Bread enriched with phytosterols with or without curcumin modulates lipoprotein profile in hypercholesterolaemic individuals. A randomised controlled trial

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Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; CC, curcumin; CVD, cardiovascular disease; CYP7A, cholesterol 7 alpha-hydroxylase; HDL-C, HDL-cholesterol; HDL-P, HDL-particle; HDL-Z, HDL particle size; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; LDL-C, LDL-cholesterol; LDL-P, LDL-particle; LDL-Z, LDL particle size; LXRα, liver X receptor alpha; MESA, Multi-Ethnic Study of Atherosclerosis; NPC1L1, Niemann Pick C1-Like 1; NMR, nuclear magnetic resonance;

25 PCSK9, proprotein convertase subtilisin /kexin type 9; PL, placebo group; PL-C, placebo and
26 curcumin group; PS, phytosterols; PS-C, phytosterol and phytosterol-curcumin group; PS-
27 CC, phytosterols and curcumin; RCT, randomised controlled trial; TC, total cholesterol;
28 TC:HDL, total cholesterol-to-HDL ratio; TG, triglycerides; TRL-P, triglyceride rich
29 lipoprotein particle; TRL-Z, triglyceride rich lipoprotein particle size.

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31 The trial was registered with the Australian New Zealand Clinical Trials Registry at
32 http://www.anzctr.org.au/ (ACTRN 12618001960246).

ABSTRACT

We previously demonstrated that the combination of phytosterols (PS) and curcumin administered as dietary supplements significantly lowers LDL-cholesterol (LDL-C) more than either treatment alone. The aim of this study was to investigate the effects of this combination in a novel food (bread) on plasma lipid profile in hypercholesterolaemic individuals.

In a double-blinded, placebo-controlled, 2x2 factorial trial, participants were randomised to receive bread fortified with placebo (PL), 2.3g PS (PS), 228mg curcumin (CC) or a combination of 2.3g PS and 228mg CC (PS-CC) daily for four weeks. Primary outcome was fasting plasma lipids [total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG)] and secondary outcomes were plasma LDL-particle (LDL-P) profile: LDL-P number and LDL-P size. Cardiovascular disease (CVD) risk (Framingham Risk Algorithm) was also explored.

There was no significant difference between PL and CC or PS and PS-CC on blood lipids or CVD risk; therefore, groups were pooled for final analysis: PL and CC group (PL-C, n=36) and PS and PS-CC group (PS-C, n=39). PS-C significantly lowered TC (-0.52 mmol/L, $p<0.0001$), LDL-C (-0.49 mmol/L, $p<0.0001$) and CVD risk (-1.1 absolute %, $p=0.0005$) compared to PL-C group. Reductions from baseline in the PS-C group compared to PL-C were 7.6% and 10.6% for TC and LDL-C respectively and statistically significant ($p<0.0001$). CVD-risk in the PS-C group reduced significantly (-12.7%) compared to PL-C ($p=0.0005$). HDL-C and TG remained unchanged. LDL-P number significantly decreased in the PS-C group by 124.33 nmol/L compared to PL-C ($p=0.005$) and both groups significantly decreased in LDL-P size ($p<0.01$), however, the absolute nm change in LDL-P size did not

differ between groups and the percent change in LDL-P size in the PS-C group was borderline significant (-0.89%, p=0.05) compared to PL-C.

Regular consumption of PS-enriched bread with or without curcumin lowers blood cholesterol, however, curcumin alone did not influence blood lipids. Bread may be a convenient means of delivering PS with greater compliance for reducing blood cholesterol concentration.

Keywords: cholesterol, LDL cholesterol, phytosterols, curcumin, hypercholesterolemia, cardiovascular disease

Highlights:

- Study findings confirm the cholesterol-lowering ability of phytosterols
- Bread enriched with phytosterols is an effective food delivery vehicle
- Cholesterol-lowering effects were not modulated by curcumin
- Findings support the development of a functional food for enhancing heart health
- Further research is required to investigate the lipidaemic effects of curcumin

INTRODUCTION

Cardiovascular disease (CVD) is the leading global burden of disease, claiming 31% of all deaths in 2016 [1]. Abnormal circulating blood lipids (dyslipidaemia) remains a key risk factor for CVD and is prevalent in over two thirds of the Australian adult population [2]. Dyslipidaemia is characterised by low concentrations of HDL-cholesterol (HDL-C) and elevated concentrations of total cholesterol (TC), triglycerides (TG) and LDL-cholesterol (LDL-C) [2]. A major concern is raised levels of small-dense LDL particles, as these are rich in TGs [3], reside longer in circulation [4] and are able to penetrate the arterial lumen where they are readily oxidized, thus increasing its atherogenic potential [5, 6]. In recent years, concentrations of LDL particle (LDL-P) number measured by nuclear magnetic resonance (NMR) spectroscopy has been shown to be more indicative of an individual's LDL-based risk of CVD when concentration of LDL-P and LDL-C are discordant [7, 8]. Changes to diet and lifestyle can modestly improve blood lipids, however, pharmacological intervention is often indicated to assist with achieving blood lipid targets. Unfortunately, long-term adherence and complexity of such regimes along with adverse side effects and cost-burden can serve as barriers to sustainable lipid management in the long-term [9]. Therefore simple, safe and effective strategies are required to give patients self-efficacy and to maximise their heart health.

Phytosterols (PS) have been widely employed as an effective strategy for managing elevated LDL-C and can be used as an adjunct and/or alternative to pharmacological interventions [10-12]. PS are naturally found in the diet from plant foods such as fruit, nuts, seeds, vegetables and oils of vegetables, nuts and seeds. It has been established that 2g/d of PS via fortified products lowers circulating LDL-C by 8-10% in four weeks with no effects on HDL-C and no or modest TG lowering effects [13-16]. PS are structurally similar to cholesterol, allowing their effective reduction of cholesterol absorption [17]. The most widely

accepted mechanism of action by PS for cholesterol-lowering is micellar displacement of dietary and biliary cholesterol in the gut, resulting in increased cholesterol excretion [18].

Curcumin, found in the underground rhizomes (turmeric) of the *Curcuma longa* plant has been shown to modulate several molecular targets giving rise to its diverse range of health benefits such as anti-inflammatory, anti-aggregatory, anti-oxidant, anti-apoptotic, anti-proliferative and anti-cancer [19]. Mild lipid-modulatory effects have been demonstrated in preclinical studies such as the reduction of endogenous cholesterol synthesis by down-regulation of key lipogenic factors [10, 20-22], stimulation of the secretion and clearance of bile [23, 24] and reduction of lipid accumulation in adipose tissue [25, 26]. Very minimal amounts of curcumin are absorbed in the gut, as it has poor aqueous solubility and bioavailability. Any absorbed curcumin undergoes rapid degradation by liver enzymes [19] with the majority recovered in the faeces [27]. Various methods have been developed to improve the bioavailability of curcumin so it can be used therapeutically such as encapsulation as polymer nanoparticles with piperine and liposome- and phospholipid-curcumin complexes [28].

