Anti-inflammatory and cardioprotective effects of n-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals

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**Abstract**

Background: Risk factors of cardiovascular disease such as lipid aberrations, hypertension, abdominal adiposity and elevations in systemic inflammation, are prominent aetiologies in hyperlipidemia. Supplementation with n-3 PUFA is associated with a reduction in cardiovascular events through its hypotriglyceridemic, anti-aggregatory and anti-inflammatory properties. Plant sterols have potent hypcholesterolemic properties, although their effect on the inflammatory cascade is uncertain. This study investigated the effect of combined supplementation with n-3 PUFA and plant sterols on cardiovascular risk factors, blood pressure, body composition, markers of systemic inflammation and overall risk, in hyperlipidemic individuals.

Methods: The study was a 3-week randomised, double-blind, placebo-controlled, 2 \(\times\) 2 factorial design, in four parallel groups. Sixty hyperlipidemic participants were randomised to receive either sunola oil or 1.4 g/d n-3 PUFA capsules with or without 2 g plant sterols per day.

Results: The combination of n-3 PUFA and plant sterols reduced several inflammatory markers. High sensitivity C-reactive protein (hs-CRP) was reduced by 39\% (\(P=0.009\)), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) by 10\% (\(P=0.02\)), interleukin-6 (IL-6) by 10.7\% (\(P=0.009\)), leukotriene B\(_4\) (LTB\(_4\)) by 29.5\% (\(P=0.01\)) and adiponectin was increased by 29.5\% (\(P=0.05\)). Overall cardiovascular risk was reduced by 22.6\% (\(P=0.006\)) in the combination group.

Conclusion: We have demonstrated, for the first time that dietary intervention with n-3 PUFA and plant sterols reduces systemic inflammation in hyperlipidemic individuals. Furthermore, our results suggest that reducing inflammation provides a potential mechanism by which the combination of n-3 PUFA and plant sterols are cardioprotective.

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1. Introduction

The cardioprotective effects of n-3 polyunsaturated fatty acids (PUFA) as evidenced by lower rates of mortality, are associated with greater fish oil consumption. Several mechanisms have been proposed to explain how n-3 PUFA might beneficially affect risk factors implicated in the pathogenesis of atherosclerosis and thrombotic disease. These include improving vascular reactivity, decreasing platelet aggregation, lowering plasma triglycerides, decreasing blood pressure, preventing arrhythmias and reducing inflammation [1].

Current evidence supports a central role for inflammation in all phases of the development of atherosclerosis [2]. Circulating markers of inflammation, such as C-reactive protein (CRP), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and some interleukins (IL-6, IL-1) correlate with propensity to develop cardiovascular events. The relationship between inflammation and plasma lipid aberrations, as seen in hyperlipidemia, is not as yet known. The tendency of cardiovascular risk factors to cluster in hyperlipidemic individuals, would suggest that many of these patients are likely to benefit from interventions targeted at multiple risk factors. Therefore, to explore this relationship, we examined the changes in inflammatory markers following plasma lipid modification, by supplementing the diet with n-3 PUFA and/or plant sterols in hyperlipidemic individuals.

Fish oils, rich in the long-chain n-3 PUFA, eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), have hypotriglyceridemic and anti-arrrhythmic properties [3]. An increased consumption of n-3 PUFA results in increased proportions of those fatty acids in immune cell phospholipids, partly at the expense of arachidonic acid [4]. The functional significance of this, is that mediators formed from n-3 PUFA are deemed to inhibit

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early atherogenic events, by reducing cytokine-induced expression of pro-atherogenic/inflammatory proteins in the endothelium [5]. Epidemiological evidence shows the consumption of n-3 PUFA protects against CVD within Western populations [6] and has the capacity to amend several cardiovascular risk factors, including elevated blood pressure, plasma lipid profile, platelet aggregation and endothelial dysfunction.

