

## Independent and interactive effects of plant sterols and fish oil *n*-3 long-chain polyunsaturated fatty acids on the plasma lipid profile of mildly hyperlipidaemic Indian adults

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The present study was designed to evaluate the independent and interactive effects of a once-a-day yoghurt drink providing 2 g plant sterols/d and capsules providing 2 g fish oil *n*-3 long-chain (LC) PUFA/d on plasma lipids, apolipoproteins and LDL particle size. Following a 2-week run-in period, 200 mildly hypercholesterolaemic Indian adults aged 35–55 years were randomised into one of four groups of a 2 × 2 factorial, double-blind controlled trial. The 4-week treatments consisted of (1) control yoghurt drink and control capsules, (2) control yoghurt drink and fish oil capsules, (3) plant sterol-enriched yoghurt drink and control capsules, or (4) plant sterol-enriched yoghurt drink and fish oil capsules. Blood was drawn before and after the 4-week intervention. Changes in health status, lifestyle and dietary habits, and daily compliance were recorded. The main effects of plant sterols were a 4.5% reduction in LDL-cholesterol and a 15% reduction in TAG without a significant change in HDL-cholesterol. Overall, fish oil *n*-3 LC-PUFA did not significantly affect cholesterol concentrations but reduced TAG by 15% and increased HDL-cholesterol by 5.4%. The combination significantly lowered TAG by 15% *v.* control. No significant interaction between plant sterols and *n*-3 LC-PUFA was observed on plasma cholesterol concentrations. In conclusion, once-a-day intake of 2 g plant sterols/d in a yoghurt drink, 2 g fish oil *n*-3 LC-PUFA/d in capsules, and their combination had beneficial effects on the lipid profile of mildly hypercholesterolaemic Indian adults. The potent hypotriacylglycerolaemic effect of plant sterols observed in the present study and this population warrants additional investigation.

**Plant sterols: Fish oil: *n*-3 Fatty acids: Plasma lipids: LDL-cholesterol: TAG: Interactions**

The burden of chronic degenerative diseases, especially CVD, is high the world over and especially in India<sup>(1–3)</sup>. Disorders of plasma lipoprotein metabolism (dyslipidaemia) play a major role in the initiation and progression of atherosclerotic CVD. Though several causative factors are implicated in the development of CVD, dyslipidaemia characterised by high LDL-cholesterol (LDL-C), low HDL-cholesterol (HDL-C) and high TAG play a major role in the development and progression of atherosclerotic CVD<sup>(4,5)</sup>. Cholesterol lowering remains the main target for lowering CVD risk and the lifestyle modification approach including a healthy diet, weight control and increased physical activity is the cornerstone for prevention at a population level<sup>(6)</sup>. As part of their dietary guidelines, several expert panels<sup>(7–9)</sup> recommend the consumption of 2 g phytosterols (plant sterols or stanols)

per d as an adjunct to a heart-healthy diet to lower elevated LDL-C concentrations. At a dose of 2 g/d, phytosterols have been shown to lower plasma LDL-C concentrations by 10% on average<sup>(10)</sup>. Phytosterols incorporated in various food formats such as spread and margarines consumed in multiple daily intakes<sup>(10)</sup>, but also low-fat or non-fat foods<sup>(11–13)</sup>, and single daily-dose yoghurt drinks<sup>(14–16)</sup> have been shown to significantly lower LDL-C.

Nutrients that are well known for their hypotriacylglycerolaemic effect are EPA and DHA, the long-chain (LC) *n*-3 PUFA from fish oil<sup>(17,18)</sup>. *n*-3 LC-PUFA from fish also have anti-arrhythmic, anti-inflammatory and anti-thrombotic properties which are believed to contribute to their cardioprotective effects<sup>(19)</sup>. For this reason, the American Heart Association recommends for the general population the consumption of

**Abbreviations:** ANCOVA, analysis of covariance; HDL-C, HDL-cholesterol; LC, long-chain; LDL-C, LDL-cholesterol; TC, total cholesterol.

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oily fish and, for hypertriacylglycerolaemic individuals, the daily consumption of 2–4 g *n*-3 LC-PUFA from fish in the form of supplements<sup>(19)</sup>.

Phytosterols have been reported to exert little or no effect on plasma TAG in normo- and hypercholesterolaemic, but otherwise healthy, individuals<sup>(10)</sup>, although more recent evidence suggests that they may lower plasma TAG in subjects with higher baseline TAG concentrations<sup>(20)</sup>. Since *n*-3 LC-PUFA from fish oil exert a potent hypotriacylglycerolaemic effect without improving total cholesterol (TC) and LDL-C concentrations<sup>(17,18)</sup>, it would be reasonable to suggest that the combination of phytosterols and *n*-3 LC-PUFA may have complementary or even synergistic effects in reducing CHD risk. At the time the present study was undertaken, the approach which had been investigated was the use of fish oil esters of plant sterols<sup>(21–23)</sup>. One trial suggested that these esters could have, overall, a more beneficial impact on lipid profile than the traditional vegetable oil esters of plant sterols<sup>(21)</sup>, while another study unexpectedly showed no significant impact on plasma LDL-C and TAG<sup>(23)</sup>. A simpler alternative for simultaneously lowering LDL-C and TAG could be the combined use of readily available phytosterol ester-enriched foods and fish oil supplements. To our knowledge, only one recent study used such an approach<sup>(24)</sup>. LDL-C concentrations were lowered more by the combination of phytosterols (provided in a rapeseed oil-based spread) and *n*-3 LC-PUFA (as tuna oil capsules) than by phytosterols alone, despite a rising effect of the *n*-3 LC-PUFA on LDL-C, suggesting a beneficial interaction between phytosterols and *n*-3 LC-PUFA<sup>(24)</sup>.

The present trial was thus designed with the objective of evaluating the independent and interactive effects of 2 g plant sterols per d provided in a once-a-day yoghurt drink and 2 g EPA and DHA per d provided in capsules on plasma LDL-C and TAG in Indian adults with mildly elevated blood lipid concentrations. The effects on TC, HDL-C, TC:HDL-C ratio, apoB:apoA1 ratio and LDL particle size were also evaluated.