Our previous work has shown that dietary supplementation with this novel combination of PS and curcumin via vegetable fat spread and tablets (respectively), significantly lowered plasma LDL-C of hypercholesterolaemic individuals by 15% in four weeks [15]. We hypothesised that the enrichment of a single food with both bioactives could enhance the cholesterol-lowering effects of the combined therapy with improved dietary adherence. Findings from this study could support the use of bread, a common global staple food, as an effective delivery mode for PS with or without curcumin as a strategy for managing dyslipidaemia and improve overall heart health.

MATERIALS AND METHODS

Study participants

Recruitment was conducted in the Hunter region (NSW, Australia) from mid-January 2018 to August-end 2018. Participants with hypercholesterolaemia were reached via media exposure (newspaper articles and radio interviews), flyers placed on local community noticeboards and word of mouth. Eligibility was assessed over the phone or in person by the lead investigator. Eligible participants were: healthy adults aged 18 to 70 years old; fasting plasma TC ≥ 5.5 mmol/L; not taking lipid- or glucose-lowering medications; no chronic disease such as CVD, diabetes mellitus, kidney/liver/gastrointestinal conditions, neurological conditions or untreated hypertension (≥ 140/95 mm Hg); not consuming PS-enriched products, curcumin supplements or any other supplements known to influence blood lipids (e.g. fish/krill/flaxseed oils, coenzyme Q10, fibre supplements); BMI < 40 kg/m^2; not pregnant or lactating; non-smoker and no strong food aversion and/or intolerance or allergy to gluten or wheat. The study protocol was approved by the Human Research Ethics Committee, University of Newcastle (H-2015-0162) and all procedures were conducted in accordance with the 1975 Declaration of Helsinki as revised in 1983. The trial was registered with the Australian New Zealand Clinical Trials Registry at http://www.anzctr.org.au/ (ACTRN 12618001960246).

Study design and intervention

This study was a four-week, double-blinded, randomised, placebo-controlled trial with a 2x2 factorial design in four parallel groups. The senior investigator allocated treatment groups using a computer-generated block randomisation method (Random Allocation Software version 1.0.0). Participants were randomly allocated to one of four treatments: two slices of bread per day containing either placebo (PL; no PS or curcumin), phytosterol (PS; 2.3g/d PS), curcumin (CC; 228mg/d curcumin) or a combination of PS and curcumin (PS-

142 CC; 2.3g/d PS + 228mg/d curcumin) for four weeks. All four intervention breads were
143 identical in sensory characteristics and bread bags were labelled with four colour-coded kwik
144 locks upon packaging by the manufacturer; therefore, all investigators and participants were
145 blinded to the treatment allocation. Breads were prepared and packaged by George Weston
146 Foods under standard bread-making procedures and GMP conditions. The dough was baked
147 at 220ºC for 30 minutes and proofed for approximately 40-50 minutes. Basic ingredients
148 included in the bread were plain white flour, yeast, improver, softener, water and salt. The
149 nutritional profile of the bread was similar to the commercially available white sandwich
150 bread and all breads were comparable for nutritional content except for the enrichment of PS,
151 curcumin and respective placebo ingredients (**Table 1**). For fortification of the bread,
152 Vegapure® 67WDP (BASF) is a mixture of plant sterol fatty acid esters spray dried into a
153 water dispersible powder and was the source of PS. The carrier system consists of sodium
154 caseinate, glucose syrup and antioxidants such as tocopherols and ascorbate. Vegapure®
155 67WDP is intended for use in dietary supplements and food applications. Meriva® (Indena®)
156 powder is a patented PHYTOSOME® curcumin formulation and was the source of curcumin.
157 In Meriva®, curcumin and soy lecithin are formulated in a 1:2 weight ratio with the addition
158 of microcrystalline cellulose to improve flowability. Canola oil was used as a placebo for PS
159 in the PL and CC bread since canola fat-based products have been used previously as a
160 placebo to PS, demonstrating minimal (if any) non-significant lowering of blood cholesterol
161 [15, 29]. Lucarotin 1 CWD/Y (BASF) and apo carotenal 2% (BASF) was used to dye the PL and
162 PS breads. Participants were instructed to consume one serving of study bread i.e. two slices
163 of bread each day at lunch time as part of a meal for the entirety of the study duration. They
164 were instructed not to change their diet and lifestyle habits and they were permitted to eat
165 other bread if desired. Compliance was monitored by a daily bread consumption log
166 completed by participants and all remaining and consumed bread was counted by the lead

investigator at the final appointment. In addition, habitual dietary intake pre- and post-intervention was also analysed to assess compliance (FoodWorks, Xyris ®, Professional Edition Version 8.0.3551).

Clinical measures

Participants attended clinical trial facility at the Nutraceuticals Research Program located at the University of Newcastle, Callaghan, NSW Australia following an overnight fast (10 hours) at baseline and post-intervention. Anthropometric measures, blood pressure, medical history, habitual dietary intake, physical activity patterns and fasting blood samples were collected for plasma TC, LDL-C, HDL-C, TC:HDL ratio, TG and Framingham Risk Algorithm. The Framingham Risk Algorithm includes the following parameters as predictors: age; gender; TC, HDL-C, systolic blood pressure and status for smoking, diabetes mellitus and treatment for blood pressure status (yes/no) [30].

Anthropometry and body composition

Anthropometry such as height, waist circumference, weight and BMI and body composition were measured with participants wearing light clothing. Shoes and all metal and/or electronic devices on participant's body was asked to be removed for all measurements. Height (cm), waist circumference (cm) and weight (kg) were collected to the nearest 0.1 units. A wall-mounted stadiometer with a movable head piece (Seca 206 Bodymeter Wall Height Measure Ruler) was used to measure height (cm). A tensible tape measure positioned midway between the lower rib margin and the iliac crest (approximately in line with the belly-button) horizontally was used to measure waist circumference (cm). Weight (kg) and other body composition parameters (skeletal muscle mass, fat mass) were measured in the standing position using bioelectrical impedance which utilises two different frequencies (InBody230, Biospace Co.). Blood pressure was measured in the seated position using a digital sphygmomanometer (Microlife®, BP3AD1-A Heerbrugg, Switzerland). Three

192 serial measurements with 1-minute rest in between, of systolic blood pressure (SBP) and
193 diastolic blood pressure (DBP) were taken in the supported left arm of a rested participant (5-
194 10 minutes). The arm was positioned at the same height as the heart. The first measurement
195 was discarded and an average of the remaining two were considered as the final
196 measurement. Participants refrained from alcohol consumption and vigorous physical activity
197 for 24 hours prior to their appointments and had fasted overnight (10 hours).