A recently renewed interest in plant sterols as a ‘nutraceutical’, stems from its efficacious lipid-lowering property and heightened by its commercial availability, now readily added to fat spreads, yoghurt and milk products [7,8]. It is well accepted that plant sterols lower plasma total and low-density lipoprotein (LDL) cholesterol, by competing with dietary and biliary cholesterol for intestinal absorption, but research on their effect in the inflammatory process, is scarce [9]. Given that patients with elevated inflammatory levels are at an increased risk of developing diabetes, hypertension and CVD, the effect of lipid-lowering foods on circulating inflammatory markers warrants investigation. The possibility that plant sterols may improve cardiovascular risk factors is speculative, but deserves further consideration.

The present study was conducted to evaluate the cardioprotective effects as measured by improvements in blood pressure, body composition, markers of systemic inflammation and overall CVD risk, subsequent to plasma lipid-lowering by combined supplementation of n-3 PUFA and plant sterols in hyperlipidemic individuals.

2. Subjects and methods

A detailed methodology of this study has previously been described, along with participant demographics, plasma fatty acid concentration and plasma lipid profiles [10].

2.1. Study design and intervention

Participants were randomised into one of four groups, using permuted block randomisation, stratified for gender. Two groups took 4 g placebo (sunola oil, SO) capsules per day and two groups took 4 g tuna oil capsules (NuMega Ingredients Pty Ltd., QLD, Australia) per day, providing 80 mg 20:5n-3 and 280 mg 22:6n-3 in each 1 g capsule, in a triacylglycerol form. One of the groups assigned to each oil treatment also consumed 25 g/d of a plant sterol-enriched spread (Logicol® original) containing 2 g/d plant sterols. The four groups were identified as: placebo (SO; n = 15), fish oil (FO; n = 15), plant sterol (SOP; n = 15) and fish oil plus plant sterol combination (FOP; n = 15). The study period was 3 consecutive weeks and participants were instructed to maintain their habitual diet throughout.

Capsule containers were labelled with a blind code, so that neither principle researchers nor volunteers knew what capsules were being consumed. Participants were instructed to take four capsules daily with their main meals. The plant sterol spread was provided as individually portioned tubs (25 g each), comprising predominantly of β-sitosterol, campesterol and stigmasterol. Participants were asked to consume one tub daily, which was to replace all habitual margarine/butter consumption. Compliance was monitored by weighing of tubs and capsule count-back before and after the trial period, regular telephone contact, evaluation of dietary records (analysed with FoodWorks, Xyris®, QLD, Australia) and analysis of plasma fatty acid composition.

2.2. Participants

Sixty participants (male n = 27 and female n = 33) with established combined hyperlipidemia, aged 35–70 years were enrolled from the general community of Newcastle, Australia. Primary inclusion criteria included plasma total cholesterol concentration ≥ 6.0 mmol/L (231 mg/dL), triacylglycerol concentration ≥ 1.5 mmol/L (132 mg/dL), no previous cardiovascular events, diabetes mellitus, chronic inflammatory disease, hypertension (≥ 140/95 mm Hg) or liver/renal disease, not taking anti-inflammatory or hypolipidemic medication, not consuming a plant sterol-enriched spread and/or fish oil supplements, no strong aversion or any known allergies/intolerances to the foods involved in the study, and a usual weekly consumption of no more than two fatty fish meals per week. Nutrient composition of the plant sterol-enriched spread and participant dietary intake has been reported previously [10].

Written informed consent was obtained from all participants prior to commencement. Ethical approval was obtained from the Human Research Ethics Committee, University of Newcastle and registered with the Australian New Zealand Clinical Trials Registry (trial # 00081597).

2.3. Clinical assessments

Participants attended two visits (baseline and post-intervention) where anthropometric measures, cardiovascular data and fasting blood samples were collected.

2.3.1. Anthropometry and body composition

All anthropometric measures were made with participants wearing light clothing and no shoes. Body mass index (BMI) was calculated as weight in kilograms (kg) divided by the square root of height in meters (m) to the nearest 0.1 using a calibrated balance beam scale (PCS Measurement, NSW, Australia), and waist to hip ratio was calculated as waist girth in centimeters (cm) divided by hip girth (cm). Single frequency bioelectrical impedance (BIA) was used to assess body composition (Maltron International, Essex, UK). Measurements were taken in the supine position following a ≥ 10-h fast and refrained from physical activity and alcohol consumption 24 h prior to testing. Body fat mass and fat free mass were recorded as percentage of total body mass.