## Experimental methods

### Subjects

The subjects comprised of employees from a large industry in New Delhi, India. Criteria for inclusion in the study were plasma TC concentrations between 5.0 and 8.0 mmol/l, aged 35–55 years, and BMI 18–30 kg/m<sup>2</sup>. Smokers (more than ten cigarettes per d), alcohol consumers (more than 360 ml of 40 % alcohol per week), individuals with chronic degenerative diseases such as diagnosed heart disease, stroke, chronic renal failure, gastrointestinal disease, thyroid abnormalities, or cancer or with impaired gastrointestinal function (for example, after bowel resection) were excluded. Other exclusion criteria were diabetes, fasting blood glucose higher than 6.7 mmol/l and glycosylated Hb of more than 7.5 %, plasma TAG higher than 4.0 mmol/l, and weight loss or gain of 10 % or more during the last 6 months. Individuals who reported having taken during the previous 3 months dietary supplements or medications known to alter lipid or glucose metabolism (for example, plant sterol-enriched foods or supplements, fish oil supplements, lipid-lowering drugs, steroids,

β-blockers, insulin or oral hypoglycaemic drugs) were also excluded.

All subjects provided written informed consent by completing forms that had been approved, together with the study protocol, by the Independent Ethics Committee, Mumbai, India (approval no. IEC/06/23). The protocol was also cleared by the Hindustan Unilever Research Ethics Committee (reference no. ETH 2006/N46). The participant industry provided permission to carry out the study. The study was carried out in accordance with International Conference on Harmonisation Good Clinical Practice guidelines. Adverse events were collected from spontaneous reporting. However, subjects were asked about their wellbeing during every visit. A physician was on call continually during the trial for subjects to contact in case they experienced any discomfort. A Data Safety Monitoring Board consisting of a specialised doctor (endocrinologist), public health specialist and a statistician was also constituted before the start of the trial in order to review subjects' data related to adverse events and compliance at any time point during the trial. The subjects were identified by a unique identification code used for coding all information obtained from them.

### Experimental design and diets

The participants underwent a double-blind, randomised, controlled, parallel trial with a 2 × 2 factorial design. The test products were one 100 ml portion of yoghurt drink with (active) or without (control) plant sterols in combination with three 1 g oil capsules containing either *n*-3 LC-PUFA (active) or safflower-seed oil (control) to be consumed every day with a meal (preferably lunch). After a 2-week successful run-in period during which the participants consumed the control yoghurt drink, the subjects were randomly allocated, using computer-generated random tables, to one of the four treatment groups and consumed for 4 weeks either: (1) control yoghurt drink and control capsules (control), (2) control yoghurt drink and fish oil capsules (*n*-3 LC-PUFA alone), (3) plant sterol-enriched yoghurt drink and control capsules (plant sterols alone), or (4) plant sterol-enriched yoghurt drink and fish oil capsules (plant sterols + *n*-3 LC-PUFA). The treatment code of the products was blinded and the keys linking subjects' identification codes and treatment were kept in sealed envelopes accessible only to one person not involved in the study. The randomisation codes were broken and made available for data analysis only after the blind review of the data.

The nutritional composition of the test yoghurt drinks and capsules is provided in Table 1. The control and active yoghurt drinks were supplied by Unilever (Englewood Cliffs, NJ, USA). They had a similar composition except for the presence of 2 g plant sterols (mainly β-sitosterol, campesterol and stigmasterol) in the plant sterol-enriched yoghurt drink. Plant sterols were incorporated in the yoghurt drink in the form of vegetable oil esters. The control capsules, which consisted of safflower-seed oil, and the fish oil capsules were provided by EnergyFirst™ (Manhattan Beach, CA, USA). The fish oil capsules provided approximately 2 g EPA and DHA. The intervention products were stored under refrigeration (3–7°C).

**Table 1.** Nutrient composition of the study products

	Yoghurt drink (100 g/d)		Oil capsules (three per d)	
	Control	Plant sterol-enriched	Control (safflower-seed oil)	Fish oil
Energy				
kJ	218	218	109	126
kcal	52	52	26	30
Carbohydrates (g)	8.0	8.0	0	–
Proteins (g)	1.2	1.2	0	–
Lipids (g)	1.5	1.5	3.0	3.2
PUFA (g)	0.7	0.8	2.2	2.5
<i>n</i> -3 Fatty acids (g)	0.002	0.003	0	1.890
<i>n</i> -6 Fatty acids (g)	0.73	0.81	2.20	n.a.
EPA (g)	–	–	0	1.26
DHA (g)	–	–	0	0.63
MUFA (g)	0.30	0.40	0.43	< 0.50
SFA (g)	0.28	0.22	0.19	Traces
Cholesterol (g)	0.67	0.68	0	n.a.
Plant sterols (g)	Traces	2.00	0.01	–

n.a., Not available.

The subjects were instructed not to make any changes to their habitual diet and lifestyle, except for the study products, during both the run-in and the intervention phase. They came weekly to the clinic to collect their supplements for the next week and return the unused and empty yoghurt drinks and capsule packs. Compliance was also assessed with a questionnaire filled in every day by the subjects where they reported the amount of test drink and capsules consumed, the time of intake of the yoghurt drink and capsules, and the time of the meal. Compliance was also checked and encouraged by means of phone calls to the subjects (every 2–3 d). At the end of the run-in period, a single baseline fasting (10–12 h fast) blood sample was taken for TC, LDL-C, HDL-C, TAG, glucose, LDL particle size, apoA1, and apoB measurements. Blood pressure (with Omron MX3, Kyoto, Japan), weight (to the nearest 0.1 kg on an electronic scale with light clothes) and waist circumference (to the nearest 0.1 cm with a standard tape midway between the lower rib margin and the iliac crest) were also measured. The same measurements were repeated at the end of the 4-week intervention period.

Validated 2 d (one working and one non-working day) 24 h diet recalls designed for computing energy, fat and protein intake were conducted during the intervention period. A physical activity pattern questionnaire validated for the Indian industrial population was administered once at baseline. A physical activity metabolic equivalent (MET) score was determined based on self-reported energy-consuming activities during work, at home, while travelling and at leisure time. The score was calculated by weighting each type of activity by its energy requirements defined in MET (multiples of the RMR)  $\times$  its duration (in min) to yield a score in MET-min<sup>(25)</sup>.

#### Laboratory analyses

Blood samples were collected in a plain and fluoride vacutainer (Becton & Dickinson, Franklin Lakes, NJ, USA). After 30 min the tubes were centrifuged at 1300 g for 15 min to isolate serum and plasma, respectively. Glucose was estimated from plasma and lipids were estimated from serum samples.