198 *Medical history, dietary intake and physical activity*

199 All participants completed a self-administered medical history questionnaire at baseline
200 to collect information regarding past and present medical conditions: history of blood lipid
201 profile (including any recent blood results if available), prescribed or over-the-counter
202 medication(s), habitual supplement use and habitual consumption of alcohol, PS-enriched
203 products, turmeric and/or curcumin, fibre, added sugars, fats and oils. A 3-day food diary and
204 physical activity questionnaire (International Physical Activity Questionnaire; IPAQ Long
205 Last 7 Days Self-Administered Format, October 2002) were used to collect information on
206 habitual diet and physical activity patterns at baseline and post-intervention, respectively.
207 FoodWorks, Xyris. Professional Edition Version 8.0.3551 was used for evaluation of all
208 dietary data. Physical activity data was interpreted as metabolic equivalent of task hours per
209 week (MET hrs/week) to measure the energy cost of physical activities.

210 *Blood sampling, lipid analyses and plasma LDL profile*

211 A phlebotomist collected fasted blood samples at baseline and post-intervention via
212 venepuncture into tubes pre-coated with EDTA. Samples were centrifuged (Heraeus Biofuge
213 Stratos) for 10 minutes at 3000 x *g* at 4ºC. Plasma and red blood cell fractions were aliquoted
214 and stored at -80ºC until further analysis. Blood parameters were measured on a VP auto
215 analyser using standardized reagents by the Hunter Area Pathology Service. LDL-C

concentration was determined using the Friedewald equation [31]. LDL-P number concentrations (nmol/L) was measured by proton NMR spectroscopy (LP4 NMR MetaboProfile™ Analysis), using the LipoProfile-3 algorithm at the National Heart, Lung, and Blood Institute; National Institute of Health (Bethesda, United States of America). LDL subclasses of different size (nm) was quantified from the amplitudes of their spectroscopically distinct lipid methyl group NMR signals. The detailed method for the determination of LDL-P number and LDL-P subclass size has been previously reported [32]. The size range for different lipoprotein subclasses corresponded to: very large TG-rich lipoprotein particle (TRL-P), 90 – 240 nm; large TRL-P, 50 – 89 nm; medium TRL-P 37 – 49; small TRL-P, 30 – 36 nm; very small TRL-P, 24 – 29nm; large LDL-P, 21.5 – 23 nm; medium LDL-P, 20.5 – 21.4 nm; small LDL-P, 19 – 20.4 nm; large HDL-P (H7P, H6P and H5P subspecies), 9.6 – 13 nm; medium HDL-P (H4P and H3P), 8.1 – 9.5 nm; and small HDL-P (H2P and H1P subspecies), 7.4 – 8 nm.

Statistical analysis

Sample size calculation yielded 80 participants in total (20 per group) based on previous estimates of variance in plasma TC concentration (standard deviation of 0.5) to elicit 80% power at a significance level of 0.05 for detection of a 0.50 mmol/L (~10%) reduction in TC whilst accounting for a 20% dropout rate. Normality was assessed via the Shapiro Wilk test and visual plots including histograms. All data are presented as means ± standard error of the mean (SEM) or median (25^{th} percentile, 75^{th} percentile). Comparison of baseline characteristics across treatment groups for anthropometrics, body composition, dietary intake, physical activity levels and blood parameters was assessed by ANOVA for normally distributed data and Kruskal-Wallis for non-normally distributed data for comparisons of more than two groups and Independent Samples t-test or Wilcoxon rank-sum test for comparisons of two groups. The chi-square test was used to compare gender and ethnicity

between groups at baseline. Depending on normality, paired samples t-test or Wilcoxon Signed Rank test was performed for change from baseline to post-intervention within-treatment groups. One-way ANOVA was used to investigate the differences between the four treatment groups in terms of their effects on the absolute and percent change from baseline to post-intervention for each dependent variable. Analysis of covariance was also used for each primary outcome variable (absolute and percent change in TC, LDL-C, LDL-P number and LDL-P size) with the inclusion of treatment group as a factor and the corresponding baseline values of the outcome variable as a covariate to enable adjustment of baseline measures. Any explanatory variables that were statistically significantly related with the outcome variables from bivariate analyses as well as known confounders from the literature were also included in the model as additional covariates. Overall, the covariates considered included age and baseline data for: waist-to-hip ratio, percent fat, exercise levels, dietary intake (saturated fat, trans fat, omega-6 polyunsaturated fatty acids, long chain omega-3 polyunsaturated fatty acids, fibre, alcohol and cholesterol) and baseline values for each primary outcome. Correlations were used to assess the relationship between explanatory variables and variables with correlation coefficients greater than 0.8 were assessed more closely for multicollinearity and the number of potential predictors to include in the analyses was reduced accordingly. For each outcome variable, a backward stepwise regression procedure was employed to eliminate covariates that were not statistically significant at the 0.05 significance level from the regression model after adjustment for the other predictors in the model. For all tests a significance level of 0.05 was used. StataCorp 2015 (*Stata Statistical Software: Release 14.* College Station, TX: StataCorp LP) was used to conduct all statistical analyses.

RESULTS

Baseline characteristics

Eighty-two participants were recruited during the period mid-January 2018 to August-end 2018. One participant dropped out of the trial due to personal reasons. A further six participants were excluded from the trial due to unreliable blood result (n=1) and significant outliers to the data set (1.5 x interquartile range below 25th quartile and above 75th quartile for relative change in LDL-C) (n=5). A total of 75 participants were included in the final analysis (**Figure 1**). Overall, 57% of participants were females and 79% were north-west European. On average, participants were middle-aged 55.61±1.39 y and slightly overweight with waist circumference of 92.61±1.46 cm, waist-to-hip-ratio of 0.94±0.01, weight of 77.74±1.77 kg, BMI of 26.92±0.49 kg/m^2, fat percentage of 31.29±1.13 % and skeletal muscle mass of 29.73±0.89 kg. Participants were hypercholesterolaemic at baseline with elevated levels of TC (6.74±0.11 mmol/L) and LDL-C (4.55±0.10 mmol/L), however, their HDL-C (1.48±0.04 mmol/L) and TC:HDL ratio (4.84±0.16) and median (25th percentile, 75th percentile) TG of 1.39 (1.02, 2.05) mmol/L were in the normal range. Participants had low (<10%) 10-year CVD risk 9.58 (5.04, 15.37) %. Lipoprotein particle number concentration was TRL-P 189.72±7.85 nmol/L, LDL-P 1910.21±45.96 nmol/L denoting high risk of CVD [33], HDL-P 23.99±0.31 μmol/L and average lipoprotein particle size in nm was medium in size for all: TRL-P 45.80 (41.9, 50.20); LDL-P 21.30 (20.90, 21.60) and HDL-P 9.03±0.05. There was no statistically significant difference detected between treatment groups at baseline (**Table 2 and 3**). Since the addition of curcumin to PL or PS did not influence blood lipid levels (**Supplementary Table 2**), the data for PL and CC (no phytosterols) groups were pooled (PL-C, n=36) and the data for the PS and PS-CC (both containing phytosterols) groups were pooled (PS-C, n=39) for subsequent analysis (Figure 1). After randomisation, the baseline TC

and LDL-C in the CC group were the lowest of all groups (non-significant), which may have contributed to the differential effect of CC intervention on blood lipids.