2.3.2. Blood pressure and heart rate

Blood pressure and heart rate were measured using an automated monitor (Microlife BP 3AD1-A, Heerbrugg, Switzerland) (pressure ± 3 mm Hg; pulse ± 5%) from the supported left arm of the rested (10 min), seated participant. Systolic blood pressure and diastolic blood pressure were based on the average of two separate measurements, taken by the investigating researcher.

2.3.3. Cardiovascular disease risk analysis

Ten-year risk of coronary artery disease was measured using the US National Cholesterol Education Program Adult Treatment Panel-III algorithm [11,12]. This risk factor sum model is an adaptation of the Framingham Study risk equations based on 10-year risk of hard cardiac points, including coronary artery disease and myocardial infarction in patients without diabetes mellitus or clinically evident cardiovascular disease. The prediction equation has taken the form of gender-specific equations, using continuous variables such as age, total cholesterol level (mmol/L), HDL-cholesterol level (mmol/L) and systolic blood pressure (mm Hg) and smoking as a dichotomous variable (yes/no). The model is age-adjusted for cholesterol and smoking status and corrects for treatment of blood pressure. A risk factor weighting approach is assigned to each variable and when totalled, they correspond to estimates of absolute 10-year risk % [13].
IL-6 (pg/mL) 1.8 ± 0.4

B4 (LTB4) levels were determined by an EIA kit based on the Pathology Service, Newcastle, NSW, Australia). Plasma leukotriene of <9%. High sensitivity C-reactive protein (hs-CRP) analysis was employed with a minimal detectable concentration of 0.106 pg/mL. High sensitivity tumor necrosis factor-α (hs-TNF-α) ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to determine the amount of leptin present in the sample, independent of the presence of leptin-binding proteins. Intra- and inter-assay CV were 6.4% and 9.7%, respectively, with a detection limit of 13.0 pg/mL. For all variables, samples from one participant were withdrawn at baseline and post-intervention for all anthropometric measures and a significant main effect of each independent variable (n-3 PUFA or plant sterol) in their effect on the dependent variable. High sensitivity tumor necrosis factor-α (hs-TNF-α) and interleukin-6 (IL-6) ELISA kits (Cayman Chemical Company, Ann Arbor, MI, USA) [15]. The intra- and inter-assay CV were 8.3% and 9.7%, respectively, with a detection limit of 13.0 pg/mL. For all variables, samples from one participant were determined using paired samples t-test. The effect of each treatment on the percentage change on the dependent variable (inflammatory mediator, fatty acids, CVD risk) between groups was explored using two-way ANOVA (i.e., SO compared with FO compared with SOP compared with FOP). Post hoc comparisons (Tukey’s honestly significant difference) were used when significance was found. This analysis was also used to determine whether there was a significant main effect of each independent variable (n-3 PUFA or plant sterol) by testing for between-subject effects. Also, an interaction effect was tested between the two independent variables (n-3 PUFA × plant sterol) in their effect on the dependent variable.

Table 1

Participant characteristics at baseline in the sunola oil (SO), fish oil (FO), SO and plant sterol (SOP), and FO and plant sterol (FOP) groups.

