

ENHANCING THE LIPID-LOWERING POTENTIAL OF PHYTOSTEROLS IN HYPERCHOLESTEROLAEMIC INDIVIDUALS

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Statement of originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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Thesis by publication

I hereby certify that this thesis is in the form of a series of papers. I have included as part of the thesis a written declaration from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.

Acknowledgements and dedications

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2. 'HMRI study needing volunteers for heart study' – Newcastle and Lake Macquarie Star (August 2016)
3. 'People needed for heart disease study' – Newcastle Herald (August 2016) <https://www.theherald.com.au/story/4090520/people-needed-for-heart-disease-study/>
4. 'Heart study adds spice' – Newcastle and Lake Macquarie Star (June 2016)

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4. 'Risk factors for heart disease' – 2NURFM 103.7FM (June 2016) <https://www.youtube.com/watch?v=f5KOqWp26jo>
5. 'Dietary curcumin and phytosterols for lowering cholesterol' – HIT Newcastle 106.5FM (June 2016)

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1. 'Oat Power' – Channel 9 National News reported by Gabriella Rogers (August 2017)

Table of Contents

Acknowledgements and dedications.....	2
List of research publications included in the thesis	4
Other co-authored research publications	5
Co-authored book chapters	5
Conference presentations.....	5
Scholarships.....	7
Awards.....	7
Media engagements	9
List of abbreviations	16
List of figures.....	19
List of tables	21
List of appendices.....	22
Synopsis.....	23
Thesis layout.....	26
Chapter 1 General Introduction.....	28
1.1 Overview	29
1.2 Background.....	29
1.2.1 Prevalence of cardiovascular disease	29
1.2.2 Economic burden and health consequences	29
1.3 CVD Risk Factors.....	30
1.4 Dyslipidaemia and CVD.....	30
1.4.1 Hyperlipidaemia	31
1.4.2 Lipoprotein profile	32
1.5 Chronic Inflammation and CVD	32
1.5.1 LDL-C Induced Inflammatory Cascade	33
1.6 Calculating Cardiovascular Disease Risk.....	35
1.7 Biological Targets for Lowering Blood Cholesterol	36

1.7.1 LDL-R	36
1.7.2 HMG-CoA reductase inhibitors	38
1.7.3 CETP inhibitors.....	38
1.7.4 Cholesterol absorption inhibitors	39
1.8 Management of Blood Lipids	39
1.8.1 Diet and lifestyle	39
1.8.2 Physical activity	41
1.8.3 Pharmacological therapy	42
1.9 Role of Bioactives in the Management of Blood Lipids	43
1.9.1 Phytosterols	44
1.9.2 Curcumin.....	47
1.9.3 Oat β -glucan.....	53
1.10 Summary of Literature Review and Thesis Rationale.....	56
1.11 Research Aims.....	57
1.12 Thesis Hypothesis.....	58
Chapter 2 General Methodology for Clinical Trials.....	59
2.1 Participant Recruitment, Ethics and Trial Registration	60
2.2 Study Design and Sample Size Calculation.....	60
2.3 Experimental Interventions.....	61
2.3.1 Study 1 – PAC-CVD Trial (Chapter 4).....	61
2.3.2 Study 2 – PAC-FOOD Trial (Chapter 5).....	62
2.3.3 Study 3 – BETA-GAPS Trial (Chapter 6).....	63
2.4 Randomisation.....	64
2.5 Clinical Assessment.....	64
2.5.1 Eligibility and consent.....	64
2.5.2 Medical history.....	64
2.5.3 Anthropometry	64
2.5.4 Blood pressure.....	65

2.5.5 Cardiovascular disease risk	65
2.6 Dietary Analysis	65
2.6.1 Three-day food diary	65
2.7 Physical Activity.....	66
2.8 Blood Collection and Biochemical Analysis.....	66
2.8.1 Blood collection	66
2.8.2 Biochemical parameters	66
2.8.3 Lipoprotein particle profile	66
2.9 Compliance and Safety Monitoring.....	67
2.9.1 Compliance.....	67
2.9.2 Safety Monitoring	67
2.10 Statistical Analysis	67
Chapter 3 Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs.....	69
3.1 Abstract.....	70
3.2 Introduction	71
3.2.1 Phytosterols	71
3.2.2 Dietary fats	72
3.2.3 Mechanisms by which dietary fats modulate blood lipid levels.....	73
3.2.4 Potential synergistic and complementary effect of phytosterols and dietary fats	75
3.3 Materials and Methods	75
3.3.1 Search strategy	75
3.3.2 Eligibility Criteria	76
3.3.3 Selection process and quality assessment	76
3.3.4 Data extraction	78
3.3.5 Statistical analysis	78
3.4 Results	79
3.4.1 Overview of publications	79
3.4.2 Description of publications	80

3.4.3 Effect of fat type on the phytosterol-lowering of blood cholesterol levels	90
3.5 Discussion.....	94
3.6 Conclusion.....	97
Chapter 4 Curcumin potentiates cholesterol-lowering effects of phytosterols in hypercholesterolaemic individuals. A randomized controlled trial.....	98
4.1 Abstract.....	99
4.2 Introduction	100
4.3 Materials and Methods	101
4.3.1 Recruitment	101
4.3.2 Trial design.....	101
4.3.3 Clinical assessments.....	103
4.3.4 Anthropometry and body composition.....	103
4.3.5 Medical history, dietary intake and physical activity.....	104
4.3.6 Blood sampling and serum lipid analyses	104
4.3.7 Statistical analysis	104
4.4 Results	105
4.4.1 Baseline characteristics	105
4.4.2 Dietary intake, physical activity and compliance.....	107
4.4.3 Effect of phytosterol and curcumin intervention on plasma lipid profile and glucose	108
4.5 Discussion.....	113
Chapter 5 Phytosterol bread with or without curcumin modulates lipoprotein profile in hypercholesterolaemic individuals. A randomized controlled trial.....	117
5.1 Abstract.....	118
5.2 Introduction	119
5.3 Materials and Methods	120
5.3.1 Study participants.....	120
5.3.2 Study design and intervention	120
5.3.3 Clinical measures	121

5.3.4 Anthropometry and body composition.....	123
5.3.5 Medical history, dietary intake and physical activity.....	123
5.3.6 Blood sampling, lipid analyses and plasma LDL profile	123
5.3.7 Statistical analysis	124
5.4 Results	125
5.4.1 Baseline characteristics	125
5.4.2 Anthropometry, dietary intake, physical activity and compliance	129
5.4.3 Effect of phytosterol and curcumin intervention on plasma lipid profile and cardiovascular disease risk.....	129
5.4.4 The effects of baseline data on change in blood lipid profile.....	129
5.5 Discussion.....	133
5.6 Conclusions	136
Chapter 6 High molecular weight oat β-glucan enhances lipid-lowering effects of phytosterols. A randomized controlled trial.....	137
6.1 Abstract.....	138
6.2 Introduction	139
6.3 Materials and Methods	140
6.3.1 Recruitment	140
6.3.2 Study design.....	140
6.3.3 Clinical assessments.....	142
6.3.4 Anthropometry and body composition.....	143
6.3.5 Medical history, dietary intake and physical activity.....	143
6.3.6 Blood sampling and serum lipid analyses	143
6.3.7 Statistical analysis	144
6.4 Results	145
6.4.1 Baseline characteristics	145
6.4.2 Dietary intake, physical activity and compliance.....	150
6.4.3 Effect of phytosterol and oat β -glucan intervention on plasma lipid profile.....	150
6.4.4 The effects of baseline data on change in blood lipid profile.....	153

6.5 Discussion.....	154
Chapter 7 Conclusions and Future Recommendations	159
7.1 Key findings	160
7.2 Strengths and limitations	169
7.2.1 Strengths.....	169
7.2.2 Limitations	170
7.3 Conclusions	172
References	174
Appendices.....	203

List of abbreviations

ABCG5, 8	ATP-binding cassette transporters
ACAT	Acyl coenzyme A:cholesterol acyltransferase
ALA	Alpha-linolenic acid
ANZCTR	Australian New Zealand Clinical Trials Registry
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BMI	Body mass index
CC	Curcumin
CHD	Coronary heart disease
CETP	Cholesteryl ester transfer protein
CRP	C-reactive protein
COX	Cyclo-oxygenase
CVD	Cardiovascular disease
CYP7A	Cholesterol 7- α -hydroxylase
D	Dairy fat
EDTA	Ethylenediaminetetraacetic acid
FFA	Free fatty acids
HDL-C	High-density lipoprotein cholesterol
HDL-P	High-density lipoprotein particle
HDL-Z	High-density lipoprotein particle size
HNF-4	Hepatocyte nuclear factor-4
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HMRI	Hunter Medical Research Institute
IL	Interleukin
LA	Linoleic acid
LCFA	Long-chain saturated fatty acid
LCn-3PUFA	Long-chain omega-3 polyunsaturated fatty acids
LDL-C	Low-density lipoprotein cholesterol
LDL-R	Low-density lipoprotein receptor
LDL-P	Low-density lipoprotein particle
LDL-Z	Low-density lipoprotein particle size
LOX	Lipoxygenase
LXR	Liver X receptor

LXR α	Liver X receptor alpha
MCFA	Medium-chain saturated fatty acids
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte chemoattractant protein-1
MESA	Multi-Ethnic Study of Atherosclerosis
MMP	Matrix metalloproteinases
MTTP	Microsomal triglyceride transfer protein
MUFA	Monounsaturated fatty acids
MW	Molecular weight
n-3 PUFA	Omega-3 polyunsaturated fatty acids
n-6 PUFA	Omega-6 polyunsaturated fatty acids
NF- κ B	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NPC1L1	Niemann-Pick C1-Like 1
OBG	Oat β -glucan
oxLDL	Oxidised low-density lipoprotein
PCSK9	Proprotein convertase subtilisin-kexin type 9
PL	Placebo
PL-C	Placebo + Curcumin
PPAR	Peroxisome proliferator-activated receptor
PS	Phytosterol
PS-C	Phytosterol + Curcumin
PS-CC	Phytosterol + Curcumin
PS-OBG	Phytosterol + oat β -glucan
RC	Rapeseed/Canola
RCT	Randomised controlled trial
ROS	Reactive oxygen species
SLCP	Solid lipid curcumin particle
SFA	Saturated fatty acids
SREBP	Sterol regulatory element binding protein
SS	Sunflower/Soybean
TC	Total cholesterol
TC:HDL	Total cholesterol to HDL ratio
TG	Triglycerides

TNF α	Tumour necrosis factor alpha
TRL-P	Triglyceride rich lipoprotein particle
TRL-Z	Triglyceride rich lipoprotein particle size
UON HREC	University of Newcastle Human Research Ethics Committee
VCAM-1	Vascular cell adhesion molecule-1
VLDL-C	Very low-density lipoprotein cholesterol
VLDL-P	Very low-density lipoprotein particles

List of figures

Figure 1.1: LDL-C and inflammation in atherosclerosis initiation.	34
Figure 1.2: Uptake of LDL-P into the cell and the action of the LDL-R.....	37
Figure 1.3: Molecular structure of cholesterol and the most abundant phytosterols and phytostanols.	45
Figure 1.4: Molecular structure of curcumin, demethoxycurcumin and bisdemethoxycurcumin.	48
Figure 1.5: Molecular structure of oat β -glucan depicting the $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ glycosidically bonded glucose polymer.	53
Figure 1.6: Rationale for combining PS with curcumin and OBG for modulation of blood lipids.	57
Figure 2.1: Study intervention products for PAC-CVD Trial. 25g vegetable fat spread per day (A) and two tablets per day (B).	61
Figure 2.2: Study intervention product for PAC-FOOD Trial. Daily dose of 2 slices of bread (A) and all four different study breads (B).	62
Figure 2.3: Study intervention product for BETA-GAPS Trial. One study biscuit (A) and all four different study biscuits (B).	63
Figure 3.1: Flow diagram of publication selection process starting with 2673 publications and ending with 24 RCT.	81
Figure 3.2: Effect size and 95% CI for TC concentrations according to fat type in PS fortified food.	92
Figure 3.3: Effect size and 95% CI for LDL-C concentrations according to fat type in PS fortified food.	93
Figure 4.1: CONSORT flow diagram of participant recruitment, screening and assessment.	106
Figure 4.2: Percent change in plasma TC (A), LDL-C (B), HDL-C (C), TC:HDL ratio (D), TG (E) and Glucose (F) from baseline to post-intervention in hypercholesterolaemic individuals who consumed PL, PS, CC and PS-CC for 4 weeks.	112
Figure 5.1: CONSORT schematic of participant recruitment, screening and assessment.	126
Figure 5.2: 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.	131
Figure 6.1: CONSORT schematic of participant recruitment, screening and assessment.	146
Figure 6.2: Percent change in TC, LDL-C, HDL-C, TC:HDL ratio and TG from baseline to post-intervention in hypercholesterolaemic individuals who consumed PL, PS, OBG or PS-OBG for 6 weeks.	152

Figure 6.3: *Absolute change in TG (mmol/L) when baseline TG <1.7 mmol/L or ≥1.7 mmol/L in hypercholesterolaemic individuals who consumed PL, PS, OBG or PS-OBG for 6 weeks. 154*

List of tables

Table 3.1: Inclusion and exclusion criteria for the selection of publications.....	77
Table 3.2: Overview of the publications included in the systematic review and meta-analysis (n= 32)	82
Table 3.3: Summary of absolute and relative change data for included strata (n = 42).....	87
Table 3.4: Absolute (mmol/L) and relative (%) net change per fat group.	91
Table 4.1: Nutrient composition of phytosterol and control spread. ¹	103
Table 4.2: Participant characteristics at baseline in the placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PS-CC) groups. ¹	107
Table 4.3: Reported macronutrient and fatty acid intakes of hypercholesterolaemic adults who consumed placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PSCC) at baseline and change from baseline to post-intervention. ¹	109
Table 4.4: Change in plasma outcome measures in the placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PS-CC) groups from baseline to post-intervention. ¹	110
Table 5.1: Nutrient composition of study bread. ¹	122
Table 5.2: Participant characteristics at baseline. ¹	127
Table 5.3: Change in plasma lipid concentrations (Δ) in the placebo (PL-C) and phytosterol (PS-C) groups from baseline (BL) to post-intervention (PI). ¹	128
Table 5.4: Lipoprotein profiles (total particle concentration and particle size) at baseline and post-intervention measured using nuclear magnetic resonance spectroscopy.	132
Table 6.1: Nutrient composition of study biscuits and compliance. ¹	142
Table 6.2: Participant characteristics at baseline in the placebo (PL), phytosterol (PS), oat β -glucan (OBG) and phytosterol + oat β -glucan (PS-OBG) groups. ¹	147
Table 6.3: Change in plasma outcome measures in the placebo (PL), phytosterol (PS), oat β -glucan (OBG) and phytosterol + oat β -glucan (PS-OBG) groups from baseline to post-intervention. ¹	149
Table 6.4: Reported dietary intake of hypercholesterolaemic adults who consumed placebo (PL), phytosterol (PS), oat β -glucan (OBG) and phytosterol + oat β -glucan (PS-OBG) at baseline and mean change from baseline to post-intervention (Δ). ¹	151
Table 7.1: Summary of 10-year CVD risk for intervention trials at baseline and post-intervention ¹	168
Table 7.2: ApoE genotype in individuals in the placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PS-CC) groups from the PAC-CVD Trial (chapter 4).....	171

List of appendices

Appendix 1: Proof of Copyright Clearance and Permission for Figure 1.1	204
Appendix 2: Proof of Copyright Clearance and Permission for Figure 1.2.....	206
Appendix 3: Ethics approval notification for PAC-CVD Trial	211
Appendix 4: Ethics approval notification for PAC-FOOD Trial.....	214
Appendix 5: Ethics approval notification for BETA-GAPs Trial.....	216
Appendix 6: General medical history questionnaire.....	219
Appendix 7: Three-day food and beverage diary.....	225
Appendix 8: Self-Administered International Physical Activity Questionnaire (Long Form)	231
Appendix 9: Statement of authorship	238
Appendix 10: Statement of authorship	239
Appendix 11: Supplementary Table 1 for Chapter 5	240
Appendix 12: Supplementary Table 2 for Chapter 5	241
Appendix 13: Supplementary Table 3 for Chapter 5	242
Appendix 14: Statement of authorship	244
Appendix 15: Lipoprotein profiles (total particle concentration and particle size) at baseline and post-intervention measured using nuclear magnetic resonance spectroscopy.	245

Synopsis

Cardiovascular disease (CVD) remains the leading cause of death worldwide, accounting for over one third of all deaths. Since CVD is chronic, complex and multifaceted in pathological pathways, effective risk reduction and prevention strategies ought to be multi-targeted and easily compliant. CVD encompasses a vast complexion of non-modifiable and modifiable risk factors, the latter of which can be somewhat modulated by diet, physical activity and lifestyle changes. Abnormal blood lipids (dyslipidaemia) remain a key modifiable risk factor underpinning the pathological mechanism of atherosclerosis, a key manifestation of CVD. Changes to diet and lifestyle remain the first line of treatment for managing dyslipidaemia, however, pharmacological intervention is often indicated due to poor long-term adherence and inadequate results of solo diet and lifestyle management. Pharmacological intervention is also riddled with complications that impede long-term adherence and sustainability such as side effects, intolerance to statins, poor adherence and cost. Nutraceuticals, also referred to as bioactives, are potential alternatives and/or adjunct therapies to diet, lifestyle and/or pharmacological treatments; given their ability to effectively down-regulate multiple pathological mechanisms (elevated blood lipids, chronic low-grade inflammation, glycaemic parameters, hypertension) involved in CVD development.

Phytosterols and phytostanols (PS) are a family of well-established cholesterol-lowering bioactive compounds commonly used in functional foods for the management of elevated bad cholesterol, namely low-density lipoprotein cholesterol (LDL-C). Foods enriched with 2 g PS when consumed daily can effectively lower LDL-C by 8-10%, with no effects on other blood lipids or CVD risk factors. The primary aim of my PhD project was to enhance lipid-modulating potential of phytosterols with the secondary aims of improving compliance and concurrently targeting other CVD risk factors for improved heart health.

Given the widespread use and acceptance of PS enriched foods as a therapy, the first aim of my PhD project, presented in chapter 3, was to investigate whether the type of carrier fat used in common PS enriched products (i.e. vegetable fat spreads and dairy products) was a determinant of the cholesterol-lowering potential of PS. A systematic review and meta-analysis was conducted and we observed that most PS enriched products are based on either rapeseed/canola oil, sunflower/soybean oil or dairy fat. Since the dietary fat type is known to modulate blood cholesterol levels, it was logical to hypothesize that the carrier fat used in PS enriched functional foods may contribute to the overall reduction in LDL-C. The meta-analysis revealed that PS enriched products based on rapeseed/canola oil fat type had a significantly greater reduction (0.1 mmol/L or 26%) in LDL-C when compared to the sunflower/soybean oil. This is clinically significant since an average reduction in LDL-C following 2 g/day PS via rapeseed/canola oil enriched products was -0.38 mmol/L in this study. Therefore, fat type in PS enriched products is

a determinant of the cholesterol-lowering potential of PS, and PS enriched foods that are predominately based on rapeseed/canola fat are more effective at lowering circulating cholesterol concentrations.

The second aim, presented in chapter 4, was to investigate the novel combination of PS and curcumin on circulating blood lipids in free-living hypercholesterolaemic adults (PAC-CVD Trial). Curcumin was selected as the bioactive to combine with PS due to its lipid-modulating effects and potent anti-inflammatory effects, reported in several preclinical and some human studies. A randomised, 2x2 factorial, double-blinded, placebo-controlled trial in four parallel groups was conducted to investigate the effects of PS with or without curcumin on circulating blood lipids in hypercholesterolaemic otherwise healthy individuals. PS and curcumin were consumed as a daily dose of vegetable fat spread and tablets (respectively) as part of participant's habitual diet and lifestyle for 4 weeks. Post-intervention, PS and the combined group had significantly lower fasting plasma total cholesterol (TC) and LDL-C, with more than additive reductions observed in the combined group. TC and LDL-C were 11% and 14% (respectively) lower in the combined group, resulting in a complementary and possibly synergistic action between the two bioactives, yielding a more effective hypocholesterolaemic bioactive therapy than either bioactive alone with potential for additional heart health benefits.

The above findings prompted further investigation via the development of a staple functional food containing the novel bioactive combination of PS and curcumin, presented in chapter 5 (PAC-FOOD Trial). The third aim of my PhD project was to examine the effects of a bread enriched with PS and curcumin on blood lipid profile and whether this would increase compliance and yield more effective results than our previous research on separate dietary supplementation. Therefore, a randomised controlled trial (RCT) utilising the same study design and bioactive doses as the preceding study in chapter 4, was conducted and we observed increased compliance (~95% versus 99%) with consumption of only 2 slices of enriched bread daily compared to the previous study. In contrast to the PAC-CVD Trial, the curcumin enriched breads did not demonstrate any lipid-lowering effects, nor did they provide additional cholesterol-lowering to the PS + curcumin combined bread. LDL-particle (LDL-P) number concentration significantly reduced in the PS enriched groups and LDL-P size reduced in all groups. Bioavailability of curcumin remains a key concern and barrier to its therapeutic use, however, effective formulations of curcumin in which bioavailability has been enhanced has enabled its widespread use as a supplement for various health conditions. The same curcumin formulation was used for both studies involving curcumin; both in tablet (PAC-CVD Trial) and powder (PAC-FOOD Trial) form. The thermal stability of the patented technology used in these formulations is unknown, and therefore likely that the structure of the curcumin formulation degraded during food

processing, therefore resulting in lack of clinical findings. The research presented here provides evidence warranting further investigation into the use of curcumin formulations in the food industry, as well as optimal food matrices in which to deliver future functional foods containing curcumin.

The final aim of this PhD project, presented in chapter 6, was to investigate the lipid-modulating effects of a snack food enriched with PS and high molecular weight (MW) oat β -glucan (OBG) on circulating blood lipids in free-living hypercholesterolaemic individuals. The study design was the same as the PAC-CVD and PAC-FOOD trials, however, participants were instructed to consume 8 small lightly-sweetened biscuits enriched with PS with or without OBG, OBG alone or placebo as part of their daily habitual diet and lifestyle for 6 weeks. All bioactive treatment arms significantly lowered fasting plasma TC and LDL-C and, a complementary cholesterol-lowering effect was reported in the combined group of -11.5% for TC and -13.9% for LDL-C. A non-significant 8.4% reduction in triglycerides (TG) was also observed in the combined group. This study was the first to investigate PS combined with a high-MW form of OBG and demonstrated an effective strategy for optimising the lipid-lowering effects of one another, with the potential to provide additional health benefits pertaining to the OBG, such as improved gut health, satiety and hypoglycaemic effects.

The research presented in this thesis provides novel supporting evidence for the effective use of bioactive combinations as alternatives and/or adjunct therapies for individuals with dyslipidaemia who are already at risk of CVD. Our findings from the clinical trials have direct translational capacity to the Australian adult population who have dyslipidaemia, presenting simple, efficacious and adherable strategies for lowering CVD risk. Findings from this thesis promote further investigation into the long-term effects of these combination therapies as well as supports the development of functional foods containing bioactives that can be incorporated into the average Australian diet for enhancing heart health. The work presented in this thesis provides significant gain to the Australian population, given the widespread prevalence of dyslipidaemia. Moreover, this research provides supporting evidence for safe, effective, potentially multidimensional approaches through diet intervention with bioactives to empower individuals to take better control of their blood lipid profile, with the potential to manage other CVD risk factors and/or prevent the development of CVD.

Thesis layout

This thesis by publication is organized into 7 chapters: a general introduction and literature review section, general methodology, two papers published in scientific journals, one paper accepted for publication (in press) and one paper under review and a general discussion with conclusions. Chapters are organized as follows:

Chapter 1 – General introduction and literature review

This chapter describes the prevalence, economic and health burden of cardiovascular disease; risk factors and pathological mechanisms involved in the development of atherosclerosis. The role of bioactive compounds such as phytosterols, curcumin and oat β -glucan will be discussed for the prevention and risk reduction of atherosclerosis. Excerpts of this chapter were published in the following paper: **Ferguson JJA**, Stojanovski E, MacDonald-Wicks L, Garg ML. Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs. *Progress in Lipid Research*. 2016;64:16-29. Impact factor 8.435.

Chapter 2 – General methodology

This chapter is an overview of the methodology, trial design and statistical analyses employed in all three clinical trials (Chapters 4, 5, 6).

Chapter 3 – Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs.

This chapter is presented as published paper: **Ferguson JJA**, Stojanovski E, MacDonald-Wicks L, Garg ML. Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs. *Progress in Lipid Research*. 2016, 64:16-29. Impact factor 8.435. The paper demonstrates that the type of carrier fat used in phytosterol-enriched products influences the overall cholesterol-lowering ability of phytosterols. Findings from this work provides useful evidence for optimising the hypocholesterolaemic effects of phytosterols.

Chapter 4 – Curcumin potentiates cholesterol-lowering effects of phytosterols in hypercholesterolaemic individuals. A randomised controlled trial.

This chapter is presented as published paper: **Ferguson JJA**, Stojanovski E, MacDonald-Wicks L, Garg ML. Curcumin potentiates cholesterol-lowering effects of phytosterols in hypercholesterolaemic individuals. A randomized controlled trial. *Metabolism – Clinical and Experimental*. 2018, 82:22-35. Impact factor 5.963. This paper describes the study aims, design, methods, results and discussion from the “PAC-CVD Trial” (study 1) which investigated the effects of combined supplementation with phytosterols and curcumin on blood lipids.

Chapter 5 – Phytosterol bread with or without curcumin modulates lipoprotein profile in hypercholesterolaemic individuals. A randomized controlled trial.

This chapter is presented as a paper submitted for publication: **Ferguson JJA**, Stojanovski E, MacDonald-Wicks L, Wolska A, Remaley AT, Garg ML. Phytosterol bread with or without curcumin modulates lipoprotein profile in hypercholesterolaemic individuals. A randomized controlled trial. *Food and Function*. 2019 (revised manuscript submitted 25th January 2019) Impact factor 3.289. It describes the study aims, design, methods, results and discussion from the “PAC-FOOD Trial” (study 2) which investigated the effects of a novel food enriched with phytosterols and curcumin on lipoprotein profile.

Chapter 6 – High molecular weight oat β -glucan enhances the lipid-lowering effects of phytosterols. A randomised controlled trial.

This chapter is presented as a paper accepted for publication (in press): **Ferguson JJA**, Stojanovski E, MacDonald-Wicks L, Garg ML. High molecular weight oat β -glucan enhances lipid-lowering effects of phytosterols. A randomized controlled trial. *Clinical Nutrition*. 2019 doi.org/10.1016/j.clnu.2019.02.007. Impact factor 5.496. It describes the study aims, design, methods, results and discussion from the “BETA-GAPs Trial” (study 3) which investigated the effects of a novel food enriched with phytosterols and/or high molecular weight oat β -glucan on blood lipids.

Chapter 7 – Conclusions and Future Recommendations

This chapter is an overall discussion of all results from the presented work including influence on cardiovascular disease risk measured by the Framingham risk algorithm, overall strengths, limitations, significance, future implications and conclusions of this research in the field of nutrition and cardiovascular disease prevention.

Chapter 1

General Introduction

1.1 Overview

An introduction to the global and national prevalence of CVD along with dyslipidaemia, a major risk factor of CVD, begins this chapter. The biological development of atherosclerosis as a key cause of CVD with an emphasis on the pathobiology of dyslipidaemia and inflammation is then discussed. The background then moves to explore current diet, lifestyle and pharmacological interventions and the barriers associated in relation to reducing CVD risk. This highlights the need for more effective, safe, compliant alternatives and/or adjunct therapies and thus the significance of nutraceuticals to fulfil this gap. Namely PS; a type of nutraceutical, is discussed as a key agent for lowering blood cholesterol along with their limitation of widespread lipid targets. Following this, a rationale is presented for ways to optimise their cholesterol-lowering ability by combining them with other bioactives; curcumin and OBG for targeting multiple lipid-based risk factors. This leads to a discussion of the current literature surrounding curcumin and OBG whereby the chapter concludes by outlining the aims and hypothesis of this thesis.

1.2 Background

1.2.1 Prevalence of cardiovascular disease

CVD is a non-communicable disease that encompasses an umbrella of conditions affecting the heart and blood vessels including stroke, heart failure, congenital heart disease, peripheral vascular disease, cardiomyopathy and coronary heart disease (also known as ischaemic heart disease) [1]. Ischaemic heart disease occurs as heart attack or angina and is the most common form of CVD. CVD is the leading cause of death worldwide with an estimated 31% of all deaths attributed to CVD [2]. The Australian Bureau of Statistics 2014-15 National Health Survey reported that one in six (4.2 million) Australians aged 18 years and over had at least one form of CVD, based on the self-reported data [3]. The prevalence of CVD among adults was similar for males and females, however, increased with age. Two-thirds (66%) of older adults aged ≥ 75 years had CVD compared to half (53%) of those aged 65-74 years old [3].

1.2.2 Economic burden and health consequences

The global economic burden of CVD is on the rise, with estimates that by 2030, the total global cost of CVD will be a staggering US \$1,044 billion; a 22% increase from 2010 (US \$863 billion) [4]. Just over half the total economic burden is due to direct healthcare costs, the remainder due to productivity losses from disability or premature death, or time lost from work due to illness or time to seek care. CVD remains Australia's most costly disease to treat, costing \$1.45 billion in medications per year including Australian Government expenditure and patient contributions in

2015-16 [5]. The most recent data (2012-13) in Australia reports \$5 billion was spent providing healthcare to hospital admitted patients with CVD. This was the highest share of all major disease groups accounting for 11.1% of total expenditure, followed by injuries at \$4.1 billion (9% of total expenditure) [6].

The mortality of CVD remains a dire burden of the disease as one Australian dies from CVD every 12 minutes [7]. These statistics are alarming particularly given 43,477 Australian lives were claimed due to the disease in 2017 [7]. Ischaemic heart disease was the main CVD-attributed cause of death in 2017, claiming 11.6% of all deaths in that year [7].

1.3 CVD Risk Factors

CVD can be, in part, preventable since most risk factors are modifiable such as: lifestyle choices including poor diet, excessive alcohol consumption, tobacco smoking, recreational drugs and inadequate physical activity. As a result, key biomedical factors can manifest such as abnormal blood lipid concentrations, chronic inflammation, high blood pressure (≥ 140 mmHg systolic and ≥ 90 mmHg diastolic [8]), overweight (body mass index, BMI; 25-29.99kg/m²) and obesity (BMI ≥ 30 kg/m²); which all play a major role in the development of CVD [1]. Non-modifiable risk factors include age, family history, genetic predisposition, gender and ethnic background. Overall, the risk factors for CVD are numerous and 90% of the Australian adult population are estimated to have at least one [9].