Anthropometry, dietary intake, physical activity and compliance

Anthropometric measures, blood pressure and physical activity were not statistically significantly different between groups at post-intervention and changes in these parameters were not different across groups (data not shown). There were also no statistically significant differences in dietary intake at baseline between groups (**Supplementary Table 1**) and nor were there any differences in the mean change of dietary parameters from baseline to post-intervention across groups. There was no statistically significant change in physical activity levels from baseline to post-intervention within- or across groups. Compliance to bread consumption was excellent and well tolerated by participants (98.98±0.35%).

Effect of phytosterol and curcumin intervention on plasma lipid profile and cardiovascular disease risk

Dietary supplementation with PS-C for four weeks significantly reduced TC (-0.44±0.07 mmol/L or -6.1%; $p<0.0001$) and LDL-C (-0.44±0.06 mmol/L or -8.8%; $p<0.0001$) (**Table 3, Figure 2)**. These absolute and percent changes were significantly different from the PL-C group ($p<0.0001$). TC:HDL ratio was significantly reduced in the PS-C group [-0.30 (-0.60, 0.00) or -5.4%; $p<0.001$) and these changes were significantly different to PL-C group ($p<0.01$). HDL-C and TG concentrations remained unaltered from baseline and nor were the changes significantly different between treatment groups. Ten-year CVD risk calculated using the Framingham Risk Algorithm significantly reduced post-intervention in the PS-C group by -0.64 (-1.53, -0.09) % or -8.1%; ($p<0.001$) from baseline and this was significantly lower than PL-C ($p<0.001$) group. LDL-P number was significantly reduced in the PS-C group ($p<0.01$) and this change was statistically significant compared to the PL-C group ($p<0.01$) (**Table 4)**. The mean particle diameter of LDL-P was significantly lower in PL-C

(p<0.01) and PS-C (p<0.0001) post-intervention and when analysed as percent change from baseline, it just reached statistical difference between groups (p<0.05). Lipoprotein particle concentration and subclass size diameter of TRL-P and HDL-P was not significantly altered by either intervention.

The effects of baseline data on change in blood lipid profile

Baseline data including age, percent fat, waist-to-hip-ratio, TC and LDL-C concentration, exercise levels and dietary intake of saturated fats, trans fats, omega-6 polyunsaturated fatty acids, long chain omega-3 polyunsaturated fatty acids, fibre, alcohol and cholesterol were assessed as potential confounders to the primary outcomes (absolute- and percent change in TC, LDL-C, LDL-P number and LDL-P size) in a multiple regression using the backward stepwise regression procedure by including them as predictor variables in the original model. The final reduced models revealed that treatment remained a significant predictor of the change in all primary outcomes (TC and LDL-C, p<0.001; LDL-P number, p<0.05; LDL-P size, p<0.05) (**Supplementary Table 3**). Baseline TC and LDL-C were also predictors (p<0.001) of the change in TC and LDL-C, respectively. Dietary cholesterol intake was also predictor of the absolute change in TC and LDL-C as well as the relative change in LDL-C (p<0.05). Baseline TC, LDL-C and LDL-P number were significant predictors (p<0.05) for absolute change in LDL-P number and the same was reported for percent change with the addition of dietary trans-fat (p<0.05). Significant predictors of change in LDL-P size included waist-to-hip ratio, TC, dietary intake of saturated and trans-fat (p<0.05) and LDL-C (p<0.01).

DISCUSSION

PS are well established hypocholesterolaemic agents with LDL-C-lowering achieved by 8-10% when administered at 2g per day for as short as 3-4 weeks [13]. Our findings show that PS effectively lowered TC by ~6% and LDL-C by ~9% in free-living hypercholesterolaemic adults supplemented with two slices of PS-enriched bread daily for four weeks. In addition, we demonstrated a significant reduction in 10-year CVD risk, LDL-P concentration and LDL-P diameter following PS supplementation. However, contrary to our previous work [15], curcumin fortification alone and in combination with PS did not provide any additional cholesterol-lowering effects in the current study.

The reductions in plasma cholesterol reported in our study are consistent with previous studies of similar dose, duration and subject characteristics [13, 34], and our findings were not affected after adjusting for potential confounders. In contrast, a study that compared food matrices (milk vs yoghurt vs bread vs cereal) for delivery of 1.6 g/d PS found that cereal and bread were less efficacious for LDL-C-lowering (5.4 – 6.5% respectively) [35]; whereas a systematic review and meta-analysis found no efficacious difference between dairy vs. non-dairy and fat-based vs. non-fat based food matrices [13]. Such discrepancies between trials investigating the LDL-C-lowering response to PS intervention could be related to individual's genotypic variation in apolipoprotein E and cholesterol 7 alpha-hydroxylase (CYP7A1) isoforms [36] and this might also apply to curcumin supplementation. There are various mechanisms of action reported for the cholesterol-lowering ability of PS in addition to micellar incorporation [18], some of which could bear potential synergistic action when paired with curcumin. Preclinical studies have shown that both PS and curcumin (independently) lower the activity of acyl coenzyme A cholesterol acyltransferase (ACAT), thus inhibiting/reducing esterification of cholesterol and subsequent uptake and/or transport in the intestine [37, 38]. In addition, PS and curcumin have been reported to potently activate and

upregulate (respectively) expression of liver X receptor alpha (LXRα), a key regulator of cholesterol transporters (ABCA1, ABCG5 and ABCG8) and mediator for free sterol efflux from enterocytes [20, 39, 40]. There are also mechanisms reported to suggest complementarity between the two bioactives such as curcumin pertaining statin-like properties by way of down-regulating 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) [20]; reduced cholesterol esterification via indirect inhibition of Niemann Pick C1-Like 1 (NPC1L1) expression [41]; increased LDL-C uptake, receptor level and activity via down-regulation of proprotein convertase subtilisin /kexin type 9 (PCSK9) gene expression [42]; and stimulation of bile secretion via enhanced gene expression of CYP7A mRNA [23, 24].