<table>
<thead>
<tr>
<th></th>
<th>SO (n = 15)</th>
<th>FO (n = 15)</th>
<th>SOP (n = 15)</th>
<th>FOP (n = 15)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>7 (46)</td>
<td>6 (40)</td>
<td>7 (46)</td>
<td>7 (46)</td>
<td>–</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.6 ± 1.0</td>
<td>26.4 ± 1.3</td>
<td>27.4 ± 1.4</td>
<td>27.3 ± 0.9</td>
<td>0.47</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.94 ± 0.03</td>
<td>0.90 ± 0.02</td>
<td>0.91 ± 0.02</td>
<td>0.96 ± 0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>33.6 ± 1.5</td>
<td>32.3 ± 2.4</td>
<td>33.1 ± 2.5</td>
<td>34.2 ± 1.7</td>
<td>0.67</td>
</tr>
<tr>
<td>Fat free mass (%)</td>
<td>66.3 ± 1.5</td>
<td>67.6 ± 2.4</td>
<td>66.9 ± 2.5</td>
<td>65.8 ± 1.7</td>
<td>0.64</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>137 ± 3.9</td>
<td>133 ± 4.0</td>
<td>134 ± 3.7</td>
<td>131 ± 3.8</td>
<td>0.98</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>84.2 ± 2.6</td>
<td>83.3 ± 2.6</td>
<td>83.4 ± 2.4</td>
<td>82.8 ± 2.8</td>
<td>0.21</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>71.0 ± 2.5</td>
<td>71.4 ± 2.3</td>
<td>68.6 ± 2.8</td>
<td>66.5 ± 2.3</td>
<td>0.80</td>
</tr>
<tr>
<td>Adiponectin (μg/mL)</td>
<td>2.1 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>0.72</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>15.1 ± 2.7</td>
<td>19.1 ± 2.3</td>
<td>14.4 ± 3.2</td>
<td>19.7 ± 4.7</td>
<td>0.14</td>
</tr>
<tr>
<td>hs-CRP (μg/mL)</td>
<td>3.2 ± 1.0</td>
<td>2.1 ± 0.4</td>
<td>2.9 ± 0.9</td>
<td>2.9 ± 0.5</td>
<td>0.76</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>3.8 ± 0.4</td>
<td>3.0 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>3.8 ± 0.5</td>
<td>0.26</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.8 ± 0.4</td>
<td>1.7 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>0.13</td>
</tr>
<tr>
<td>LTB4 (pg/mL)</td>
<td>20.3 ± 5.2</td>
<td>27.2 ± 3.5</td>
<td>24.9 ± 3.0</td>
<td>19.6 ± 3.4</td>
<td>0.46</td>
</tr>
</tbody>
</table>

hs-CRP: high sensitivity C-reactive protein; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; LTB4: leukotriene B4.

2.4. Biochemical analysis

2.4.1. Blood sample collection

Fasting (≥10 h) blood samples were collected into tubes pre-coated with EDTA by venipuncture at baseline and post-intervention. Samples were prepared by centrifuging (Heraeus Biofuge Stratos) for 10 min 3000 × g at 4 °C. Plasma, buffy coat and red blood cell sub-fractions were collected and stored at −80 °C until further analysis.

2.4.2. Determination of inflammatory markers

Adiponectin was quantitated by sandwich enzyme-linked immunosorbent assay (ELISA) (SPI-bio, Montigny le Bretonneux, France). This assay measures total circulating concentration of adiponectin in the presence of an adiponectin-specific antibody [14]. Intra- and inter-assay coefficients of variation (CV) were 6.4% and 7.3%, respectively, and detection range was 0.1–100 μg/mL. Plasma leptin levels were determined using a commercial double-antibody enzyme immunoassay kit (EIA) (Cayman Chemical Company, Ann Arbor, MI, USA). Plasma leptin levels were determined using an immunoturbidimetric method (Hunter Area Pathology Service, Newcastle, NSW, Australia). Plasma leptin concentration (IL-6 ELISA kits (R&D Systems, Minneapolis, MN, USA) were employed with a minimal detectable concentration of 0.106 pg/mL, and 0.039 pg/mL, respectively, and an intra- and inter-assay CV of <9%. High sensitivity C-reactive protein (hs-CRP) analysis was carried out using an immunoturbidimetric method (Hunter Area Pathology Service, Newcastle, NSW, Australia). Plasma leptin concentration (IL-6 ELISA kits (R&D Systems, Minneapolis, MN, USA) were employed with a minimal detectable concentration of 0.106 pg/mL, and 0.039 pg/mL, respectively, and an intra- and inter-assay CV of <9%. High sensitivity C-reactive protein (hs-CRP) analysis was carried out using an immunoturbidimetric method (Hunter Area Pathology Service, Newcastle, NSW, Australia). Plasma leptin concentration (IL-6 ELISA kits (R&D Systems, Minneapolis, MN, USA) were employed with a minimal detectable concentration of 0.106 pg/mL, and 0.039 pg/mL, respectively, and an intra- and inter-assay CV of <9%. High sensitivity C-reactive protein (hs-CRP) analysis was carried out using an immunoturbidimetric method (Hunter Area Pathology Service, Newcastle, NSW, Australia). Plasma leptin concentration (IL-6 ELISA kits (R&D Systems, Minneapolis, MN, USA) were employed with a minimal detectable concentration of 0.106 pg/mL, and 0.039 pg/mL, respectively, and an intra- and inter-assay CV of <9%. High sensitivity C-reactive protein (hs-CRP) analysis was carried out using an immunoturbidimetric method (Hunter Area Pathology Service, Newcastle, NSW, Australia).