1.4 Dyslipidaemia and CVD

One of the major risk factors that contribute to the pathobiology of CVD is abnormal concentrations of circulating blood lipids, also known as dyslipidaemia [10]. Dyslipidaemia encompasses a wide range of abnormalities in blood lipid profile which are modifiable such as: increased flux of free fatty acids (FFA), raised concentrations of small, dense LDL-C, TG and apolipoprotein B (apoB), and reduced concentrations of high-density lipoprotein cholesterol (HDL-C) [11]. Desirable levels of circulating plasma lipids for the general Australian adult population are as follows: TC < 5.5 mmol/L, LDL-C \leq 3.5 mmol/L [10], HDL-C > 1.0 mmol/L for men and >1.2 mmol/L for women and TG < 2.0 mmol/L [12]. Targets for those at risk of developing CVD are more conservative: TC < 4.0 mmol/L, LDL-C < 2.0 mmol/L, HDL \geq 1.0 mmol/L, TG < 1.7 mmol/L and non-HDL-C < 2.5 mmol/L [13].

Over 63% of the Australian adult population have dyslipidaemia, making it a highly prevalent biological risk factor for CVD [14]. In 2011-12, a large proportion (7.6 million) of those who had dyslipidaemia were not taking lipid-modifying medication [15]. Diet and lifestyle habits remain a key contributor to changes in blood lipid concentrations, with elevated blood lipids being the strongest predictive factor for CVD, indicated by a large case-control study conducted across 52 countries [16]. This was followed by smoking, hypertension, diabetes, visceral obesity, poor fruit and vegetable intake, excessive alcohol intake and lack of habitual physical activity, all of which are precursors to abnormal blood lipids [16].

1.4.1 Hyperlipidaemia

Hyperlipidaemia is a form of dyslipidaemia involving multiple aetiologies and is often a consequence of lifestyle and dietary patterns in the body. Hyperlipidaemia is characterised by elevated levels of non-esterified FFA as a result of insufficient esterification. This generates a surplus of fatty acid pool, retention of fatty acids in the adipose tissue are decreased leading to an increased flow of FFA returning to the liver. In turn, hepatic synthesis of TG is stimulated, as well as the formation and secretion of very low-density lipoprotein cholesterol (VLDL-C) and apoB. This process raises plasma concentrations of TG, promotes TG/cholesterol ester exchange between LDL-P and HDL particles (HDL-P) via cholesteryl ester transfer protein (CETP), forming HDL-P that are rich in TG and highly catabolic. As a result, plasma concentrations of HDL-C become diminished [17].

Hyperlipidaemia is also characterised by raised LDL-C concentrations, particularly those that are small and dense. CETP mediates the exchange of cholesteryl esters with TG to form these small dense LDL-P which are rich in TG [17]. Increased atherogenic potential of small, dense LDL-P is suggested by increased binding ability to arterial proteoglycans [18], greater propensity for uptake by arterial tissue suggestive of enhanced transendothelial transport [19], reduced receptor-mediated uptake [20], and increased susceptibility to oxidative modification [21, 22].

At a population level the primary focus for prevention and treatment of hyperlipidaemia is to lead a healthy lifestyle governed by maintaining a healthy weight for age, gender and height, consuming a healthy diet rich in fresh fruits, vegetables, lean sources of meat, adequate amounts of fibre, limited consumption of saturated fat, processed meats and alcohol, avoid smoking and to engage in regular physical activity. Individuals at 'high risk' (>15%) of CVD with fasting serum TC of >7.5 mmol/L are advised to adhere to intensive lifestyle advice, commence lipid-lowering therapy simultaneously with anti-hypertensive therapy where indicated and to test blood lipids every 12 months [23].

1.4.2 Lipoprotein profile

Lipoprotein profile encompasses lipoprotein particle number, size and subclass distribution and provides a thorough description of lipoprotein status. Abnormal lipoprotein profiles have been shown to be associated with atherosclerosis [24, 25]. Atherosclerotic disease risk increases with larger VLDL particle (also referred to as triglyceride rich lipoprotein particles, TRL-P) sizes, smaller LDL-P and HDL-P sizes and elevated concentrations of small LDL-P and large TRL-P [26, 27]. Changes to diet and lifestyle can modulate lipoprotein profiles [28, 29] independent of adiposity and other lifestyle factors [30]. The density of LDL subfractions can be divided into two phenotypes: pattern A, characterised by larger, buoyant LDL-P; and pattern B, characterised by smaller, dense LDL-P [31]. The latter is associated with features of metabolic syndrome such as elevated concentrations of TG, insulin, glucose, apolipoprotein B; high blood pressure; insulin resistance and low concentrations of HDL-C [32].

There are various methods used to measure lipoprotein subfractions such as: vertical ultracentrifugation, electrophoresis gradient gel, high-resolution polyacrylamide gel tubes and nuclear magnetic resonance (NMR) spectroscopy [26]. NMR spectroscopy has been shown to be more indicative of lipoprotein profile as it exploits spectral differences between lipoprotein subspecies to directly quantify particle size and concentration [29, 33]. More specifically, the protein NMR spectroscopy (LP4 NMR MetaboProfile™ Analysis) using LipoProfile-3 algorithm enables the determination of LDL-P number as well, which has been shown to be more indicative of LDL-based risk of CVD when concentrations of LDL-P and LDL-C are discordant [24, 34]. Therefore, in addition to targeting concentrations of LDL-C, it is important that these additional markers of lipoprotein profile are also measured in order to better understand, target and optimise lipoprotein profile to then effectively lower lipid-induced atherosclerotic risk.

1.5 Chronic Inflammation and CVD

Inflammation is a biological process essential for healing and removing harmful stimuli including pathogens and damaged or mutated cells. Acute inflammation is body's first line of defence, however, if this remains unresolved and uncontrolled for a long period of time it can lead to extensive damage to host tissues and chronic diseases such as diabetes, CVD, obesity and even cancer. Molecular mechanisms resulting in inflammation are mediated by various enzymes, cytokines, chemokines and polypeptide hormones, including: cyclo-oxygenase (COX), 5-lipoxygenase (LOX), tumour necrosis factor alpha (TNF α), interleukins (IL)-1, 1- β , -6, -8, -12, -17, -21 and -23 and monocyte chemoattractant protein-1 (MCP-1) [35], which are regulated by

transcription factor nuclear factor kappa B (NF- κ B) [36]. Inflammation plays a pivotal role in all stages of atherosclerosis.

1.5.1 LDL-C Induced Inflammatory Cascade

Atherosclerosis involves fatty lesion accumulation in the arterial wall, and over time can become large enough to protrude into the arterial lumen and occlude blood flow [37]. When the lumen becomes narrowed by fatty lesion deposits, the blood is forced through with increased pressure. This combined with unstable fatty lesions in the lumen can lead to plaque rupture, clot formation, and complete occlusion of the artery thereby inducing a heart attack or stroke [37]. The process of atherosclerosis begins with damage to the arterial wall, which can be caused by infection, injury, smoking, foods rich in saturated fat or response to small, dense LDL-P. Initiation of inflammation commences by the entrapment of small dense LDL-P in the sub-endothelial layer of the arterial tunica intima. The LDL-P binds to proteoglycan and resides in the intima proteoglycans [18]. Small dense LDL-P have a greater propensity for uptake by arterial tissue suggestive of enhanced transendothelial transport [19] and an increased susceptibility to oxidative modification [21, 22]. Once they reside in the arterial intima, they undergo chemical and enzymatic modification by various biochemical mediators such as reactive oxygen species (ROS) and various enzymes. It has been reported that these agents initiate and regulate the oxidation of LDL-P in the intima (Figure 1.1). The protein portions of the oxidized LDL (OxLDL) become antigenic, causing recruitment of T lymphocytes to the arterial wall [37]. OxLDL is a potent inflammatory agent and as it builds up in the tunica intima it stimulates inflammatory signalling by endothelial cells which express cell adhesion molecules to promote the migration of monocytes into the intima, which would otherwise resist contact with the epithelium in a healthy individual [37]. Cytokines such as MCP-1 and macrophage colony-stimulating factor (M-CSF) are secreted as the monocytes differentiate into macrophages, this recruits more macrophages to the arterial wall and enhances the permeability of the endothelium; facilitating further influx of LDL into the intima [38].

ARTERIAL LUMEN

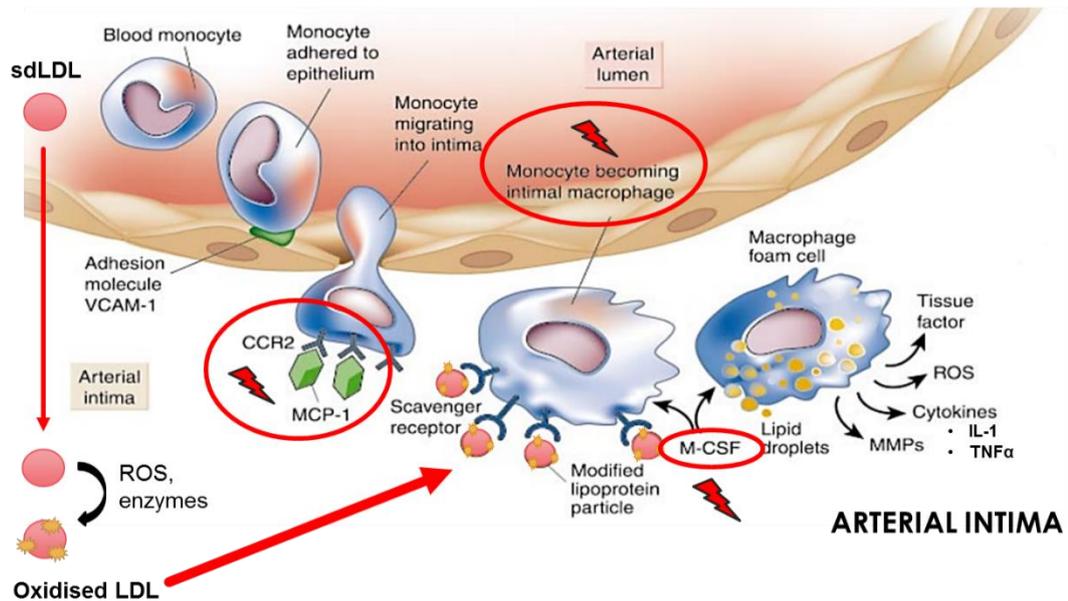


Figure 1.1: LDL-C and inflammation in atherosclerosis initiation.

Red arrow, circle and lightning bolt indicate key areas by which OxLDL stimulate this inflammatory cascade of atherosclerosis.

Reprinted and adapted by permission from Springer Nature Customer Service Centre GmbH: Nature <https://www.nature.com/>. Libby, P. Inflammation in atherosclerosis. Nature. 2002;420(6917):68-74 [38] (see Appendix 1 for proof of permission). CCR2, C-C chemokine receptor type 2; IL-1, interleukin-1; sdLDL, small dense LDL; M-CSF, macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; MMPs, matrix metalloproteinases; OxLDL, oxidized LDL; ROS, reactive oxygen species; TNF α , tumour necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1.

OxLDL also promotes the differentiation of monocytes into macrophages, therefore increasing its chance of being engulfed. OxLDL follow a different uptake pathway to native LDL-P, instead of slow, regulated uptake via the LDL-receptor (LDL-R), OxLDL undergo rapid, unregulated engulfment by macrophages via scavenger receptors [39, 40]. Thus, uptake is fast and contributes to the lipidation of macrophages and subsequent formation of foam cells, a hallmark of an arterial lesion. These foam cells secrete inflammatory cytokines such as IL-1, TNF α and ROS that amplify this inflammatory response and increase the permeability of the inner endothelial layer; facilitating entry of more small, dense LDL-P and monocytes to continue this cascade [37, 38].

As the inflammatory cascade elevates, the foam cells mature into fatty streaks and smooth muscle cells lining the tunica intima undergo anatomical changes whereby they shift from being cells that are dormant and contractile to migratory and proliferative. The transformed smooth muscle cells migrate to the fatty lesion where they form an extracellular matrix that acts as a fibrous cap,

covering the accumulating fatty lesion embedded on the arterial lumen [37, 40]. Continuation of this inflammatory state facilitates the increase in cytokine secretion on a systemic scale, namely IL-6 which activates the production of acute-phase proteins: C-reactive protein (CRP), fibrinogen, serum amyloid A and coagulants [37].

1.6 Calculating Cardiovascular Disease Risk

Framingham Risk Equation: The Framingham Heart Study led to the identification of major risk and predisposing factors for CVD (elevated blood pressure, elevated blood cholesterol, TG, obesity, smoking, diabetes, physical inactivity, low HDL-C levels, gender, age, and psychosocial issues) and subsequent development of several risk prediction algorithms indicating risk of CVD outcomes such as: atrial fibrillation, CVD, congestive heart failure, coronary heart disease, diabetes, hypertension, intermittent claudication and stroke [41]. The two key risk prediction algorithms for CVD include a 10-year and 30-year risk prediction.

The 10-year risk prediction algorithm includes predicting the risk for coronary death, myocardial infarction, coronary insufficiency, angina, ischemic stroke, transient ischemic attack, haemorrhagic stroke, peripheral artery disease and heart failure. This algorithm is targeted at individuals aged 30-74 years without CVD at baseline examination. The predictors involve: age, gender, diabetes mellitus, smoking, treated/untreated systolic blood pressure, TC, HDL-C, and BMI. This algorithm includes two versions; one using lipids as predictors, the other replaces lipids with BMI (simpler model) and the regression coefficients and hazard ratios used in the algorithms can be found on the Framingham Heart Study website [42]. The 30-year risk predicts the same outcomes except the facets of CVD are divided into either 'hard CVD' (acute myocardial infarction and death due to coronary cause and fatal/non-fatal stroke) or 'full CVD' (all hard CVD outcomes plus coronary insufficiency, angina pectoris, transient ischaemic attack, intermittent claudication and congestive heart failure) [43].

This risk algorithm has not been validated for all population groups and may underestimate the CVD risk of certain groups of individuals such as: elderly (> 74 years), Aboriginal and Torres Strait Islanders, individuals with diabetes between the ages of 45 and 60 years and adults with depression and socioeconomic deprivation [13]. Interpretation of the risk score with individuals belonging to any of the above groups must be done with caution.

Australian Absolute Cardiovascular Risk Charts: The Australian Cardiovascular risk charts were devised based on guidelines that were developed by the National Disease Prevention Alliance, released in May 2012 and approved by the National Health and Medical Research Council. The

guidelines upon which the charts are based are known as ‘Guidelines for the Assessment of Absolute CVD risk (2009)’ and adapted with permission from New Zealand Guidelines Group (New Zealand Cardiovascular Guidelines Handbook: A Summary Resource for Primary Care Practitioners. 2nd Edition, Wellington, NZ: 2009) [13]. The risk charts are specific for adults aged > 45 years (35 years if Aboriginal or Torres Strait Islander) without known CVD. This method of assessing CVD risk focuses on depicting an individual’s overall risk of CVD but calculating one’s absolute risk, that is, the probability of a CVD event occurring within 5 years. This method is opposed to the traditional method of risk assessment which considers risk factors for CVD in isolation [44]. The risk assessment includes separate sections to attend to individuals with diabetes and chronic disease.

1.7 Biological Targets for Lowering Blood Cholesterol

1.7.1 LDL-R

LDL-C is the primary transport vehicle that delivers cholesterol to cells by utilising the LDL-R [45]. The protein portion of the LDL-P binds to the LDL-R on the cell surface. The LDL-P is then engulfed into the cell via endocytosis and begins to breakdown into amino acids and free cholesterol [45]. The cholesterol portion is then utilised by the cell to form cholesteryl esters. During this process, the receptor protein is recycled and returns to the cell surface ready to bind with more LDL-P. Cholesterol supply in excess suppresses the synthesis of LDL-R, reducing the number of LDL-R and leads to an accumulation of cholesterol circulating the bloodstream [45]. Oversupply of cholesterol in the bloodstream can also reduce the production and activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA) reductase as well as increase the activity of acyl coenzyme A:cholesterol acyltransferase (ACAT, the rate-limiting enzyme of cholesterol absorption), thus promoting storage of cholesterol [46] (Figure 1.2). The particle size of LDL-C influences its atherogenic effects such that small, dense LDL-P are pro-atherogenic because they are not easily taken up by the LDL-R and therefore reside in circulation for longer [39]. Prolonged retention time of small, dense LDL-P is problematic as it remains trapped in the subendothelial layer of the arterial wall for longer, increasing the opportunity to inflict pro-atherogenic damage [39]. This coupled with its ability to be readily oxidised [47], further heightens the atherogenicity of this particle. Therefore, inhibition of LDL-R is a target for lipid-lowering.

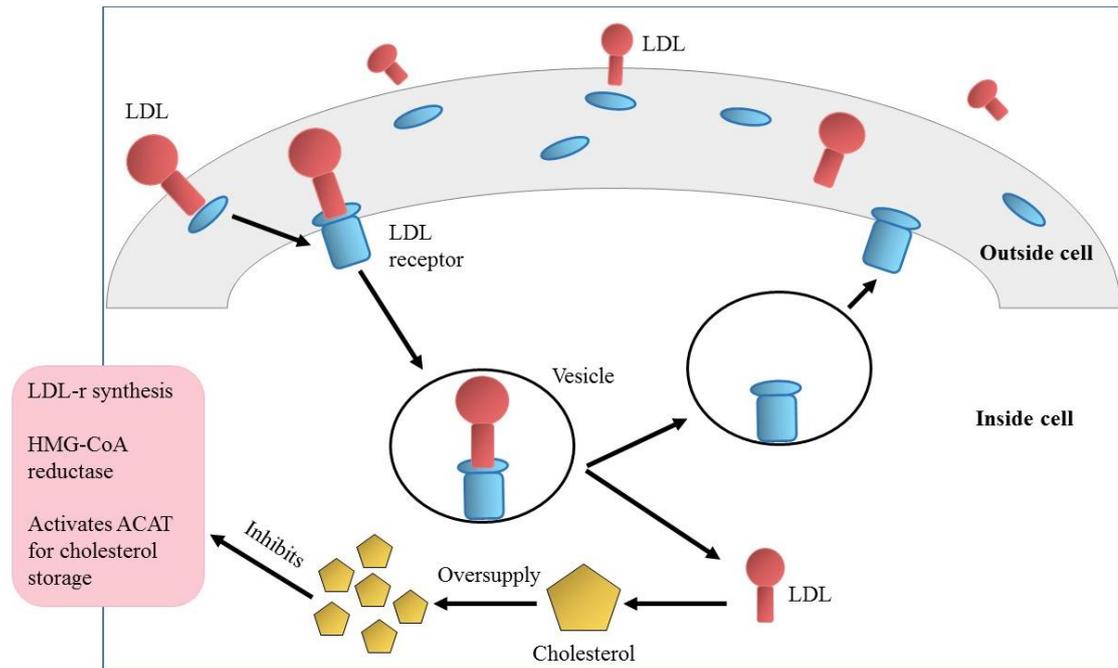


Figure 1.2: Uptake of LDL-P into the cell and the action of the LDL-R.

Biochemistry by Campbell, Mary K.; Farrell, Shawn O. Reproduced with permission of Brooks/Cole in the format Republish in a thesis/dissertation via Copyright Clearance Centre (see Appendix 2 for proof of permission). Campbell and Farrell, Chapter 8: Lipids and proteins are associated in biological membranes. In: Campbell J, Bitter E, Lum C, editors. Biochemistry. 5th ed. Belmont, California: Thomson Brooks/Cole; 2006. p. 203 [45]. ACAT, acyl-CoA:cholesterol acyltransferase; HMG-CoA reductase, hydroxymethylglutaryl-CoA reductase; LDL-r, low-density lipoprotein receptor.

Proprotein convertase subtilisin-kexin type 9 (PCSK9) inhibitors (evolocumab and alirocumab) are a relatively new class of lipid medication that lower LDL-C. PCSK9 inhibitors are a biological drug known as monoclonal antibodies that inactivate the protein PCSK9 found in the liver [48]. This protein itself inhibits LDL-R on the surface of hepatocytes, causing limited uptake of LDL-P and therefore accumulation of LDL-C in the bloodstream. Inhibition of PCSK9 leads to increased recirculation of the LDL-R to the surface of hepatocytes; increasing the availability of LDL-R to capture LDL-P for correct metabolism and removal from circulation [48]. PCSK9 inhibitors are recommended to be administered in combination with statins to treat patients with familial hypercholesterolaemia and/or patients who cannot tolerate statin medications [48]. LDL-C reductions as high as 50-60% were reported in PCSK9-statin combination patients [49]. Currently the medication is administered via subcutaneous injection 1-2 times per month and is expensive, costing approximately \$7000-\$12000 each year [50].

1.7.2 HMG-CoA reductase inhibitors

HMG-CoA reductase is the rate-limiting enzyme involved in cholesterol synthesis [51]. The manipulation of HMG-CoA reductase activity is the basis for how statins operate to lower plasma cholesterol. HMG-CoA reductase inhibitors (statins) act on the rate-limiting step of cholesterol synthesis. Statins inhibit the conversion of HMG-CoA to mevalonic acid, subsequently leading to a reduction in intracellular cholesterol synthesis [51]. Statins are considered one of the most successful agents for lowering LDL-C levels, with additional health benefits including improved endothelial function, moderate anti-inflammatory action, prevention of thrombogenic response and anti-hypertensive action in some cases [52]. Numerous studies have reported adverse side effects including myalgia, skeletal myotoxicity, acute kidney injury, myopathy, myositis and even fatal rhabdomyolysis [52]. Some trials have reported cancer, diabetes and cognitive impairment as a result of long-term statin therapy [53]. Studies have also shown that differences exist between statins such that some (e.g. atorvastatin) can increase blood markers of cholesterol absorption such as campesterol and β -sitosterol [54]. A meta-analysis of 27 RCTs reported reductions in LDL-C as well as reduced risk of major vascular events irrespective of age, previous vascular disease, baseline LDL-C and all-cause mortality (RR 0.79, 95% CI 0.77-0.81, per 1 mmol/L reduction) in individuals administered statins compared to those who were not medicated [53].

1.7.3 CETP inhibitors

CETP is a plasma protein which aids in the transfer of cholesteryl esters and TG between lipoproteins, namely HDL-C and LDL-C [55, 56]. There are currently four CETP inhibitors under clinical development: torcetrapib, dalcetrapib, anacetrapib and evacetrapib [55]. Inhibition of CETP and subsequent elevation of HDL-C is an attractive target for reducing CVD risk. The targeted mechanism with CETP inhibitors harnesses the important role HDL-C plays in reverse transport of cholesterol and subsequent cholesterol efflux capacity [55]. Inhibition of CETP leads to raised concentrations of HDL-C which promotes further recycling of cholesterol, and removal of cholesterol from foam cells present in atherosclerotic plaques to the liver. Torcetrapib and dalcetrapib have failed to present beneficial effects, however, the remaining two drugs in this category have shown promising results in human phase I and II clinical trials with confirmation of their success after completion of 2 large studies (REVEAL Study, ClinicalTrials.gov Identifier: NCT01252953 and the ACCELERATE Study ClinicalTrials.gov Identifier: NCT01687998) [55].

Other inhibiting medications that have been investigated include ACAT and microsomal triglyceride transfer protein (MTTP) inhibitors. In addition to re-esterification of cholesterol, ACAT is responsible for hepatic secretion of VLDL-C and macrophage-mediated storage of cholesterol esters in the arterial lumen. Therefore, inhibition of ACAT activity could lead to

subsequent reductions in plasma cholesterol levels as well as limit the accumulation of cholesterol esters in atherosclerotic lesions [57]. The two isoforms are present in the endoplasmic reticulum of certain cells including foam cells (ACAT-1), and exclusively in intestinal cells and hepatocytes (ACAT-2) [58]. The efficacy of ACAT inhibitors is conflicting in humans [57]. Avasimibe is the only bioavailable oral ACAT inhibitor and has inhibited ACAT in vitro and in vivo, however, despite promising results in animal studies, most human trials have failed to present changes in lipid profile and markers for CVD [58]. The question remains as to whether there is still merit for ACAT inhibitors, since in theory they may have favourable effects on the early stages of atherosclerosis by limiting fatty streak formation.

MTTP is a lipid transfer protein essential for the assembly of hepatic VLDL-C and intestinal chylomicrons [57]. MTTP is responsible for the transport of TG, cholesteryl esters and phosphatidylcholine between intracellular membranes. Therefore, inhibition of MTTP leads to diminished secretion of intestinal chylomicrons and hepatic VLDL-C producing a favourable reduction in plasma LDL-C, VLDL-C and TG in addition to preventing fatty acid build-up in the liver [57]. Human studies are limited, however, preclinical studies have shown promising results with noticeable reductions in both plasma cholesterol and TG [57]. Common side effects include gastrointestinal upsets and increased liver transaminases [59].

1.7.4 Cholesterol absorption inhibitors

Ezetimibe selectively inhibits cholesterol absorption by binding to the Niemann-Pick C1-Like 1 (NPC1L1) protein at the intestinal brush border blocking the transport of cholesterol across the intestinal lumen [54, 60]. This leads to a compensatory up-regulation of cell surface LDL-R, increasing LDL-C uptake and subsequent reduction of plasma LDL-C concentrations up to 15-20% [54, 59]. Unlike statins, ezetimibe reduces markers of cholesterol absorption, however, elicits an increase in markers of cholesterol synthesis [57]. Ezetimibe remains the only drug currently available that inhibits absorption of exogenous cholesterol without affecting the absorption of other lipids such as TG, fat-soluble vitamins and bile acids [59]. Side effects include gastrointestinal upsets, myalgia and myopathy, hepatitis, headaches and rhabdomyolysis and is contraindicated during active liver disease [59].

1.8 Management of Blood Lipids

1.8.1 Diet and lifestyle

The American Heart Association in partnership with the American College of Cardiology in their updated version of the ATP guidelines on lifestyle management to reduce CVD risk, emphasize

the consumption of a dietary pattern that focuses on the intake of fruits, vegetables, wholegrains, lean sources of meat and poultry, fish, include low-fat dairy products and limit sweets, sugar-sweetened beverages and red meats [61]. Specific to dietary fats it is also recommended to reduce calories from saturated to no more than 5-6% of total energy and to reduce calories from trans fats. With regards to dietary fats and in addition to the above guidelines, the National Heart Foundation of Australia currently advises the Australian population to choose foods containing healthier fats to assist with balancing blood cholesterol levels and reducing the risk of CVD in addition to avoiding tobacco smoking and limiting alcohol intake [62].

Animal studies by Fernandez and colleagues have suggested that dietary fatty acids regulate plasma LDL-C via the action of LDL-R activity, protein and mRNA abundance, more specifically, that these are reduced with dietary saturated fatty acids (SFA) and increased with unsaturated fatty acids [63]. This theory implies that SFA-rich diets may inhibit hepatic esterification of cholesterol and therefore reduced concentrations of esterified cholesterol [64, 65]. In addition, LDL-C is raised in plasma as a result of reduced LDL-R activity via diminished levels of LDL-R mRNA in the liver [64].

SFA have been classically deemed the major causative factor in the development of coronary heart disease and source of morbidity and mortality in the Western world over the past 50 years. Despite this commonly held belief in both public and scientific domains including an abundance of epidemiological and intervention studies; conclusive evidence defining a link between dietary SFA intake and circulating blood cholesterol concentrations has not yet been established [64]. Evidence for raised blood cholesterol and TG has been shown in animal models administered a SFA-rich diet deficient in omega-3 polyunsaturated fatty acids (n-3 PUFA) [66, 67]. Recent human studies rely on the basis of dated studies conducted in the 1950-60s whereby the relationship between SFA and blood cholesterol was born, however, these studies have failed to assess the interactive effects of SFA with other dietary fats, namely n-3 PUFA on serum lipids [64]. Some human studies that have investigated the effects of SFA-rich diets supplemented with n-3 PUFA have reported favourable outcomes in TG, VLDL-C and HDL-C compared to non-supplemented [68, 69].

Dietary trans fats are well known for their detrimental effects on circulating lipids so as to raise plasma LDL-C and lipoprotein (a) and lower HDL-C [63]. Less than 1% of total energy intake should derive from trans fat and Australians are leading the world by currently consuming only 0.6% total energy from trans fats [62]. Clinical evidence shows cis monounsaturated fatty acids (MUFA) and omega-6 polyunsaturated fatty acids (PUFA) reduce LDL-C [70-74], whilst long-chain omega-3 polyunsaturated fatty acids (LCn-3PUFA) modestly increase HDL-C and dose-dependently raise LDL-C concentrations; a consequence of increased LDL-P size conversion

[75]. This is reflected in the current Australian National Heart Foundation guidelines whereby dietary sources of MUFA (avocados, almonds, cashews, peanuts and plant/seed derived oils such as olive, canola, peanut) and PUFA (fish, tahini, linseed, chia seeds, pine nuts, brazil nuts, walnuts and oils/margarines derived from soybean, sunflower and safflower) are encouraged as healthy fats that are to replace unhealthy fats in the diet. Unhealthy dietary fats are identified as saturated and trans fats and the National Heart Foundation encourages Australians to limit consumption of food rich in these fat sources such as meat fat, coconut products, palm oil products, full-fat dairy products and processed and/or takeaway foods.

Interestingly, it has been reported that dietary SFA and cholesterol may not be as detrimental to heart health as previously thought, with no effects reported for LDL-C [76-78]. Although the link between dietary trans fats and raised lipid concentrations with subsequent mortality from coronary heart disease is established [63], the same cannot be deemed for dietary SFA. A recent systematic review and meta-analysis by our research group has shown that diets rich in medium-chain saturated fatty acids (MCFA) significantly raise HDL-C and apolipoprotein A-I concentrations compared to long-chain saturated fatty acid (LCFA) rich diets, with no effects on other blood lipids [79]. Moreover, consumption of MCFA delivered via coconut oil resulted in lower postprandial TG excursions compared to short-chain saturated fatty acids (butter) and LCFA (lard) despite identical fat and kilojoule content of the test meals in healthy adults (*accepted for publication, in press*). The inconsistent evidence arising about the association between dietary SFA and plasma lipids and subsequent coronary heart disease risk suggests that SFA consumption as an isolated risk factor may not be the main culprit for elevating blood cholesterol, but rather a holistic approach investigating the interaction between different dietary fats and types of SFA is warranted to elucidate the optimum fat profile for the prevention and management of hyperlipidaemia and subsequent reduction of CVD risk.