The dietary combination of PS and curcumin was first investigated in an RCT by our group whereby a vegetable fat spread and tablets (respectively) served as the dietary delivery using a similar dose, duration and subject characteristics as the present study [15]. We found that dual supplementation of PS and curcumin led to a significant 11% reduction in TC and 14.4% in LDL-C, whereas smaller reductions were reported in the individual PS (-4.8% and -8.1% respectively) and curcumin groups (-2.3 % and -2.6% respectively), suggesting more than just complementarity between the two bioactives. In both studies we used Meriva® a bioavailability enhanced formulation of curcumin complexed with phosphatidylcholine as a curcumin-PHYTOSOME® complex [43]. In this formulation, curcumin is on the surface of the phytosome and possibly less protected and unstable at higher temperatures (170 ºC), such as during the bread baking process. A thermal degradation kinetics study concluded that food processing temperatures using curcumin as a food colouring agent should not exceed 190 ºC, to avoid thermal decomposition [44]. It is possible that during baking, either the PHYTOSOME® complex or the curcumin bound on the surface was mildly destroyed or damaged. Perhaps solid lipid curcumin particle (SLCP™) formulation where curcumin is

383 encapsulated into a lipid core, would be more robust for future food preparations involving
384 heat exposure [45].

385 In recent years, increased attention has been drawn to measuring the number and size of
386 LDL-P as these are more predictive of CVD risk than LDL-C concentrations [7, 46]. It has been
387 shown in the Framingham offspring study [47] and Multi-Ethnic Study of Atherosclerosis
388 (MESA) [8] that LDL-C and LDL-P are equally associated with coronary artery disease risk
389 when LDL-C and LDL-P were concordant, however, if discordant (occurs in patients with
390 metabolic syndrome and type 2 diabetes); the LDL-attributable atherosclerotic risk is better
391 indicated by LDL-P. In the present study, individuals not only had elevated LDL-C
392 concentrations, but they also had a high LDL-P level of ~1910 nmol/L (1600-2000 nmol/L);
393 indicating a higher risk of CVD [8, 33]. LDL-P was significantly reduced following PS
394 supplementation in our study and to the best of our knowledge there is only one other study
395 which has measured LDL-P via NMR following PS supplementation, and they reported
396 similar findings [48]. In this study, Matvienko et al reported a borderline significant reduction in
397 particle number (p=0.058) following consumption of ground beef enriched with 2.7 g/day PS
398 in young mildly hypercholesterolaemic males [48]. Participants did, however, have a much
399 lower baseline LDL-C (4.02 mmol/L) compared to our participants (4.55 mmol/L), which we
400 have showed predicts change in LDL-P number. Moreover, their LDL-P at follow-up was
401 much lower (1467 nmol/L, borderline-high risk) than ours (1863 nmol/L, high risk),
402 suggesting they had a lower baseline LDL-P (baseline LDL-P was not reported) [48]. The
403 amount of cholesterol per LDL-P varies depending on the size, with smaller LDL-P carrying
404 less. Increased atherogenic potential of small LDL-P is suggested by increased binding ability
405 to arterial proteoglycans [49], greater propensity for uptake by arterial tissue suggestive of
406 enhanced transendothelial transport [50], reduced receptor-mediated uptake [51], and increased
407 susceptibility to oxidative modification [52, 53]. Overall findings are inconsistent with respect to

particle size modulation by PS, with studies reporting reductions in the concentration of small LDL-P [54] and studies reporting no effect on subclass concentrations [55] or shifts in subclass size [48]. The average mean diameter of LDL-P in our study was ~21 nm, which is medium subclass size and significantly reduced in both treatment groups, but more prominently in the PS-C group. Previous findings suggest that beneficial shifts in subclass are more likely in individuals with metabolic syndrome who tend to have elevated TG and low HDL-C [32, 46], which was not the case in this study.

We reported a significant 8% reduction from baseline in 10-year CVD risk (Framingham Risk Algorithm) in only four weeks. It is unknown whether PS affect CVD endpoints, as randomised controlled trial evidence with hard end-points are lacking. Several observational studies have explored the association between atherosclerosis and circulating PS in the general population. Gylling et al report that earlier studies have found positive associations between PS and vascular disease whereas others an inverse or lack of association [56]. The longest trial duration administering PS is 85 weeks [57], therefore, large-scale outcome trials and longer trial duration of PS-enriched foods are warranted to substantiate our CVD risk findings and ascertain the long-term heart health effects of PS.

Chronic low-grade inflammation is another risk factor for CVD that operates in congruence with dyslipidaemia to initiate and exacerbate atherosclerosis [6]. There is some evidence for anti-inflammatory and immune-modulatory effects of PS in preclinical [58] and human studies [59, 60], however, the consensus on this remains inconsistent [61]. Curcumin is a well-known potent anti-inflammatory agent, which is mediated by its ability to down-regulate the activation of nuclear factor kappa-B [62, 63], a transcription factor largely responsible for gene expression of pro-inflammatory cytokines. The addition of curcumin to PS therapy has the potential to not only enhance lipid-lowering but lower chronic inflammation associated with the initiation of atherosclerosis thereby maximising cardio-protection.

The double-blinded randomised study design, excellent compliance, safety of PS [56] and curcumin [64]; and production of a high-quality intervention product are key strengths of this study as well as adequately powered sample size. Participants were also free-living individuals who continued about their habitual diet and lifestyle whilst participating in the trial, yet compliance was excellent, therefore, our findings are highly transferable to the Australian adult population who have elevated cholesterol levels. The study duration was adequate to demonstrate a modulation in plasma cholesterol as per previous PS studies; however, a longer duration could further substantiate our findings as well as provide further insight into the longer-term dietary supplementation of curcumin. Another possible limitation involves the handling of curcumin during the bread production. It is unknown whether the production process, particularly the baking temperature; damaged the curcumin and/or PHYTOSOME® complex, but further investigation into the stability of curcumin formulations during various food processing should be explored in the context of functional food development.

CONCLUSIONS

Findings from this study provides evidence for the use of bread as a safe, efficacious and compliable food format for PS with or without curcumin for lowering cholesterol, 10-year CVD risk score and modulating lipoprotein profile in hypercholesterolaemic individuals. These findings support the development of a functional food that may serve as an efficacious adjunct therapy to classic pharmacological interventions targeted at dyslipidaemia. It also serves as a safe alternative for individuals who are statin intolerant and require moderate lowering of their blood cholesterol. Although not explored in this paper, this novel combination has potential added heart health benefits attributed to by curcumin such as anti-inflammatory, anti-oxidant, anti-cancer, anti-aggregatory [19] and mild hypoglycaemic effects [15, 65]. Further research is warranted to investigate the non-lipid modulatory roles curcumin has to offer when combined with PS in a functional food as well as the optimal delivery medium of bioavailability-enhanced curcumin for food processing.