The analyses were carried out at the Department of Cardiac Biochemistry at the All India Institute of Medical Sciences in New Delhi. The laboratory is externally validated by the UK National External Quality Assessment Service. TC, TAG and glucose were measured by enzymic methods using the cholesterol oxidase/*p*-aminophenazone (CHOD-PAP), the glycerol-3-phosphate oxidase/*p*-aminophenazone (GPO-PAP) and the glucose oxidase/*p*-aminophenazone (GOD-PAP) kits by Sentinel Diagnostics (Milan, Italy), respectively. LDL-C and HDL-C were measured by the elimination method using kits from Sentinel Diagnostics (Milan, Italy). The LDL particle size was determined by gel electrophoresis using the method of Krauss & Burke with modifications<sup>(26)</sup>. ApoB and apoA1 were measured by the immunoturbidimetry method using kits from Sentinel Diagnostics (Milan, Italy) and HbA1c was estimated by an Immunoturbidimetric latex method (Agappe Diagnostics, Kerala, India).

#### Statistical analyses

The study was designed as a double-blind randomised study with three test groups and one control group, with the key outcome parameters being LDL-C and TAG. A power calculation based on an 8% (0.24 mmol/l) decrease in LDL-C and a 15% (0.3 mmol/l) decrease in TAG with a 14% drop-out rate suggested a sample of 50 subjects in each arm in order to identify the main effects of plant sterols and fish oil as well as interactions between fish oil and plant sterols on LDL-C. This would yield 80% power at the 5% level of significance. This power calculation was made assuming that one-sided tests would be done.

Results are expressed as mean values and either standard deviations or standard errors where appropriate, and  $P < 0.05$  was considered significant. The main analysis for the present study was a 'quasi' intention-to-treat analysis<sup>(27)</sup> including all randomised subjects for whom the end-of-intervention data for the primary response variable were available ( $n$  178). A per-protocol analysis excluding non-compliant subjects (eleven out of 178) was also performed. Non-compliant subjects were defined as having consumed less than 75% of

the scheduled portions of yoghurt drinks and capsules. Mean compliance was calculated separately for yoghurt drinks and capsules and then averaged to determine the overall compliance.

Differences in group means of baseline subject characteristics (age, sex, anthropometric parameters, blood pressure, plasma glucose, dietary intake, physical activity scores and proportion of vegetarians) were tested using the ANOVA  $F$  test for continuous variables and  $\chi^2$  or Fisher's exact test for categorical variables.

Differences in group means of plasma lipid concentrations were determined by an analysis of covariance (ANCOVA) using a model including sex and baseline age, BMI, plasma glucose, and the respective baseline lipid concentrations as covariates when significantly contributing to the model. The ANCOVA is particularly useful in case of small differences in mean values of baseline characteristics between groups, because it takes into account the baseline levels in estimating the effects of the treatments<sup>(28–31)</sup>. The Dunnett–Hsu method was used for comparing the adjusted means of active treatment groups *v.* control. Tukey–Kramer adjustments were used to obtain the 95% CI of the differences between treatment groups. For TAG values, a logarithmic transformation was used because their distributions were skewed. Model selection for the covariance analysis was done using a 'stepdown' procedure starting with all adjusting variables and then keeping in the model only the significant variables. For this 2 × 2 design, we first looked for second-order interactions between plant sterols and *n*-3 LC-PUFA. Once the interaction effect was deemed insignificant, we calculated the (pooled) main effects of plant sterols and *n*-3 LC-PUFA compared with their respective control counterpart. This strategy was applied to all outcome variables except LDL particle size. LDL particle size was categorised into three categories: A (>26.3 nm), intermediate (25.8–26.3 nm) and B (<25.8 nm)<sup>(26)</sup>. We tested for associations between follow-up LDL particle size category and treatment groups using the Mantel–Haenszel mean score test statistic. Rank ANCOVA was also carried out on the follow-up LDL particle size adjusting for baseline LDL particle size. All statistical analyses were conducted using SAS software (version 9.1; SAS Institute Inc., Cary, NC, USA).

## Results

### Subjects

The participant flow diagram is shown in Fig. 1. After a screening of medical records obtained from the annual health check of the employees, 518 individuals were considered as potentially eligible to participate in the trial. Out of these 518 subjects, 230 met the inclusion and exclusion criteria after interview and biochemical analyses. Of these 230 subjects, 200 were enrolled in the run-in phase; twenty-six dropped-out before the end of the run-in, and four were excluded for non-compliance. A total of 200 subjects were randomised to one of the four treatment groups. Out of the 200 randomised, twenty-two subjects dropped out post-randomisation, yielding 178 subjects (of which nineteen were women) who had both their baseline and follow-up measurements done. Reasons for post-randomisation drop-outs

included refusal to participate further ( $n$  8), job change ( $n$  1), travel ( $n$  2), vacationing ( $n$  2), adverse event ( $n$  5) and non-compliance ( $n$  4) and were not related to treatment group. Adverse events that resulted in discontinuation of participation were reported by seven subjects: two during the run-in period and five post-randomisation. The two subjects who dropped out during the run-in period reported nausea. The adverse events reported during the intervention showed the following distribution: three were observed in the *n*-3 LC-PUFA group (bloating ( $n$  1), weakness ( $n$  1) and lacrimation ( $n$  1)) and one each in the control group (itchiness ( $n$  1)) and plant sterols-alone group (upper respiratory tract infection, stomach upset and fever ( $n$  1)). Given the low number of reported adverse events, no conclusion towards a relationship with a specific intervention could be drawn.

The baseline characteristics of the 178 subjects who completed the study (quasi-intention-to-treat population) are presented in Table 2. Eleven subjects were non-compliant with respect to the consumption of the test products (<75% of the scheduled doses consumed). Thus the per-protocol analyses were done using 167 subjects. As the results obtained from the analyses of 178 and 167 subjects were similar, only the results obtained from the quasi-intention-to-treat population ( $n$  178) are presented. The unadjusted pre- and post-intervention mean lipid and apolipoprotein values are shown in Table 3 while the adjusted post-intervention mean lipid and apolipoprotein values are presented in Tables 4 and 5.