2.4.3. Plasma fatty acid analysis

The fatty acid composition of plasma lipids was determined according to a modification in the method of Lepage and Roy [16], using an acetyl chloride methylation procedure, as detailed previously [10]. Fatty acid methyl esters were quantified using a Hewlett Packard 6890 gas chromatograph and quantified by comparison with fatty acid methyl ester standards (Nu Check Prep).

2.5. Statistical methods

Statistical analysis was performed using SPSS, Version 15.0 for Windows (SPSS, Inc., Chicago). Based on previous estimates of variance in plasma total cholesterol concentration, 60 participants provide 80% power at P < 0.05 for detection of a 0.60 mmol/L (10%) change in total cholesterol. All data are presented as means ± S.E.M. Significance was set at P-value < 0.05. Baseline characteristics of each group were compared using two-way ANOVA to test for homogeneity of variance violation. Changes from baseline were determined using paired samples t-test. The effect of each treatment on the percentage change on the dependent variable (inflammatory mediator, fatty acids, CVD risk) between groups was explored using two-way ANOVA (i.e., SO compared with FO compared with SOP compared with FOP). Post hoc comparisons (Tukey’s honestly significant difference) were used when significance was found. This analysis was also used to determine whether there was a significant main effect of each independent variable (n-3 PUFA or plant sterol) by testing for between-subject effects. Also, an interaction effect was tested between the two independent variables (n-3 PUFA × plant sterol) in their effect on the dependent variable.

3. Experimental results

3.1. Baseline assessment

The average participant age was 55.4 ± 1.0 y, with a BMI of 26.9 ± 0.5 kg/m² and a waist-to-hip ratio of 0.93 ± 0.01. No differences were observed within and between the four groups at baseline and post-intervention for all anthropometric measures [10].

3.2. Effect of plant sterol and fish oil intervention

3.2.1. Body composition and blood pressure

Measures of body composition, blood pressure and heart rate were assessed in all 60 subjects (Table 1). Body composition did not differ among the groups at baseline, with an average body fat mass of 26.0 ± 1.2 kg (33.3 ± 1.0%) and fat free mass of 50.8 ± 1.2 kg (66.6 ± 1.0%). Participants had an average systolic blood pressure of 134 ± 1.9 mm Hg, a diastolic blood pressure of 82.1 ± 1.7 mm Hg, and a heart rate of 69.4 ± 1.2 bpm at baseline. Consistent with current literature, those treated with n-3 PUFA (FO and FOP groups) tended to exhibit reductions in systolic (3.1 ± 1.7% and 1.5 ± 1.3%) and diastolic blood pressure (4.13 ± 1.2% and 3.56 ± 1.7%) over the 3 weeks; however, this failed to reach significance. Further analysis...
3.2.2. Markers of inflammation