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STATEMENT OF AUTHORSHIP

JJAF and MLG conceptualized and designed the research. JJAF conducted research. JJAF analysed the data. AW and AR performed lipoprotein particle profile analyses. ES provided statistical support. JJAF and MLG wrote the paper; JJAF had primary responsibility for final content. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest to declare.

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666

FIGURE LEGENDS

Figure 1

CONSORT schematic of participant recruitment, screening and assessment.

* Value for primary outcome lies outside two standard deviations from the mean

Figure 2

Figure 1. Percent change in TC, LDL-C, HDL-C, TC:HDL ratio, TG and 10-year CVD risk from baseline to post-intervention in hypercholesterolaemic individuals who consumed PL-C or PS-C for 4 weeks. Data represent mean ± SEM or median (25^{th} and 75^{th} percentile) where appropriate. Symbols indicate significant changes from baseline as analysed by Independent samples t-test or Wilcoxon Rank Sum test: * $p<0.01$, ** $p<0.001$, *** $p<0.0001$. Significant changes in blood measures between the groups (Independent Samples t-test or Wilcoxon Rank Sum test) are indicated by superscript letter.

[a] Change in TC was significantly lower in the PS-C group compared to the PL-C group ($p<0.0001$)

[b] Change in LDL-C was significantly lower in the PS-C group compared to the PL-C group ($p<0.0001$)

[c] Change in TC:HDL ratio was significantly lower in the PS-C group compared to the PL-C group ($p=0.0024$)

[d] Change in 10-year CVD risk was significantly lower in the PS-C group compared to the PL-C group ($p=0.0005$)

PL-C, placebo; PS-C, phytosterols.

690 **TABLES**

691 Table 1. Nutrient composition of study bread.[1]

Dietary component	One serving of bread (two slices)
Energy	
kJ	811.7
kcal	194.2
Protein (g)	5.0
CHO (g)	32.3
Sugars (g)	4.4
Starch (g)	27.9
Total fat (g)	4.3
Saturated (g)	0.7
Trans (g)	0.1
MUFAs (g)	0.8
PUFAs (g)	2.7
Dietary fibre (g)	1.7
Sodium (mg)	299.1
For Phytosterol bread:	
Phytosterols (g)	2.3
For Curcumin bread:	
Curcumin (mg)	228

[1] Nutrient information is given for one serve (2 slices bread, ~75 g). Each participant consumed one serving of bread per day. CHO, carbohydrates; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

692

693 Table 2. Participant characteristics at baseline.[1]

	PL-C (n = 36)	PS-C (n = 39)
Sex, *n* (%)		
Male	17 (47)	15 (38)
Female	19 (53)	24 (62)
Ethnicity, *n* (%)		
North-west European	27 (75)	32 (82)
South-east European	2 (5)	4 (10)
Asian	1 (3)	0 (0)
Other [2]	6 (17)	3 (8)
Age (y)	54.00 ± 1.94	57.10 ± 1.98
Height (cm)	171.00 ± 1.77	168.01 ± 1.51
Waist circumference (cm)	91.69 ± 1.79	93.46 ± 2.28
Waist-to-hip ratio	0.93 ± 0.01	0.95 ± 0.01
Weight (kg)	77.83 ± 2.15	77.66 ± 2.81
BMI (kg/m^2)	26.53 ± 0.55	27.28 ± 0.79
Skeletal muscle mass (kg)	30.78 ± 1.24	28.77 ± 1.28
Body fat mass (g)	22.84 ± 1.44	25.91 ± 1.67
Body fat (%)	29.37 ± 1.70	33.06 ± 1.47
SBP (mmHg)	122.94 ± 2.11	129.73 ± 2.75
DBP (mmHg)	75.92 ± 1.45	79.95 ± 1.60
MET hrs/wk	97.87 ± 12.88	82.78 ± 14.47

[1] Values are reported as means ± SEM for continuous measures and as n (%) for categorical measures.

[2] Other races include Oceanian; North African and Middle Eastern; Other (combination of races). Independent samples t-test was used to compare baseline data for normally distributed data and Wilcoxon rank sum for non-normally distributed data.

DBP, diastolic blood pressure; MET hrs/wk, metabolic equivalent of task hours per week; PL-C, placebo; PS-C, phytosterols; SBP, systolic blood pressure.

694

Table 3. Change in plasma lipid concentrations (Δ) in the placebo (PL-C) and phytosterol (PS-C) groups from baseline (BL) to post-intervention (PI).[1]

	PL-C (n = 36)	PS-C (n = 39)	*p* ^
TC			
BL	6.60 ± 0.17	6.86 ± 0.14	
PI	6.68 ± 0.16	6.42 ± 0.11***	
Δ mmol/L[3]	0.08 ± 0.06	-0.44 ± 0.07	<0.0001
LDL-C			
BL	4.47 ± 0.15	4.63 ± 0.13	
PI	4.52 ± 0.14	4.19 ± 0.10***	
Δ mmol/L[4]	0.05 ± 0.06	-0.44 ± 0.06	<0.0001
HDL-C			
BL	1.42 ± 0.06	1.54 ± 0.06	
PI	1.44 ± 0.06	1.52 ± 0.06	
Δ mmol/L	0.02 ± 0.03	-0.02 ± 0.02	0.388
TC:HDL			
BL	4.75 (3.8, 5.75)	4.40 (3.70, 5.50)	
PI	4.70 (3.95, 5.60)	4.40 (3.40, 5.30)**	
Δ	0.00 (-0.30, 0.35)	-0.30 (-0.60, 0.00)	0.003
TG			
BL	1.39 (0.98, 2.02)	1.31 (1.05, 2.05)	
PI	1.30 (1.05, 1.95)	1.40 (0.93, 1.75)	
Δ mmol/L	-0.02 (-0.31, 0.33)	-0.05 (-0.22, 0.11)	0.436
10-year CVD risk (%)			
BL	8.43 (5.04, 13.63)	10.46 (5.04, 17.40)	
PI	9.30 (4.99, 14.41)	9.96 (4.32, 16.42)*	
Δ	0.46 (-0.31, 1.78)	-0.64 (-1.53, -0.09)	<0.001

[1] Values are reported as means ± SEM for all data except TC:HDL ratio, triglycerides and 10-year CVD risk data which is presented as median (25th and 75th percentile) due to lack of normality of the distribution. All baseline and post-intervention data are in mmol/L except for TC:HDL ratio.