### Baseline subject characteristics and compliance

No significant baseline differences in general subject characteristics were noted among the groups, except for age ( $P=0.0328$ ) and diastolic blood pressure ( $P=0.0021$ ), which were higher in the plant sterols-alone and *n*-3 LC-PUFA-alone groups compared with the other two groups. Energy intake was the lowest in the *n*-3 LC-PUFA-alone group and the highest in the plant sterols-alone group ( $P=0.044$ ). The proportion of male, baseline anthropometric measurements (weight, height and waist circumference), proportion of hypertensive and non-vegetarian subjects, dietary fat and protein intake, as well as physical activity scores did not differ significantly among the four study groups (Table 2).

Baseline plasma lipid and apolipoprotein concentrations are presented in Table 3. Mean baseline TC concentrations across the four groups were in the desirable (<5.1 mmol/l) to borderline high (5.1–6.1 mmol/l) range, while mean baseline LDL-C concentrations were above optimal to borderline high (>2.5 mmol/l and <4.1 mmol/l) according to the National Cholesterol Education Program classification (Table 3). Although the trial was randomised, there was an imbalance between the groups in baseline TC ( $P=0.0106$ ) and LDL-C ( $P=0.0278$ ) concentrations, with lower baseline cholesterol levels observed in the plant sterols-alone-treated group. Mean baseline TAG concentrations were normal (below 1.7 mmol/l) according to the National Cholesterol Education Program classification, except for the *n*-3 LC-PUFA-alone group, in which they were borderline high, indicating an imbalance between the groups at baseline ( $P=0.0204$ ). There were also imbalances in the groups at apoB and apoA1 concentrations

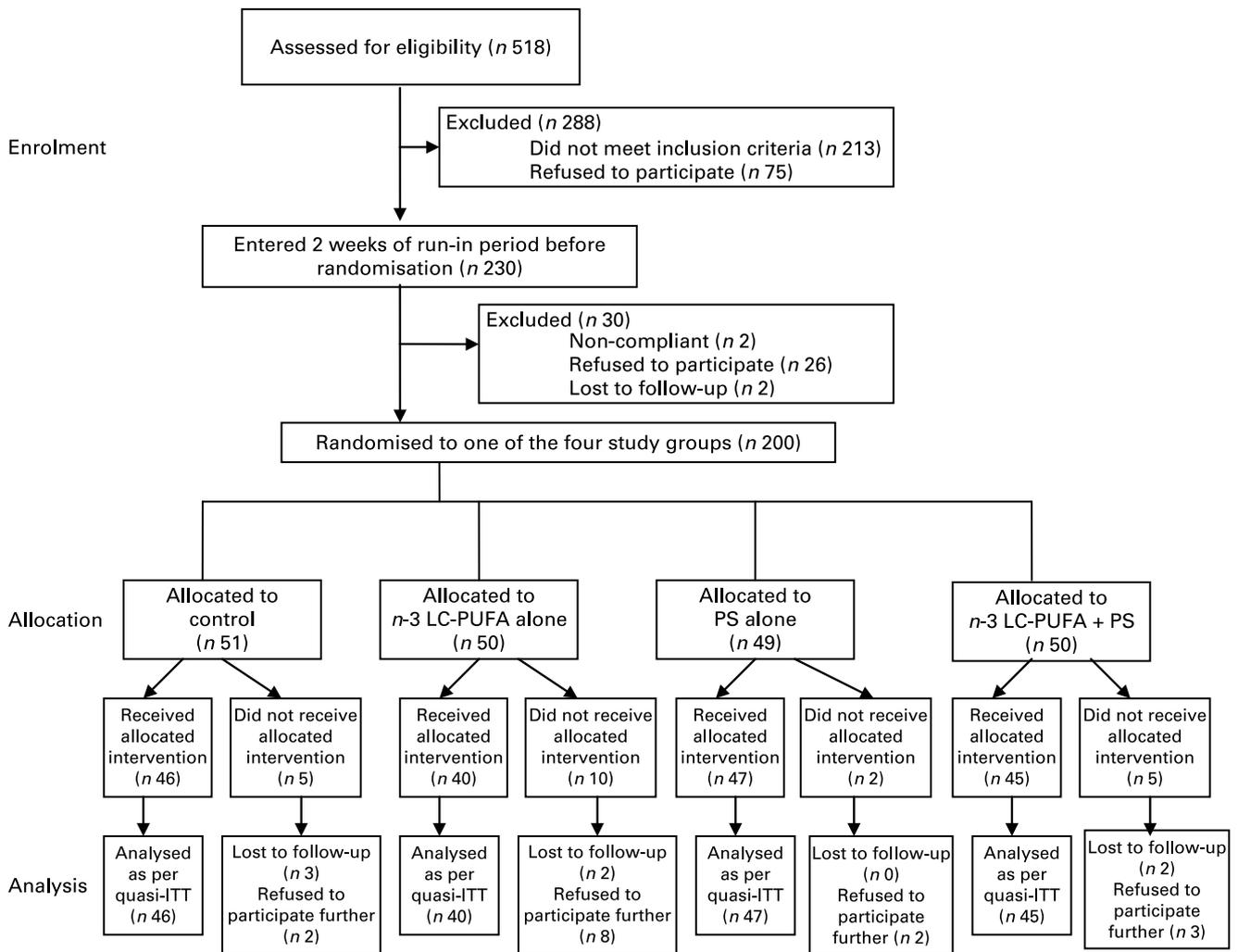


Fig. 1. Participant flow throughout the trial. LC, long-chain; PS, plant sterols; ITT, intention to treat.

(Table 3). The mean LDL particle size at baseline was similar for the four treatment groups. The largest LDL particle sizes (category A) were observed in 164 subjects, the intermediate category in nine subjects and category B (small LDL particle size) in five subjects.

Self-reported overall compliance for the yoghurt drink and capsules was good in all groups. The average compliance for the yoghurt drink and capsules was 96.7 and 95.2%, respectively. The average proportion of subjects in all the study arms who consumed  $\geq 75\%$  of the scheduled portions of yoghurt drink and capsules was 97.8 and 95.0%, respectively. No noticeable changes in weight or BMI were observed over the intervention period, with post-intervention values varying by not more than 0.7 kg or 0.1 kg/m<sup>2</sup>, respectively in each of the treatment groups.