There were no significant differences in inflammatory markers between each of the four groups at baseline (Table 1). Percentage change from baseline was examined for each group (Fig. 1A–F). Plasma inflammatory markers did not significantly change in the SO group. In the FO group, hs-CRP and TNF-α were significantly decreased from baseline (2.1 ± 0.4 μg/mL to 1.7 ± 0.3 μg/mL, P = 0.02 and 3.0 ± 0.2 μg/mL to 2.8 ± 0.2 μg/mL, P = 0.002, respectively). IL-6, LTB₄ and leptin decreased (1.7 ± 0.1 μg/mL to 1.5 ± 0.1 μg/mL, P = 0.14; 27.2 ± 3.5 pg/mL to 24.2 pg/mL ± 3.3 P = 0.30; 191 ± 5.2 ng/mL to 161 ± 4.6 ng/mL, P = 0.33, respectively), whilst adiponectin increased (15 ± 0.2 μg/mL to 1.7 ± 0.2 μg/mL; P = 0.39) in the FO group, however, failed to reach significance. There were no significant changes in any of the inflammatory markers in the SOP group. Conversely, for the FOP group there was a significant reduction in hs-CRP (2.9 ± 0.5 μg/mL to 2.6 ± 0.5 μg/mL, P = 0.009), TNF-α (3.8 ± 0.5 pg/mL to 3.4 ± 0.5 pg/mL, P = 0.02), IL-6 (1.7 ± 0.2 pg/mL to 1.5 ± 0.2 pg/mL, P = 0.009), and LTB₄ (19.6 ± 3.4 pg/mL to 16.2 ± 2.6 pg/mL, P = 0.01) and an increase in adiponectin (1.7 ± 0.2 μg/mL to 1.9 ± 0.1 μg/mL, P = 0.05). There was no change in leptin.

The change in hs-CRP in the FO and FOP groups were significantly different to the SO group (P = 0.007, 95% CI, 0.76–100.1 and P = 0.004, 95% CI, –1.6 to 82.7, respectively). No other between-group differences were found. Post hoc analysis found a significant main effect of fish oil for hs-CRP and TNF-α (P < 0.0001 and P = 0.05, respectively).

3.2.3. Plasma fatty acid concentration

Percentage fatty acid composition of plasma lipids was analysed at baseline and post-intervention. There were no differences at baseline between groups. Total saturated and monounsaturated fatty acids did not change significantly across the four interventions (data not shown). Plasma C20:5n-3 was significantly increased in the FO (1.1 ± 0.1 to 1.5 ± 0.1, P = 0.002) and FOP (1.2 ± 0.1 to 2.0 ± 0.1,
P = 0.003) groups (Fig. 2A). In the n-3 PUFA supplemented groups, a significant increase in C22:6n-3 was observed (FO = 2.3 ± 0.2 to 3.5 ± 0.2, P = 0.02 and FOP = 2.2 ± 0.1 to 4.7 ± 0.2, P < 0.0001) (Fig. 2B). These respective changes demonstrate participant compliance to the n-3 PUFA supplementation.

Furthermore, an exploration of treatment effects on C20:5n-3 and C22:6n-3 concentration between-groups was undertaken. Changes in C20:5n-3 in the FOP group was significantly different to that seen in the SO (P = 0.001, 95% CI, 26.3–127.6) and SOP (P = 0.007, 95% CI, 13.7–115.0) groups. Also, changes in C20:5n-3 in the FO group was significantly different to the SO group (P = 0.001, 95% CI, 26.4–127.8). Changes in C22:6n-3 in the FOP group were significantly different to that of the SO (P < 0.0001, 95% CI, 64.8–160.2), and SOP (P = 0.0001, 95% CI, 49.9–145.4) groups. Changes seen in the FO group were significantly different to the SO (P = 0.001, 95% CI, 26.9–122.4) and SOP (P = 0.008, 95% CI, 12.1–107.6) groups.

Post hoc analysis showed a significant main effect of n-3 PUFA supplementation on C20:5n-3 and C22:6n-3 plasma concentration (P < 0.0001). Plant sterol consumption also showed a significant main effect on C22:6n-3 plasma concentration (P = 0.04).

3.2.4. Cardiovascular disease risk
Cardiovascular risk, as determined by the NCEP ATP-III model, showed no significant changes from baseline among the SO (P = 0.08), FO (P = 0.10) and SOP (P = 0.06) groups (Fig. 3). However, a significant risk reduction (22.6 ± 5.1%, P = 0.006) was found in the FOP group. ANOVA failed to find any significant between-group differences in percentage change from baseline and post hoc analysis did not find any fish oil × plant sterol interactions for overall risk.