Significant change from baseline, * $p<0.01$, ** $p<0.001$, *** $p<0.0001$.

^ Independent samples t-test or Wilcoxon rank sum test was used to compare change in outcome parameters across treatment groups. $P < 0.05$ indicates statistically significant difference between groups.

CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PL-C, placebo; PS-C, phytosterols; TC, total cholesterol, TC:HDL, total cholesterol-to-HDL cholesterol ratio; TG, triglycerides.

695

696 Table 4. Lipoprotein profiles (total particle concentration and particle size) at baseline and
697 post-intervention measured using nuclear magnetic resonance spectroscopy.

	PL-C	PS-C	*p* ^
Total lipoprotein particle concentration			
TRL-P (nmol/L)			
BL	194.50 ± 10.72	185.31 ± 11.50	0.5623
PI	192.61 ± 11.32	173.18 ± 12.27	0.2508
Δ nmol/L	-1.89 ± 7.27	-12.12 ± 6.63	0.3007
Δ %	2.00 ± 4.47	-8.36 (-25.22, 13.92)	0.2565
LDL-P (nmol/L)			
BL	1864.33 ± 73.24	1952 ± 57.02	0.341
PI	1899.22 ± 76.30	1863.13 ± 55.73**	0.701
Δ nmol/L	34.89 ± 31.86	-89.44 ± 28.89	**0.0050**
Δ %	2.35 ± 1.63	-4.20 ± 1.56	**0.0049**
HDL-P (µmol/L)			
BL	23.55 (21.75. 24.90)	24.20 (21.80, 25.60)	0.6636
PI	23.15 (21.95, 25.20)	23.60 (22.20, 25.40)	0.5704
Δ µmol/L	-0.08 ± 0.21	-0.32 ± 0.35	0.5607
Δ %	-0.35 ± 0.88	-0.83 ± 1.43	0.7823
Lipoprotein particle size (nm)			
TRL-Z			
BL	44.80 (42.40, 49.30)	46.10 (41.30, 50.40)	0.937
PI	46.55 (41.10, 49.90)	48.10 (42.40, 53.60)	0.451
Δ nm	1.95 ± 1.24	1.70 ± 0.97	0.873
Δ %	4.56 ± 2.65	4.48 ± 2.04	0.981
LDL-Z			
BL	21.30 (20.90, 21.60)	21.40 (20.80, 21.70)	0.799

PI	21.10 (20.80, 21.60)**	21.10 (20.50, 21.40)***	0.486
Δ nm	-0.10 (-0.20, 0.00)	-0.30 (-0.40, 0.00)	0.0568
Δ %	-0.48 (-0.96, 0.00)	-1.37 (-1.86, 0.00)	**0.0496**
HDL-Z			
BL	8.98 ± 0.08	9.08 ± 0.08	0.345
PI	8.99 ± 0.08	9.11 ± 0.08	0.310
Δ	0.02 ± 0.03	0.03 ± 0.03	0.838
Δ %	0.21 ± 0.38	0.30 ± 0.32	0.866

[1] Values are reported as means ± SEM for all data except TC:HDL ratio, triglycerides and 10-year CVD risk data which is presented as median (25th and 75th percentile) due to lack of normality of the distribution. All baseline and post-intervention data are in mmol/L except for TC:HDL ratio.

Significant change from baseline, ** p<0.001, *** p<0.0001.

[^] Independent samples t-test or Wilcoxon rank sum test was used to compare change in outcome parameters across treatment groups. $P < 0.05$ indicates statistically significant difference between groups.

BL, baseline; HDL-Z, high-density lipoprotein particle size; LDL-Z, low-density lipoprotein particle size; PI, post-intervention; PL-C, placebo; PS-C, phytosterols; TRL-Z, triglyceride-rich lipoprotein particle size.

Supplementary Table 1. Reported dietary intake of hypercholesterolaemic adults who consumed placebo (PL-C) and phytosterol (PS-C) at baseline (BL) and mean change (Δ) from baseline to post-intervention.[1]

	PL-C (n=36)		PS-C (n=39)	
	BL	Δ	BL	Δ
Energy				
kJ	8687 (7436, 10570)	-184 (-1298, 1481)	8447 (6761, 10211)	-86 (-1332, 934)
kcal	2078 (1779, 2529)	-44 (-311, 354)	2021 (1617, 2443)	-21 (-319, 223)
Protein (g)	105.06 (81.92, 123.38)	-5.31 (-25.69, 14.46)	85.94 (72.69, 106.98)	-1.08 (-12.46, 12.79)
CHO (g)	214.32 (166.80, 269.51)	7.04 (-40.38, 45.78)	187.03 (148.21, 242.29)	8.93 (-27.53, 26.42)
Sugars (g)	104.60 (61.20, 133.31)	-8.63 (-36.84, 24.02)	83.97 (64.03, 128.18)	-3.56 (-22.17, 10.45)
Starch (g)	108.16 (89.55, 139.14)	16.05 (-17.03, 35.52)	95.74 (86.83, 124.18)	12.80 (-13.55, 27.83)
Total fat (g)	78.47 (61.53, 99.90)	0.50 (-15.14, 17.18)	77.26 (63.79, 107.35)	-2.51 (-19.48, 17.32)
Saturated (g)	29.66 (20.18, 35.71)	0.01 (-5.33, 7.04)	28.04 (21.05, 36.99)	-1.22 (-8.65, 5.00)
Trans (g)	1.38 (0.79, 1.72)	0.15 (-0.17, 0.53)	1.35 (0.95, 1.68)	0.70 (-0.25, 0.62)
MUFAs (g)	28.83 (22.45, 35.41)	3.00 (-7.30, 8.87)	28.37 (24.35, 40.51)	-4.56 (-9.71, 6.25)
PUFAs (g)	11.44 (9.10, 16.08)	1.55 (-2.32, 4.39)	12.22 (9.34, 17.14)	1.37 (-1.10, 6.06)
Cholesterol (mg)	359 (256, 448)	-34 (-132, 108)	267 (217, 406)	-55 (-130, 46)

Fibre (g)	26.76 (18.82, 36.33)	-2.22 (-10.58, 2.91)	26.38 (21.79, 28.80)	-1.26 (-5.40, 3.74)
Alcohol (g)	1.33 (0.00, 17.55)	0.00 (0.00, 10.50)	3.01 (0.00, 14.77)	0.00 (-4.96, 0.49)

[1] Values are reported as median (25^{th} and 75^{th} percentile). BL, baseline; Δ, change from baseline to post-intervention; CHO, carbohydrates; MUFA, monounsaturated fatty acid; PL-C, placebo; PS-C, phytosterols; PUFA, polyunsaturated fatty acid.