#### Effects of the treatments on plasma lipids, apolipoproteins and low-density lipoprotein particle size

**Plasma lipids.** No significant interaction between plant sterols and fish oil *n*-3 PUFA was observed on plasma TC, LDL-C, HDL-C concentrations and TC:HDL-C ratio. The main effect of the plant sterol ester-enriched yoghurt

drink *v.* control was a significant 0.17 mmol/l (3.2%) reduction in TC (Table 4). Plant sterols (main effect) also significantly lowered LDL-C by 0.16 mmol/l (4.5%) compared with the control treatment. Fish oil *n*-3 LC-PUFA did not significantly lower either TC or LDL-C. Compared with the control capsules, fish oil significantly increased HDL-C (+0.05 mmol/l or 5.4%). The TC:HDL-C ratio was significantly reduced by plant sterols (3% reduction) and by fish oil *n*-3 LC-PUFA (6.3% reduction) independently (Table 4). In combination, they decreased TC:HDL by 9.1% compared with the control group (Table 5).

A significant interaction was observed between plant sterols and fish oil *n*-3 LC-PUFA regarding their effect on plasma TAG. Plasma TAG were decreased by 0.22 mmol/l (15%), 0.21 mmol/l (14.5%) and 0.22 mmol/l (15%) in the *n*-3 LC-PUFA-alone, the plant sterols-alone and the *n*-3 LC-PUFA + plant sterol groups, respectively, compared with the control group (Table 5). Therefore, both plant sterols and *n*-3 LC-PUFA independently lowered TAG significantly by about 15%, but their combination did not bring any additional benefit on TAG (Table 5).

**Apolipoproteins.** No significant interaction was observed between plant sterols and fish oil *n*-3 PUFA. The main effects

**Table 2.** Baseline characteristics of the 178 subjects who completed the study (quasi-intention-to-treat population) (Mean values with their standard errors or numbers of subjects and percentages)

	Control (n 46)		n-3 LC-PUFA alone (n 40)		PS alone (n 47)		n-3 LC-PUFA + PS (n 45)		P*
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Age (years)	46.1	0.9	48.2	0.9	45.9	0.9	49.0	0.9	0.032
Sex									0.593
Males (n)	41		38		41		39		
Males (%)	89.1		95.0		87.2		86.7		
Anthropometry									
Weight (kg)	67.3	1.7	70.8	1.8	68.6	1.7	70.0	1.7	0.498
BMI (kg/m <sup>2</sup> )	24.3	0.5	25.7	0.6	25.2	0.5	25.9	0.5	0.119
Waist circumference (cm)	88.9	1.4	93.6	1.5	91.2	1.4	92.9	1.4	0.094
Blood pressure									
Systolic (mmHg)	124.0	2.3	131.9	2.4	129.4	2.3	125.8	2.3	0.080
Diastolic (mmHg)	77.5	1.5	85.7	1.6	81.7	1.5	79.7	1.5	0.002
Hypertensive									0.452
n	2.0		4.0		2.0		5.0		
%	4.4		10.0		4.3		11.1		
Plasma glucose (mmol/l)	5.93	0.13	6.37	0.14	6.08	0.13	6.05	0.13	0.162
Dietary intake									
Energy									
kJ/d	7099.0	322.6	6536.7	350.2	7157.6	318.8	7887.3	322.6	0.044
kcal/d	1696.7	77.1	1562.3	83.7	1710.7	76.2	1885.1	77.1	0.044
Protein (g/d)	50.0	2.7	47.9	2.9	49.1	2.7	56.8	2.7	0.096
Total fat (g/d)	45.5	4.1	39.1	4.5	50.2	4.1	51.9	4.1	0.158
Non-vegetarians									0.713
n	30.0		23.0		27.0		30.0		
%	65.2		57.5		57.5		66.7		
Physical activity score (MET-min/week)	9558.6	822.0	9253.1	881.5	9994.6	813.2	8465.4	831.1	0.608

LC, long-chain; PS, plant sterols; MET, metabolic equivalent.

\* P values for testing overall differences among treatment groups using the ANOVA F test for continuous variables and  $\chi^2$  or Fisher's exact test for categorical variables.

of plant sterols ( $-2.1\%$ ) and n-3 LC-PUFA (no decrease) v. control on apoB concentrations were not statistically significant and no significant differences were observed between groups in apoB concentrations. Similarly, the main effects on apoA concentrations of plant sterols ( $+1.5\%$ ) and n-3 LC-PUFA (no increase) v. their respective control treatments were not statistically significant and no differences were observed between groups. Nevertheless, the decreases in apoB and increases in apoA observed with plant sterols v. control resulted in a significant  $4.5\%$  reduction in apoB:apoA1 ( $P=0.0407$ ). By contrast, fish oil n-3 LC-PUFA produced a non-significant increase ( $+2.4\%$ ) in the apoB:apoA1 ratio (Table 4).

**Low-density lipoprotein particle size.** No association between treatment and follow-up LDL particle size category was observed using the Mantel-Haenzel mean score statistics adjusting for baseline LDL particle size category ( $P=0.1880$ ). For rank ANCOVA, the Mantel-Haenzel zero-correlation test statistic was used to compare the mean value of the residuals in the four treatment groups. No significant difference ( $P=0.8829$ ) was noted.

#### Secondary outcomes – impact of baseline characteristics on treatment effects

The results of the ANCOVA performed by adjusting for differences in baseline variables between groups showed that age significantly ( $P=0.0183$ ) influenced the impact of plant

sterols on plasma TC concentrations. The response to plant sterols was more pronounced in younger subjects, with a  $6.3\%$  decrease ( $P=0.0011$ ) at the 25th percentile of age (42 years) and non-significant  $2.0\%$  ( $P=0.1008$ ) and  $0.2\%$  ( $P=0.4673$ ) decreases at the 50th (49 years) and 75th percentile (52 years) of age, respectively. In addition, a significant ( $P=0.0072$ ) three-way interaction between plant sterols, n-3 LC-PUFA and sex was observed on LDL-C concentrations. Thus an analysis stratified by sex was performed on LDL-C. In males ( $n 159$ ), a significant interaction between plant sterols and baseline blood glucose ( $P=0.0011$ ) was observed. The difference in LDL-C with plant sterols v. control was  $-0.01\%$  ( $P=0.4986$ ),  $-4.13\%$  ( $P=0.0390$ ) and  $8.44\%$  ( $P=0.0003$ ) in individuals with baseline glucose concentrations at the 25th percentile ( $5.5$  mmol/l), median ( $6.0$  mmol/l) and 75th percentile ( $6.6$  mmol/l), respectively. Interactions were also observed between age and plant sterols ( $P=0.0011$ ), as well as between baseline blood glucose and plant sterols ( $P=0.0251$ ) in their effect on HDL-C. A beneficial effect of plant sterols on HDL-C was observed in older individuals, with significant  $4.5\%$  ( $P=0.0134$ ) and  $7.6\%$  ( $P=0.0012$ ) increases in HDL-C levels observed in individuals aged 49 years (50th percentile) or 52 years (75th percentile), respectively. In addition, for subjects with lower baseline glucose concentrations, plant sterols increased HDL-C levels  $+5.9\%$  ( $P=0.0087$ ) for baseline glucose of  $5.4$  mmol/l corresponding to the 25th percentile and  $+3.5\%$  ( $P=0.0408$ ) for baseline glucose of  $5.9$  mmol/l corresponding