4. Discussion
We have demonstrated that dietary intervention with combined n-3 PUFA and plant sterols significantly reduces the inflammatory markers hs-CRP, TNF-α, IL-6 and LTB4 and significantly increases adiponectin in hyperlipidemic individuals. More importantly, the data demonstrates a 22.6% reduction in overall cardiovascular risk. Together, these findings suggest that reducing systemic inflammation in hyperlipidemia represents an important mechanism by which n-3 PUFA and plant sterols confer their putative cardiovascular benefits.

We demonstrated a 39% reduction in circulating hs-CRP, 10% reduction in TNF-α, 10.7% reduction in IL-6, 15.3% reduction in LTB4 and a 29.5% increase in adiponectin levels, in response to combined n-3 PUFA and plant sterol supplementation for 3 weeks. Evidence suggests that the n-3 PUFA C22:6n-3 and to a lesser extent C20:5n-3, inhibit early atherogenic events by reducing cytokine expression of pro-inflammatory proteins in the endothelium, however, it is not yet clear whether this effect is associated with individual or combined effects of the two fatty acids [17]. In a study by Thies et al. [18] comparing the effects of supplementation with fish oil (1 g/d C20:5n-3 + C22:6n-3), highly purified C22:6n-3 (720 mg/d) and a placebo oil on lymphocyte proliferation in healthy subjects, it was shown that the fish oil combination significantly reduced lymphocyte proliferation, while C22:6n-3 alone had no effect. Another study by Halvorsen et al. [19] compared the effects of supplementation with 3.8 g/d C20:5n-3 and 3.6 g/d C22:6n-3 on phagocytic activity of monocytes, reporting no effect. These findings may be taken to suggest that neither C20:5n-3 nor C22:6n-3 alone is responsible for an immunomodulatory effect alone, but required together for effective treatment. In a more recent study [20] on purified C20:5n-3 and C22:6n-3 (5 g/d for 4 weeks), it was found that neither fatty acid affected monocyte nor neutrophil phagocytosis, however, C22:6n-3 did appear to decrease the production of some inflammatory cytokines. In our study, we supplemented hyperlipidemic participants with a C22:6n-3 rich tuna oil, which was effective in reducing several inflammatory markers. Interestingly, when plant sterols were combined with n-3 PUFA, a greater anti-inflammatory effect was seen, whereas plant sterols alone had no effect.
Although it is hard to speculate on the exact mechanism by which plant sterols are anti-inflammatory, it should be noted that plant sterol supplementation did have a significant main effect on C22:6n-3 concentration (P = 0.04). The plant sterol-enriched spread used in this study provided an additional 1.5 g/d of n-3 PUFA as C18:3n-3. The authors speculate that the conversion of C18:3n-3 to C22:6n-3 may be a possible mechanism by which the combination of the two supplements elicits anti-inflammatory effects. We acknowledge the relatively short duration of this intervention trial, however, changes in inflammatory markers in response to n-3 PUFA and plant sterol supplementation have been highly responsive.

Our current knowledge of the interaction between plant sterols and inflammatory markers is poor. In a recent study by Clifton et al. [21], hypercholesterolemic individuals were supplemented with 1.6 g/d plant sterols for 3 weeks, followed by 3 g/d plant sterols for 3 weeks, compared to a control. In this study, no significant changes in hs-CRP were found, although there was a modest reduction trend (P = 0.07). In a similar study with hypercholesterolemic men, a 4-week supplementation period with a plant sterol-enriched spread (2.0 g/d) did not significantly change CRP levels [22]. More recently, De Jong et al. [23] provided statin treatment patients with 2.5 g/d of plant sterols as a margarine for 16 weeks. No effects were found for soluble adhesion molecules, CRP or monocyte chemotactic protein-1 concentrations. These studies support our findings of a non-significant reduction in hs-CRP with 2 g/d plant sterols for 3 weeks. Conversely, a study by Devaraj et al. [24] a median reduction in hs-CRP of 12% (P = 0.02) was found with 2 g/d plant sterol supplementation provided as a reduced-calorie orange juice beverage, perhaps due to large inter-individual variations. The role of plant sterols in the inflammatory process is largely unknown, however, given their interaction with the regulation of cholesterol influx and efflux at the phospholipid membrane, there is some degree of interaction with receptor activity. Potentially these could influence gene expression of COX-2 and IL-6, thereby having a direct impact on plasma markers of inflammation [25].