Supplementary Table 2. Change in plasma outcome measures in the placebo (PL), phytosterols (PS), curcumin (CC) and phytosterol + curcumin (PS-CC) groups from baseline to postintervention.[1]

	PL (n = 18)	PS (n = 19)	CC (n = 18)	PS-CC (n=20)
TC				
BL	6.88 ± 0.27	6.73 ± 0.17	6.32 ± 0.19	6.99 ± 0.22
PI	6.83 ± 0.27	6.28 ± 0.17***	6.53 ± 0.18*	6.55 ± 0.15*
Δ mmol/L	-0.05 ± 0.11^{a}	-0.44 ± 0.07^{b}	0.21 ± 0.05^{ac}	-0.44 ± 0.12^{bd}
LDL-C				
BL	4.77 ± 0.24	4.43 ± 0.15	4.16 ± 0.15	4.82 ± 0.20
PI	4.70 ± 0.24	4.08 ± 0.15***	4.34 ± 0.14*	4.30 ± 0.12**
Δ mmol/L	-0.08 ± 0.10^{ab}	-0.35 ± 0.06^{ac}	0.18 ± 0.07^{b}	-0.52 ± 0.11^{c}
HDL-C				
BL	1.44 ± 0.09	1.69 ± 0.08	1.41 ± 0.08	1.39 ± 0.09
PI	1.44 ± 0.08	1.66 ± 0.08	1.44 ± 0.08	1.39 ± 0.09
Δ mmol/L	0.00 ± 0.05	-0.04 ± 0.04	0.03 ± 0.03	0.01 ± 0.03
TC:HDL				
BL	5.05 ± 0.35^{ab}	4.12 ± 0.21^{a}	4.73 ± 0.29^{ab}	5.44 ± 0.38^{b}
PI	5.00 ± 0.34^{ab}	3.95 ± 0.20^{a}	4.77 ± 0.29^{ab}	5.06 ± 0.33^{***b}
Δ	-0.05 ± 0.20	-0.17 ± 0.11	0.03 ± 0.10	-0.38 ± 0.08
TG				
BL	1.39 (1.02, 1.95)	1.21 (0.98, 1.48)	1.40 (0.88, 2.39)	1.65 (1.17, 2.50)
PI	1.39 (1.02, 1.82)	1.16 (0.84, 1.51)	1.12 (1.05, 2.11)	1.55 (1.10, 2.59)
Δ mmol/L	0.01 (-0.25, 0.42)	-0.04 (-0.19, 0.12)	-0.09 (-0.32, 0.24)	-0.05 (-0.32, 0.10)
CVD risk (%)				
BL	11.03 (7.24, 13.82)	7.60 (3.88, 15.69)	7.31 (3.04, 13.43)	13.09 (8.40, 24.77)
PI	10.27 (7.32, 15.11)	6.96 (3.67, 12.83)*	7.50 (3.72, 12.93)	11.87 (7.18, 23.38)**

Δ	0.63 (-0.23, 1.86)[a]	-0.50 (-1.42, 0.07)[ab]	-0.15 (-0.50, 1.63)[ab]	-0.95 (-2.21, -0.10)[b]

[1] Values are reported as means ± SEM for all plasma concentrations except triglycerides and 10-year CVD risk data which is presented as median (25^{th} and 75^{th} percentile) due to lack of normality of the distribution. All baseline and post-intervention data are in mmol/L except for TC:HDL ratio. Significant change from baseline, * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

One-way ANOVA was used to compare change in outcome parameters across treatment groups. Tukey's HSD post-hoc analyses were used to compare differences in mean change between groups when significance was found. Kruskal-Wallis test was conducted for triglycerides and CVD risk. Values with different superscript letters in each row indicate statistically significant differences between corresponding groups at a p-value < 0.05.

Supplementary Table 3. Effects of baseline data on the change in blood lipid profile.[1]

	Coefficient[2]	t	*p*
Absolute change in TC (mmol/L)			
Dietary cholesterol	0.0004 ± 0.0002	2.10	0.039
TC	-0.21 ± 0.04	-4.64	<0.001
Treatment	-0.45 ± 0.08	-5.46	<0.001
Relative change in TC (%)			
TC	-2.40 ± 0.63	-3.84	<0.001
Treatment	-6.99 ± 1.18	-5.94	<0.001
Absolute change in LDL-C (mmol/L)			
Dietary cholesterol	0.0004 ± 0.0002	2.41	0.018
LDL-C	-0.25 ± 0.04	-5.69	<0.001
Treatment	-0.43 ± 0.07	-5.89	<0.001
Relative change in LDL-C (%)			
Dietary cholesterol	0.006 ± 0.003	2.14	0.036
LDL-C	-4.21 ± 0.84	-4.98	<0.001
Treatment	-9.67 ± 1.43	-6.75	<0.001
Absolute change in LDL-P (nmol/L)			
LDL-P	-0.20 ± 0.09	-2.18	0.033
TC	-150.75 ± 66.83	-2.26	0.027
Treatment	-103.12 ± 41.32	-2.50	0.015
LDL-C	222.27 ± 78.03	2.85	0.006
Relative change in LDL-P (%)			
Trans fat	4.02 ± 1.79	2.24	0.028
Treatment	-5.15 ± 2.07	-2.49	0.015
LDL-P	-0.01 ± 0.005	-2.58	0.012
TC	-9.14 ± 3.35	-2.73	0.008

LDL-C	13.43 ± 3.91	3.44	0.001
Absolute change in LDL-Z (nm)			
Saturated fat	0.01 ± 0.005	2.08	0.041
TC	0.23 ± 0.10	2.36	0.021
Trans fat	-0.21 ± 0.09	-2.40	0.019
Treatment	-0.16 ± 0.06	-2.51	0.015
WHR	1.59 ± 0.63	2.52	0.014
LDL-C	-0.35 ± 0.11	-3.19	0.002
Relative change in LDL-Z (%)			
Saturated fat	0.05 ± 0.02	2.04	0.046
TC	1.07 ± 0.46	2.33	0.023
Trans fat	-0.97 ± 0.41	-2.36	0.021
WHR	7.39 ± 2.98	2.48	0.016
Treatment	-0.73 ± 0.30	-2.46	0.016
LDL-C	-1.63 ± 0.51	-3.17	0.002

[1] Baseline data including known confounders were included in a multiple regression utilising a backward stepwise procedure to eliminate covariates that were not statistically significant at the 0.05 significance level from the regression model. Final reduced models from the backward regression procedure containing statistically significant predictors are presented.

[2] Data are reported as coefficient ± SEM.

LDL-C, low-density lipoprotein cholesterol; LDL-P, low-density lipoprotein particle number; LDL-Z, low-density lipoprotein particle size; TC, total cholesterol; WHR, waist-to-hip ratio