1.8.2 Physical activity

It is well established that regular physical activity is beneficial for overall health, including heart health. The guidelines by the American Heart Association in partnership with the American College of Cardiology advise adults to engage in aerobic physical activity at least 3-4 sessions per week for a duration of at least 40 minutes at moderate-to-vigorous intensity to improve blood lipids [61]. Likewise, The Australian Physical Activity & Sedentary Behaviour Guidelines for Adults (18-64 years) recommends weekly participation in at least 150 to 300 minutes of moderate intensity- or 75 to 150 minutes of vigorous intensity activity, or an equivalent combination of both intensities [80]. Muscle strengthening exercises are also recommended on at least two days per week. The mechanisms by which physical activity modulates blood lipids remains uncertain. A review conducted by Ahmed et al in 2012 reported at least 30-60 minutes of moderate-intensity

exercise on three to five days of the week long-term leads to increased HDL-C and decreased TG, even in the absence of weight loss [28]. A systematic review and network meta-analysis of 14 clinical trials reported combined aerobic and resistance training was the most effective regarding its beneficial impact on TG, HDL-C and other cardiometabolic measures in individuals with type 2 diabetes [81]. The current evidence regarding the effects of physical activity on TC and LDL-C levels are less defined, as the majority of studies reporting reductions in TC and LDL-C with exercise were not independent of weight loss [28]. Interestingly, lower concentrations of small, dense pro-atherogenic LDL-P is associated with regular physical activity, despite no change in LDL-C concentrations. LDL-P size appears to increase with the inclusion of 25 minutes/day of aerobic exercise, irrespective of weight loss or training intensity [28]. Although regular aerobic exercise does not seem to directly reduce LDL-C, it does assist with the management of blood lipids, given a transformation to anti-atherogenic LDL-P size, increased HDL-C and reduced TG concentrations.

1.8.3 Pharmacological therapy

The Australian National Vascular Disease Prevention Alliance recommends simultaneous lipid and blood pressure pharmacotherapy for individuals clinically diagnosed at high risk of CVD and/or who score an absolute risk of >15% for the development of CVD [13]. In addition to pharmacotherapy intervention, patients are advised to: cease smoking (if smoker), consume a diet rich in fresh fruits, vegetables, low in salt, saturated and trans fat, limit alcohol intake and participate in at least 30 minutes of physical activity on most/all days of the week [13]. Diet and lifestyle intervention independent of pharmacotherapy are recommended for individuals at moderate 5-year risk (10-15%) of CVD for 3-6 months before commencing pharmacological agents if dietary intervention failed to reduce the risk of CVD.

Statins are the most popular pharmacological agent used for lipid-lowering [52] and reducing vascular inflammation [40]. Statins reduce LDL-C by ~20% (with reductions observed as high as 55%, depending on the statin dose and type), modestly increase HDL-C and decrease TG concentrations [82]. Statin therapy inhibits endogenous synthesis of cholesterol in macrophages, thus reducing macrophage activity and generation of foam cells. Statins do pose serious adverse side effects, the most common being muscle pains known as myalgia which is common among 10% of statin-therapy patients [83] and the most severe (and rare) being rhabdomyolysis, which is the degeneration of muscle tissue experienced by one in a million patients receiving statins [84]. The side effects of pharmacotherapy for the treatment of CVD and/or alleviation of the associated risk factors pose great concern for the long-term health of patients. Also, the long-term economic burden of statin therapy in Australia remains a significant issue as approximately 2.2 million Australians are taking statins at the government's expense of \$346 million collective costs

for rosuvastatin, atorvastatin and simvastatin + ezetimibe in 2015-16. This was second to the first most expensive drug (Ledipasvir + Sofosbuvir) [85].

Bile acid sequestrants (resins) are positively charged, allowing them to bind to bile acids in the intestines, forming a complex that is insoluble and therefore excreted in stool [86]. As a result, hepatic levels of bile acids become reduced, activating the rate-limiting enzyme 7- α -hydroxylase (CYP7A) which facilitates the synthesis of bile acid from cholesterol. As a result, hepatic and circulatory concentrations of cholesterol are reduced [86]. Clinical studies have shown bile acid sequestrants effectively lower LDL-C by 15-30% with small increases in HDL-C (3-5%) and no change in TG [59]. Studies have reported additional health benefits such as improved glycaemic control in type 2 diabetics as well as a trend towards lower concentrations of glycosylated haemoglobin A_{1c} [86].

1.9 Role of Bioactives in the Management of Blood Lipids

In recent years, dietary bioactives have surfaced as popular supporting therapies for lowering blood lipids, particularly for individuals whose blood lipids are moderately elevated but not high enough to require pharmacological intervention [87]. For most bioactives, lipid modulation occurs through multiple mechanisms, potentially acting simultaneously on multiple stages of lipid-induced vascular damage. This has led to the emergence of their widespread use in combination with changes to diet or in combination with medications or other bioactives [88]. Bioactives provide an efficacious alternative for patients who are intolerant to lipid-lowering medications such as statins; with a large body of epidemiological and clinical evidence supporting the tolerability and safety of many bioactives [89].

A wide range of bioactives target blood lipids from a diverse range of molecular targets. Common lipid-modulating bioactives which inhibit intestinal cholesterol absorption include: phytosterols and phytostanols (PS), soluble fibres (β -glucan, psyllium and glucomannan) and chitosan [87, 88]. Bioactives which inhibit endogenous cholesterol synthesis include: red yeast rice extract; garlic (*allium sativum*); bergamot (*citrus bergamia*); policosanols; berberine; soy, lupin and green tea extracts. N-3 PUFA, curcumin (*curcuma longa*), gamma-oryzanol, spirulina, anthocyanins and others have mixed mechanisms of action [87, 88].

The scientific literature highlights three dietary bioactives (PS, curcumin and oat β -glucan, (OBG)) that play key roles in lipid modulation which might provide not only additional health benefits when co-administrated, but amplified effects as a result of complementarity and/or synergy.

1.9.1 Phytosterols

Dietary sources and intake

PS (comprising phytosterols and their saturated form phytostanols) are well established cholesterol-lowering agents derived from the diet and are naturally found in all plant based foods such as vegetables, nuts, seeds and their oils, fruits, legumes and vegetable fat spreads [90]. On average, Western populations are estimated to consume 150-450 mg/day of naturally occurring PS from the diet and only 20-50 mg/day of phytostanols [91]. Higher intakes of 2 g/day can be achieved via consumption of enriched products such as vegetable fat spreads, low-fat milk and wheat biscuit cereals in Australia. PS lower circulating LDL-C concentrations by inhibiting the intestinal absorption of cholesterol, made possible by their structural similarity to cholesterol [90].

Structure

PS differ from cholesterol by containing an extra ethyl (β -sitosterol) or methyl (campesterol) group at C-24 or an extra ethyl group plus a double bond (stigmasterol) at C-22 [17] (Figure 1.3). Phytostanols are saturated derivatives of PS and thus do not contain any double bonds.

Absorption and bioavailability

Incorporation into transport vehicles known as micelles is a pre-requisite for sterol absorption. Micelles interact with the brush border membrane of the intestinal lumen for sterol uptake, which is facilitated by transport protein NPC1L1 [92]. PS are poorly bioavailable compared to cholesterol and minimal amounts of PS enter circulation as the majority are selectively pumped back into the gut lumen along with unesterified cholesterol by ATP-binding cassette (ABC) transporters ABCG5 and ABCG8 [92]. As a result, concentrations of PS are ~200 times lower than cholesterol concentrations in individuals consuming their habitual diet [93].

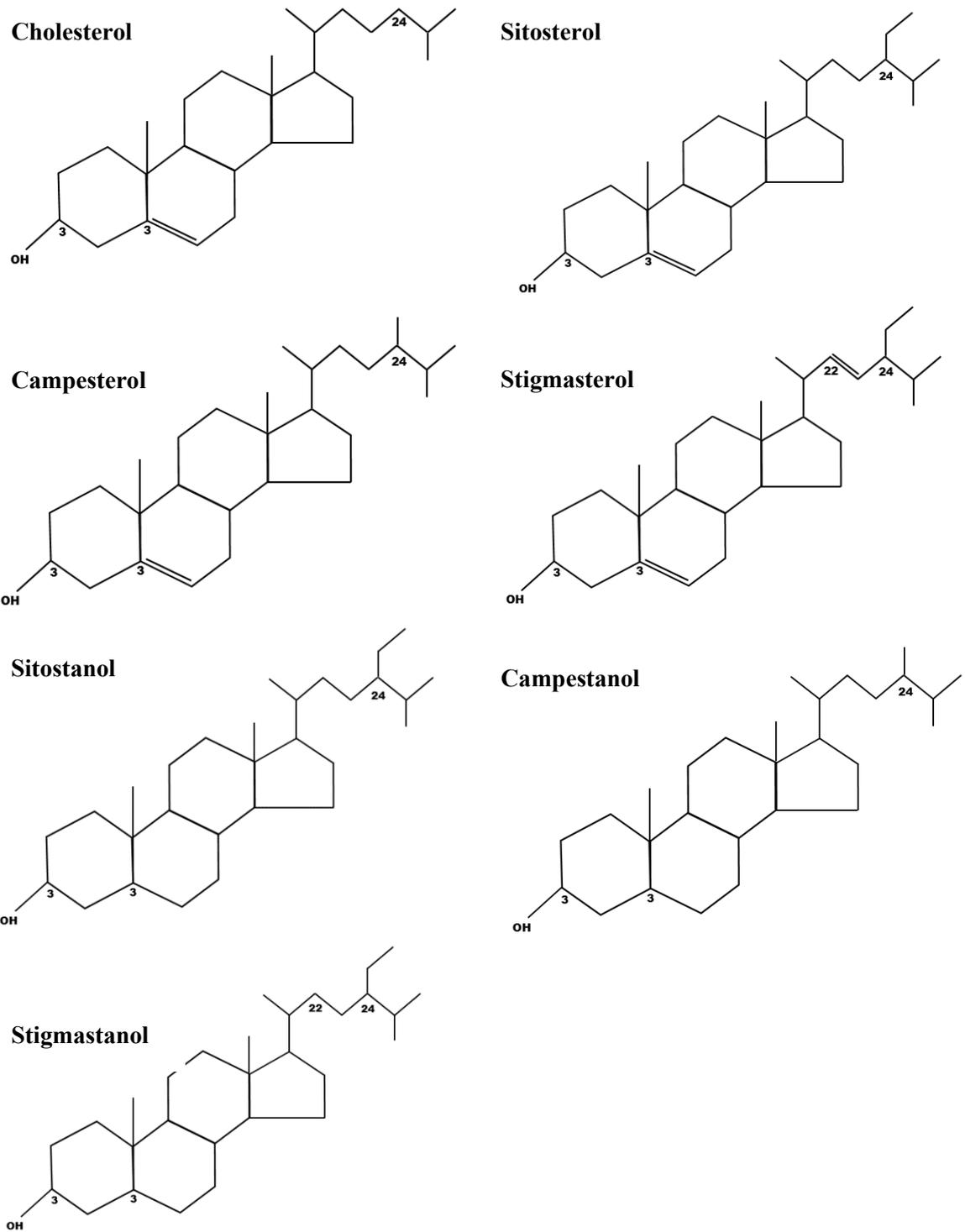


Figure 1.3: Molecular structure of cholesterol and the most abundant phytosterols and phytostanols.

Health implications and existing preclinical and clinical evidence

PS are well known cholesterol-lowering agents, to which they operate in a dose-dependent manner. The dose-response is well established with reductions in LDL-C by up to 10% following dietary supplementation of 2-2.5 g/day PS via enriched products in at least 4 weeks with no significant effects on HDL-C [94]. Mild TG-lowering effects have been reported, with most findings inconsistent. Findings appear inconsistent since most studies reporting mild or non-significant TG-lowering effects were not powered to assess change in TG [95] and/or it appears that TG-lowering induced by PS is more likely in individuals who have higher baseline TG such as individuals with type 2 diabetes or metabolic syndrome [96, 97].

Hypolipidaemic mechanism of action

Reduced cholesterol absorption: PS reduce the intestinal solubility of cholesterol by competing for incorporation in the micelle. PS have a greater affinity for micellar incorporation, therefore absorption of dietary cholesterol in the presence of PS causes micellar displacement of some cholesterol and then subsequent precipitation into the gut lumen where its fate is excretion from the body via stool [90]. Intestinal absorption of cholesterol can be reduced by up to 50% following PS supplementation [98]. This has been confirmed in human trials whereby higher faecal excretion of cholesterol was reported in a controlled feeding study of individuals administered a PS enriched diet (512 mg/day) vs PS poor diet (140 mg/day) [99]. This was an extension of Nestel's findings whereby a compensatory increase in cholesterol synthesis was observed in congruence with increasing plasma lathosterol (a marker of endogenous cholesterol synthesis) and plasma PS concentrations following 2.4 g/day PS supplementation for 4 weeks in hypercholesterolaemic adults [100]. Preclinical findings have also reported that PS treatment suppresses ACAT activity and thus inhibit esterification of cholesterol [90], thus leads to a reduction in the intestinal uptake and/or transport of cholesterol.

Liver X receptor alpha (LXR α) is a key regulator of lipid synthesis and activator of cholesterol transporters ABCA1, ABCG5 and ABCG8 responsible for mediating efflux of free sterols from enterocytes. PS have also been shown to promote the expression of ABCA1 transporter in micelles treated with phytosterols in caco-2 cells [101], thereby contributing to reduced cholesterol absorption. Preclinical studies have shown that certain PS (but not phytosterols) are potent activators of LXR, however, only in regards to agonising LXR in enterocytes [102]. Moreover, the metabolites of PS i.e. oxidised plant sterols have been identified as natural ligands for LXR [103]. It is in this way PS may interact with intracellular cholesterol sensors like LXR to indirectly modulate transporter activity and reduce cholesterol absorption via disruption and

potential competition for cholesterol transporters [90]. Preclinical studies conclude NPC1L1 does not appear to be involved in the cholesterol-lowering mechanisms of PS [90].

Safety

PS are well documented for their safety, tolerability and acceptability with no adverse effects for up to 85 weeks; the longest human PS-enriched trial [104]. A rare (one in five million) genetic condition associated with PS known as Phytosterolaemia (or sitosterolaemia) involves dysfunction in genes coding for ABCG5 and ABCG8 [105]. This results in severe accumulation of cholesterol and PS secondary to hyper-absorption as well as suppressed biliary excretion. The condition is characterised by high serum concentrations of plant sterols/stanols (up to 1.3 mmol/L and 0.2 mmol/L, respectively) [106]. Clinical characteristics include severe hypercholesterolaemia, haemolysis, xanthomas [92], premature development of atherosclerosis with PS accumulation in atherosclerotic lesions and subsequent angina pectoris, myocardial infarction and sudden death in severe cases [105]. Individuals with phytosterolaemia are encouraged to avoid consuming foods rich in PS, and ezetimibe has been recognised as a suitable treatment due to minimal side effects, inhibition of PS and cholesterol absorption at the intestinal brush border and noticeable reductions in plasma PS concentrations [107]. Further investigation into the long-term effects particularly examining the contribution of natural and therapeutic intake of PS are warranted to elucidate the potential risk of PS and those with phytosterolaemia must also be considered.

- ❖ Introduction to PS has been previously published as part of this thesis. Please refer to section 3.2 for further review of this literature.

1.9.2 Curcumin

Dietary sources and intake

Curcumin is found in the perennial herb *Curcuma Longa* (turmeric) which belongs to the ginger family and has been cultivated for centuries in Asia [108]. Turmeric has been widely used for cosmetics, as a preservative, colouring agent and medicinal ingredient in these countries for centuries [108]. Curcumin is widely consumed via turmeric in India, South Asia and Japan and is the main curcuminoid compound (>80%) extracted from turmeric roots [109]. This hydrophobic phytochemical is responsible for the intense yellow-orange colour of turmeric, sparingly soluble in water and soluble in oils, acetone and ethanol. Turmeric contains smaller amounts of other curcuminoids such as demethoxycurcumin, and bisdemethoxycurcumin [108]. The year 1937 marks the first study of curcumin administration in human disease and since then, over 3000

studies have demonstrated various medicinal benefits and properties [108]. In India, the average daily intake of turmeric is up to 2000 mg, providing roughly 125 mg per day of curcumin [110].

Structure

Curcumin is a polyphenol commonly known as diferuloylmethane and contains two ferulic acid portions bound with an additional methane on the two carboxyl side groups (Figure 1.4) [111]. The molecular formula for curcumin is $C_{21}H_{20}O_6$ and its melting point is approximately 170-175°C [109].

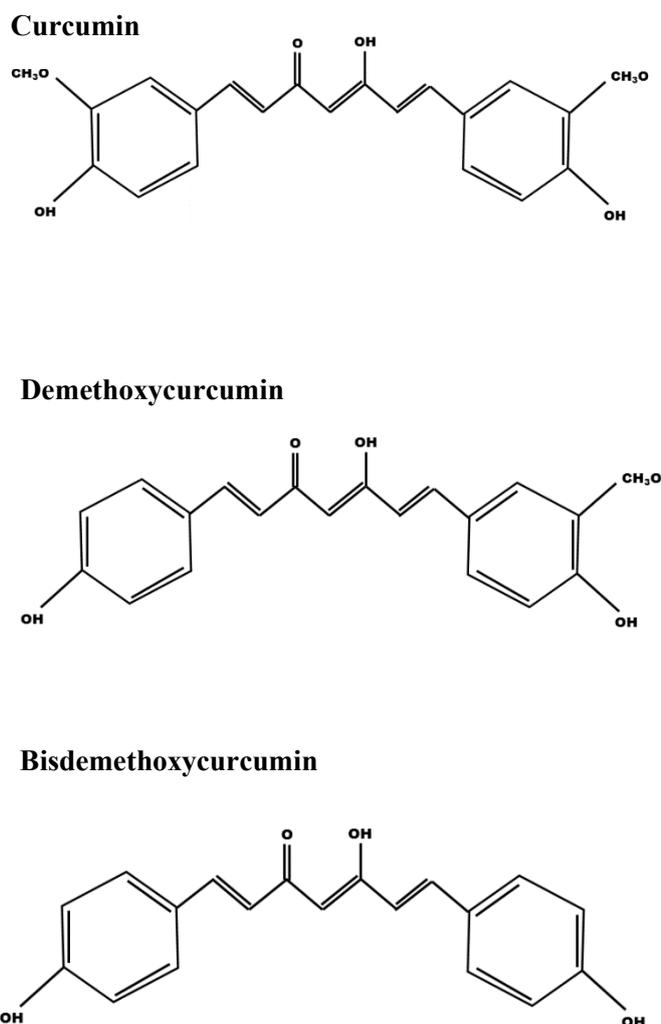


Figure 1.4: Molecular structure of curcumin, demethoxycurcumin and bisdemethoxycurcumin.

Absorption and bioavailability

Curcumin has limited bioavailability with less than 1% of curcumin present in the blood after oral consumption [112]. When administered orally, curcumin is rapidly conjugated in the liver to form metabolites such as glucuronide and sulfate esters. These metabolites undergo reduction to form

tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin [111]. Both conjugated and reduced metabolites being hydrophobic, have been reported to be biologically inactive [113]. The reduced form of metabolites are then excreted in the faeces via bile. In humans, serum concentrations of curcumin remain low even after doses as high as 10-12 g per day [114]. The highest plasma concentrations of curcumin are typically 1-2 hours post ingestion [114] and are only detectable when consumed in gram quantities [109]. This is indicative of curcumin's limited systemic bioavailability and poor intestinal absorption. The poor systemic bioavailability of curcumin is attributed to the rapid conjugation of curcuminoids [115], yielding curcumin glucuronide and sulfates that are excreted in faeces via bile [111, 116]. Like other compounds with low systemic bioavailability, curcumin has limited tissue distribution, low serum concentrations, rapid metabolism and a short half-life of approximately 8 hours in the bloodstream [117]. A wide range of formulations have been developed to overcome the poor bioavailability of curcumin: metal ion complexes such as Se^{2+} , Zn^{2+} , Mg^{2+} , and Cu^{2+} ; combining with serum albumin [118]; water-soluble curcumin (polyvinyl pyrrolidone); adjuvants such as liposomal encapsulations; curcumin nanoparticles; phytosome complexed with phosphatidylcholine [111]; combined with piperine [115] and solid lipid curcumin particle (SLCP) [119].

Health Implications

Several preclinical and clinical studies have demonstrated the diverse range of molecular targets curcumin acts upon either by binding or by directly or indirectly modulating their activity [108]. Targets of curcumin include growth factors, transcription factors, kinases, enzymes and inflammatory mediators such as cell adhesion molecules, cytokines and apoptosis-related proteins. Clinical evidence for the hypolipidaemic effects of curcumin is currently somewhat inconsistent. A recent systematic review and meta-analysis of seven RCTs ranging from 4 weeks to 6 months duration, conducted in individuals with either type 2 diabetes or metabolic syndrome; found curcumin and turmeric significantly lowered serum LDL-C (-0.3 mmol/L) and TG (-0.21 mmol/L). Despite these beneficial findings, the authors report only two of the included studies used enhanced formulations of curcumin (amorphous and nanoparticles) [120]. The studies included were heterogenous as different formulations of curcumin were used and all participants were Asian, thus limiting the transferability of findings. A recent study in subjects with metabolic syndrome using a bioavailability-enhanced form of curcumin reported significant improvements in blood lipids, however, subjects were also receiving standard care including pharmacological therapy [115]. Further clinical trials with bioavailability-enhanced curcumin administered to dyslipidaemic individuals is warranted to not only confirm the hypolipidaemic properties of curcumin but the optimal dose and duration. In vivo and in vitro studies have demonstrated several molecular and cellular mechanisms by which curcumin acts as a lipid-modulating agent [121],

with the majority of findings supporting lowering of TC, LDL-C, TG and FFA concentrations. There is some preclinical evidence to suggest curcumin favourably increases HDL-C concentrations [56, 115] and hepatic apoA-1 expression [56], however, this is yet to be replicated in human trials [120, 122].

Reduced cholesterol biosynthesis: Curcumin has been shown to reduce hepatic cholesterol biosynthesis in a similar manner to statins, that is, via the inhibition of HMG-CoA reductase [56, 123, 124]. Additionally, curcumin treatment in in vitro studies down-regulated the expression of sterol regulatory element binding proteins -1 and -2 (SREBP-1, SREBP-2); key nuclear receptors involved in mediating lipid metabolism [125, 126]. Plasma and hepatic cholesterol and apoB concentrations significantly reduced following curcumin therapy due to transcriptional inhibition of HMG-CoA reductase, and the degree of reduction was similar to lovastatin treatment in LDL-R knockout mice fed a high cholesterol diet for 18 weeks [56]. Interestingly, the cholesterol-lowering ability of curcumin via transcriptional inhibition of HMG-Co-A reductase appears to be independent of changes in hepatic ACAT expression or activity [56]. In both wild type (fed a high fat diet) and leptin-receptor deficient mice (fed a standard diet), curcumin treatment led to reduced ACAT activity in the liver [127, 128]. In addition, curcumin has also inhibited NPC1L1 expression in intestinal Caco-2 monolayer via the inhibition of SREBP-2, resulting in reduced cholesterol esterification [129]. The inhibition of HMG-CoA reductase and NPC1L1, the two key molecular targets of statins and ezetimibe respectively, characterises curcumin as a potentially powerful cholesterol absorption inhibitor, alluding to complementary and possibly synergistic effects when coupled with PS.

Reduced cholesterol absorption: Curcumin treatment has been shown to upregulate LXRA expression therefore indirectly modulating transporter activity as well as compete for cholesterol transporters, this is also associated with reduced hepatic TG accumulation [56, 130]. Moreover, inhibition of NPC1L1 in intestinal caco-2 monolayer was exhibited following curcumin treatment via the inhibition of SREBP-2; thus lowering cholesterol esterification [126]. Activity of hepatic ACAT has been shown to be significantly reduced in type 2 diabetic *db/db* mice supplemented with curcumin (0.2g/kg body weight) for 6 weeks, causing significant reductions in plasma cholesterol, FFA and TG as a consequence of lowered cholesterol esterification and subsequent uptake and/or intestinal transport [128]. Reduced hepatic ACAT activity has also been reported in both wild type (fed a high fat diet) and leptin-receptor deficient mice (fed standard diet) administered curcumin intervention [127, 128].

Increased clearance of cholesterol: Curcumin promotes the clearance of lipids from the blood by up-regulating the LDL-R in HepG2 cells and the liver [130, 131], including a sevenfold increase

in LDL-R mRNA of human HepG2 cells [130]. These modulatory effects on the LDL-R subsequently leads to the removal of cholesterol and fatty acids from the blood. The enhanced clearance of cholesterol via bile is a result of the activation and up-regulation of hepatic gene expression of CYP7A mRNA by curcumin; the rate-limiting enzyme involved in bile biosynthesis from cholesterol [121, 132-134]. Furthermore, CYP7A is further enhanced by curcumin via the activation and upregulation of ABCA1 and LXR in subcutaneous adipocytes and the liver [135].

Many animal studies report LDL-C-lowering following curcumin treatment in a variety of diet-induced disease models, all with wild-type rodents. Curcumin treatment led to reductions in LDL-C and apoB in LDL-R knockout mice fed a high cholesterol diet for 18 weeks, these reductions were similar to the mice treated with lovastatin [56]. The authors conclude that the increased clearance of LDL-C following curcumin treatment indicates that LDL-C production is reduced or that other receptors which can detect apoB-containing lipoproteins such as scavenger receptors, cell surface proteoglycans and LDL-R-related protein may be compensating for the lack of LDL-R present [56]. A review article conducted by Zingg et al (2013) found that hepatic and skeletal muscle CD36 scavenger receptor/fatty acid transporter has been shown to be up-regulated following curcumin treatment in LDL-R^{-/-} mice, resulting in the removal of FFA and TG from plasma, making them available for β -oxidation in tissues [121]. Preclinical studies have demonstrated that curcumin treatment induces significant inhibition of CETP activity [56]. It is important to note that changes observed in CETP activity are reported to be driven by changes in plasma TC, independent of LDL-R, therefore, it is uncertain as to whether curcumin directly modulates CETP activity [56].

Increased LDL-C uptake: The number of LDL-R is enhanced with curcumin treatment via down-regulation of PCSK9 expression [136], leading to enhanced LDL-P uptake and correct metabolism and removal from circulation.

Modulation of free fatty acids: Alterations in the transcription of nuclear receptors mediate the hypolipidaemic effects of curcumin. Peroxisome proliferator activated receptor (PPAR) is a major nuclear receptor responsible for regulating the expression of key proteins and enzymes involved in lipid and energy homeostasis such as: increased TG-rich lipoprotein hydrolysis; production of TG and VLDL; fatty acid storage in adipose tissue; reduction of FFA, and promotion of β -oxidation [63]. Animal studies have shown that curcumin treatment upregulates PPAR α expression by ~30%, leading to increased fatty acid catabolism and a significant reduction in hepatic TG accumulation [56]. Upregulation of PPAR α also results in diminished expression of apoCIII, a key inhibitor of lipoprotein lipase, and thus an indirect mechanism for TG-lowering by curcumin [137, 138]. Activation of PPAR γ and differential regulation of SREBP gene expression in hepatocytes have been shown in vitro after curcumin treatment, leading to a

subsequent increase in hepatic fatty acids and TG [139], reduction of plasma FFA, TG and glucose [140] and a reduction in hepatic cholesterol via the reduction of LDL-R gene expression [139]. In contrast, PPAR γ expression is reduced in adipocytes following curcumin, resulting in suppressed lipogenesis and adipogenesis and enhanced cholesterol efflux from adipocytes [135]. In association with upregulated LXR α following curcumin, a reduction in TG accumulation in the liver was also reported [56, 130]. Moreover, a review article conducted by Zingg et al (2013) found that hepatic and skeletal muscle CD36 scavenger receptor/fatty acid transporter has been shown to be up-regulated following curcumin treatment in LDL-R $^{-/-}$ mice, resulting in the removal of FFA and TG from plasma, making them available for β -oxidation in tissues [121].

Anti-inflammatory effects

There is an abundance of preclinical research to suggest that curcumin treatment mediates anti-inflammatory effects to lower CVD risk. The anti-inflammatory properties are partly mediated via down-regulation of NF- κ B [36, 109], leading to a subsequent reduction in gene expression of inflammatory proteins. Stimuli such as viral and bacterial infections, lipopolysaccharides or inflammatory cytokines activate NF- κ B [109, 141], resulting in translocation to the nucleus and initiation of various target gene expression such as TNF α and IL-1 β [109]. It is well known that curcumin interrupts the NF- κ B signalling pathway at multiple points; including suppressed AP-1 expression [109, 142], reduced activity of I κ B kinase and thus suppressed phosphorylation of I κ B in intestinal epithelial cells [109]; resulting in a net down-regulation of NF- κ B activation and diminished gene expression. Ultimately, this leads to suppressed activity of key inflammatory cytokines involved in inflammation [109] such as IL-1, -1 β , -6, -8 and -12 [143], TNF α [143, 144], MCP-1 and cell adhesion molecules. In addition, curcumin has also been shown to inhibit independent mitogen-activated protein kinase pathways [145] and pro-inflammatory enzymes such as matrix metalloproteinases [143, 146], COX-2 [143, 146] and 5-LOX which are involved in generating lipid mediators implicated in producing an inflammatory response via arachidonic acid metabolism. In animals, the cardio protective properties of curcumin are partly attributed to the down-regulation and/or inhibition of TNF α [35]. The review by Aggarwal et al reported suppressed TNF α production from macrophages that have been activated by various stimuli, in addition to inhibited expression of a range of stimuli that upregulate TNF α following curcumin administration [35]. There is no established dose for optimal anti-inflammatory effects, however, recent research findings suggest a daily dose of 150-300 mg of bioavailability-enhanced curcumin is sufficient to elicit a clinically significant reduction in pro-inflammatory cytokines such as IL-6 and TNF α in humans [35, 147].

Safety

The safety and tolerance of curcumin in humans is well documented and regarded as safe. Oral supplementation with curcumin has been tolerated with no toxicity in doses as high as 8000 mg/day for 3 months [114]. Longer trials at high doses such as the latter are warranted to confirm the safety of curcumin supplementation in the long-term.

