blood cells or others tissues, as it has been shown with other antioxidants [34]. In our study, only serum cryptoxanthin concentration was significantly reduced following consumption of PS. Data on cryptoxanthin concentrations following addition of PS to a healthy diet are inconsistent, with some studies reporting no effect [11,19] and with others observing a decrease in serum levels [35]. These discrepancies could be explained by seasonal consumption of citrus fruits (the main source of β-cryptoxanthin) [36], gender differences (a more marked decrease has been reported in women after PS ingestion) [37] and the implication of the apolipoprotein E genotype in the variability of β-cryptoxanthin concentrations [38]. In contrast, the concentration of β-carotene increased in the control group (which adhered to the standard healthy diet), but remained unaltered in the group supplemented with PS. Colgan et al. [19] demonstrated that the β-carotene concentration in hypercholesterolemic subjects after the NCEP Step 1 diet tended to rise, whereas it fell after 3 weeks of PS consumption. The discrepancies between our study and Colgan et al.'s study are probably due to differences in the duration of the dietary therapies. Results similar to ours were reported by Noakes et al. [39], who observed that plasma β-carotene concentrations were maintained when subjects consumed several daily rations of a high-carotenoid vegetable or fruit, in addition to intake of spreads containing sterol or stanol esters. Many studies have shown that when serum β-carotene concentrations are standardized according to LDL cholesterol concentration, there is no significant effect of PS on lipid-standardized β-carotene [11,15,19]. However, the fact that ApoB was unaltered in our trial indicates that the number of LDL cholesterol particles did not change.

Oxidative stress is implicated in the development of atherosclerosis and other chronic pathologies [40]. It has been suggested that even a minor decrease in β-carotene concentration can be proatherogenic [41]. However, antioxidants include numerous substances or enzymes and liposoluble vitamins that may interact synergistically. Analysis of one component independently of the others ignores a possible combined action and, therefore, does not allow systemic oxidative stress to be accurately estimated. For that reason, we evaluated the effect of PS on total antioxidant status, on the capacity of *in vitro* antioxidants to protect LDL cholesterol particles against oxidation and on lipid peroxidation. Interestingly, *in vitro* LDL cholesterol oxidation resistance was higher among the subjects who did not receive the PS supplement, in accordance with the increase in the concentration of liposoluble vitamins (β-carotene) associated with healthy diets enriched with fruits and vegetables. There is no consistent effect of diet on the susceptibility of LDL cholesterol to oxidation. In one previous study, a vegetarian diet improved lag time to values similar to those achieved in our study [42], whereas in another report, no effect was obtained with a low-fat diet enriched with vegetable oils [43]. In our study, addition of PS did not alter the susceptibility of LDL cholesterol to oxidation, as reported in a previous publication [44]. As far as we know, ours is the first study in which total antioxidant status has been evaluated after addition of PS to a healthy diet. Despite changes in the concentration of serum liposoluble antioxidants and the capacity of *in vitro* antioxidants to protect LDL cholesterol particles against oxidation, no changes in serum total antioxidant status or oxidative stress (assessed as lipid peroxidation) were detected. There is scarcity of evidence regarding the effect of PS on markers of lipid peroxidation levels, and the results that do exist are controversial. Studies spanning relatively short periods of time (4–8 weeks) have reported a decrease in lipid peroxidation parameters [32,45,46], whereas no changes were observed when PS were consumed over 16 weeks [17], in line with our data. Thus, our results suggest that the slight decrease in the liposoluble antioxidant concentration observed with PS did not affect the antioxidant capacity of LDL cholesterol particles, total antioxidant status or lipid peroxidation.

In summary, our results confirm and extend the positive effect of including a PS supplement in dietary measures by demonstrating that it reduces the lipoprotein-mediated risk of cardiovascular disease. The

PS-mediated decrease in fat-soluble antioxidants does not lead to a global impairment of antioxidative defenses or an enhancement of oxidative stress, although it does impede improvement in the resistance of LDL cholesterol particles to oxidation associated with dietary therapy. Subjects did not report any adverse effect after 3 months of daily consumption of PS within a low-fat matrix. Thus, this approach would seem to represent a useful additional therapeutic measure to the classic low cholesterolemic diet when aiming to reduce cardiovascular risk.

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