The relationship between plasma lipids and inflammatory cytokines, suggests that hyperlipidemia and enhanced inflammation are separate but interactive processes [26]. We have previously reported significant reductions in plasma total- and LDL-cholesterol and triglycerides and increases in HDL-cholesterol after 3 weeks of n-3 PUFA and plant sterol supplementation [10]. Further exploration of the data found no correlation between reductions in inflammatory markers and reductions in plasma lipid profile (data not shown), suggesting that the anti-inflammatory effects of combined n-3 PUFA and plant sterol.

To a large extent, reducing the inflammatory milieu to provide benefit among cardiovascular risk factors is yet to be fully understood in the context of hyperlipidemia.

It is evident that markers of systemic inflammation such as hs-CRP, TNF-α, IL-6 and several adipokines are elevated in hyperlipidemic individuals [27]. The relevance of this remains in the setting of primary prevention, as many large-scale studies have shown baseline levels of such markers can independently predict future CVD events, many with adequate power after adjustment for all Framingham covariates [28].

An additional objective of this study was to attenuate a possible reduction in overall cardiovascular risk. A growing body of evidence indicate an association between the consumption of fish and relative risk of sudden death. In the US Physicians Health Study, an inverse relationship between plasma levels of n-3 PUFA and risk of sudden death in men without a history of CVD was found [29]. In the Diet and Reinforcement Trial (DART) a 29% reduction in all-cause mortality over 2 years in male myocardial infarction survivors was found, following an increase of oily fish intake (200–400 g/week) [30]. In one of the largest randomised controlled trials, the GISSI-Prevention Study with 11,324 patients with pre-existing coronary heart disease randomised to 300 mg vitamin E, 850 mg n-3 PUFA, both or neither [31]. After 3.5 years, the n-3 PUFA alone group experienced a 15% reduction in the primary end point of death, non-fatal myocardial infarction and non-fatal stroke (P = 0.02), compared with the control.

In our study we showed a 22.6% (P = 0.006) reduction in overall cardiovascular risk using the combination of n-3 PUFA and plant sterols. To date, this is the first study to investigate the combined cardioprotective effects of these two functional foods in hyperlipidemic individuals with no history of cardiovascular events. We speculate that the improvement in cardiovascular risk is primarily representative of subsequent improvements in plasma lipid profile, given we did not see significant changes in blood pressure [10], a risk factor usually affected by n-3 PUFA supplementation [32]. Dietary supplementation longer than 3 weeks in duration may be needed to significantly influence other cardiovascular risk factors such as blood pressure, which merits further investigation.

Since cardiovascular risk factors such as increased inflammation and elevated plasma lipid levels rarely occur in isolation, combined therapy with anti-inflammatory agents such as n-3 PUFA and lipid-lowering agents such as plant sterols may provide greater risk reduction compared to either of the supplements alone. Collectively, our data is supportive of the cardioprotective benefits of combined n-3 PUFA and plant sterol supplementation for hyperlipidemic individuals. It is the first study to demonstrate overall lipid-lowering benefits, reduce markers of systemic inflammation and reduce overall cardiovascular risk, using a non-pharmacological dietary approach. This makes the combined n-3 PUFA and plant sterol therapy an ideal alternative or adjunct to pharmacological treatment, for maximum cardioprotection in high risk individuals.

Conflict of interest

The authors’ responsibilities were as follows—MAM was responsible for the conduct of the clinical trial, biochemical analyses, data collection, statistical analysis and manuscript preparation; MLG was involved in obtaining funding, the concept development and planning of the study, the overall supervision of the trial and interpretation and writing of the final draft of the paper. None of the authors had a personal or financial conflict of interest.

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