1.9.3 Oat β -glucan

Dietary sources and intake

Oat β -glucan (OBG) is a soluble fibre derived from the bran of oats. The bran of an oat kernel is rich in fibre and minerals and has a layered structure comprising aleurone, the outer bran layer and the sub-aleurone layer, the inner bran; both of which form the cell walls of the endosperm [148]. β -glucan is most richly found in the sub-aleurone layer, and thinly in the outer aleurone layer [149]. β -glucan is also richly found in barley and lesser amounts in rye, maize, rice, sorghum and millet. The levels of β -glucan are influenced by genetics and the cultivating environmental [150]. On average, 25-30 g dietary fibre, predominately sourced from grains is recommended for adults for optimal gut function and heart health [151]. In 2011-12, only 30% of Australian's aged 2 years and over consumed the recommended servings of grains and only one third of all grains consumed were wholegrain or high in fibre [152]. In 2011-12, Australian Aboriginal and Torres Strait Islanders consumed on average 18 g/day dietary fibre which was less than non-Indigenous Australians who consumed 22 g/day on average [153]; both groups falling short of the recommended guidelines.

Structure

OBG is a linear glucose polymer bonded by glycosidic linkages $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ (Figure 1.5) [154]. The β -glucan found in oats is similar to that found in barley with some differences with respect to MW [155], possibly solubility [156] and the ratio of (1 \rightarrow 3) and (1 \rightarrow 4) linkages present in OBG compared to barley β -glucan [157].

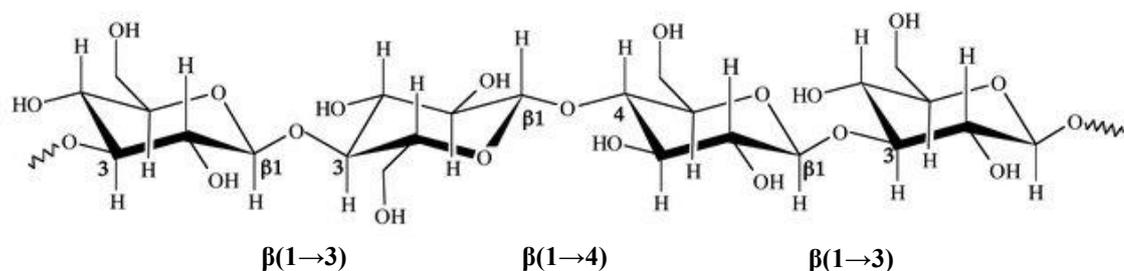


Figure 1.5: Molecular structure of oat β -glucan depicting the $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ glycosidically bonded glucose polymer.

Adapted and modified from: Pillai, R. Redmond, M and Rödning, J. Int J Cosmet Sci. 2005;27(5):291-3 [149]

Absorption and bioavailability

OBG is stable at varying pH's, resists digestion in the stomach and enters the small intestine where it increases the viscosity of intestinal contents as it absorbs fluid while passing through the length of the intestine [158]. Its eventual fate is excretion in stool. The key physiological property of OBG is its viscosity, which is determined by its MW and solubility [154, 159]. The higher the MW, the greater the viscous capacity of OBG and therefore greater capacity to increase the viscosity of the upper gastrointestinal tract [159]. The MW and solubility of OBG can be altered by food processing methods, storage [160] and β -glucanase enzymes present in wheat flour. Native unprocessed bran is naturally high in MW (~1730-2800 kDa) [161, 162], however, this can be reduced to as low as ≤ 100 kDa during extreme food processing, extrusion techniques and storage [162]. Typical MW of commercial products containing β -glucan in snacks, beverages and grain foods that have been tested range from as low as 40-80 kDa [154]. The solubility of β -glucan is known to be reduced in foods that have low water availability as well as storage of hydrated β -glucan at cooler temperatures [161]. It has been shown that these limitations can be controlled for during food processing such as varying the water content, extrusion temperatures, shear rate and opting for baking, however, the MW of β -glucan in a food cannot be increased if it is already low in MW prior to food processing [154].

Health Implications

OBG has been shown to be a useful tool in reducing a diverse range of cardiometabolic risk factors such as lowering cholesterol concentrations, delaying gastric emptying [163] and reducing postprandial blood glucose concentrations [164]. It has also been shown to enhance satiety and thus possibly play a role in weight management [165, 166]. Moreover, OBG supplementation has been shown to improve gut health by promoting colonic fermentation and production of short-chain fatty acids (SCFA) [167-169] which plays various roles including inhibiting cholesterol biosynthesis [167, 170] and mediating caloric intake [171, 172].

It has been reported that consumption of ≥ 3 g/day OBG significantly lowers TC and LDL-C concentrations by 0.30 mmol/L and 0.25 mmol/L respectively, with no significant changes to HDL-C or TG concentrations [161]. A systematic review and meta-analysis reported that a high-MW (2210 kDa) and medium-MW (530 kDa) OBG induced a greater LDL-C reduction compared to low-MW (210 kDa) and control (low-MW and low solubility wheat bran) [154]. In hypercholesterolaemic individuals, a beverage enriched with 5 g/day OBG of low-MW (70 kDa) significantly lowered TC (-7.4%) in 8 weeks as well as 30 minute postprandial blood glucose and insulin concentrations compared to control (rice starch) [173]. The same dose of barely β -glucan; with a much lower MW (40 kDa) had no effect. There remains a degree of reported lack of effect

from oat products on cholesterol concentrations, however, many studies have used different doses and/or varying degrees of MW of OBG, with many studies not documenting the MW used in oat products [161]. Discrepancies may also be explained by differing preparation, food manufacturing and storage techniques of OBG intervention products as well as diverse origins and climates upon which the oat kernels are grown; all of which could impact the physiological effects of OBG.

Reduced intestinal bile acid reabsorption: Irrespective of the MW, the most widely accepted mechanism of action by OBG is its bile acid sequestering ability [174, 175]. Bile is normally recovered in the distal end of the small intestine and recycled after facilitating the digestion and absorption of dietary fats [174]. The adjacent side chains on the OBG polymer structure form cross links enabling it to swell as it absorbs fluid whilst passing through the length of the small intestine [175]. It has been reported that the resultant gel acts as a highly gelatinous net which traps and binds to bile leaving it unable to be reabsorbed and thus it is excreted in stool [174]. The homeostasis of bile acid metabolism is disrupted, causing a cascade of changes to cholesterol metabolism: bile acid synthesis is increased utilising cholesterol from both the circulation and the liver, which as a result is supplied by raised cholesterol biosynthesis evidenced by increased concentrations of lathosterol and 7 α -hydroxy-4-cholesten-3-one (a marker of bile acid synthesis) [176-178] and upregulation of LDL-R expression thus enhancing clearance of LDL-C from circulation for use in bile acid synthesis [154, 174]

Increased production of SCFAs: OBG has a prebiotic effect in the gut, thereby promoting colonic fermentation and it has been reported that OBG significantly increases the production of SCFA. The most abundant SCFA include propionate, acetate and butyrate [179]. This has been shown in a model of intestinal fermentation using human faecal inoculum to represent microflora [180], in animal studies [181] and human studies [169, 170]. Faecal total SCFA significantly increased in mildly hypercholesterolaemic individuals who consumed a breakfast cereal enriched with 3 g/day high-MW barley β -glucan compared to 3 g/day low-MW barley β -glucan and control [169]. Preclinical and human studies have demonstrated a suppression of endogenous cholesterol synthesis following SCFA treatment or intervention [169, 182]. Propionate has been reported to play a key role in SCFA-mediated cholesterol-lowering by suppressing endogenous cholesterol synthesis via reduced activity of HMG Co-A reductase; a similar mechanistic action to statins. Elevated concentrations of propionate have been demonstrated in in vitro gut fermentation models treated with concentrated OBG [183]. In addition, the elevation in butyrate following OBG treatment, has been shown to promote Verrucomicrobia population expansion; resulting in a prebiotic effect in the caecal microbiota protection against high-fat/cholesterol-induced atherogenesis in apolipoprotein E (apoE) deficient mice [181].

Interference with lipid digestion: Little is known regarding the degree of direct impact on lipid digestion by OBG, however, since it increases the viscosity of the small intestine, it likely diminishes the efficiency of emulsification, thereby limiting accessibility of dietary fats by digestive enzymes. In vitro studies have reported that OBG interfered with lipid emulsification, lipolysis of triacylglycerols [184, 185] and lipase activity [186], thus contributing to its overall mechanistic role in bile acid sequestering and disruption of cholesterol homeostasis. Furthermore, dietary supplementation with soluble fibres has been shown to promote ileal and faecal fat excretion in humans [187, 188] and several animal studies have demonstrated increased faecal fat excretion [189, 190].

Safety

OBG is reported to be safe, however, at high doses of high-MW OBG it is possible for individuals to suffer from mild gastrointestinal discomfort such as bloating, abdominal cramping and flatulence, particularly in individuals who have a low-moderate soluble fibre intake prior to supplementation. It is advised that fluid intake is increased when increasing soluble fibre intake.

1.10 Summary of Literature Review and Thesis Rationale

Dyslipidaemia is a major modifiable risk factor underpinning the pathobiological development of atherosclerosis. Although diet and lifestyle changes can assist with managing dyslipidaemia, often pharmacological interventions are required to effectively achieve blood lipid targets. Side effects, medication interactions and adverse events can impede the long-term sustainability of pharmacological interventions, with some individuals intolerant to some medications. PS have been shown to be a good alternative and/or adjunct therapy for such individuals, however, only provide a moderate cholesterol-lowering effect with no significant changes to other blood lipids. Their heart health benefits also stop short at just blood cholesterol, which is only one facet of the multidimensional CVD. As it has been discussed in this literature review, curcumin and OBG could amplify the cholesterol-lowering effects of PS when administered in combination, as each bioactive acts via distinct mechanisms to modulate blood lipids (Figure 1.6) and provide additional heart health benefits, to therefore maximise CVD risk reduction in hypercholesterolaemic individuals.

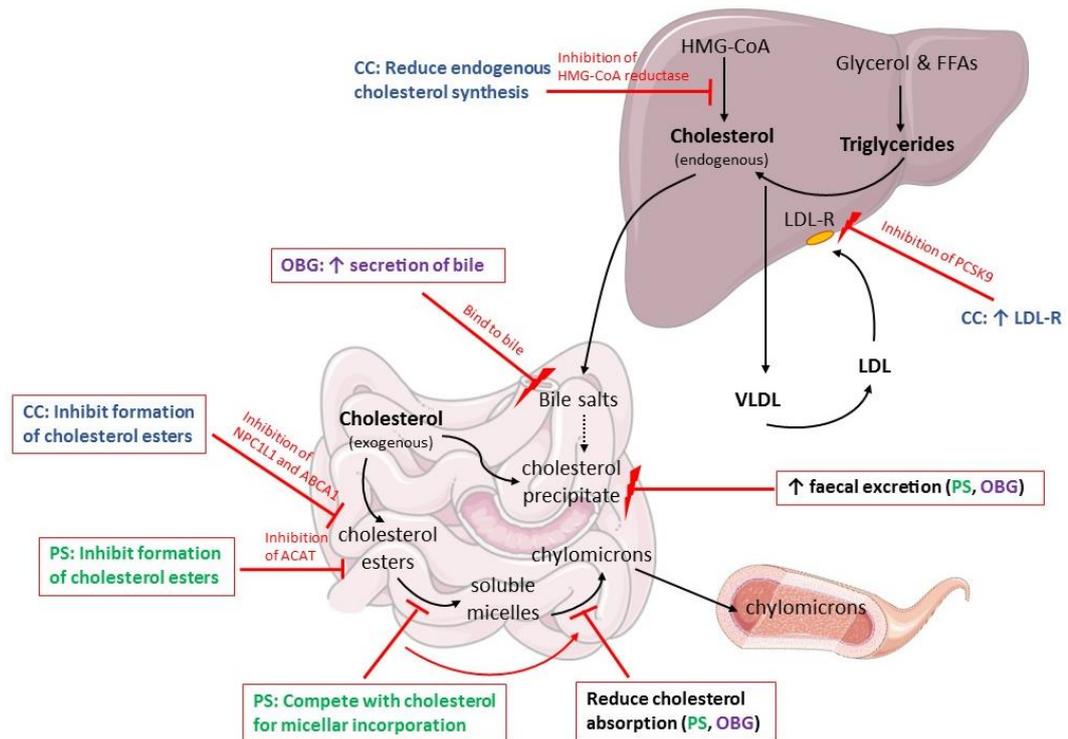


Figure 1.6: Rationale for combining PS with curcumin and OBG for modulation of blood lipids. ABCA1, ATP-binding cassette transport; ACAT, acyl coenzyme A:cholesterol acyltransferase; CC, curcumin; FFA, free fatty acids; HMG-CoA, hydroxymethylglutaryl-CoA; LDL-R, low-density lipoprotein receptor; NPC1L1, Niemann-Pick C1-Like1; OBG, oat β -glucan; PCSK9, proprotein convertase subtilisin-kexin type 9; PS, phytosterols; VLDL, very low-density lipoprotein.

1.11 Research Aims

1. To determine whether the type of carrier fat used in PS enriched fat spreads and dairy products is a determinant of the hypocholesterolaemic effects of PS enriched products (chapter 3).
2. To investigate the effects of dietary supplementation with PS with or without curcumin on circulating blood lipids in hypercholesterolaemic individuals (chapter 4).
3. To investigate the effects of dietary supplementation with a novel functional food enriched with PS with or without curcumin on blood lipid profile and CVD risk in hypercholesterolaemic individuals (chapter 5).
4. To investigate the effects of dietary supplementation with PS with or without high-MW OBG on circulating blood lipids in hypercholesterolaemic individuals (chapter 6).

5. To explore the effects of the dietary combination of PS plus curcumin and PS plus OBG on CVD risk in hypercholesterolaemic individuals (chapter 5, 7).

1.12 Thesis Hypothesis

It is hypothesised that the lipid-lowering potential of PS can be heightened by combining with other bioactive compounds with the likelihood of alleviating other cardio-metabolic disease risk factors concurrently.

Chapter 2

General Methodology for Clinical Trials

The following description of general methodology relates to all three clinical trials included in this thesis unless otherwise specified. Further detail regarding trial methodology is reported in the respective chapter for each trial.

2.1 Participant Recruitment, Ethics and Trial Registration

Participants were recruited from the Newcastle, Hunter and Central Coast regions of New South Wales, Australia. Various recruitment methods were used: media advertisements (radio interviews, newspaper articles, website advertisements), social media (Facebook and Twitter), word of mouth and the Hunter Medical Research Institute Volunteer Register. Recruitment flyers approved by the University of Newcastle Human Research Ethics Committee (UON HREC) were placed on notice boards within the University of Newcastle located in buildings, pathology centre, health service, pharmacy as well as general practise centres (with permission) and local shopping centres in the Hunter area. Prior to consenting to participate, interested volunteers were screened over the phone or in person by the lead investigator (JF) using an eligibility checklist. Interested and eligible participants were provided with a study information statement outlining the details of the study and all participants provided written informed consent in order to be enrolled in the trials. All clinical trials presented in this thesis were approved by the UON HREC as well as registered on the Australian New Zealand Clinical Trials Registry (ANZCTR). The respective ethics approval and trial registration numbers are reported in the following chapters (4, 5 and 6) specific to each trial. See appendices 3-5 for ethics approval notification.

2.2 Study Design and Sample Size Calculation

A double-blinded, randomized, placebo-controlled trial with a 2x2 factorial design in 4 parallel groups was employed for all trials. Sample size calculations yielded 80 participants in total (20 per treatment group) based on previous estimates of variance in plasma TC (standard deviation of 0.5) to detect a 0.5 mmol/L (~10%) reduction in TC at 80% power whilst accounting for a 20% dropout rate.

2.3 Experimental Interventions

2.3.1 Study 1 – PAC-CVD Trial (Chapter 4)

Participants were randomly allocated to one of four treatments which they had to consume daily for 4 weeks:

- **Placebo (PL):** 25 g canola fat spread plus two placebo tablets matching for curcumin
- **Phytosterol (PS):** 25 g canola fat spread enriched with 2 g PS plus two placebo tablets matching for curcumin
- **Curcumin (CC):** 25 g canola fat spread plus two tablets providing 200 mg curcumin
- **Phytosterol plus Curcumin (PS-CC):** 25 g canola fat spread enriched with 2 g PS plus two tablets providing 200 mg curcumin

The PS enriched fat spread was commercially available (Logicol Original, MeadowLea) and predominately based on canola oil and the placebo fat spread (Gold’N Canola, MeadowLea) was equivalent in nutritional profile but devoid of PS (Figure 2.1A). Each 1 g of curcumin tablet contained 500 mg curcuma phospholipid (Meriva® Indena) plus excipients; delivering 100 mg curcumin. Placebo tablets were comparable but did not contain curcuminoids and soy lecithin, but more microcrystalline cellulose (Figure 2.1B). All fat spreads were identical in nutrient profile except for PS content, and fat spreads and tablets were identical in sensory characteristics. Product containers were labelled with a code prior to study commencement and packaged by a research assistant impartial to the study to ensure neither investigators nor volunteers could determine intervention allocation.



Figure 2.1: Study intervention products for PAC-CVD Trial. 25g vegetable fat spread per day (A) and two tablets per day (B).

2.3.2 Study 2 – PAC-FOOD Trial (Chapter 5)

Participants were randomly allocated to one of four treatments which they had to consume daily for 4 weeks:

- **Placebo (PL)**: 2 slices bread providing no PS or curcumin
- **Phytosterol (PS)**: 2 slices bread providing 2.3 g PS and no curcumin
- **Curcumin (CC)**: 2 slices bread providing no PS and 228 mg curcumin
- **Phytosterol plus Curcumin (PS-CC)**: 2 slices bread providing 2.3 g PS plus 228 mg curcumin

Breads were prepared and packaged by George Weston Foods (North Ryde, New South Wales, Australia) under standard bread-making procedures and GMP conditions. All study breads were comparable for sensory characteristics and nutrient profile which matched that of a commercially available white sandwich bread. PS were delivered via Vegapure® 67WDP; a water dispersible powder containing PS fatty acid esters and curcumin was delivered via Meriva® Indena powder (Figure 2.2). Bread bags were labelled and colour-coded by kwik locks upon packaging by the manufacturer to ensure participants and all investigators were blinded to treatment allocation.

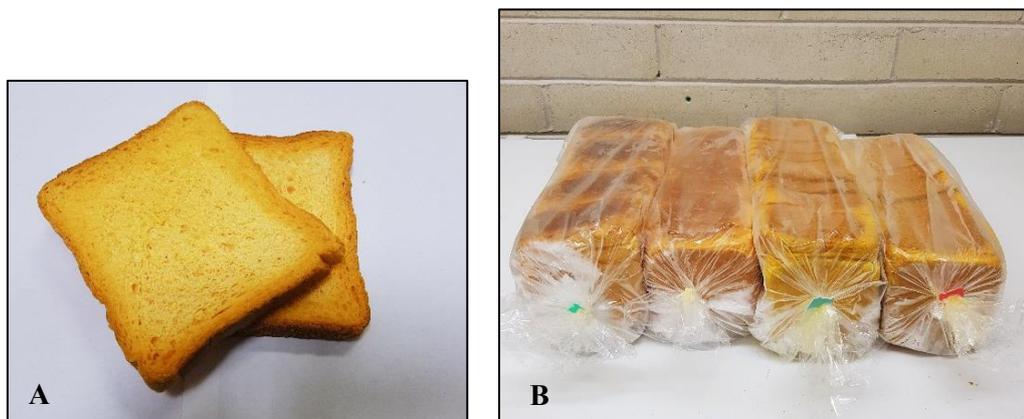


Figure 2.2: Study intervention product for PAC-FOOD Trial. Daily dose of 2 slices of bread (A) and all four different study breads (B).

2.3.3 Study 3 – BETA-GAPS Trial (Chapter 6)

Participants were randomly allocated to one of four treatments which they had to consume daily for 6 weeks:

- **Placebo (PL):** 8 small sweet biscuits providing no PS or high-MW OBG
- **Phytosterol (PS):** 8 small sweet biscuits providing 2 g PS and no high-MW OBG
- **High-MW Oat β -Glucan (OBG):** 8 small sweet biscuits providing no PS and 3 g high-MW OBG
- **Phytosterol plus High-MW Oat β -Glucan (PS-OBG):** 8 small sweet biscuits providing 2 g PS and 3 g high-MW OBG

Biscuits were prepared and packaged by Sweethings Pasticceria Wholesale Bakery (Heatherbrae, New South Wales, Australia) under GMP conditions. PS were provided by a commercially available enriched fat spread (Logicol Original, MeadowLea) predominately based on canola oil and placebo-matched fat spread devoid of PS (Gold’N Canola, MeadowLea); these spreads are the same as those used for study 1. High-MW OBG was provided by high-MW (2000-2500 kDa) oat bran powder derived from Scandinavian oats (SWEOAT®, Prorsum Healthsacre AB, Sweden) and plain wholemeal flour as the placebo. All study biscuits were comparable for sensory characteristics and nutrient profile except for additional soluble fibre provided by OBG in the OBG enriched biscuits and PS in the PS enriched biscuits (Figure 2.3).



Figure 2.3: Study intervention product for BETA-GAPS Trial. One study biscuit (A) and all four different study biscuits (B).

2.4 Randomisation

Allocation to treatment groups was conducted using a computer-generated block randomisation method and participants were stratified by gender in block sizes of 8 to achieve target sample sizes for each group (Random Allocation Software 1.0.0). Participants were de-identified and assigned alpha-numeric codes. Trial food intervention packaging was colour-coded and/or labelled by the senior investigator (MG) to ensure double-blinding. Participant codes were held under strict confidence by the senior investigator and locked in a filing cabinet until the codes were broken after the final participant returned.

2.5 Clinical Assessment

All data for clinical trials were collected at two clinic visits: baseline and post-intervention. Participants were dressed in light clothing, without shoes and had fasted overnight (10 hours) for all measurements. Participants also refrained from vigorous physical activity and alcohol consumption for 24 hours prior to all measurements.

2.5.1 Eligibility and consent

All participants were screened for eligibility by the lead investigator (JF) using an eligibility checklist. Eligibility screening was conducted over the phone or in person. Written and signed consent was provided by participants for enrolment into the trial and was provided prior to their first clinical appointment. Written consent was mandatory for study participation.

2.5.2 Medical history

A general medical history questionnaire was collected from all participants at the baseline clinical appointment (Appendix 6). The questionnaire included medical history, medication and supplement use, habitual intake of alcohol, sugar and fat.

2.5.3 Anthropometry

Height (cm), weight (kg) and waist circumference (cm) were collected to the nearest 0.1 units. Height was measured using a wall mounted stadiometer with movable headpiece (Seca 206 Bodymeter Wall Height Measure Ruler). Waist circumference was measured horizontally using a tensible tape measure positioned midway between the iliac crest and lower rib margin (approximately in line with the belly-button). Weight and body composition parameters such as skeletal muscle mass, body fat mass, percent body fat, BMI and waist-to-hip ratio were measured using bioelectrical impedance which utilised two different frequencies (InBody 230, Biospace Co., Ltd. Seoul, Korea). Participants were standing for all measurements, wore light clothing and

all shoes, socks, jewellery and any metal removed. All anthropometry measurements were conducted by an Accredited Practising Dietitian (JF).

2.5.4 Blood pressure

Blood pressure and pulse were measured using a digital sphygmomanometer (Microlife®, BP3AD1-A Heerbrugg, Switzerland) in the seated position. The height of the chair was adjustable to ensure the cuffed arm was positioned in line with the heart, resting on a table by the side of the torso. Legs were uncrossed and placed perpendicular to the floor with feet placed flat on the floor or stool. Serial measures (three) were conducted with a 1-minute rest in between each. The first measure was discarded and an average of the second and third readings was considered as the final measurement and used for analyses.

2.5.5 Cardiovascular disease risk

Ten-year CVD risk was measured using the Framingham Risk Algorithm as described in section 1.6. It is used to predict the risk of developing coronary artery disease within the next 10 years. Predictors used were: age, gender, diabetes mellitus, smoking, treated/untreated systolic blood pressure, TC and HDL-C. All data required for the algorithm was collected during clinic appointments. A low risk is considered $\leq 10\%$, increased risk 10-20% and high risk $> 20\%$.

2.6 Dietary Analysis

Participants were instructed to maintain their habitual diet, lifestyle and exercise patterns for the duration of the trial. They did not receive any diet or lifestyle counselling at any point of the trial.

2.6.1 Three-day food diary

Participants were instructed to complete a 3-day food diary, encompassing detailed food and beverage intake over 2 weekdays and 1 weekday (Appendix 7) leading up to each clinic appointment. Participants received examples for how to complete the food diary as well as detailed instructions from the lead investigator (JF). Food diaries were checked by the lead investigator (also a Dietitian) during clinic appointments in order to ensure diaries were completed in detail. Food diaries were analysed using a food database system (FoodWorks, Xyris, Professional Edition Version 8.0.3551) to estimate habitual dietary intake. Participants were instructed to include the study intervention products in the diaries where relevant.

2.7 Physical Activity

Habitual physical activity levels were captured by the Self-Administered International Physical Activity Questionnaire (Long Form) (Appendix 8) before every clinic appointment. The questionnaire is a validated tool designed to capture the frequency, duration and intensity of physical activity undertaken in the previous 7 days. Participants were instructed to maintain their habitual physical activity patterns for the duration of the trial and to avoid dramatically altering their current exercise regime (or lack thereof).

2.8 Blood Collection and Biochemical Analysis

2.8.1 Blood collection

A 25 mL fasting blood sample (10 hours) was collected via venepuncture by a trained phlebotomist at baseline and post-intervention at the Nutraceuticals Research Program trial facility. Blood samples were collected in various tubes: ethylenediaminetetraacetic acid (EDTA), fluoride oxalate and serum clot separation activator coated tubes. Samples collected into EDTA were prepared by centrifuging (Heraeus Biofuge Stratos) for 10 minutes at 3000 x g at 4 °C. Plasma, red blood cell and buffy coat subfractions were aliquoted and stored at -80 °C until further analysis. Blood collection was terminated if a participant experienced any adverse reaction or requested termination of blood collection. In case of any adverse event, the chief investigator (MG) was notified as soon as possible.

2.8.2 Biochemical parameters

Plasma TC, HDL-C, TG, TC:HDL ratio and glucose concentrations were measured by VP auto analyser using standardized reagents by Hunter Area Pathology Service. LDL-C concentration was calculated by Hunter Area Pathology Service and determined using the Friedewald equation [191].

2.8.3 Lipoprotein particle profile

LDL-P concentrations 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). Different sizes of LDL sub classes in nanometres was quantified from the amplitudes of their spectroscopically distinct lipid methyl group NMR signals. A detailed methodology for the determination of LDL-P number and LDL-P subclass size has been previously reported [33].

2.9 Compliance and Safety Monitoring

2.9.1 Compliance

A study food consumption log was provided to participants to record their consumption of the trial intervention product each day for the duration of the trial. Participants were given instructions on how to correctly complete the log including date, time of day consumed and how much of the trial product was consumed. Reasons for not consuming the trial product was also recorded. They were also informed to retain all trial intervention food packaging for collection by the lead investigator (JF) at the end of the trial. Participants returned all empty and unfinished food packaging at their final post-intervention clinic appointment. Returned items including any leftover intervention foodstuffs was cross-checked against their study food consumption log by the lead investigator (JF). Data from participants with $\geq 80\%$ compliance were included in the final analysis.

2.9.2 Safety Monitoring

In the case of all adverse- and serious adverse events, the lead investigator (JF) informed the chief investigator (MG) as soon as possible. The chief investigator would then follow procedures for unblinding as necessary and notify relevant bodies e.g. participant's general practitioner. Depending on the nature of the event, the participant would be withdrawn from the study if necessary. All serious adverse events would be documented in a serious adverse event form and immediately reported to the UON HREC. If there was to be a serious adverse event relating to the investigative intervention with risk to participants, the trial would be ceased, investigation performed, and findings generated before recommencement of the trial.

2.10 Statistical Analysis

Statistical analyses were conducted using StataCorp 2015 (*Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP). All data are presented as mean \pm SEM or median (IQR or 25th, 75th percentile) where appropriate. The significant levels for all statistical tests was set at 0.05 unless otherwise stated. Normality was assessed via Shapiro Wilk test and visual plots including histograms and bar graphs. Baseline characteristics across treatment groups were compared using ANOVA for normally distributed data or Kruskal-Wallis for non-normally distributed data for comparisons between more than 2 groups. Gender and ethnicity were compared across groups using chi-square test. Within-group changes from baseline to post-intervention were compared using paired samples-test or Wilcoxon Signed Rank test for normally- and non-normally distributed data, respectively. Where only 2 treatment groups are

compared, independent samples t-test or Wilcoxon Rank Sum test for normally- and non-normally distributed data (respectively) were used to examine within-treatment group changes. Absolute change was calculated as post-intervention value minus baseline value and relative change (%) was calculated as absolute change divided by baseline value, then multiplied by 100. Therefore, for change data, a negative sign denotes a reduction from baseline.

The effect of treatment on absolute and percent change from baseline to post-intervention on the dependent variables (e.g. blood lipids) between groups was explored using one-way ANOVA or Kruskal-Wallis test. Two-way ANOVA was used to determine whether there was a significant main effect for each independent variable (e.g. bioactive) and an interaction term was tested to investigate potential synergy. Tukey's honestly significant difference or Kruskal-Wallis with post hoc tests was used to perform post hoc comparisons on any significant effects.

The effects of potential confounders were explored using multiple linear regression in a backward stepwise design. This was considered for each response variable (absolute- and percent change in outcomes) with treatment group and corresponding baseline values or covariates included as explanatory variables. Correlations between potential baseline explanatory variables were assessed and variables with correlation coefficients above 0.8 were identified as potentially multicollinear and the number of potential predictors to include in the regression was reduced accordingly. The backward stepwise procedure was used for each regression to determine the optimal set of predictors for each model.

- ❖ For more detail regarding statistical methodology please refer to statistical methods section discussed in each respective chapter.

Chapter 3

Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs

The contents of this chapter have been published as the following manuscript:

Ferguson JJA, Stojanovski E, MacDonald-Wicks L, Garg ML. Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs. *Progress in Lipid Research*. 2016, 64:16-29. Impact factor 8.435.

Statement of authorship is attached as Appendix 9

3.1 Abstract

The most common form of phytosterol (PS) fortified foods are fat spreads and dairy products. The predominant fats used are soybean/sunflower (SS) or rapeseed/canola (RC) oils and animal fat (D) in dairy products. This review aimed to investigate whether the carrier fat is a determinant of the hypocholesterolaemic effects of PS fortified foods. Databases were searched using relevant keywords and published RCTs from 1990 investigating the effects of dietary PS intervention (≥ 1.5 g per day) on total cholesterol and LDL-C were included. After methodological quality assessment and data extraction, a total of 32 RCTs (RC, n=15; SS, n=9; D, n=8) were included. As expected, all fat groups significantly reduced TC and LDL-C ($p < 0.01$). When compared across different carrier fats, RC as the main carrier fat, reduced LDL-C significantly more than the SS spreads ($p = 0.01$). Therefore, a combination of monounsaturated fatty acid rich spread with adequate amounts of omega-3 fatty acids (as evident in RC spreads) may be the superior carrier fat for the delivery of PS for optimal blood cholesterol-lowering. The findings of this research provide useful evidence for optimising the hypocholesterolaemic effects of PS and support further investigation into the possible mechanisms behind these findings.