**Table 3.** Pre- and post-intervention unadjusted plasma lipid and apolipoprotein concentrations for 178 subjects who completed the study (quasi-intention-to-treat population) (Mean values and standard deviations)

	Control (n 46)				n-3 LC-PUFA alone (n 40)				PS alone (n 47)				n-3 LC-PUFA + PS (n 45)			
	Pre		Post		Pre		Post		Pre		Post		Pre		Post	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TC (mmol/l)	4.94	0.78	5.06	0.83	5.26	0.68	5.22	0.75	4.75	0.69	4.71	0.75	5.09	0.77	4.96	0.70
LDL-C (mmol/l)	3.35	0.66	3.47	0.70	3.57	0.64	3.67	0.74	3.18	0.76	3.19	0.75	3.57	0.80	3.49	0.69
HDL-C (mmol/l)	0.93	0.25	0.91	0.22	0.93	0.16	0.95	0.18	0.97	0.18	0.96	0.20	0.96	0.23	0.98	0.23
TC:HDL-C	5.48	0.87	5.69	0.84	5.71	0.80	5.63	0.94	5.00	0.92	5.10	1.96	5.49	1.04	5.19	0.86
Apo A1 (mg/l)	1230	201.3	1210	186.4	1240	226.2	1210	200.0	1270	239.0	1270	228.2	1250	266.3	1230	231.1
Apo B (mg/l)	1070	210.6	1070	188.8	1140	175.7	1150	202.2	980	163.0	990	184.7	1110	204.6	1080	181.0
ApoB:apoA1	0.90	0.23	0.90	0.20	0.95	0.19	0.97	0.21	0.79	0.19	0.81	0.22	0.93	0.27	0.90	0.20
TAG (mmol/l)	1.59	0.67	1.70	0.90	1.87	0.79	1.60	0.70	1.41	0.68	1.28	0.45	1.56	0.57	1.40	0.54
LDL particle size (nm)	26.96	0.07	26.96	0.49	26.87	0.08	26.95	0.39	26.95	0.07	27.03	0.43	26.95	0.07	27.03	0.47

LC, long-chain; PS, plant sterols; TC, total cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol.

**Table 4.** Post-intervention plasma lipid and apolipoprotein concentrations adjusted for baseline values obtained by analysis of covariance (ANCOVA) in the 178 subjects who completed the study (quasi-intention-to-treat population): main effects of plant sterols (PS) and n-3 long-chain (LC)-PUFA v. their respective controls\* (Mean values with their standard errors)

	Control PS (n 86)		Active PS (n 92)			Control n-3 LC-PUFA (n 93)		Active n-3 LC-PUFA (n 85)		
	Adjusted mean	SEM	Adjusted mean	SEM	P†	Adjusted mean	SEM	Adjusted mean	SEM	P†
Total cholesterol (mmol/l)	5.06	0.06	4.90	0.05	0.018	5.00	0.05	4.96	0.05	0.298
LDL-cholesterol (mmol/l)	3.53	0.05	3.37	0.05	0.017	3.45	0.05	3.45	0.05	0.518
HDL-cholesterol (mmol/l)‡	0.95	0.01	0.96	0.01	0.1808	0.93	0.01	0.98	0.01	0.0004
Total cholesterol:HDL-cholesterol	5.30	0.08	5.14	0.07	0.019	5.39	0.07	5.05	0.07	0.0001
ApoB (mg/l)	1080	13.3	1060	12.8	0.110	1070	12.9	1070	13.5	0.602
ApoA1 (mg/l)	1250	18.6	1270	17.3	0.164	1260	17.5	1260	18.5	0.677
ApoB:apoA1	0.88	0.02	0.84	0.02	0.041	0.85	0.02	0.87	0.02	0.766
TAG (mmol/l)§	–	–	–	–	–	–	–	–	–	–

\* In addition with the respective baseline lipid parameter, sex, baseline BMI, age and glucose concentrations were included in the model when significant.

† P value for the one-sided comparison v. the control treatment.

‡ Excluding three outliers: two from n-3 LC-PUFA+PS and one from n-3 LC-PUFA alone.

§ Interaction between PS and n-3 LC-PUFA was considered significant (P=0.0566) and therefore the main effects of PS and n-3 LC-PUFA on this parameter are not presented.

**Table 5.** Post-intervention plasma lipid and apolipoprotein concentrations adjusted for baseline values obtained by analysis of covariance (ANCOVA) in the 178 subjects who completed the study (quasi-intention-to-treat population): interaction effects – comparisons between each of the four treatment groups\* (Mean values with their standard errors)

	Control (n 46)			n-3 LC-PUFA alone (n 40)			PS alone (n 47)			n-3 LC-PUFA + PS (n 45)		
	Adjusted mean	SEM	P†	Adjusted mean	SEM	P†	Adjusted mean	SEM	P†	Adjusted mean	SEM	P†
Total cholesterol (mmol/l)	5.10	0.08	0.448	5.02	0.08	0.448	4.90	0.08	0.074	4.89	0.08	0.072
LDL-cholesterol (mmol/l)	3.53	0.07	0.713	3.52	0.08	0.713	3.36	0.07	0.109	3.38	0.07	0.1576
HDL-cholesterol (mmol/l)‡	0.92 <sup>a</sup>	0.02	0.051	0.97 <sup>a,b</sup>	0.02	0.051	0.93 <sup>a,b</sup>	0.02	0.603	0.99 <sup>b</sup>	0.02	0.004
Total cholesterol:HDL-cholesterol§	5.49 <sup>a</sup>	0.09	0.002	5.12 <sup>b,c</sup>	0.10	0.002	5.29 <sup>a,b</sup>	0.09	0.090	4.99 <sup>c,d</sup>	0.09	0.0001
ApoB (mg/l)	1070	18.0	0.939	1090	19.7	0.939	1060	18.4	0.631	1060	18.3	0.458
ApoA1 (mg/l)	1250	21.9	0.712	1250	24.1	0.712	1280	21.6	0.263	1260	21.7	0.601
ApoB:apoA1	0.86	0.02	0.975	0.90	0.03	0.975	0.84	0.02	0.469	0.84	0.02	0.442
TAG (mmol/l)	1.45 <sup>a</sup>	0.07	0.009	1.23 <sup>b</sup>	0.06	0.009	1.24 <sup>b</sup>	0.06	0.011	1.23 <sup>b</sup>	0.06	0.007

LC, long-chain; PS, plant sterols.

a–d Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ; Tukey's test).