Keywords: Phytosterols, Rapeseed, Canola, Sunflower, Dairy, Cholesterol

3.2 Introduction

Cardiovascular disease (CVD) remains the leading cause of mortality, claiming 31% of all deaths worldwide in 2012 [192]. The global economic impact of CVD is estimated to be US \$906 billion dollars in 2015 and is expected to rise by 22% by the year 2030 [4]. Elevated total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations in the blood are major diet-related risk factors for the development of CVD [193].

3.2.1 Phytosterols

It is well established that phytosterols and their saturated form, phytostanols, are key cholesterol-lowering agents. PS (comprising phytosterols and phytostanols) are non-nutritive compounds similar in structure to cholesterol, however, are produced by plants and are essential components of cell membranes. PS are solely derived from the diet and are naturally found in plant based foods such as vegetable oils, fruits, nuts, seeds, legumes and fat spreads [90]. Western populations are estimated to consume 150-450 mg/day of phytosterols in the diet, with Japanese and vegetarian people consuming up to 50% more. Moreover, only 20-50 mg/day of phytostanols are consumed from foods on a regular basis [91]. In addition to natural sources, staple foods have been fortified with PS, the most common being fat spreads and low-fat dairy products [90]. Other products include breakfast cereals, cereal bars, orange juice, chocolate, muffins, croissants, breads, vegetable oils, salad dressings, mayonnaise and tortilla chips [94]. Intestinal absorption of PS compared to dietary cholesterol is markedly less (0.5-2% vs 55-60%) [92, 105], with the majority being rapidly excreted by the liver [17].

PS differ from cholesterol by containing an extra ethyl (β -sitosterol) or methyl (campesterol) group at C-24 or an extra double bond (stigmasterol) at C-22 [17]. Dietary and biliary PS and cholesterol must be solubilised in micelles prior to digestion and intestinal absorption. The micelle interacts with the brush border membrane of the intestinal lumen for sterol uptake, which is facilitated by transport protein Niemann-Pick C1-Like1 (NPC1L1) [92]. ATP-binding cassette proteins (ABCG5 and ABCG8) shuttle any unesterified cholesterol and PS back to the intestinal lumen [92].

Dietary PS can reduce cholesterol absorption by 30 to 50% [98]. Several mechanisms have been proposed, however, the most widely accepted mechanism involves PS displacement of cholesterol in the micelle due to the greater affinity of PS [194]. This causes cholesterol to precipitate out into the lumen, hence limiting intestinal solubility of cholesterol and hydrolysis of cholesterol esters in the intestines [90, 195]. PS may also enhance the efflux of cholesterol via inducing the expression of ABCA1 transporter which cannot differentiate between cholesterol and PS [196];

in addition, PS may suppress acyl coenzyme A:cholesterol acyltransferase (ACAT) activity leading to a subsequent reduction in cholesterol uptake/transport in the intestine [17].

PS were first reported to play a regulatory role in serum cholesterol concentrations in 1951 [197] following by a number of clinical trials to establish that 2.0-2.5 g/day of PS elicit a 10-15% reduction in plasma LDL-C concentrations within 2-3 weeks [17, 198]. Given that sterols require fat for solubilisation, a practical mode of delivery for PS esters has been their dispersion into fat spreads. This elicits an optimum transport vehicle which increases lipid solubility and promotes PS incorporation into micelles [17]. A meta-analysis of 124 randomised control trials (RCT) showed that a PS intake of 0.6-3.3 g per day dose-dependently lowers LDL-C concentrations by 6-12% [199]. This reduction is considered clinically significant as it has been demonstrated that a 1% reduction in LDL-C leads to a 1% decrease in coronary heart disease risk [200].

There are some factors known to influence PS efficacy such as dosage, baseline LDL-C concentrations, lipid-lowering medication and combination therapy with other nutraceuticals such as omega-3 polyunsaturated fatty acids (n-3PUFA). As mentioned above, PS dose-dependently lower LDL-C concentrations with greater reductions seen in those with higher baseline LDL-C concentrations [94]. A meta-analysis on continuous dose-response relationships of the LDL-C-lowering effect of PS showed that the total fat content of the meal, PS type (sterols vs stanols) and dairy vs non-dairy food vehicles, did not significantly affect the LDL-C lowering efficacy of PS [94]. Combining PS supplementation (~2.5 g/day up to 5.0 g/day for 4 to 8 weeks) with statin therapy resulted in additive reductions in LDL-C by 10-15% [201-203] compared to a 5-7% reduction when the statin dose was doubled [204].

Long-term clinical trials are yet to report any serious adverse side effects of long-term PS consumption, however, a common observation is a modest reduction of plasma carotenoid levels by up to 10% [205]. This is offset by increasing fruit and vegetable consumption by one extra serve per day whilst consuming the cholesterol-lowering dosage of PS [205]. A rare condition associated with PS is called phytosterolaemia, involving dysfunction in genes coding for the ATP-binding cassette transporters ABCG5 and ABCG8, key regulators of PS absorption.

3.2.2 Dietary fats

It is well known that dietary fats modulate circulating lipid concentrations and are associated with CVD risk [63]. The classically held belief backed by over half a century of interventional and epidemiological studies acknowledges saturated fats as the major causative factor for the development of coronary heart disease and source of morbidity and mortality in the Western world [64]. Despite this public and scientific belief, conclusive evidence confirming the exact relationship between dietary saturated fatty acids (SFA) and blood cholesterol concentrations

remains controversial [64]. Recent studies have suggested that the food source and type of SFA are more important determinants of their lipid modulating potential [64]. For instance, dairy fats containing short chain fatty acids (less than 6 carbon atoms) may not be as hypercholesterolaemic as tallow or lard providing longer chain fatty acids (greater than 14 carbon atoms) [206-209]. A number of studies have shown that trans fatty acids (TFA) increase plasma LDL-C and lipoprotein (a) and lower HDL-C [63, 210] with subsequent mortality from coronary artery disease [63]. On the other hand, cis isomers of monounsaturated fatty acids (MUFA) have been shown to reduce LDL-C and increase HDL-C concentrations [70-74] as well as modulate some markers of inflammation [70]. PUFA of the omega-6 (n-6) family are more effective in lowering circulating TC and LDL-C concentrations, but have no effect on TG or HDL-C [63]. Some researchers have raised concerns about the health effects of n-6PUFA due to their potential to promote pro-inflammatory mediators [17]. Conversely n-3PUFA, particularly of marine origin (eicosapentaenoic acid and docosahexaenoic acid) are potent lipid (TG)-lowering agents and possess mild LDL-C and HDL-C raising effects while shifting the LDL-P size to larger, buoyant and less atherogenic particles [17, 63].

The importance of exploring interactions between various classes of dietary fats rather than the effects of a single dietary fat in isolation for optimal blood lipid profile and subsequent reduced CVD risk cannot be undermined.

3.2.3 Mechanisms by which dietary fats modulate blood lipid levels

The current evidence exploring the mechanisms by which dietary fats modulate circulating blood lipids is largely limited to animal models. Fernandez et al [63] demonstrated that dietary fatty acids modulate circulating LDL-C by interfering with LDL-R activity, protein and mRNA abundance. Moreover, dietary fatty acids modulate plasma cholesterol indirectly via regulation of four families of transcription factors: peroxisome proliferator activated receptors (PPARs), liver X receptors (LXRs), hepatic nuclear factor-4 (HNF-4) and sterol regulatory element binding proteins (SREBPs) [63].

Dietary SFA have been shown to impose their LDL-C-raising effects by inhibiting LDL-R activity and promoting apolipoprotein (apo) B-containing lipoprotein production [211]. The degree of LDL-C raising appears to be more pronounced with higher dietary cholesterol intake [211]. Longer chain SFA (e.g. stearic acid) have shown no effects on blood lipids, however, SFA of shorter chain length such as myristic and palmitic acid have been shown to induce a rise in LDL-C [63, 208, 212]. Negligible effects on LDL-C clearance and concentrations in non-human primates administered a high SFA diet are evident in the absence of dietary cholesterol and adequate intake of PUFA [213]. LDL-R activity is suppressed in humans and animals by SFA

(particularly palmitic acid) upon an energy dense and dietary cholesterol-rich, diet [213]. The LDL-R plays an important role in the delivery of cholesterol to the cell, therefore, any reduction in LDL-R activity results in a subsequent accumulation of cholesterol in circulation. SFA are known to suppress the rate-limiting enzyme known as ACAT, which is involved in regulating cholesterol esterification. *In vitro* and animal studies have suggested that dietary fatty acids and cholesterol modulate LDL-R activity in the liver via cholesteryl ester and unesterified cholesterol pools [65, 214, 215]. Given ACAT's regulatory role in cholesterol pools, and SFA suppression of its activity, a larger proportion of cholesterol may remain in these pools, therefore reducing LDL-R activity and subsequently contribute to elevated levels of LDL-C as observed in SFA-rich diets [63]. It is important to note that the heterogeneity of responses to dietary SFA intake may be explained by intrinsic differences in the regulation of lipid metabolism for e.g. obesity, insulin resistance, female gender, hypertriglyceridaemia and apoE polymorphism [212].

PUFAs have been shown to increase LDL-R activity, protein and mRNA abundance via possible modulation of hepatocyte membrane fluidity [63]. In humans and non-human primates, dietary MUFA reduced LDL-C without lowering HDL-C, and when compared to SFA and PUFA, dietary MUFA result in lowest LDL: HDL ratio [74, 216, 217]. Human studies have reported mixed results regarding the TC and LDL-C lowering effects of MUFA compared to PUFA. Some report comparable cholesterol-lowering effects, whilst others have reported a greater effect induced with PUFA [218]. A MUFA-rich diet derived from plant sources may offer additional benefits to lipid profile by improving the quality of LDL-C. Human studies have shown that LDL-C in individuals consuming MUFA-rich diets were less susceptible to oxidation than those who consumed a PUFA diet, irrespective of the total fat content in the diet i.e. low-fat vs high-fat [219].

Dietary n-6PUFA play an independent regulatory role on LDL-R expression, with an upregulation of LDL-R seen in animals fed a diet containing 0.25% total energy as cholesterol. Compared to SFA (palmitic acid), n-6PUFA upregulated LDL-R protein relative to controls on a low-fat/cholesterol-free or cholesterol-only diet [220]. LXR regulates intracellular cholesterol levels by inducing the expression of cholesterol 7 α -hydroxylase (CYP7); the rate-limiting enzyme required for the conversion of cholesterol to bile acids. PUFA have been shown to induce LXR expression in rats [221], thus resulting in an indirect increase in CYP7 activity causing elimination of cholesterol from the liver and a subsequent reduction in plasma cholesterol levels. Animal and *in vitro* studies have demonstrated a reduction in LDL-C apoB pool size by 50% and a two-fold increase in LDL-C fractional catabolic rate following administration of PUFA when compared to SFA, hence having an overall favourable effect on LDL-C clearance [63]. Dietary n-3PUFA confer additional regulatory benefits during cholesterol metabolism. Many human studies report

decreased residence time of very low-density lipoprotein cholesterol (VLDL-C) in serum after dietary supplementation with n-3PUFA [63, 222].

The majority of the available evidence for the mechanistic properties of dietary fats and their modulation of blood lipids is based on animal or cellular models conducted up to 30 years ago. Recent human data are warranted to elucidate the exact mechanisms involved in dietary fat modulation of blood lipids in order to confirm the previous observations of animal and experimental models. Rigour in study design including appropriate placebo-controlled groups, focus on whole-food consumption, adjustment for confounders and the assessment and analysis of diet is crucial for the determination of how dietary fats are regulating circulating blood lipid concentrations.

3.2.4 Potential synergistic and complementary effect of phytosterols and dietary fats

The mechanisms by which PS lowers blood cholesterol levels are different from those of dietary fats. Moreover, the efficacy of PS to reduce circulating levels of cholesterol may depend on the fat content of the meal and fat spreads are convenient means of delivering the two together. The predominant fats used in the PS enriched fat spreads are soybean/sunflower oil (SS) or rapeseed/canola oil (RC) and dairy fat (D) in dairy products. SS oil based spreads are predominately composed of linoleic acid (LA) and RC oil based spreads are predominately composed of oleic acid with considerably higher amounts of alpha-linolenic acid (ALA) than the SS oil based spreads [223]. Dairy based PS enriched food products are composed predominately of SFA, with relatively small amounts of PUFA or MUFA.

The purpose of this systematic review and meta-analysis was to determine whether the type of carrier fat used in PS enriched fat spreads and dairy products, is a determinant of the hypocholesterolaemic effects of PS enriched products. The systematic review aims to underpin the mechanisms by which PS and dietary fats influence blood cholesterol levels, with particular reference to the interactions between the two. The meta-analysis of the RCTs conducted using the various PS fortified food products, will provide an in-depth understanding of how circulating lipid levels may be optimised by using PS in combination with different dietary fats.

3.3 Materials and Methods

3.3.1 Search strategy

A research librarian assisted with the planning of the systematic search for publications in May 2015. Four electronic databases (EMBASE, MEDLINE, CINAHL and Cochrane Library) were searched from end-May 2015 to mid-August 2015. The following Medical Subject Headings

(terms), words and/or their combinations were searched: phytosterol, phytostanol, plant sterol(s), plant stanol(s), sitosterol, sitostanol, campesterol, campestanol, stigmasterol, stigmastanol; and randomised controlled trial, randomized controlled trial, intervention study, intervention studies, clinical trial(s), random*, group*, trial*. Keywords were searched in the title, abstract or topic as free text and combined using the Boolean operator 'AND'. To optimise publication retrieval, the internationally broad MeSH terms and spelling were used. Limits included publications from 1990 to present, English language, humans and adults. Additional publications were identified from the reference lists of included papers and systematic reviews retrieved by the initial search. Outcomes were divided into two groups: TC and LDL-C.

3.3.2 Eligibility Criteria

Table 3.1 shows the inclusion and exclusion criteria for the selection of publications. Publications that did not report cholesterol and/or LDL-C as an outcome measure were excluded from this review. Publications that reported blood cholesterol values in mg/dL were converted to mmol/L using the standardised conversion method (multiply mg/dL by 0.0259) [224]. When fat type of the intervention product was not stated in the paper, authors of publications were contacted. Publications could not be included in this review if the fat type was unable to be ascertained and when authors of the paper could not be contacted or could not provide or confirm the fat type.

3.3.3 Selection process and quality assessment

The title and abstract of publications were screened (JF) for the first selection process based on the eligibility criteria (Table 3.1). Studies that included a form of cholesterol-lowering therapy (i.e. statin) were only selected if they involved PS-alone treatment arms (i.e. PS) as well as a control or placebo group without cholesterol-lowering therapy. The full text of all publications that appeared to meet the eligibility screening process were retrieved and a second selection assessment was undertaken. Any discrepancies in the assessment and/or the decision-making of selection were resolved in discussion with another independent research investigator (MG). Two independent research investigators (JF, LMW) assessed the methodological quality of the selected full texts using the Quality Criteria Checklist for Primary Research in the American Dietetic Association Evidence Analysis Manual [225].

Table 3.1: Inclusion and exclusion criteria for the selection of publications

Inclusion	Exclusion
RCT	Observational studies (i.e. cross-sectional, longitudinal), case control, quasi-randomised control trials, non-randomised control trials, any other that was not an RCT
Humans	Children, animal, <i>in vivo</i> and <i>in vitro</i> studies
Adults (aged ≥ 18 years)	Studies using food format other than margarine or dairy ²
Blood cholesterol concentrations as primary or secondary outcomes	Studies where fat type could not be ascertained ³
PS dosage ≥ 1.5 g/day	Co-intervention with cholesterol-lowering therapy without PS-placebo treatment arm ⁴
Publication published year 1990 to present	Insufficient relevant blood cholesterol data
Publications published in English	PS consumed for ≤ 3 weeks
Predominant fat type of food product was stated ¹	Non-English

¹ Publications which reported 40% or greater of total fatty acids as linoleic acid were included in the SS group (n=3).

² One publication was removed because it the intervention was a dual food format including both margarine and milk enriched with PS.

³ Fat type was not ascertained when the authors of the publication had not specified the fat type the product was based on and when authors could not confirm the predominant fat type or could not be contacted.

⁴ Publications that included a PS-placebo group (i.e. PS was administered in isolation of cholesterol-lowering therapy) were included, along with the control group.

The Quality Criteria Checklist aided in critically appraising the quality constructs of each publication as well as to determine the relevance and validity of the selected publications. The checklist contains 10 structured validity questions including additional sub-questions specific to different research designs. The validity questions address the scientific quality, soundness, consideration of bias and confounding, the appropriateness of intervention, follow-up measures, data collection methods and statistical analyses. An overall systematic and objective rating (i.e. ‘positive’, ‘negative’ or ‘neutral’) was assigned to each publication. A study was deemed ‘positive’ if it met all priority criteria and most of the validity criteria. Priority criterions specifically address the methodology in relation to participant selection and recruitment; comparability of study groups; provision of adequate detail regarding the intervention and data

collection process; use of valid and appropriate measurement tools and/or methods for study outcomes and whether potential confounders were considered. A ‘Neutral’ rating indicates publications that have met most of the validity criteria but have not met one or more of the priority criterion, indicating that the study is not entirely strong, while a ‘negative’ rating indicates that publications have failed to meet 6 or more of the validity criteria and are therefore excluded from this review. Any discrepancies between the two independent research investigators were resolved through discussion where both parties came to an agreement.

3.3.4 Data extraction

Relevant data from included publications were extracted. The following data were collected: study identification (author, year, country), study design (cross-over or parallel, level of blinding), duration, sample size of each group, participant characteristics (age, gender, BMI, health status), intervention characteristics (PS dose and regime, PS type, food matrix), predominate oil used in food matrix (if margarine), baseline and post-intervention blood cholesterol outcomes (TC and LDL-C), compliance measures and study quality. Variance measures of all relevant data were also extracted. Actual PS intake calculated via compliance measures was reported for PS dose, not the intended dose. Mean and variance measures such as standard deviations, standard errors or 95% confidence intervals (CI) were collected where possible. Authors of publications were contacted to obtain missing data wherever required.

3.3.5 Statistical analysis

The main outcome variables for the meta-analysis were the absolute (mmol/L) and relative (%) change in TC and LDL-C concentrations due to PS intervention. TC and LDL-C concentrations were compared across the three classes of fat types. The within-trial variance measures for the absolute and relative changes in cholesterol were reported as standard errors (SE) and derived from standard deviations (SD) or 95% CI when they were not available. When the absolute and relative changes in TC and LDL-C were not reported, they were calculated using formulae that are summarised in a systematic review conducted by Demonty et al [94].

Pooled effect sizes were calculated for the absolute and relative changes in TC and LDL-C following PS intervention using a random-effects model described by DerSimonian and Laird [226] which takes into account within and between study variation. Between study heterogeneity was quantified using the I^2 statistic, which measures the between study variation that can be attributed to heterogeneity as opposed to random variation, with the intent to assess whether studies share a common effect size. I^2 values of approximately 25%, 50% and 75% are considered to show low, moderate and high levels of heterogeneity, respectively [227].

Sensitivity analyses were conducted to assess whether any single study elicited any undue influence on the overall results. This was conducted by excluding one study at a time from the analyses and recalculating the effect size each time. Publication bias was visually assessed using a funnel plot and formally using Begg's rank correlation test [228]. The comparison between the mean reductions in TC and LDL between fat groups was compared using confidence intervals for the difference between two means.

Statistical analysis was conducted using the statistical software package STATA version 13 (StataCorp. 2013 *Stata Statistical Software: Release 13*; College Station, TX: StataCorp LP). STATA has been widely used by researchers for meta-analyses including papers that are published in reputed scientific journals [229-235]. STATA is a command line-driven programmable statistical package with commands for conducting meta-analyses. In addition to the core in-built commands, several user-written add-ons have been added and documented in the Stata Technical Bulletin (STB) [236]. All data are reported as mean \pm standard error of the mean (SEM). *P*-values <0.05 were considered statistically significant.

3.4 Results

3.4.1 Overview of publications

A total of 2673 publications were retrieved from the database search and one publication from hand searches. Of these, 139 publications met the inclusion criteria based on the title and abstract content. After the full texts were assessed, and further exclusions applied based on exclusion criteria, 57 publications were assessed for methodological quality. During this step, given the critical nature of assessing the quality of each publication, further exclusions were made and 35 publications were subjected to data extraction. Three publications were excluded from the meta-analysis due to insufficient data available and therefore a total of 32 studies (Figure 3.1) published from 1993 to 2015 comprising 49 strata were included in this systematic review (Table 3.1) and only 42 strata were included in the final meta-analysis (Table 3.2). Only the first cross-over period was included for cross-over publications in order to prevent carry-over bias [237]. The only exception to this was Geelen et al. which involved a cross-over design involving two groups which served as their own control, but differed by apoE polymorphism [238]. This study was treated as a parallel study in the meta-analysis whereby the two groups were included as separate strata. The majority of included publications were positive in quality ($n=28$) and only four were neutral. There were no studies with a negative quality rating.

3.4.2 Description of publications

A total of 2157 participants were included. The majority of publications included European participants who were otherwise healthy apart from their baseline blood cholesterol levels. Most trials were double-blinded (n=27, where both study participants and investigators are unaware of group assignment) with a mixture of parallel (n=19, participants are randomised to one of two or more intervention groups) and cross-over (n=13, participants are their own control by receiving each intervention one after the other in a random order) designs. Twenty two studies included participants who were mild-moderate hypercholesterolaemic (≥ 5.5 mmol/L [193]) whereas eight included normocholesterolaemic (< 5.5 mmol/L [193]) and two a combination of both hyper- and normocholesterolaemic participants. Mean age ranged from 25.0 ± 1.46 to 58.0 ± 1.06 years and mean BMI ranged from 22.2 ± 0.53 to 31.0 ± 0.52 kg/m². Trials ranged from three to 52 weeks in duration, with the majority of trials lasting 3 or 4 weeks. PS dosage ranged from 1.5 to 4.0 g/day. Baseline mean TC ranged from 4.97 ± 0.12 to 7.69 ± 0.16 mmol/L and mean LDL-C ranged from 2.81 ± 0.24 to 5.41 ± 0.15 mmol/L (Table 3.2). In the majority of strata included in the meta-analysis, the fat type comprised margarine (n=32) followed by yoghurt (n=4), cheese (n=3), butter (n=2) and milk (n=1) (Table 3.3). Twenty three strata were RC fat type, nine were SS and ten were D.

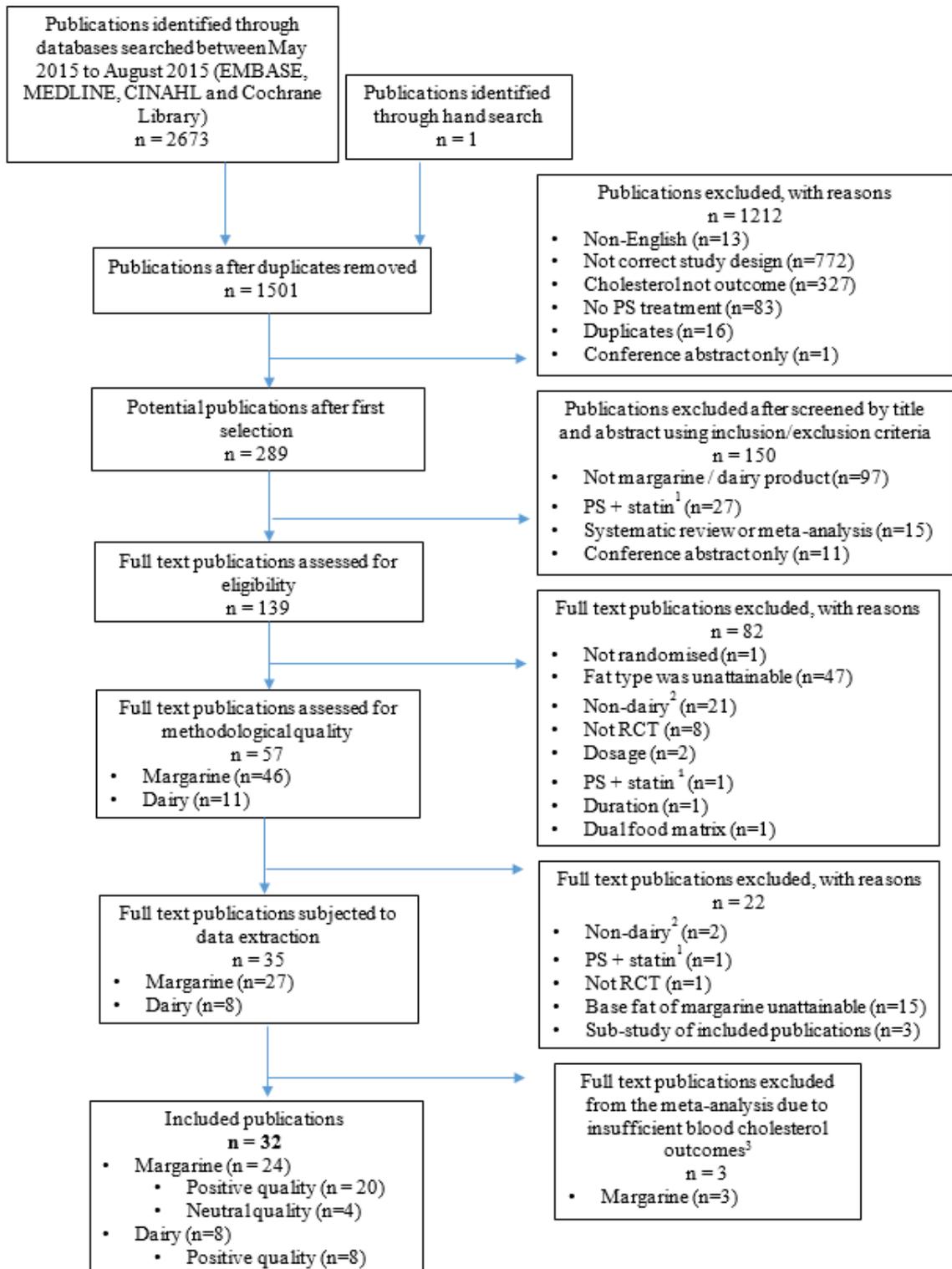


Figure 3.1: Flow diagram of publication selection process starting with 2673 publications and ending with 24 RCT.

¹ PS plus cholesterol-lowering therapy without PS-placebo (i.e. PS alone) treatment arm. ² Fat type of ‘dairy’ product was non-representative of commercial animal fat-based dairy products. ³ These include publications which insufficient data available which were not able to be provided by the authors.

Table 3.2: Overview of the publications included in the systematic review and meta-analysis (n= 32)

Author, publication year, quality score ¹ and reference	Location and study design	Subject Characteristics						Treatment Characteristics			
		Sample size		Mean age (y) ²	Mean baseline BMI (kg/m ²) ²	Mean baseline TC (mmol/L) ³	Mean baseline LDL-C (mmol/L) ³	Food matrix	Fat type	Duration (weeks)	PS dose (g/d)
		C	T								
Alhassan et al. 2006 (+) [239]	USA, Parallel	9	17	53.0 ± (8.0)	27.0 ± (1.0)	5.23 ± (0.21)	3.32 ± (1.18)	Margarine	RC	4	3.0
Baumgartner et al. 2013 (+) [240]	Netherlands Cross-over		43	41 ± (2.74)	24.8 ± (0.43)	5.72 ± (0.17) ^a	3.53 ± (0.16) ^a	Margarine	RC	4	3.0
Blomqvist et al. 1993 (ø) [241]	Finland, Parallel	3	34	NR	NR	5.84 ± (0.13)	3.35 ± (0.12)	Margarine	RC	4	3.4
Buyuktuncer et al. 2013 (+) [242]	Netherlands, Parallel	3	35	45.5 ± (1.19) ^b	27.9 ± (0.53) ^b	6.24 ± (0.11)	4.09 ± (0.11)	Yoghurt	D	4	1.9
Charest et al. 2004 (+) [243]	Canada, Cross-over		14	48.6 ± (1.82)	30.9 ± (1.39)	6.0 ± (0.29)	4.0 ± (0.21)	Butter	D	3	1.8
- Stratum 1						6.2 ± (0.24)	4.1 ± (0.19)	Butter	D	3	1.8
- Stratum 2						6.4 ± (0.35)	4.2 ± (0.27)	Butter	D	3	1.8
Christiansen et al. 2001 (+) [244]	Finland, Parallel		1								
- Stratum 1		7	19	50.7 ^c	24.9 ± (0.88)	6.66 ± (0.07) ^a	4.29 ± (0.06) ^a	Margarine	RC	24	1.5
- Stratum 2			16	50.7 ^c	25.2 ± (0.68)			Margarine	RC	24	3.0
Clifton et al. 2004 (+) [245]	Australia		5								
- Stratum 1	Cross-over	8	40 ^d	54 ± (1.05)	26.2 ± (0.39)	6.24 ± (0.10) ^a	4.03 ± (0.09) ^a	Milk	D	3	1.6
- Stratum 2			40 ^d					Yoghurt	D	3	1.6

Gagliardi et al. 2010 (+) [246]	Brazil, Parallel	1 6	19	47.4 ± (1.33)	31.3 ± (0.47)	5.31 ± (0.18) ^c	3.26 ± (0.19) ^c	Margarine	SS	5	2.4
Geelan et al. 2002 (ø)	Netherlands, Cross-over		31	26 ± (2.16)	23 ± (0.54)	5.2 ± (0.16)	–	Margarine	RC	3	3.2
- Stratum 1			57	25 ± (1.46)	23 ± (0.26)	4.6 ± (0.12)	–	Margarine	RC	3	3.2
- Stratum 2											
Gylling et al. 1997 (+) [247]	Finland, Parallel	1 1	11	51 ± (1)	26.0 ± (0.7)	6.26 ± (0.20)	3.85 ± (0.17)	Margarine	RC	7	3.0
Gylling et al. 2013 (+) [248]	Finland, Parallel	4 6	46	50.9 ± (1.4) ^b	25.4 ± (0.6) ^b	5.48 ± (0.12)	3.52 ± (0.12)	Margarine	RC	24	3.0
Hallikainen et al. 1999 (+) [249]	Finland Parallel		1								
- Stratum 1		7	18	43.2 ± (1.93) ^b	25.6 ± (0.94) ^b	6.55 ± (0.18)	4.54 ± (0.17)	Margarine	RC	8	2.31
- Stratum 2			20	40.8 ± (2.08) ^b	24.2 ± (0.67) ^b	6.13 ± (0.18)	4.25 ± (0.19)	Margarine	RC	8	2.16
Hallikainen et al. 2000 i (+) [250]	Finland Cross-over										
- Stratum 1			34	48.8 ± (1.39)	24.9 ± (0.41)	6.24 ± (0.14)	4.43 ± (0.14)	Margarine	RC	4	2.04
- Stratum 2								Margarine	RC	4	2.01
Hallikainen et al. 2000 ii (+) [251]	Finland, Cross-over										
- Stratum 2			22	50.5 ± (2.49)	26.2 ± (0.75)	6.87 ± (0.27) ^a	4.81 ± (0.23) ^a	Margarine	RC	4	1.6
- Stratum 3								Margarine	RC	4	2.4
- Stratum 4								Margarine	RC	4	3.2
Hallikainen et al. 2006 (+) [252]	Finland, Cross-over	3									
- Stratum 1		7	39	50.0 ± (2.1) ^b	25.7 ± (0.6) ^b	5.52 ± (0.18)	3.31 ± (0.16)	Margarine	RC	10	1.92

- Stratum 2								Margarine	RC	10	1.97
Hallikainen et al. 2008 (+) [253]	Finland, Parallel	8	11	39.6 ± (4.2) ^b	24.5 ± (1.0) ^b	5.26 ± (0.07)	2.81 ± (0.24)	Margarine	RC	12	2.0
Hendriks et al. 1999 (+) [254]	Netherlands, Cross-over	80		37 ± (1.12)	22.8 ± (0.28)	5.10 ± (0.11) ^a	2.97 ± (0.09) ^a	Margarine	SS	3.5	1.61
- Stratum 1											
- Stratum 2								Margarine	SS	3.5	3.24
Hyun et al. 2005 (+) [255]	Korea Parallel	2	23	28.7 ± (0.7)	22.6 ± (0.4)	4.97 ± (0.12)	3.08 ± (0.12)	Yoghurt	D	4	2.0
		8									
Jauhiainen et al. 2006 (+) [256]	Finland, Parallel	3	33	43.3 ± (1.08)	NR	5.65 ± (0.09)	3.58 ± (0.09)	Cheese	D	5	2.0
		4									
Korpela et al. 2006 (+) [257]	Finland, Parallel	2	33	57.6 ± (1.00) ^b	27.1 ± (0.43) ^b	6.42 ± (0.08) ^a	4.05 ± (0.08) ^a	Hard cheese	D	6	2.0
- Stratum 1		9									
		2	24					Fresh cheese	D	6	2.0
- Stratum 2		8									
- Stratum 3		2	25					Yoghurt	D	6	2.0
		5									
Lottenberg et al. 2003 (ø) [258]	Brazil, Cross-over	3	30	55.8 ^c	26.4 ^b	7.0 ± (0.10) ^a	5.0 ± (0.09) ^a	Margarine	SS	4	1.68
		0									
Miettinen et al. 1995 (+) [259]	Finland, Parallel										
		5									
- Stratum 1		1	51	49 ± (2)	NR	6.01 ± (0.10)	3.96 ± (0.10)	Margarine	RC	52	1.8
- Stratum 2			51	51 ± (1)	NR	6.06 ± (0.10)	4.14 ± (0.10)	Margarine	RC	52	2.6
Nigon et al. 2001 (+) ^f [260]	France, Cross-over	34		55 ± (2.23)	22.8 ± (0.43)	6.73 ± (0.11)	4.56 ± (0.10)	Margarine	SS	8	1.6

Plat et al. 2000 (+) [261]	Netherlands,	4										
- Stratum 1	Parallel	2	36	33 ± (2.5) ^b	22.2 ± (0.78) ^b	4.99 ± (0.12)	2.94 ± (0.12)	Margarine	RC	8	3.8	
- Stratum 2			34	33 ± (2.5) ^b	23.4 ± (0.53) ^b	4.98 ± (0.16)	2.94 ± (0.15)	Margarine	RC	8	4.0	
Ras et al. 2014 (+) [262]	Sweden,	6	64	57.9 ± (0.6)	25.0 ± (0.1)	6.39 ± (0.10)	3.91 ± (0.09)	Margarine	SS	4	2.5	
	Parallel	2										
Ras et al. 2015 (+) [263]	Germany,	1	113	53.2 ± (0.44)	25.3 ± (0.18)	5.75 ± (0.09)	3.89 ± (0.06)	Margarine	SS	12	3.0	
	Parallel	0										
		5										
Simons 2002 (+) [264]	Australia	3	39	58 ± (1.60) ^b	27.6 ± (0.58) ^b	7.69 ± (0.16)	5.41 ± (0.15)	Margarine	SS	4	2.0	
	Parallel	8										
Theuwissen et al. 2009 (+) [265]	Netherlands,	1	14	54 ± (1.51)	28 ± (0.57)	7.35 ± (0.18)	4.96 ± (0.19)	Margarine	SS	3	2.5	
	Parallel	4										
Turpeinen et al. 2012 (+) [266]	Finland	4	51	49 ± (0.84) ^b	28.3 ± (0.66) ^b	6.0 ± (0.10)	3.8 ± (0.08)	Margarine	RC	10	2.0	
	Parallel	9										
Vanstone et al. 2002 (+) [267]	Canada,											
	Cross-over											
- Stratum 1			15	47.8 ± (1.9)	30.8 ± (1.3)	5.97 ± (0.26)	4.0 ± (0.20)	Butter	D	3	1.8	
- Stratum 2						6.23 ± (0.26)	4.11 ± (0.18)	Butter	D	3	1.8	
- Stratum 3						6.40 ± (0.31)	4.18 ± (0.23)	Butter	D	3	1.8	
Vasquez-Trespalacios et al. 2014 (+) ^g [268]	USA,		40	37.9 ± (1.34)	25.0 ± (0.54)	NR ^g	NR	Yoghurt	D	4	4.0	
	Cross-over											
Visser et al. 2000 (ø) ^h	Netherlands,											
- Stratum 1	Cross-over		60	NR	NR	NR	NR	Margarine	SS	3	2.1	

C, Control group; T, Treatment group; RC, Rapeseed/canola group; D, Dairy group; SS, Sunflower/soybean group; NR, not reported.

Stratum refers to a single treatment group.

- ^a Data reported for total sample size only.
- ^b Data only reported separate for control and intervention group. Therefore value presented is for intervention group only.
- ^c Measure of spread not reported.
- ^d Total sample size was N = 58, however, N=58 completed control period and only 40 completed the study for the milk and yoghurt intervention group.
- ^e SE calculated from the range using formula: $SD = (\text{maximum} - \text{minimum})/4$. SD converted to SE using formula described in section 2.5.
- ^f Only stratum 1 included (plant-sterol alone therapy). Stratum 2 excluded for fibrate and plant sterol co-therapy.
- ^g Authors only included moderately hypercholesterolaemic individuals (5.2-7.5 mmol/L) [268].
- ^h Only one strata was included as only this strata met the inclusion criteria. Authors only included normolipidaemic individuals [269].
- ¹ American Dietetic Association's Quality Criteria Checklist quality score given in brackets: (+) = positive, (ø) = neutral and (-) negative.
- ² Data is reported for total sample size unless otherwise specified.
- ³ Mean baseline TC (mmol/L) and LDL-C (mmol/L) data are reported for treatment group only unless otherwise specified.

Table 3.3: Summary of absolute and relative change data for included strata (n = 42)

Fat Group	Author, publication year and reference	Blood cholesterol outcomes							
		TC (mmol/L)				LDL-C (mmol/L)			
		Net Δ (mmol/L) ¹	95%CI	Relative Δ (%) ¹	95%CI	Net Δ (mmol/L) ¹	95%CI	Relative Δ (%) ¹	95%CI
RC	Alhassan et al. 2006 [239]	-0.650	(-1.490, 0.190)	-13.0	(-30.0, 5.1)	-0.650	(-1.030, -0.270)	-20.4	(-42.0, 1.2)
	Baumgartner et al. 2013 [240]	-0.300	(-0.450, -0.150)	-5.3	(-8.4, -2.2)	-0.290	(-0.420, -0.160)	-8.1	(-13.0, -3.3)
	Blomqvist et al. 1993 [241]	-0.370	(-0.900, 0.160)	-6.0	(-16.0, 3.0)	-0.330	(-0.780, 0.120)	-10.0	(-22.0, 2.0)
	Christiansen et al. 2001 [244]								
	- Stratum 1	-0.620	(-1.030, -0.210)	-8.9	(-14.0, -3.0)	-0.470	(-0.860, -0.078)	-11.3	(-17.0, -5.0)
	- Stratum 2	-0.580	(-0.990, -0.170)	-8.3	(-13.2, -4.2)	-0.450	(-0.880, -0.020)	-10.6	(-16.0, -5.0)
	Geelan et al. 2002 [238]								
	- Stratum 1	-0.310	(-0.450, -0.170)	-6.0	(-15.0, 3.0)	-0.310	(-0.480, -0.140)	-9.9	(-18.0, -1.6)
	- Stratum 2	-0.360	(-0.440, -0.280)	-8.0	(-15.0, -0.7)	-0.340	(-0.470, -0.210)	-12.4	(-20.0, -5.0)
	Gylling et al. 1997 [247]	-0.550	(-1.350, 0.250)	-8.0	(-16.0, 0.0)	-0.530	(-0.760, -0.300)	-14.5	(-42.0, 13.0)
	Gylling et al. 2009 [270]	NR	NR			NR	NR	NR	NR
	- Stratum 1			-4.4	(-7.3, -1.5)				
	- Stratum 2			-4.2	(-7.1, 2.9)				
	Gylling et al. 2013 [248]	-0.360	(-0.580, -0.140)	-6.6	(-10.3, -2.9)	-0.350	(-0.530, -0.170)	-10.2	(-19.0, -4.1)
	Hallikainen et al. 1999 [249]								
	- Stratum 1	-0.730	(-1.080, -0.380)	-10.6	(-17.0, -4.3)	-0.610	(-0.960, -0.260)	-13.7	(-25.0, -2.1)
- Stratum 2	-0.500	(-0.850, -0.150)	-8.1	(-16.0, -0.1)	-0.350	(-0.710, -0.010)	-8.6	(-18.0, -0.8)	
Hallikainen et al. 2000 i [250]	-0.460	(-0.790, -0.130)	-7.3	(-10.4, -4.2)	-0.440	(-0.590, -0.280)	-10.4	(-14.1, -6.7)	
Hallikainen et al. 2000 ii [251]	-0.450	(-0.980, -0.060)	-6.8	(-11.0, -2.7)	-0.270	(-0.800, 0.260)	-5.6	(-11.2, 0.6)	

	Hallikainen et al. 2006 [252]	-0.370	(-0.820, 0.080)	-6.0	(-9.6, -2.4)	-0.410	(-0.820, 0.000)	-9.0	(-14.0, -3.6)
	Hallikainen et al. 2008 [253]	-0.590	(-1.500, 0.330)	-10.8	(-22.0, 0.0)	-0.520	(-1.520, 0.480)	-18.6	(-4.6, 9.0)
	Miettinen et al. 1995 [259]								
	- Stratum 1	-0.520	(-0.910, -0.130)	-9.0	(-16.0, -0.9)	-0.440	(-0.620, -0.260)	6.2	(-6.3, 19.0)
	- Stratum 2	-0.670	(-1.060, -0.280)	-11.0	(-20.0, -2.4)	-0.540	(-0.750, -0.360)	-15.0	(-25.0, -5.0)
	Plat et al. 2000 [261]								
	- Stratum 1	-0.390	(-0.880, 0.100)	-8.0	(-17.0, 2.0)	-0.370	(-0.510, -0.220)	-12.6	(-24.0, -0.8)
	- Stratum 2	-0.390	(-0.940, 0.160)	-8.0	(-18.0, 2.0)	-0.340	(-0.510, -0.180)	-11.6	(-23.0, 0.2)
	Turpeinen et al. 2012 [266]	-0.410	(-0.700, -0.116)	-7.0	(-14.0, 0.3)	-0.340	(-0.590, -0.085)	-8.9	(-17.0, -1.1)
SS	Gagliardi et al. 2010 [246]	-0.450	(-1.450, 0.550)	-0.8	(-1.9, 0.3)	-0.050	(-0.910, 0.810)	-14.7	(-26.0, -3.3)
	Hendriks et al. 1999 [271]	-0.310	(-0.410, -0.200)	-5.9	(-11.0, -0.4)	-0.260	(-0.360, -0.150)	-8.5	(-17.0, -0.7)
	Lottenberg et al. 2003 [258]	-0.300	(-0.720, 0.119)	-6.9	(-9.0, -4.7)	-0.300	(-0.410, -0.190)	-6.3	(-8.7, -4.0)
	Nigon et al. 2001 [260]	-0.260	(-0.630, 0.110)	-5.9	(-8.6, -3.2)	-0.240	(-0.550, 0.070)	-24.0	(-55.0, 7.0)
	Ras et al. 2014 [262]	-0.570	(-0.740, -0.400)	-9.0	(-17.0, -1.2)	-0.450	(-0.590, -0.320)	-11.7	(-21.0, -3.0)
	Ras et al. 2015 [263]	-0.260	(-0.460, -0.070)	-4.5	(-12.3, 3.0)	-0.260	(-0.400, -0.120)	-26.0	(-40.0, -12.0)
	Simons 2002 [264]	-	-	-6.7	(-10.0, -4.0)			-8.1	(-12.0, -4.0)
	Theuwissen et al. 2009 [265]	-0.490	(-1.370, 0.390)	-6.7	(-13.0, -0.8)	-0.430	(-1.270, 0.410)	-9.5	(-18.6, -0.4)
	Vissers et al. 2000 [269]	-0.190	(-0.310, -0.070)	NR	NR	-0.200	(-0.300, -0.100)	NR	NR
D	Buyuktuncer et al. 2013 [242]	-0.450	(-0.660, -0.240)	-4.5	(-8.3, -0.8)	-0.260	(-0.412, -0.108)	-6.3	(-9.0, -3.7)
	Charest et al. 2004 [272]	-0.600	(-1.290, 0.086)	NR	NR	-0.500	(-1.050, 0.050)	-13.1	(-31.0, 5.0)
	Clifton et al. 2004 [245]	-0.530	(-0.800, -0.260)	-9.7	(-19.5, 0.1)	-0.530	(-0.810, -0.250)	-12.4	(-22.0, -0.3)
	Hyun et al. 2005 [255]	-0.150	(-0.460, 0.160)	-4.4	(-14.2, 5.0)	-0.240	(-0.430, -0.060)	-7.8	(-20.0, 4.0)
	Jauhiainen et al. 2006 [256]	-0.320	(-0.500, -0.150)	-5.8	(-8.8, -2.8)	-0.360	(-0.530, -0.180)	-10.3	(-14.9, -5.7)
	Korpela et al. 2006 [257]								
	- Stratum 1	-0.460	(-0.750, -0.170)	-6.9	(-11.0, -3.0)	-0.460	(-0.710, -0.210)	-11.2	(-17.0, -5.0)

- Stratum 2	NR	NR	NR	NR	-0.560	(-0.800, -0.320)	-13.7	(-19.6, -8.0)
- Stratum 3	NR	NR	NR	NR	-0.320	(-0.580, -0.060)	-7.8	(-14.0, -1.9)
Vanstone et al. 2002 [267]	-0.610	(-1.260, 0.040)	-7.8	(-17.6, 2.0)	-0.410	(-0.650, -0.170)	-10.2	(-22.0, 2.0)
Vasquez-Trespalacios et al. 2014 [268]	-0.410	(-0.698, -0.122)	-7.2	(-11.1, -3.3)	-0.320	(-0.560, -0.070)	-10.3	(-14.3, -6.4)

NR, not reported. Additionally, not enough information was provided in order to estimate absolute change and / or relative change.

Only the first period of cross-over studies were included in the meta-analysis as current practice [237].

RC, rapeseed/canola; SS, sunflower/soybean; D, dairy

Stratum refers to a single treatment group.

¹ Net change calculated by subtracting the mean change in control group from the mean change in the treatment group. Mean change = blood cholesterol value at the end of intervention – blood cholesterol value at baseline).

3.4.3 Effect of fat type on the phytosterol-lowering of blood cholesterol levels

The absolute net change in TC reported in studies ranged from -0.73 (95% CI: -1.08, -0.38) to -0.15 (95% CI: -0.46, 0.16) and LDL-C from -0.65 (95% CI: -1.03, -0.27) to -0.05 (95% CI: -0.91, 0.81). The relative net change in TC ranged from -13.0% (95% CI: -30.0, 5.1) to -4.4% (95% CI: -14.2, 5.0) and LDL-C from -26.0% (95% CI: -40.0, -12.0) to -5.6% (95% CI: -11.2, 0.6).

The overall pooled mean TC reduction was -0.38 mmol/L (95% CI: -0.42, -0.33; $p < 0.001$) and the relative reduction -6.4% (95% CI: -7.3, -5.5; $p < 0.001$) (Table 3.4). The overall pooled LDL-C reduction was -0.34 mmol/L (95% CI: -0.41, -0.28; $p < 0.001$) and the relative reduction -9.3% (95% CI: -10.4, -8.2; $p < 0.001$). Between-trial heterogeneity, assessed using the I^2 statistic was not statistically significant for the absolute or the relative changes in TC and LDL-C ($p > 0.05$).

By fat group, overall TC reduced by -0.39 mmol/L (95% CI: -0.49, -0.30; $I^2 = 0\%$, $P = 0.62$), -0.38 mmol/L (95% CI: -0.44, -0.33; $I^2 = 0\%$, $P = 0.86$), and -0.32 mmol/L (95% CI: -0.43, -0.21; $I^2 = 48\%$, $P = 0.06$) in the D, RC and SS groups respectively. All reductions were statistically significant ($p < 0.01$). LDL-C reduced by -0.36 mmol/L (95% CI: -0.43, -0.29; $I^2 = 0\%$, $P = 0.48$), -0.38 mmol/L (95% CI: -0.43, -0.34; $I^2 = 0\%$, $P = 0.85$), and -0.28 mmol/L (95% CI: -0.35, -0.22; $I^2 = 25\%$, $P = 0.23$) in the D, RC and SS groups respectively. Reductions in LDL-C for each fat group were statistically significant ($p < 0.01$) (See Figures. 3.2 and 3.3).

The absolute mean change in LDL-C induced by the RC spreads was significantly greater compared to the SS spreads by -0.101 mmol/L (95% CI: -0.887, -0.097; $p = 0.01$). Other comparisons across fat types were not statistically significant.

Low to moderate between-trial heterogeneity was evident in the SS group only for the TC measure ($I^2 = 48\%$, $P = 0.06$). Subgroup analysis was conducted to investigate this borderline heterogeneity using predefined criteria known to influence circulating cholesterol levels such as PS dosage, baseline LDL-C concentrations and BMI. Categorisation of PS dose into the following categories: < 2.0 g/d; 2.0-2.4 g/d; 2.5-2.9 g/d and > 3.0 g/d, demonstrated that a dose of 2.5-2.9 g/d had the greatest effect on TC (-0.57; 95% CI: -0.73, -0.40), whereas studies were more similar in terms of the effect size within the remaining PS dosages < 2.0 g/d (-0.31; 95% CI: -0.40, -0.21), 2.0-2.4g/d (-0.19; 95% CI: -0.31, -0.08) and > 3.0 g/d (-0.26; 95% CI: -0.46, -0.07). This is to be expected given the known dose-response relationship between PS and cholesterol-reduction. The range of PS dose across the trials for the TC measure appears to be accounting for some of the heterogeneity. Baseline LDL-C was categorised according to ATPIII [273] and does not appear to explain the observed heterogeneity given similar mean effect sizes across categories. Only one category (borderline high LDL-C) demonstrated significantly large heterogeneous effect sizes. Similarly, BMI does not appear to influence the heterogeneity, given similar effect sizes across

the BMI categories (ES ranging from -0.31; 95% CI: -0.41, -0.21 to ES -0.45; 95% CI: -1.45, 0.55).

Table 3.4: Absolute (mmol/L) and relative (%) net change per fat group.

Fat group		TC ES (95% CI)		LDL-C ES (95% CI)	
RC	mmol/L	-0.383	(-0.435, -0.330)	-0.384	(-0.431, -0.337)
	%	-6.7	(-7.8, -5.6)	-9.7	(-11.4, -8.1)
SS	mmol/L	-0.322	(-0.433, -0.211)	-0.283	(-0.346, -0.220)
	%	-5.5	(-8.3, -2.7)	-9.5	(-12.7, -6.3)
D	mmol/L	-0.395	(-0.491, -0.299)	-0.357	(-0.428, -0.287)
	%	-6.1	(-7.9, -4.4)	-8.9	(-10.6, -7.3)

Pooled absolute and relative net change in TC and LDL-cholesterol assessed using random effects model.

TC, total cholesterol; ES, estimate; CI, confidence interval; LDL, low-density lipoprotein cholesterol

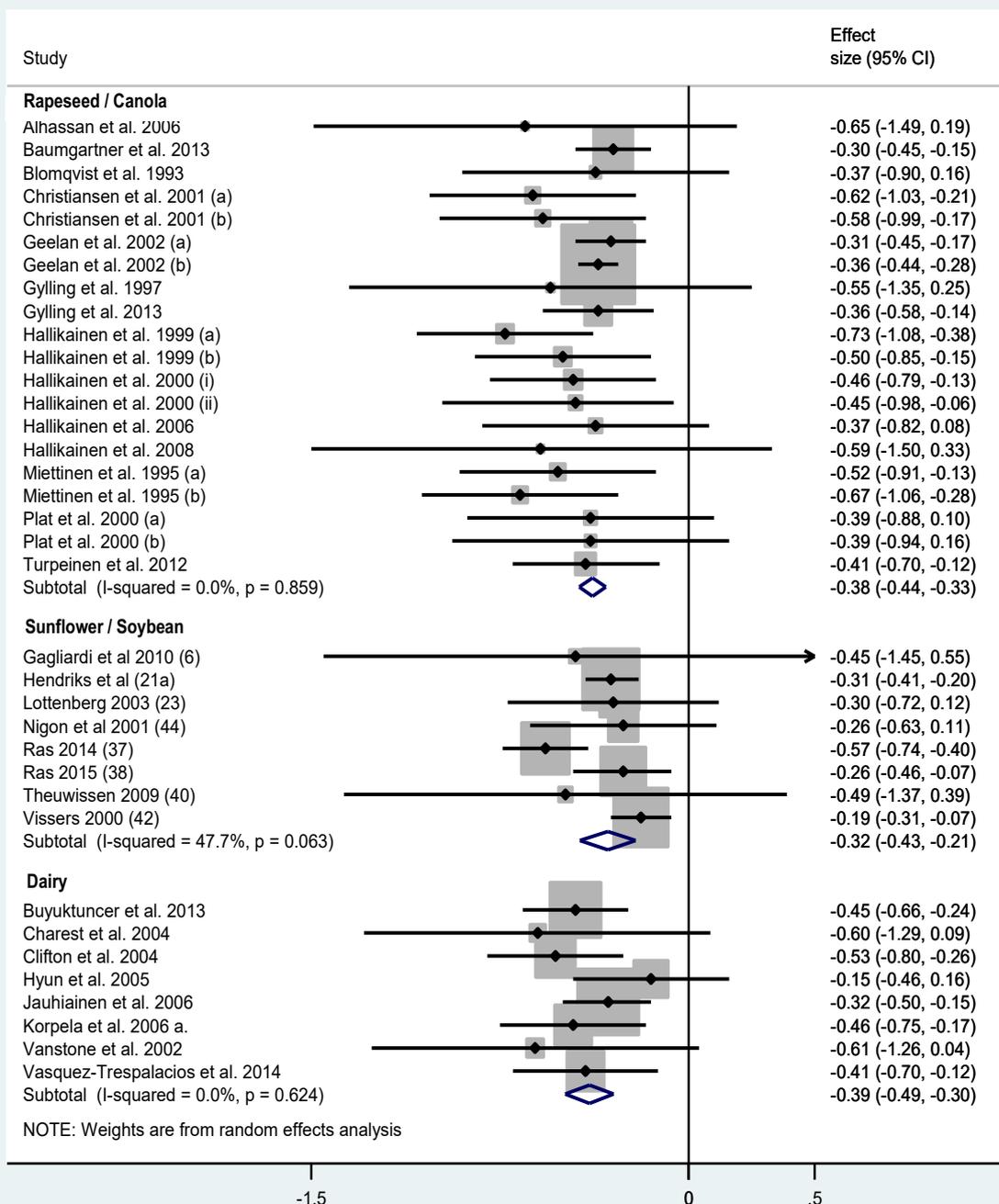


Figure 3.2: Effect size and 95% CI for TC concentrations according to fat type in PS fortified food.

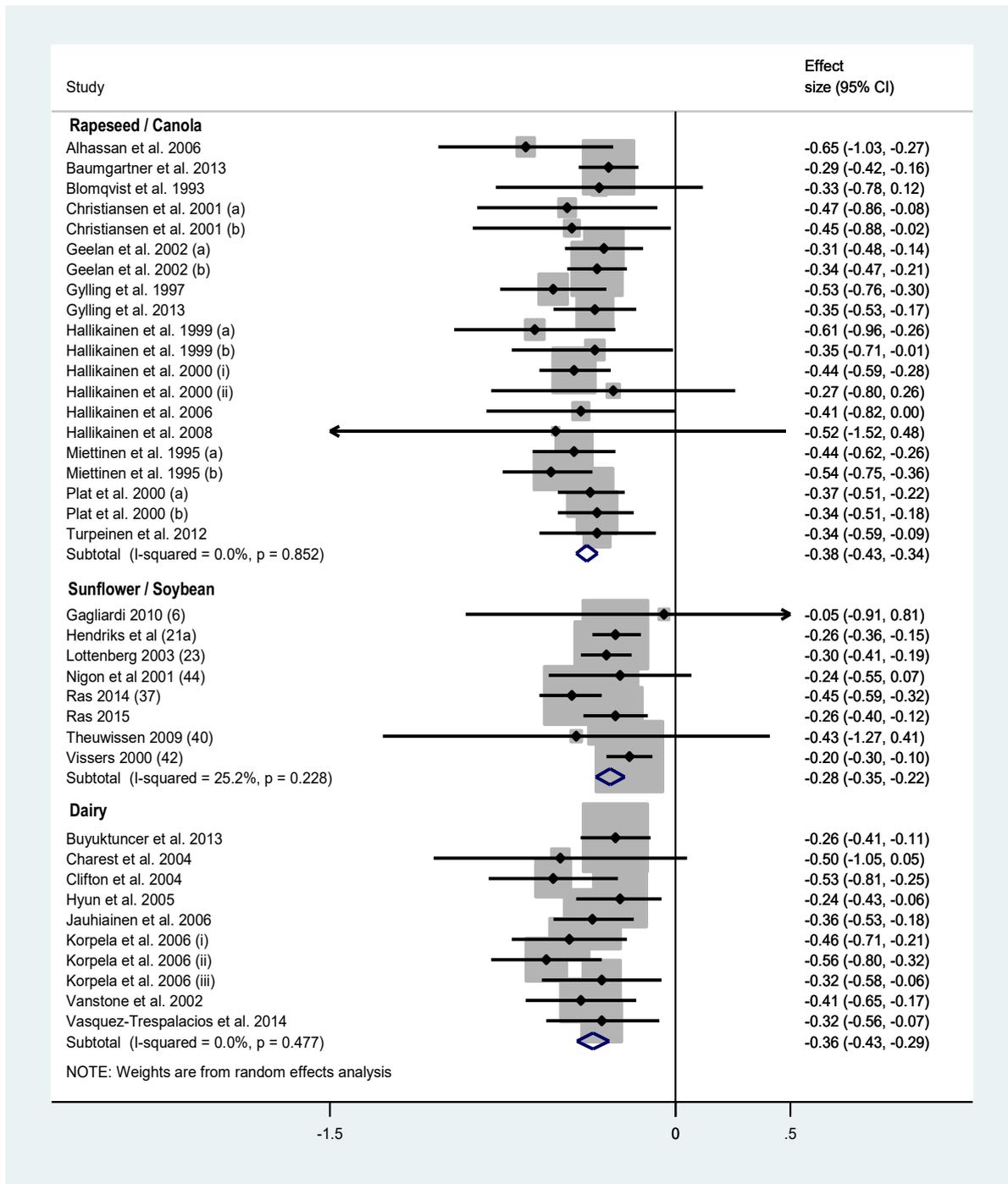


Figure 3.3: Effect size and 95% CI for LDL-C concentrations according to fat type in PS fortified food.

3.5 Discussion

This systematic review and meta-analysis has shown that PS-enriched spreads with RC as a carrier fat is superior for lowering LDL-C compared to SS. The PS-fortified products included in this review reflect those that are voluntary fortified in Australia, that is, fat spreads and dairy products e.g. low-fat milk. As expected, PS with all carrier fats significantly reduced TC and LDL-C independently, however, when compared against each other, only the RC fat type was found to reduce LDL-C significantly more than the SS group. It is noteworthy that compared to the RC and SS groups, the D group contains a diverse range of products with differing fat content; including butter (~80%), cheese ($\leq 17\%$), yoghurt ($\leq 2\%$) and low-fat milk ($\leq 1.8\%$). Moreover, compared to the SS and RC group, the D group is complex since the level of SFA varies greatly, making it less of a homogenous group to include in the comparison against RC and SS. Therefore, plasma cholesterol reduction in the D group may have been due to a combination of factors including PS and the meal containing low fat and low SFA. It is also noteworthy that the greater heterogeneity due to varying fat content in the D group resulted in a larger confidence interval and consequently the difference did not reach a significant level compared to SS or RC groups. Given the diversity of the D group studies, the role of dairy fat in modulating the cholesterol-lowering effects of PS cannot be clearly established in the present analysis, however, the fat content of SS- and RC-containing PS products was comparable, providing a valid rationale for comparing their hypocholesterolaemic effects. The addition of PS to dairy products may be acting synergistically to improve the efficacy of their overall cholesterol-lowering ability.