\*In addition with the respective baseline lipid parameter, sex, baseline BMI, age and glucose concentrations were included in the model when significant.

†P value for the comparison with the control group.

‡Excluding three outliers: two from n-3 LC-PUFA+PS and one from n-3 LC-PUFA alone.

§Excluding one outlier from n-3 LC-PUFA alone.

||ANCOVA on log-transformed variables. Adjusted means and SEM are back-transformed.

to the 50th percentile. However, for subjects with higher blood glucose (75th percentile, or 6.6 mmol/l), practically no change in HDL-C was observed.

## Discussion

To our knowledge, this double-blind, randomised controlled trial is the first study reporting the individual and combined effects of plant sterols and fish oil n-3 LC-PUFA in mildly hyperlipidaemic Indian adults. Consumption, once per d, of 2 g plant sterols/d in a yoghurt drink format resulted in an overall 4.5 % reduction in LDL-C. Supplementation of 2 g n-3 LC-PUFA/d from fish oil provided in capsules did not significantly affect cholesterol concentrations. No significant interaction between plant sterols and fish oil n-3 LC-PUFA was observed on plasma TC and LDL-C concentrations, with the combination of plant sterols and n-3 LC-PUFA resulting in a 4.2 % decrease in LDL-C. There was a significant interaction between plant sterols and n-3 LC-PUFA in their TAG-lowering effect. Plant sterols alone and n-3 LC-PUFA alone both lowered TAG by 15 % compared with control, but their combination did not result in an additive effect on TAG, with a 15 % decrease observed in the combination group as well.

There is substantial evidence from a vast number of randomised controlled trials that plant sterols and stanols incorporated in fat-based foods consumed in multiple daily intakes lower TC and LDL-C with, on average, reductions in LDL-C ranging from 7.4 to 10.5 % for doses of 2.0–2.4 g/d<sup>(10)</sup>. Plant sterols and stanols have also been shown to significantly lower LDL-C by 2.9–12.2 % for doses of about 2 g/d when incorporated in low-fat yoghurt or yoghurt drinks consumed in single daily intakes<sup>(12,14–16,32)</sup>. The 4.5 % LDL-C-lowering effect observed in the present study falls in the range reported in previous trials with once-a-day food formats, though this effect is less than could be expected for a 2 g/d dose based on the meta-analysis by Katan *et al.*<sup>(10)</sup>. One of the factors that have been shown to affect the efficacy of a single daily-dose yoghurt drink is the time of intake, with a larger efficacy observed when the yoghurt drink was consumed with a meal than when consumed after an overnight fast<sup>(33)</sup>. In the present study, the participants were instructed to consume the yoghurt drink with a main meal, preferably lunch, in order to optimise efficacy. Although from their daily diaries it appears that the participants followed this advice, the possibility that some of them may not have followed this instruction closely enough cannot be excluded. Nevertheless, the results from the present study confirm that a single daily intake of 2 g plant sterols/d in a yoghurt drink format can significantly lower LDL-C by about 5 % and the TC:HDL-C ratio by 3 %, which may contribute to significantly reducing coronary artery disease risk at a population level. On an individual basis, the results of the present study suggest that younger individuals could benefit more from plant sterol consumption than older subjects in terms of plasma TC reductions. However, age did not independently affect plant sterol efficacy in lowering LDL-C, and it might be that the larger effects observed in older subjects could simply be related to higher baseline LDL-C concentrations in older individuals, as suggested by Katan *et al.*<sup>(10)</sup>. Further, the possibility that the age-related interaction on TC

concentrations may have resulted from the interplay of chance cannot be excluded.

The finding of a larger LDL-C-lowering efficacy of plant sterols, coupled with a lower HDL-C-raising effect in subjects with higher baseline plasma glucose concentrations, suggests that abnormalities of glucose metabolism may modulate the effects of plant sterols on LDL-C and HDL-C concentrations. Previous trials have shown that plant sterols significantly lower LDL-C concentrations in diabetic individuals<sup>(34–38)</sup>. Preliminary data also suggest that plant sterols may improve not only LDL-C but also HDL-C and TAG concentrations in subjects with the metabolic syndrome<sup>(39,40)</sup>. The results of the present trial are of particular interest especially in the context of the high burden of CHD in younger Indians and the significantly higher rates of CHD in younger Indians compared with Western countries. However, as these data originate from a non-pre-hypothesised secondary analysis, they should be considered as hypothesis-generating findings warranting further evaluation by additional studies.

The absence of a beneficial effect of *n*-3 LC-PUFA on TC and LDL-C but an increase in HDL-C observed in the present study are in accordance with previous meta-analyses and reviews<sup>(17,18)</sup> and large-scale clinical trials<sup>(41)</sup>. In the present study, no interaction was observed between plant sterols and *n*-3 LC-PUFA in their effect on LDL-C. However, the recent study by Micallef & Garg<sup>(24)</sup> suggested that plant sterols and *n*-3 LC-PUFA could interact and result, when combined, in larger LDL-C decreases than those observed with plant sterols alone, despite the LDL-C-raising effect of fish oil *n*-3 LC-PUFA. The authors suggested that the coexistence of *n*-3 LC-PUFA and phytosterols in the gastrointestinal tract may cause greater micellar displacement of cholesterol, resulting in greater inhibition of cholesterol absorption<sup>(24)</sup>. The results of the present study, showing a similar LDL-C reduction in the plant sterol–*n*-3 LC-PUFA combination group to the reduction due to plant sterols alone, do not provide support to the hypothesis of an interaction of plant sterols and *n*-3 LC-PUFA in the inhibition of cholesterol absorption.