Findings of this review are consistent with current scientific literature and health claims associated with PS induced cholesterol reduction [94, 245]. The overall pooled reduction in LDL-C (both absolute and relative change) reported in this meta-analysis is similar to the findings of a previous systematic review and meta-analysis conducted by Demonty et al [94], involving analysis of 84 trials including 141 strata. Demonty et al reported a similar pooled reduction in LDL-C; -0.34 mmol/L (95% CI: $-0.36, -0.31$) and a relative reduction of 8.8% (95% CI: $-9.4, -8.3$) [94], which is comparable to the LDL-C reduction reported in the current meta-analysis.

Indeed factors that may influence the efficacy of cholesterol-lowering by PS must not be overlooked. The proportion of females and males was similar in all three groups, with less than half of the population comprising males. The age of participants was similar between the RC and D group, however, the mean age of the SS group was approximately 10 years higher. Studies have shown no direct influence of age on the efficacy of PS, however, the direct relationship between increasing age and increasing LDL-C concentrations is well established [94, 274, 275]. Since it has been shown that higher baseline cholesterol levels result in greater absolute reductions in cholesterol [94, 276], it is important to consider whether this may be influencing the findings of

this review. All publications included in the meta-analysis involved participants with elevated LDL-C of >2.00 mmol/L [277]. The SS fat type had participants with the highest baseline TC and LDL-C levels, followed by D and lastly RC. The majority of publications (n=22) included participants with high TC concentrations at baseline i.e. ≥ 5.50 mmol/L [193]. In all three groups, the proportion of publications with baseline cholesterol concentrations >5.00 mmol/L was similar. Each fat type contained one publication including baseline TC concentrations below 5.00 mmol/L. Although the number of total publications included per fat type are not evenly distributed in this review, the significantly greater reduction in LDL-C observed with the RC group compared to the SS group is likely to be independent of baseline cholesterol levels. Most of the studies assessed background dietary intake via three- or seven-day food records with or without validation by a Dietitian. Majority reported no change in dietary intake (including fat) pre- and post-intervention period, therefore it is unlikely that the background diet is influencing the efficacy of cholesterol-lowering induced by PS. Both sterol and stanol derived PS were included in the publications, however, it is unlikely that these are influencing the findings of this review given a meta-analysis on the continuous dose-response relationship of the LDL-C-lowering effect of PS showed that the PS type (i.e. sterols vs stanols) did not significantly affect the dose-dependent LDL-C-lowering efficacy of PS [94].

Previous studies have explored the effects of diets and/or oils rich in MUFA, PUFA and SFA on circulating cholesterol concentrations, independent of PS. The mechanisms by which PS interact with different dietary fats to influence circulating LDL-C remain to be established. The structural similarity between PS and cholesterol allows PS to compete with dietary cholesterol for intestinal absorption, however, fat plays a major role in the solubilisation of PS for micelle formation. Although the fat content of RC and SS spreads are similar, the fatty acid composition vary greatly with RC providing higher amounts of MUFA (~61%, namely oleic acid), n-3PUFA (~11%, ALA), reasonable amounts of n-6PUFA (~21%) but low levels of SFA (~7%) [72, 223]. While SS contains noticeably higher levels of n-6PUFA (~60-70%) and SFA (~13%) and relatively smaller amounts of MUFA (~20-30%) and n-3PUFA (0.5-5%) [223]. It is well known that dietary fatty acids affect circulating cholesterol levels differently, independent of PS [70-72, 218, 278, 279]. Although not previously examined, it is likely that the fatty acid composition of carrier fats may influence the degree of cholesterol displacement from the micelle, accounting for the differences in their cholesterol-lowering potential. The fat profile of RC spreads could facilitate the delivery and incorporation of PS into the micelle, thereby enhancing PS affinity for solubilisation into the micelle and heightening cholesterol displacement and overall reduction in circulating concentrations [90]. This could possibly explain the observations reported in this

meta-analysis, however, mechanisms by which different dietary fats may influence micellar formation and cholesterol displacement by PS, merits further examination.

Minimal evidence from human, animal and *in vitro* studies suggest PS may elicit a regulatory role in LDL-C metabolism. A four-week study in polygenic hypercholesterolaemic individuals administered 1.6 g/day of PS in yoghurt observed a near 10% significant increase in LDL-R affinity as well as a marked decrease (18%) in CD36 expression in addition to the expected significant decrease in plasma LDL-C (-4.3%) [280]. This observation reflects potential additional anti-atherogenic properties of PS. The potential influence of PS on LDL-R affinity could provide a complementary and/or synergistic effect between PS and carrier fats used in PS enriched fat spreads, given the upregulation of LDL-R activity elicited by PUFAs. Experimental studies are yet to report any effect of PS on LDL-R activity. Animal and human *in vitro* studies administered PS have reported increased activity of hepatic HMG-CoA reductase as well as the inhibition of NPC1L1 expression in enterocytes via reduction in their mRNA levels [281, 282]. These observations provide insight into other potential targets for optimising PS efficacy for cholesterol-lowering and overall anti-atherogenic outcomes.

The present systematic review and meta-analysis is the first to investigate whether the carrier fat in PS fortified products is a determinant of the overall hypocholesterolaemic effects of PS. This review provides an in-depth understanding of how circulating cholesterol levels may be optimised by using PS in combination with different dietary fats. A key limitation worthy of note was that several publications were excluded due to insufficient data for example type of fat used in PS fortified product and outcome data to calculate absolute and/or relative change when this was not provided. Several authors were unable to provide further information, clarification and/or did not respond to our queries, thus limiting the inclusion of additional publications. The authors of the current review decided that to assume the base fat of unidentified PS investigational products used would weaken the validity and transparency of the results. Therefore publications were only included when the base fat could be clearly classified either through a clear description of the PS fortified product stating the type of predominant fat or if the authors reported the fatty acid profile of the PS fortified product. Previous studies have investigated the effect of using a 'dairy' product with PS, however, the fatty acid profile is not consistent with that of a true dairy-based product. Commonly the 'dairy' products used in these studies contained added vegetable fats, often in amounts that override the typical SFA composition of a regular commercial low-fat milk based on cow's milk. Therefore, in this meta-analysis we have only included dairy fat-based product studies which have a nutritional profile representative of commercial dairy milks.

Visual inspection of funnel plots and statistical assessment [228] indicated non-significant results and thus no evidence of publication bias for all fat groups and outcomes ($p > 0.1$), except for

relative change in LDL-C for the SS group only ($p=0.019$). Based on the relative change calculations, this could potentially result in a reduction in the effectiveness of SS in lowering LDL-C. It should be noted, however, that when comparing absolute change scores, the superiority of RC over SS was demonstrated for LDL-C.

If the effect of SS on relative changes in LDL-C is overestimated due to publication bias, this could potentially enhance the effect of RC relative to the SS group. Due to the relatively smaller number of trials available for the SS group, a trim and fill analysis to estimate the publication bias adjusted effect for relative change in LDL-C in the SS group was unable to be conducted, however, this is noted as a potential limitation in the present study. The present systematic review underpins the potential mechanisms by which PS and dietary fats influence cholesterol levels and how circulating levels might be optimised by using PS in combination with different dietary fats.

3.6 Conclusion

To the best of our knowledge, this systematic review and meta-analysis is the first to demonstrate that the carrier fat used in PS-fortified foods is a determinant of the cholesterol-lowering potential of PS. We report novel findings such that the carrier fat of common PS fortified foods does appear to modulate the cholesterol-lowering potential of PS. Furthermore, we have demonstrated that RC appear to be the superior carrier fat for optimum PS functionality. In addition to the cholesterol-lowering potential of PS fortified RC fat spreads, the health benefits of MUFA and n-3PUFA (which are the predominant fats found in RC fat spreads) support RC as an ideal carrier fat for PS, providing additional cardio-protective action to improve the lipid profile of atherogenic LDL-C by lowering its oxidation susceptibility [283-287]. Our systematic review and meta-analysis provides the first evidence that RC based PS spreads may offer dual benefit for greater cholesterol reduction as well as to facilitate the role of PS for cholesterol-lowering in hypercholesterolaemic individuals. The findings of this review suggest the importance of considering the fat composition used for PS fortified products in order to yield optimum cholesterol-lowering outcomes in individuals with hypercholesterolaemia.

Chapter 4

Curcumin potentiates cholesterol-lowering effects of phytosterols in hypercholesterolaemic individuals. A randomized controlled trial

The contents of this chapter have been published as the following manuscript:

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Statement of authorship is attached as Appendix 10

4.1 Abstract

Background: Dietary phytosterols (PS) are well-known hypocholesterolaemic agents. Curcumin elicits hypolipidaemic and anti-inflammatory effects in preclinical studies, however, consistent findings in humans are lacking.

Objective: Concurrent PS and curcumin supplementation may exhibit enhanced hypocholesterolaemic and anti-inflammatory effects to optimise cardio-protection. The objective of this trial was to investigate the effects of dietary intervention with PS with or without curcumin on blood lipids (primary outcome) in hypercholesterolaemic individuals.

Methods: A double-blinded, randomised, placebo-controlled, 2x2 factorial trial was conducted in hypercholesterolaemic individuals. Participants received either placebo (PL, no phytosterols or curcumin), phytosterols (PS, 2g/d), curcumin (CC, 200mg/d) or a combination of PS and curcumin (PS-CC, 2g/d-200mg/d respectively) for four weeks. Primary outcomes included fasting total cholesterol (TC), LDL-cholesterol, HDL-cholesterol, triglycerides (TG), TC-to-HDL-C ratio (TC:HDL-C). Secondary outcomes included anthropometrics and fasting blood glucose concentrations.

Results: Seventy participants with a mean (\pm SEM) fasting TC concentration of 6.57 ± 0.13 mmol/L completed the study (PL, n=18; PS, n=17; CC, n=18; PS-CC, n=17). PS and PS-CC supplementation significantly lowered TC, LDL-cholesterol and TC:HDL-C post-intervention ($p<0.05$). Reductions from baseline in the PS group were 4.8% and 8.1% for TC and LDL-cholesterol respectively ($p<0.05$). CC exhibited non-significant reduction (2.3% and 2.6%) in TC and LDL-C respectively, however, the PS-CC resulted in a greater reduction in TC (11.0%) and LDL-cholesterol (14.4%) than either of the treatments alone ($p<0.0001$). The reduction in the PS-CC treatment was significantly greater compared to those for CC ($p<0.05$) or PL ($p<0.01$) alone. Plasma HDL-cholesterol and TG concentrations remained unchanged across all groups. No adverse side effects were reported.

Conclusions: The addition of curcumin to phytosterol therapy provides a complementary cholesterol-lowering effect that is larger than phytosterol therapy alone. Implications of these findings include the development of a single functional food containing both the active ingredients for enhanced lipid-lowering and compliance in hypercholesterolaemic individuals.

ANZCTR identifier: 1261500095650

Keywords: cholesterol, lipids, phytosterols, curcumin, hypercholesterolemia, cardiovascular disease.

4.2 Introduction

Cardiovascular disease (CVD) remains the number one killer worldwide claiming 31% of all deaths [288]. In Australia one in six people are affected by CVD and 45,392 lives were claimed by the disease in 2015 [289]. These statistics are alarming since every 12 minutes, one Australian dies from CVD [290]. CVD is Australia's most expensive condition to treat, costing \$1.8 billion per year for CVD medications [291] and \$18.3 billion in total economic costs in 2014 [292]. CVD poses several risk factors and 90% of the Australian population are estimated to have at least one [9]. Dyslipidaemia, as indicated by elevated concentrations of TC, LDL-cholesterol (LDL-C) and TG, as well as low concentrations of HDL-cholesterol (HDL-C), continues to be a major CVD risk factor [16]. In 2011-12, one third of the Australian adult population had abnormal or elevated LDL-C concentrations and 23% had low concentrations of HDL-C [14]. Whilst lifestyle and pharmacological therapies have proven useful for managing dyslipidaemia, it is evident that simple, safe, free of serious side effects, cost-effective and more efficacious strategies are required.

Phytosterols (PS) are non-nutritive compounds naturally found in foods of plant origin that are structurally analogous to cholesterol and are well known for their cholesterol-lowering ability [194]. In Australia, PS have been added to common foods such as vegetable fat spreads and dairy milk in order to assist individuals with achieving therapeutic doses. An average daily dose of 2g phytosterols lowers plasma LDL-C by approximately 0.31-0.34 mmol/L or 8-10% within 3-4 weeks [94, 274]. Their ability to lower LDL-C concentrations through reduced intestinal absorption has been well documented. The most widely accepted mechanism underlying this property is attributed to competitive displacement of dietary and biliary cholesterol in mixed micelles due to higher affinity for PS [90]. Dietary supplementation of PS are safe and measured absorption [293] and plasma concentrations are very small with the majority recovered in the stool [294]. Curcumin is a polyphenol compound found in the perennial herb *Curcuma Longa* (turmeric) which belongs to the ginger family. Curcumin is well known for its abundance of health benefits, namely anti-inflammatory, anti-proliferative, anti-oxidant and anti-apoptotic effects [108]. In regards to cholesterol homeostasis, preclinical studies have shown that curcumin modulates hepatic gene expression, inhibits cholesterol biosynthesis via down-regulation of major lipogenic factors [56, 87, 125, 126, 295], stimulates bile acid secretion, enhances clearance of cholesterol as bile [133, 134] and mobilises and decreases accumulation of lipids from adipose tissue [133, 296]. Curcumin is poorly bioavailable as it rapidly undergoes degradation in the liver [108]. Incorporation of curcumin into liposomes and phospholipids [297], encapsulation as polymer nanoparticles and complexed with piperine [298], a known inhibitor of hepatic and intestinal glucuronidation have been shown to improve bioavailability. Curcumin

supplementation is safe in humans with no adverse side effects or events reported, even at higher doses for over 12 months [14, 108, 299].

Since elevated blood lipids and inflammation are the two major CVD risk factors, concurrent PS and curcumin therapy has the potential to provide a safe and efficacious means of protection against the development of CVD. PS and curcumin are well tolerated in humans and do not bear any serious side effects even at high doses [114, 205]. Both nutraceuticals are recognised as lipid-lowering tools in clinical practice; PS for their major role as a cholesterol absorption inhibitor and curcumin as an inhibitor of hepatic cholesterol synthesis [87]. The aim of this study was to examine the effects of dietary intervention with PS with or without concurrent supplementation with curcumin on circulating blood lipids and to investigate whether curcumin complements or acts in synergy with PS to modulate lipid markers.

4.3 Materials and Methods

4.3.1 Recruitment

Participants with hypercholesterolaemia were recruited from the Hunter region (NSW, Australia) by radio announcements, newspaper articles and advertisements placed around the local community. Volunteers were assessed for eligibility by a study investigator if they were: healthy adults aged between 18 to 70 years; fasting plasma total cholesterol > 5.5 mmol/L; no CVD, diabetes mellitus, kidney/liver conditions, chronic inflammatory diseases, neurological conditions or untreated hypertension ($\geq 140/95$ mm Hg); not taking lipid-lowering or anti-inflammatory medication; not consuming PS-enriched products or curcumin/turmeric supplements or any other supplements known to influence study outcomes e.g. fish oil, krill oil, flaxseed oil, coenzyme Q10, fibre; BMI < 40 kg/m²; not pregnant or lactating; non-smoker; and no strong aversion and/or intolerance/allergy to the foods used in the study. The study protocol was approved by the Human Research Ethics Committee, University of Newcastle (protocol no. H-2015-0162) and was conducted in accordance with the 1975 Declaration of Helsinki as revised in 1983. All participants provided written informed consent prior to inclusion in the study. Participants were de-identified at point of enrolment and identified by alphanumeric codes. The trial was registered with the Australian and New Zealand Clinical Trials Registry at <http://www.anzctr.org.au/> as 1261500095650.

4.3.2 Trial design

Subjects participated in a four-week, double-blinded, randomised, placebo-controlled, 2x2 factorial intervention in four parallel groups. Allocation to treatment groups was conducted by

the lead investigator (JF) using a computer-generated block randomisation approach with subjects stratified by gender (Random Allocation Software version 1.0.0). Blocks of size eight were used to achieve the goal sample size in each group. Participants were de-identified and assigned alphanumeric codes for identification. The treatment allocations were concealed and revealed at the conclusion of the study by the senior investigator (MG). Food product containers were labelled before study commencement with a code to ensure that neither the investigators nor volunteers could determine intervention allocation.

Participants were randomised to one of four treatment combinations: placebo (PL; 25 g/d fat spread plus two placebo tablets), phytosterol-only (PS; 25 g/d PS-enriched fat spread containing 2 g/d PS plus two placebo tablets), curcumin-only (CC; or 25 g/d fat spread plus two tablets containing 200 mg/d curcumin) or a combination of PS plus curcumin (phytosterol + curcumin, PS-CC). Each 1 gram curcumin tablet contained 500 mg curcuma phospholipid (Meriva ® Indena) plus excipients; delivering 100 mg curcumin. The placebo tablet was comparable but devoid of both curcuminoids and soy lecithin, containing more microcrystalline cellulose. Participants were instructed to consume two tablets each day (one with morning meal and one with evening meal). The PS-enriched vegetable fat spread was commercially available (Logicol Original) and predominately based on canola oil and the placebo spread was equivalent in nutritional profile including fats but devoid of PS fortification (MeadowLea Canola), see Table 4.1. The main source of PS esters used in Australian PS-fortified food products is derived from soybean oil or tall (pine) oil [198]. The fat spreads were provided to participants in individually portioned containers (25 g/d) and participants received detailed instruction to displace all habitual margarine and/or butter consumption with the study spread. Compliance was monitored by evaluating the consumption log, weighing returned tubs, tablet count pre- and post-intervention and evaluation of dietary records (analysed with FoodWorks, Xyris ®, Professional Edition Version 8.0.3551). Irregular use of medications or illness experienced were also logged for the duration of study participation.

Table 4.1: Nutrient composition of phytosterol and control spread.¹

Dietary component	Control spread	Phytosterol spread
Energy (kJ)	600	600
Total fat (g)	16.25	16.00
Saturated (g)	4	4
Monounsaturated (g)	9.00	7.75
Polyunsaturated (g)	3.25	4.25
Omega-3 (ALA) (g)	1.00	1.05
Phytosterols (g)	0	2
Sodium (mg)	87.5	87.5
Potassium (mg)	12.5	10.0

¹ Nutrient information is given for one serving (25 g). Each participant had to consume one serving of spread per day. Data provided by manufacturer (MeadowLea Foods) and Goodman Fielder.
ALA, alpha-linolenic acid.

4.3.3 Clinical assessments

Participants attended two visits (baseline and post-intervention) at the University of Newcastle, Callaghan, NSW in an overnight fasted state (≥ 10 h) where anthropometric measures, medical history, dietary intake, physical activity patterns and fasting blood samples were collected for primary (plasma TC and LDL-C, HDL-C, TC:HDL ratio, TG) and secondary outcome measures (glucose).

4.3.4 Anthropometry and body composition

Anthropometrics (height, weight, waist circumference, weight, BMI) and body composition was collected at baseline and post-intervention. Participants were measured wearing light clothing and asked to remove shoes and all metal and/or electronic devices on person for all measurements. Height (cm), waist circumference (cm) and weight (kg) were collected to the nearest 0.1 units. Height was measured using a wall mounted stadiometer with a movable head piece. Waist circumference was measured using a tensible tape measure positioned midway between the lower rib margin and the iliac crest (approximately belly-button) horizontally. BMI was calculated as

weight/height² (kg/m²). Weight, along with other body composition parameters (skeletal muscle mass, fat mass, total body water etc) were measured using bioelectrical impedance (BIA) utilising two different frequencies (InBody230, Biospace Co.). Measurements were taken in the standing position following a ≥ 10 h fast and participants refrained from vigorous physical activity and alcohol consumption 24 h prior to testing.

4.3.5 Medical history, dietary intake and physical activity

A self-administered medical history questionnaire was completed by all participants at baseline to collect information regarding past and present medical conditions; prescribed/over-the-counter medication(s); use of supplements and habitual intake of alcohol, PS-enriched products, curcumin/turmeric supplements and habitual use of fats, oils and added sugars. A three-day food diary and physical activity questionnaire (International Physical Activity Questionnaire; IPAQ Long Last 7 Days Self-Administered Format, October 2002) were used to collect measures of habitual dietary intake and physical activity levels (respectively). Dietary data was entered into a food database system (FoodWorks, Xyris. Professional Edition Version 8.0.3551). Physical activity data was interpreted as metabolic equivalent of task minutes per week (MET/week) to measure the energy cost of physical activities.

4.3.6 Blood sampling and serum lipid analyses

Fasting blood samples (10 h) were collected into tubes pre-coated with EDTA via venepuncture by a trained phlebotomist at baseline and post-intervention. Samples were prepared by centrifuging (Heraeus Biofuge Stratos) for 10 minutes at 3000 x g at 4°C. Plasma, buffy coat and red blood cell sub-fractions were aliquoted and stored at -80 °C until further analysis. Plasma TC, HDL-C, TG and glucose concentrations were measured on a VP auto analyser using standardized reagents by Hunter Area Pathology Service. LDL-C concentration was determined using the Friedewald equation [191].

4.3.7 Statistical analysis

Statistical analysis was conducted using StataCorp. 2015 (*Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP). All data are presented as means \pm SEM (standard error of the mean) unless otherwise specified and the significance level for all statistical tests was set at 0.05. Sample size was calculated based on previous estimates of variance in plasma TC concentration (standard deviation 0.5) yielding 80 participants in total (20 in each group) to provide 80% power at a 0.05 significance level for detection of a 0.50 mmol/L (~10%) reduction in TC and accounting for a 20% dropout rate. At baseline, comparability of treatment groups for age, height, weight, BMI, body composition, dietary intake, fasting lipid profile and change in

dietary parameters were assessed by ANOVA for normally distributed data and Kruskal-Wallis when the assumption of normality was not met. Comparability between treatment groups for ethnicity and gender was evaluated by the chi-square test. Within-treatment comparisons from baseline to post-intervention were performed using the paired samples t-test. Absolute change (mmol/L) was calculated as post-intervention value minus baseline value and the percent change (%) was calculated as the absolute change divided by the baseline value, then multiplied by 100. Therefore, for change data, a negative sign denotes a reduction. The effect of each treatment on the absolute and percent change from baseline to follow up on the dependent variables (lipid profiles) between groups was explored using one-way ANOVA. Two-way ANOVA was used to determine whether there was a significant main effect for each independent variable. An interaction term [PS x curcumin] was tested between the two independent variables to investigate their effect on the dependent variables. For significant effects, Tukey's honestly significant difference was used to perform post hoc comparisons to test for complementarity and/or synergy between PS and curcumin. A separate multiple linear regression model was then considered for each response variable (absolute- and percent change in TC and LDL-C) with treatment group and the corresponding baseline values of the response variables included as explanatory variables. The effect of multiple explanatory variables were also adjusted for in the model by their inclusion as explanatory variables (age, BMI, baseline LDL-C concentrations, baseline dietary total fat, fibre, alcohol and LCn-3PUFA). Correlations between potential baseline explanatory variables were assessed and variables with correlation coefficients above 0.8 were identified as potentially multicollinear and the number of potential predictors to include in the regression was reduced accordingly. The backward stepwise procedure was used for each regression to select the optimal set of predictors for each model.

4.4 Results

4.4.1 Baseline characteristics

This study recruited 76 participants during the period from end-October 2015 to end-October 2016. Four participants dropped out of the study due to inability to comply with daily fat spread consumption (n=3) and illness (n=1). A further two participants were excluded from the study (poor compliance with study products, n=1 and unreliable (abnormally high TG) blood test results, n=1). A total of 70 participants (57% females) were included in the final analysis (PL, n=18; PS, n=17; CC, n=18; PS-CC, n=17) with a mean age of 50.70 ± 1.51 y, BMI of 27.16 ± 0.50 kg/m², waist circumference of 92.05 ± 1.32 cm, waist-to-hip ratio of 0.93 ± 0.01 , skeletal muscle mass of 30.04 ± 0.90 kg and fat percentage of 31.82 ± 1.00 . The majority of study participants were

north-west European (79%). Participant allocation is presented in the CONSORT diagram (Figure 4.1). At baseline, study participants were hypercholesterolaemic with TC of 6.57 ± 0.13 mmol/L, LDL-C of 4.38 ± 0.11 mmol/L, HDL-C of 1.51 ± 0.05 mmol/L, TC:HDL ratio of 4.61 ± 0.15 and median (IQR) TG of 1.29 (0.78) mmol/L. All participants were comparable on baseline characteristics since there were no statistically significant differences between treatment groups (Table 4.2).

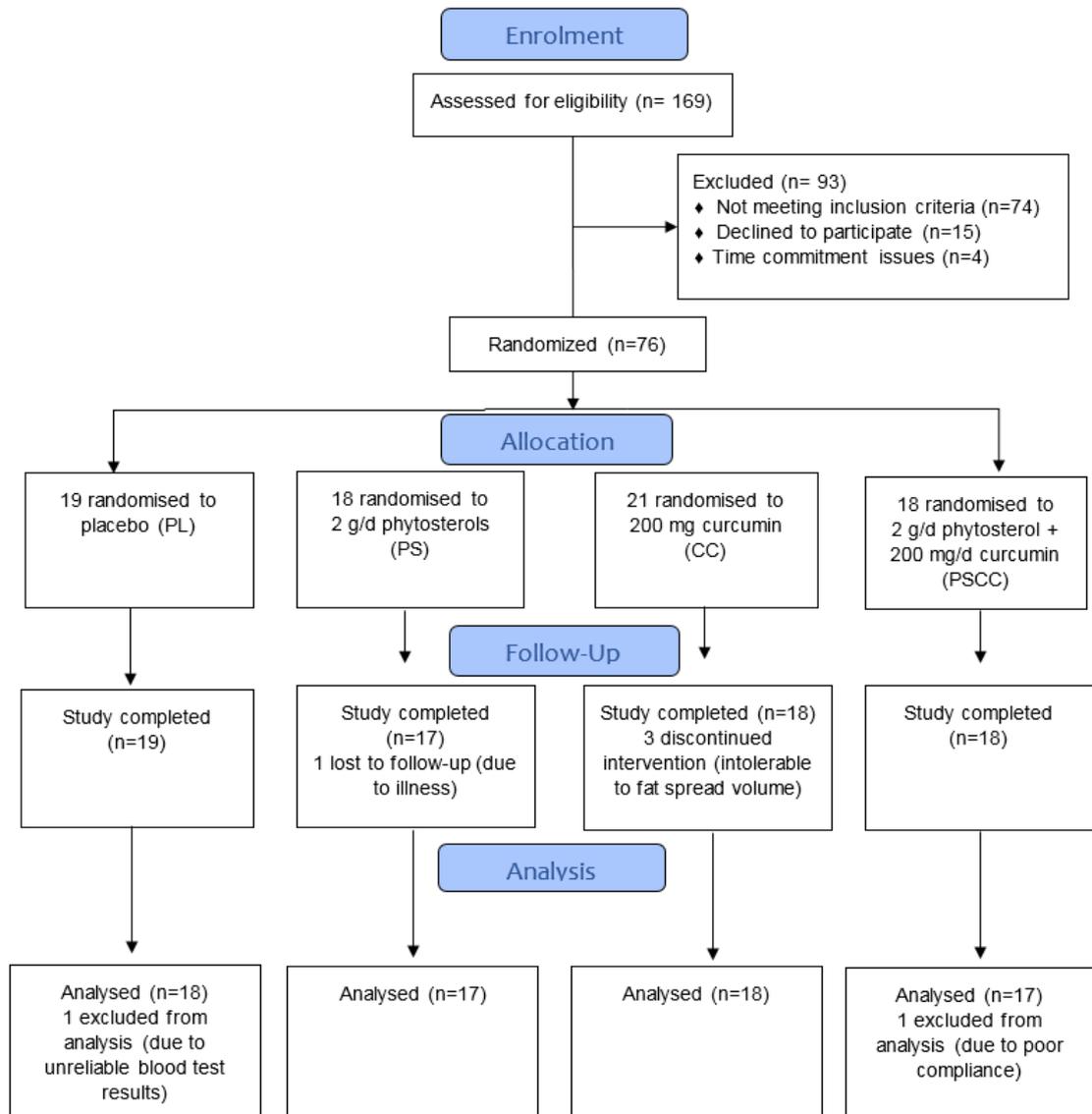


Figure 4.1: CONSORT flow diagram of participant recruitment, screening and assessment.

Table 4.2: Participant characteristics at baseline in the placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PS-CC) groups.¹

	PL (n = 18)	PS (n = 17)	CC (n = 18)	PS-CC (n = 17)
Sex, n (%)				
Male	7 (39)	8 (47)	8 (44)	7 (41)
Female	11 (61)	9 (53)	10 (56)	10 (59)
Ethnicity, n (%)				
North-west European	13 (72)	11 (65)	15 (83)	14 (82)
South-east European	0 (0)	4 (24)	2 (11)	0 (0)
Other²	5 (28)	2 (11)	1 (6)	3 (18)
Age (y)	50.11 ± 2.96	51.35 ± 3.62	51.00 ± 2.34	50.35 ± 3.36
Height (cm)	168.64 ± 2.77	171.56 ± 2.08	170.60 ± 2.43	169.30 ± 2.21
Waist (cm)	87.79 ± 2.47	92.72 ± 2.16	96.54 ± 2.65	91.14 ± 3.00
WHR	0.93 ± 0.01	0.93 ± 0.01	0.95 ± 0.01	0.93 ± 0.02
Weight (kg)	74.36 ± 3.72	80.61 ± 2.19	83.86 ± 3.98	76.64 ± 3.83
BMI (kg/m²)	25.94 ± 0.86	27.31 ± 0.43	28.79 ± 1.31	26.57 ± 1.12
SMM (kg)	28.14 ± 1.93	31.21 ± 1.53	31.56 ± 1.95	29.29 ± 1.72
Body fat (%)	31.99 ± 2.33	31.19 ± 1.94	32.95 ± 2.04	31.09 ± 1.78

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; South-east Asian; North-east Asian; Southern and central Asian; Sub-Saharan Africa; Other (n=1 unsure, n=1 combination of races).
CC, curcumin; PL, placebo; PS, phytosterols; PS-CC, phytosterol-curcumin; SMM, skeletal muscle mass; WHR, waist-to-hip ratio

4.4.2 Dietary intake, physical activity and compliance

All groups were comparable at baseline for dietary intake (Table 4.3). Comparisons between groups showed no significant differences in the mean change of dietary parameters from baseline to post-intervention. Since there was no significant change in dietary fat intake or body weight, it is likely participants replaced habitual fat intake with the intervention fat spread.

Mean compliance with study product intake was excellent for both fat spreads (94.58 ± 1.14 %) and tablets (95.77 ± 0.83 %) in all groups. The intervention was tolerated well, and no adverse events were reported. Physical activity levels did not significantly change from baseline to post-intervention nor was there any statistically significant differences between groups at both time points (data not shown).