An interesting finding of the present trial is the pronounced (–15%) hypotriacylglycerolaemic effect of plant sterols on plasma TAG. Until recently, the effect of phytosterols on plasma TAG in normo- and hypercholesterolaemic individuals was considered as minimal and non-significant<sup>(10)</sup>. In fact, although plasma TAG concentrations were measured and reported in most previous plant sterol and stanol consumption studies, only a very limited number reported changes in TAG<sup>(32–35,42–48)</sup> out of which only a few showed decreases ranging from –1.3 to –17.4% for plant sterol or stanol doses of 1.6–3.2 g/d<sup>(34,45–48)</sup>. Additional evidence for a hypotriacylglycerolaemic effect of plant sterols has been recently published by Naumann *et al.*<sup>(20)</sup> who performed a meta-analysis of individual subject data from five of their previous plant stanol studies. They showed larger reductions in plasma TAG in subjects having higher baseline TAG levels<sup>(20)</sup>. For a daily intake of 2 g plant stanols/d, moderate reductions in plasma TAG of 1.0, 3.8 and 4.7% were observed with baseline TAG concentrations of 1.0, 2.0 and 3.0 mmol/l, respectively<sup>(20)</sup>. In contrast, with baseline concentrations of 1.4 mmol/l, a reduction of about 19% (when adjusted for changes in the control group) was observed in subjects fed

plant sterols alone in the trial by Micallef & Garg<sup>(24)</sup>. Although this effect was not statistically significant, it is of the same magnitude as the effect observed in the present study for the same dose of plant sterols. Moreover, preliminary data in subjects with the metabolic syndrome have shown a 28% reduction in TAG with the consumption of 2 g plant stanols/d, despite a mean baseline TAG concentration of less than 1.5 mmol/l<sup>(39)</sup>. In the present study, the subjects were not affected by the metabolic syndrome, but the substantial 15% decrease in plasma TAG suggests that plant sterols may have particularly beneficial effects on this parameter in the Indian population, which is known for its high prevalence of hypertriacylglycerolaemia and low HDL-C concentrations<sup>(49,50)</sup>.

Fish oil *n*-3 LC-PUFA when used in doses of 2 g EPA and DHA/d have been reported to lower TAG concentrations, on average, by about 15%<sup>(18)</sup> or 0.18 mmol/l<sup>(17)</sup>, which is in accordance with the effects observed in the present study. *n*-3 LC-PUFA from fish are believed to lower plasma TAG by reducing VLDL secretion, accelerating chylomicron TAG clearance, increasing the conversion of VLDL to LDL, depressing LDL synthesis and reducing postprandial lipaemia<sup>(18)</sup>. Although plant sterols also reduced plasma TAG by about 15%, the combination of plant sterols and *n*-3 LC-PUFA did not result in an additional TAG-lowering effect. The absence of additivity in effects of plant sterols and *n*-3 LC-PUFA suggest that these two compounds may interact in their TAG-lowering properties by an unknown mechanism. Preliminary results suggest that one of the mechanisms involved in the TAG-lowering effect of phytosterols would be a reduction in VLDL1 secretion<sup>(40)</sup>. Reductions in postprandial remnant-like particle-TAG have also been observed in diabetic subjects who consumed plant stanols<sup>(40)</sup>. Although *n*-3 LC-PUFA and plant sterols may share common mechanisms of actions to lower plasma TAG, a threshold in TAG lowering is unlikely in light of the large reductions (>15%) which have been observed in some high-dose fish oil trials<sup>(18)</sup>. From the present data, it is impossible to determine what the respective contribution of plant sterols and *n*-3 LC-PUFA to the hypotriacylglycerolaemic effect observed with the combination is, but it can be concluded that plant sterols and *n*-3 LC-PUFA may interfere with each other in modulating TAG concentrations. The data by Micallef & Garg<sup>(24)</sup> may provide support to this hypothesis: despite a non-significant reduction of about 19% with the consumption of 2 g plant stanols/d *v.* control, the TAG-lowering effects of plant sterols and *n*-3 LC-PUFA were not additive, with control-adjusted TAG reductions of about 33 and 37% in the fish oil and plant sterols–*n*-3 LC-PUFA combination groups, respectively. However, no significant interaction between plant sterols and *n*-3 LC-PUFA on plasma TAG<sup>(24)</sup> was observed; it is thus possible that their study was not sufficiently powered to detect an interaction between plant sterols and *n*-3 LC-PUFA on plasma TAG. On the other hand, the dose of EPA and DHA and the size of the lipid-lowering effects of plant sterols and *n*-3 LC-PUFA were different in that study and ours. Therefore, further investigations on the mechanisms and conditions (doses, study population) which may be involved in the interactive effects of plant sterols and *n*-3 LC-PUFA on plasma TAG would be worthwhile.

Nevertheless, due to the beneficial impact of the plant sterol–fish oil *n*-3 PUFA combination on the TC:HDL-C ratio (–9.1% *v.* the control group) and the lowering effect of low-dose fish oil on heart events<sup>(41)</sup> despite modest TAG lowering, it may be considered that the combination of plant sterols and fish oil may bring complementary benefits in view of reducing CVD risk. Although the present data are generalisable to men only, due to the small number of women included in the present study, previous results have reported no impact of sex on the effects of plant sterols<sup>(10)</sup> and fish oil *n*-3 LC-PUFA<sup>(17,18)</sup> on plasma lipid concentrations.

### Conclusion

Changes in dietary habits have been involved in the high burden of CVD and increased vulnerability of Indians to CVD<sup>(51,52)</sup>. Reductions of intermediate risk factors such as plasma lipids by means of dietary approaches should putatively reduce the risk of CVD. The results of the present study, showing that 2 g plant sterols/d consumed once per d in a yoghurt drink format lowered plasma LDL-C by about 5% and TAG by 15%, suggest that plant sterol-enriched foods are a promising addition to interventions aimed at lowering heart disease risk in the Indian population. The TAG-lowering (–15%) and HDL-C-raising (+5.4%) effects of *n*-3 LC-PUFA, together with the beneficial impact of the plant sterol–*n*-3 LC-PUFA combination on the TC:HDL-C ratio, suggest that fish oil *n*-3 LC-PUFA may be a useful adjunct to plant sterols for reducing CVD risk despite the lack of additive effect on plasma TAG. Additional investigations are warranted for the potent hypotriacylglycerolaemic effect of plant sterols and the relationship between baseline glucose concentrations and the effects of plant sterols on LDL-C and HDL-C observed in the present study.

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The other authors have no conflicts of interest to declare.

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