4.4.3 Effect of phytosterol and curcumin intervention on plasma lipid profile and glucose

After four weeks intervention TC, LDL-C and TC:HDL ratio were all significantly reduced both in terms of absolute- and relative change values: -0.34 ± 0.11 mmol/L ($p=0.008$) and $-4.8 \pm 1.7\%$ ($p=0.013$), -0.38 ± 0.10 mmol/L ($p=0.002$) and $-8.1 \pm 2.4\%$ ($p=0.004$) and -0.36 ± 0.11 ($p=0.004$) and $-7.2 \pm 2.2\%$ ($p=0.006$) respectively in the PS group and -0.74 ± 0.16 mmol/L ($p=0.0002$) and $-11.0 \pm 1.9\%$ ($p<0.0001$), -0.63 ± 0.12 mmol/L ($p=0.001$) and $-14.4 \pm 2.3\%$ ($p<0.0001$) and -0.48 ± 0.14 ($p<0.01$) and $-9.6 \pm 2.2\%$ ($p=0.0004$) respectively in the PS-CC group (Table 4.4, Figure 4.2). Blood cholesterol parameters did not significantly change from baseline in the PL and CC group. Across all four treatment groups, there was a significant difference in absolute and percent change in TC ($p<0.01$), LDL-C ($p<0.01$), TC:HDL ratio ($p<0.05$) and plasma glucose ($p<0.05$). Post-hoc analyses showed that for TC, PS-CC had a significantly larger reduction in absolute (-0.62 ± 0.19 mmol/L, $p=0.011$) and percent change ($-8.70 \pm 2.80\%$, $p=0.015$) compared to CC, and compared to PL (-0.66 ± 0.19 mmol/L, $p=0.006$ and $-9.78 \pm 2.80\%$, $p=0.005$, respectively). The same trend was evident for LDL-C, whereby the PS-CC group had significantly larger reductions in absolute (-0.56 ± 0.17 mmol/L, $p=0.012$) and percent change ($-11.86 \pm 3.96\%$, $p=0.020$) compared to CC, and PL (-0.56 ± 0.17 mmol/L, $p=0.010$ and $-13.53 \pm 3.96\%$, $p=0.006$). The reduction in TC:HDL ratio was significantly larger in the PS-CC group compared to the CC group only (-0.58 ± 0.19 mmol/L, $p=0.020$ and -11.57% , $p=0.019$). Absolute and percent change in plasma glucose concentrations significantly reduced in the CC group compared to PL group only (-0.34 ± 0.13 mmol/L, $p=0.038$ and $-6.94 \pm 2.51\%$, $p=0.036$). There was no statistically significant differences in HDL-C or TG concentrations between groups. When the data were analysed using two-factor ANOVA, PS x curcumin interactions were not significant. Additionally, there was no significant main effect of curcumin on post-treatment absolute or percent TC and LDL-C values, however, there was a significant ($p<0.01$) main effect of PS on post-intervention absolute and percent TC and LDL-C values. Baseline data including BMI, LDL-C concentration and dietary intake of total fat, fibre, alcohol and LCn-3PUFA were investigated as potential confounders using multiple linear regression models. The final reduced models revealed that treatment was the only significant predictor of the change in TC and LDL-C.

Table 4.3: Reported macronutrient and fatty acid intakes of hypercholesterolaemic adults who consumed placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PSCC) at baseline and change from baseline to post-intervention.¹

	PL (n=18)		PS (n=17)		CC (n=18)		PSCC (n=17)	
	BL	Δ	BL	Δ	BL	Δ	BL	Δ
Energy (kJ)	8744.85 ± 548.93	-68.72 ± 386.68	8816.17 ± 558.83	152.30 ± 411.20	9015.90 ± 620.12	-224.29 ± 362.04	8820.07 ± 354.72	734.18 ± 505.65
Protein (g)	99.57 ± 7.80	-6.34 ± 6.31	96.09 ± 8.86	-9.71 ± 8.12	106.07 ± 7.67	-5.60 ± 4.03	96.38 ± 5.64	-2.46 ± 5.32
CHO (g)	224.14 ± 15.51	-14.35 ± 9.48	224.14 ± 15.31	-11.22 ± 12.29	203.78 ± 19.04	-12.72 ± 11.63	207.69 ± 13.64	6.28 ± 16.67
Sugars (g)	99.30 ± 10.56	-16.94 ± 6.68	89.90 ± 7.52	-2.73 ± 6.62	88.32 ± 8.74	-7.05 ± 8.33	89.12 ± 8.20	3.62 ± 7.70
Starch (g)	122.76 ± 8.76	2.21 ± 5.43	132.45 ± 11.71	-9.01 ± 9.34	113.10 ± 11.76	-4.29 ± 8.24	115.83 ± 9.28	2.09 ± 9.91
Total fat (g)	78.29 ± 5.67	4.67 ± 5.01	77.53 ± 6.12	16.51 ± 6.88	86.40 ± 6.58	6.10 ± 3.78	86.34 ± 6.08	18.17 ± 6.11
Saturated (g)	28.16 ± 2.55	0.19 ± 2.71	26.17 ± 2.29	4.36 ± 2.43	31.39 ± 2.43	-0.36 ± 2.25	31.40 ± 3.00	4.79 ± 2.42
Trans (g)	1.28 ± 0.13	0.04 ± 0.20	1.16 ± 0.14	0.18 ± 0.15	1.38 ± 0.17	-0.08 ± 0.16	1.48 ± 0.17	0.11 ± 0.20
MUFAs (g)	29.38 ± 2.03	3.71 ± 2.11	30.75 ± 2.80	7.26 ± 3.04	34.27 ± 2.96	3.94 ± 1.75	33.41 ± 2.80	7.23 ± 3.06
PUFAs (g)	13.45 ± 1.56	1.06 ± 1.01	13.99 ± 1.53	4.12 ± 1.51	13.56 ± 1.35	2.46 ± 0.92	14.42 ± 1.89	5.27 ± 1.43
Cholesterol (mg)	320.82 ± 30.60	-27.20 ± 46.46	260.63 ± 51.36	-11.02 ± 50.02	348.25 ± 30.61	-61.72 ± 26.60	324.69 ± 43.58	-25.21 ± 31.80
Fibre (g)	28.54 ± 2.47	2.52 ± 2.52	31.51 ± 2.65	-3.02 ± 1.86	26.36 ± 2.08	-0.88 ± 1.46	25.88 ± 1.95	1.94 ± 2.27
Alcohol (g)	4.22 ± 2.12	2.74 ± 1.46	9.18 ± 4.02	-2.08 ± 2.90	12.04 ± 2.89	-4.20 ± 2.86	8.81 ± 3.63	-0.34 ± 0.89

¹ Values are reported as means ± SEM. BL, baseline; CC, curcumin; Δ, change from baseline to post-intervention; CHO, carbohydrates; PL, placebo; PS, phytosterols; PS-CC, phytosterol-curcumin.

Table 4.4: Change in plasma outcome measures in the placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PS-CC) groups from baseline to post-intervention.¹

	PL	PS	CC	PS-CC	<i>p</i> ²
TC					
BL	6.63 ± 0.18	6.39 ± 0.25	6.72 ± 0.36	6.51 ± 0.19	
PI	6.55 ± 0.21	6.05 ± 0.21*	6.60 ± 0.42	5.76 ± 0.16**	
Δ mmol/L³	-0.08 ± 0.11 [†]	-0.34 ± 0.11	-0.12 ± 0.16 ^γ	-0.74 ± 0.16 ^{†γ}	0.004
LDL-C					
BL	4.55 ± 0.18	4.23 ± 0.21	4.42 ± 0.31	4.32 ± 0.15	
PI	4.48 ± 0.19	3.85 ± 0.18*	4.35 ± 0.36	3.69 ± 0.16**	
Δ mmol/L⁴	-0.07 ± 0.12 [†]	-0.38 ± 0.10	-0.07 ± 0.14 ^γ	-0.63 ± 0.12 ^{†γ}	0.004
HDL-C					
BL	1.49 ± 0.11	1.43 ± 0.10	1.59 ± 0.10	1.54 ± 0.09	
PI	1.52 ± 0.11	1.46 ± 0.11	1.54 ± 0.12	1.51 ± 0.07	
Δ mmol/L	0.02 ± 0.04	0.04 ± 0.04	-0.05 ± 0.04	-0.03 ± 0.04	0.386
TC:HDL					
BL	4.79 ± 0.34	4.74 ± 0.31	4.47 ± 0.30	4.44 ± 0.28	
PI	4.62 ± 0.30	4.38 ± 0.29*	4.57 ± 0.37	3.96 ± 0.20*	
Δ⁵	-0.17 ± 0.14	-0.36 ± 0.11	0.10 ± 0.16 ^γ	-0.48 ± 0.14 ^γ	0.022
TG					
BL	1.27 (0.57)	1.50 (0.71)	1.23 (0.65)	1.24 (0.65)	
PI	1.08 (0.59)	1.41 (0.78)	1.46 (0.93)	0.96 (1.02)	
Δ mmol/L	-0.02 (0.54)	0.01 (0.49)	-0.02 (0.5)	-0.06 (0.3)	0.578
Glucose					
BL	4.93 ± 0.09	5.05 ± 0.10	5.33 ± 0.12	5.09 ± 0.16	
PI	5.14 ± 0.13	5.01 ± 0.07	5.19 ± 0.14	5.04 ± 0.15	
Δ mmol/L⁶	0.21 ± 0.11 [‡]	-0.04 ± 0.08	-0.13 ± 0.07 [‡]	-0.05 ± 0.10	0.046

¹ Values are reported as means ± SEM for all plasma concentrations except triglycerides. Triglycerides are median (IQR) due to lack of normality of the distribution. All baseline and post-intervention data is in mmol/L except for TC:HDL ratio. Significant change from baseline, * *p*<0.01, ** *p*<0.001.

² One-way ANOVA was used to compare change in outcome parameters across treatment groups. *P* < 0.05 indicates statistically significant difference between groups. Tukey's honestly significant difference post-hoc analyses were used to compare differences in mean change between groups when significance was found. Values with matching symbols in each row indicate statistically significant differences between corresponding groups.

³ TC significantly reduced in the PS-CC group compared to the PL (Δ mmol/L, $p=0.006$) and the CC group (Δ mmol/L, $p=0.011$).

⁴ LDL-C significantly reduced in the PS-CC group compared to the PL (Δ mmol/L, $p=0.010$) and CC group (Δ mmol/L, $p=0.012$).

⁵ TC:HDL ratio significantly reduced in the PS-CC group compared to the CC group (Δ mmol/L, $p=0.020$)

⁶ Glucose significantly reduced in the CC group compared to the PL group (Δ mmol/L, $p=0.038$).

BL, baseline; CC, curcumin; HDL, HDL-cholesterol; LDL-C, LDL-cholesterol; PI, post-intervention; PL, placebo; PS, phytosterols; PS-CC, phytosterol-curcumin; TC, total cholesterol; TC:HDL ratio, total cholesterol-to-HDL ratio; TG, triglycerides.

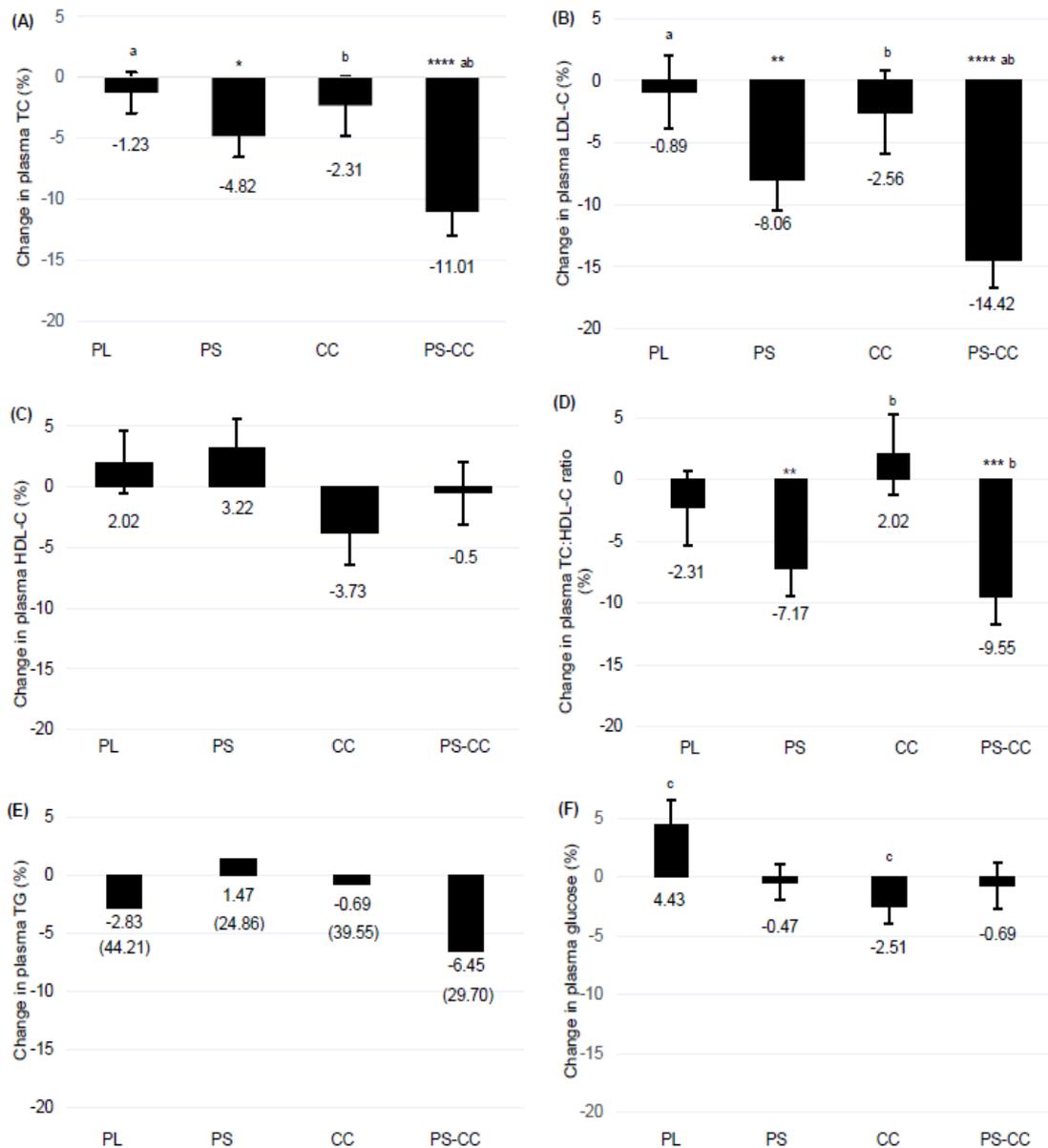


Figure 4.2: Percent change in plasma TC (A), LDL-C (B), HDL-C (C), TC:HDL ratio (D), TG (E) and Glucose (F) from baseline to post-intervention in hypercholesterolaemic individuals who consumed PL, PS, CC and PS-CC for 4 weeks.

Data represent mean \pm SEM except for TG where data are median (IQR). Symbols indicate significant changes from baseline as analysed by paired samples t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. One-way ANOVA and Tukey's honestly significant difference was used to perform post hoc comparisons of group means. Means with a common letter significantly differ: ^a PS-CC and PL significantly differ for fasting plasma TC ($p = 0.005$) and LDL-C ($p = 0.006$). ^b PS-CC and CC significantly differ for fasting plasma TC ($p = 0.015$), LDL-C ($p = 0.020$) and TC:HDL ratio ($p = 0.019$). ^c CC and PL significantly differ for fasting plasma glucose ($p = 0.036$).

CC, curcumin; HDL, HDL-cholesterol; LDL-C, LDL-cholesterol; PL, placebo; PS, phytosterols; PS-CC, phytosterol-curcumin; TC, total cholesterol; TC:HDL ratio, total cholesterol-to-HDL ratio; TG, triglycerides.

4.5 Discussion

The hypocholesterolaemic property of PS has been well established and recognised as an asset to CVD risk reduction. The potential to heighten their lipid-modulating property has been demonstrated by coupling with LCn-3PUFA [262, 300, 301] and statin therapy [203, 302-304]. The results presented in this study demonstrate the efficacy of curcumin to potentiate the cholesterol-lowering effects of PS in hypercholesterolaemic individuals. Our findings provide evidence of a complementary reduction in TC and LDL-C following concomitant supplementation with PS and curcumin. Although statistically insignificant, TC and LDL-C reduction following dual supplementation exceeded the additive effect of the two bioactives.

Reductions in TC and LDL-C following PS supplementation are consistent with previous studies of similar duration [94, 276, 305, 306]. A systematic review and meta-analysis of 84 randomised controlled trials (RCT) concluded that LDL-C reduced by -8.8% (vs -8.1% in our study) after a mean dose of 2.15 g/d of PS [94]. Higher baseline plasma LDL-C results in a greater reduction in plasma LDL-C [94, 307], however, in this study, covariate analysis showed that baseline LDL-C was not a predictor of reductions in TC or LDL-C.

In addition to competitive micellar incorporation [194], there are a number of reported mechanisms by which PS lower plasma cholesterol concentrations, some of which may be enhanced when combining PS with curcumin. Preclinical findings report PS may suppress acyl coenzyme A:cholesterol acyltransferase (ACAT) and therefore inhibit cholesterol esterification causing a reduction in cholesterol uptake and/or transport in the intestine [17]. A study in db/db mice showed that curcumin supplementation significantly lowered the activity of hepatic ACAT in addition to observed reductions in plasma concentrations of free fatty acids and cholesterol [128], suggesting curcumin may play a modulatory role in the activity of enzymes involved in lipid homeostasis. PS may also promote cholesterol efflux by initiating the expression and activity of ATP-binding cassette A1 (ABCA1) transporter which cannot differentiate between cholesterol and PS [96, 308].

Cholesterol transporters ABCA1, ABCG5 and ABCG8 are regulated by liver X receptor alpha (LXR α) and mediate efflux of free sterols from enterocytes. PS have been shown to be a potent activator of LXR in regards to agonising LXR [102, 309]. In this regard, PS metabolites have been identified as natural ligands for LXR [103]. Likewise, curcumin treatment has been shown

to upregulate the expression of LXR α in association with reduced TG accumulation in the liver [56]. It is in this way curcumin may potentiate the mechanistic hypocholesterolaemic effects of PS to interact with intracellular cholesterol sensors like LXR to indirectly modulate transporter activity as well as compete for cholesterol transporters [90].

Preclinical studies report several mechanisms by which curcumin acts as a lipid-modulating agent [56, 121, 133, 310]. Curcumin has been shown to down-regulate 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) [56], the rate limiting step in cholesterol biosynthesis. Curcumin therapy has been shown to lower plasma and hepatic cholesterol concentrations [56]. These effects were comparable to that of lovastatin treatment and were caused by transcriptional inhibition of HMG-CoA reductase. Moreover, preclinical studies have demonstrated decreased activity of hepatic ACAT after curcumin treatment in mice fed a high fat diet [127, 128], resulting in reduced cholesterol uptake and transport in the intestine [17]. In the intestinal Caco-2 monolayer, curcumin inhibited Niemann-Pick C1-Like 1 (NPC1L1) expression via the inhibition of sterol regulatory element binding protein-2 (SREBP-2) thus lowering cholesterol esterification [129]. Inhibition of HMG-CoA reductase and NPC1L1, the two key molecular targets of statins and ezetimibe respectively, characterises curcumin as a potentially powerful cholesterol absorption inhibitor, alluding to complementary effects when coupled with PS. Curcumin has also been shown to promote efflux and clearance of cholesterol by stimulating bile secretion via enhancing hepatic gene expression of cholesterol 7 α -hydroxylase (CYP7A) mRNA, the rate-limiting enzyme involved in bile acid biosynthesis from cholesterol [121, 133, 134]. CYP7A is further promoted by curcumin via the activation and upregulation of LXR and ABCA1 in the liver and subcutaneous adipocytes [135]. In addition, curcumin has been shown to decrease cholesterol biosynthesis via down-regulation of SREBP-1 and SREBP-2; key nuclear receptors involved in mediating lipid metabolism [125, 126].

Despite the promising findings from preclinical investigations, current clinical evidence remains scarce and somewhat inconsistent. A recent RCT by Panahi et al reported significant improvements in circulating blood lipids after supplementation with bioavailability-enhanced curcuminoids in metabolic syndrome individuals [115]. Curcuminoid supplementation led to significantly lower concentrations of TC, LDL-C, non-HDL-C, TG and lipoprotein(a) in addition to significant elevations in HDL-C [115]. These clinically significant trends in lipid parameters were not replicated in the CC group of the present study. It is likely that the study duration of the present trial was inadequate for curcumin to elicit significant hypolipidaemic responses. It should be noted that all subjects in Panahi et al [115] trial received standard care including administration of medications for lipid-lowering, hypotension and hyperglycaemia. Therefore, it is difficult to ascertain the precise modulatory effects of curcumin given the concurrent lifestyle intervention,

diet and pharmacological modifications. A recent systematic review and meta-analysis of five RCTs concluded curcumin did not elicit any significant effects on blood lipids in humans [122]. The included studies were largely heterogeneous; individuals were not exclusively dyslipidaemic and none of the studies used bioavailability-enhanced formulations of curcumin. These limitations possibly impeded any significant effects on blood lipids, particularly given the poor bioavailability of curcumin in native form. Since the degree of LDL-C-lowering achieved in the PS-CC group was nearly double that of the well-established reduction following PS therapy [94], complementary lipid-lowering mechanisms may be in play following the combined PS/curcumin supplementation.

Irrespective of the mechanisms, it is evident that curcumin may potentiate cholesterol-lowering effects of PS, since the PS-CC group had the greatest reduction in plasma cholesterol compared to either of the treatments alone. It is possible that in the combined group, bioavailability of curcumin is enhanced to heighten its hypolipidaemic effects as the bioavailability of curcumin has been shown to be enhanced in a fat-rich medium [311]. The short duration of the study remains a limitation, however, it is well documented that PS elicit hypocholesterolaemic effects within 3-4 weeks, less is known about the duration regarding the hypolipidaemic effects of curcumin. It is possible that a longer study duration may provoke more pronounced hypolipidaemic effects, since positive longer-term trials investigating the hypolipidaemic effects of curcumin have been reported [115, 122]. Participants in the current study were not specifically instructed to consume the vegetable fat spreads at the same time point as the tablets. Thus, some participants consumed the two products widely apart across the day. As previously mentioned, curcumin has enhanced bioavailability in a fat-rich medium, therefore, a single food matrix may likely further enhance the synergy between the two bioactive compounds, hence, the development of a single food containing the two compounds, provoking the translational aspect of these research findings. Strengths of this study include the robust study design pertaining 2x2 factorial group treatment to isolate the effects of each treatment arm as well as combined effects, in a double-blinded, placebo-controlled randomised manner in free-living individuals. We used a curcumin complexed with phosphatidylcholine (Meriva®) that ensured a high bioavailability of curcumin. This delivery matrix retains the biological effects and free-radical scavenging activity of curcumin [311]. The tablets and delivery of PS-enriched fat spread was easily compliant and transferable to the everyday diet (respectively) ensuring adequate intake of both active ingredients with no adverse effects.

The LDL-C reductions reported in the current study hold clinical significance for minimising heart disease risk since coronary heart disease (CHD) all-cause mortality is reduced by 12% for every 1 mmol/L reduction in LDL-C [312]. In the present study, LDL-C was lowered by 0.63

mmol/L representing approximately 7.6% reduction in CHD mortality after only four weeks of PS + curcumin intervention. Considering the known anti-inflammatory, anti-aggregatory and antioxidant effects [108] and safety of curcumin [147], the complementary and possibly synergistic cholesterol-lowering effects demonstrated makes the combined treatment a good candidate for a safe, effective alternative or adjunct therapy for CVD risk reduction that potentially bares a multifaceted approach. The combined PS-CC therapy bears significant implications for use as an adjunct therapy to statins and/or ezetimibe and potentially enhance lipid-lowering, reduce drug dependence and possibly reduce the dose required. The concomitant therapy of PS and statins has shown incremental reductions in LDL-C of 10-15%; a superior outcome compared to doubling the statin dose (6%) [201]. The use of PS-CC as an adjunct therapy may provide a safe and effective avenue for high-risk patients who fail to achieve LDL-C targets whilst on statin monotherapy or those who are statin intolerant and/or otherwise seeking an alternative approach to pharmacological interventions due to side effects such as chronic musculoskeletal pains [313].

In conclusion, dietary combination of PS and curcumin for four weeks significantly reduced fasting plasma TC and LDL-C in hypercholesterolaemic individuals. Compared to single supplementation of PS or curcumin, the combined group elicited a greater reduction than either treatment administered alone including a statistically greater reduction compared to PL and CC. Curcumin may potentiate the cholesterol-modulating effects of PS, suggestive of additive effects and potentially synergistic in nature. Further investigations exploring this combination are warranted to determine the optimum delivery mode and duration to maximise the cardio-protective properties of PS. Our findings initiate the need for future research exploring the potential mechanistic actions of curcumin to enhance our understanding of its role in lipid metabolism in humans and to confirm the cardio-protective benefits it may offer to PS therapy. The reason for a reduction in glucose level in the CC group but no change in the PS-CC group is not clear and should be followed up in further studies. In addition to cholesterol-lowering, concurrent PS and curcumin supplementation may exhibit anti-inflammatory effects, therefore, providing additional cardiometabolic benefits, however, investigation into circulating chronic inflammatory parameters following this combination therapy are required to confirm this hypothesis. Future investigations involve the development of a single food containing PS and curcumin for ease of consumption, improved compliance and bioavailability, and ultimately enhanced lipid-lowering in hypercholesterolaemic individuals at risk of CVD.

Chapter 5

Phytosterol bread with or without curcumin modulates lipoprotein profile in hypercholesterolaemic individuals. A randomized controlled trial.

The contents of this chapter have been submitted as a revised manuscript:

Ferguson JJA, Stojanovski E, MacDonald-Wicks L, Wolska A, Remaley AT, Garg ML. Phytosterol bread with or without curcumin modulates lipoprotein profile in hypercholesterolaemic individuals. A randomized controlled trial. *Food and Function*. 2019. Revised manuscript submitted on 25th January 2019. Impact factor 3.289.

Supplementary material presented in this chapter are found in Appendices 9-11.

5.1 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

5.2 Introduction

Cardiovascular disease (CVD) is the leading global burden of disease, claiming 31% of all deaths in 2016 [2]. Abnormal circulating blood lipids (dyslipidaemia) remains a key risk factor for CVD and is prevalent in over two thirds of the Australian adult population [14]. 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) [14]. A major concern is raised levels of small-dense LDL particles, as these are rich in TGs [17], reside longer in circulation [39] and are able to penetrate the arterial lumen where they are readily oxidized, thus increasing its atherogenic potential [47, 314]. 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 [24, 34]. 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 [315]. 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 [87, 198, 316]. 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 [94, 95, 317, 318]. PS are structurally similar to cholesterol, allowing their effective reduction of cholesterol absorption [319]. 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 [90].

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 [108]. 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 [56, 87, 125, 126], stimulation of the secretion and clearance of bile [133, 134] and reduction of lipid accumulation in adipose tissue [295, 296]. 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 [108] with the majority recovered in the

faeces [320]. 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 [112].

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 [318]. 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.

5.3 Materials and Methods

5.3.1 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 \geq 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 (\geq 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/m²; 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).

5.3.2 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-CC; 2.3g/d PS + 228mg/d curcumin) for four weeks. All four intervention breads were identical in sensory characteristics and bread bags were labelled with four colour-coded kwik locks upon packaging by the manufacturer; therefore, all investigators and participants were blinded to the treatment allocation. Breads were prepared and packaged by George Weston Foods under standard bread-making procedures and GMP conditions. The dough was baked at 220°C for 30 minutes and proofed for approximately 40-50 minutes. Basic ingredients included in the bread were plain white flour, yeast, improver, softener, water and salt. The nutritional profile of the bread was similar to the commercially available white sandwich bread and all breads were comparable for nutritional content except for the enrichment of PS, curcumin and respective placebo ingredients (Table 5.1). For fortification of the bread, Vegapure® 67WDP (BASF) is a mixture of plant sterol fatty acid esters spray dried into a water dispersible powder and was the source of PS. The carrier system consists of sodium caseinate, glucose syrup and antioxidants such as tocopherols and ascorbate. Vegapure® 67WDP is intended for use in dietary supplements and food applications. Meriva® (Indena®) powder is a patented PHYTOSOME® curcumin formulation and was the source of curcumin. In Meriva®, curcumin and soy lecithin are formulated in a 1:2 weight ratio with the addition of microcrystalline cellulose to improve flowability. Canola oil was used as a placebo for PS in the PL and CC bread since canola fat-based products have been used previously as a placebo to PS, demonstrating minimal (if any) non-significant lowering of blood cholesterol [240, 318]. Lucarotin 1 CWD/Y (BASF) and apo carotenal 2% (BASF) was used to dye the PL and PS breads. Participants were instructed to consume one serving of study bread i.e. two slices of bread each day at lunch time as part of a meal for the entirety of the study duration. They were instructed not to change their diet and lifestyle habits and they were permitted to eat other bread if desired. Compliance was monitored by a daily bread consumption log 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).

5.3.3 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) [42].

Table 5.1: 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
<i>For Phytosterol bread:</i>	
Phytosterols (g)	2.3
<i>For Curcumin bread:</i>	
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.

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

5.3.5 Medical history, dietary intake and physical activity

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

5.3.6 Blood sampling, lipid analyses and plasma LDL profile

A phlebotomist collected fasted blood samples at baseline and post-intervention via venepuncture into tubes pre-coated with EDTA. Samples were centrifuged (Heraeus Biofuge Stratos) for 10 minutes at 3000 x g at 4°C. Plasma and red blood cell fractions were aliquoted and stored at -80°C

until further analysis. Blood parameters were measured on a VP auto analyser using standardized reagents by the Hunter Area Pathology Service. LDL-C concentration was determined using the Friedewald equation [191]. 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 [33]. 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.

5.3.7 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 \pm standard error of the mean (SEM) or median (25th percentile, 75th 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.

5.4 Results

5.4.1 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 5.1). 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/m², 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 [321], 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 5.2 and 5.3). As there was no significant difference in blood lipids in feeding PS in the presence or absence of curcumin (Supplementary Table 1), the data for PL and CC groups

was pooled (PL-C, n=36) and data for the PS and PS-CC groups was also pooled (PS-C, n=39) for subsequent analysis (Figure 5.1).

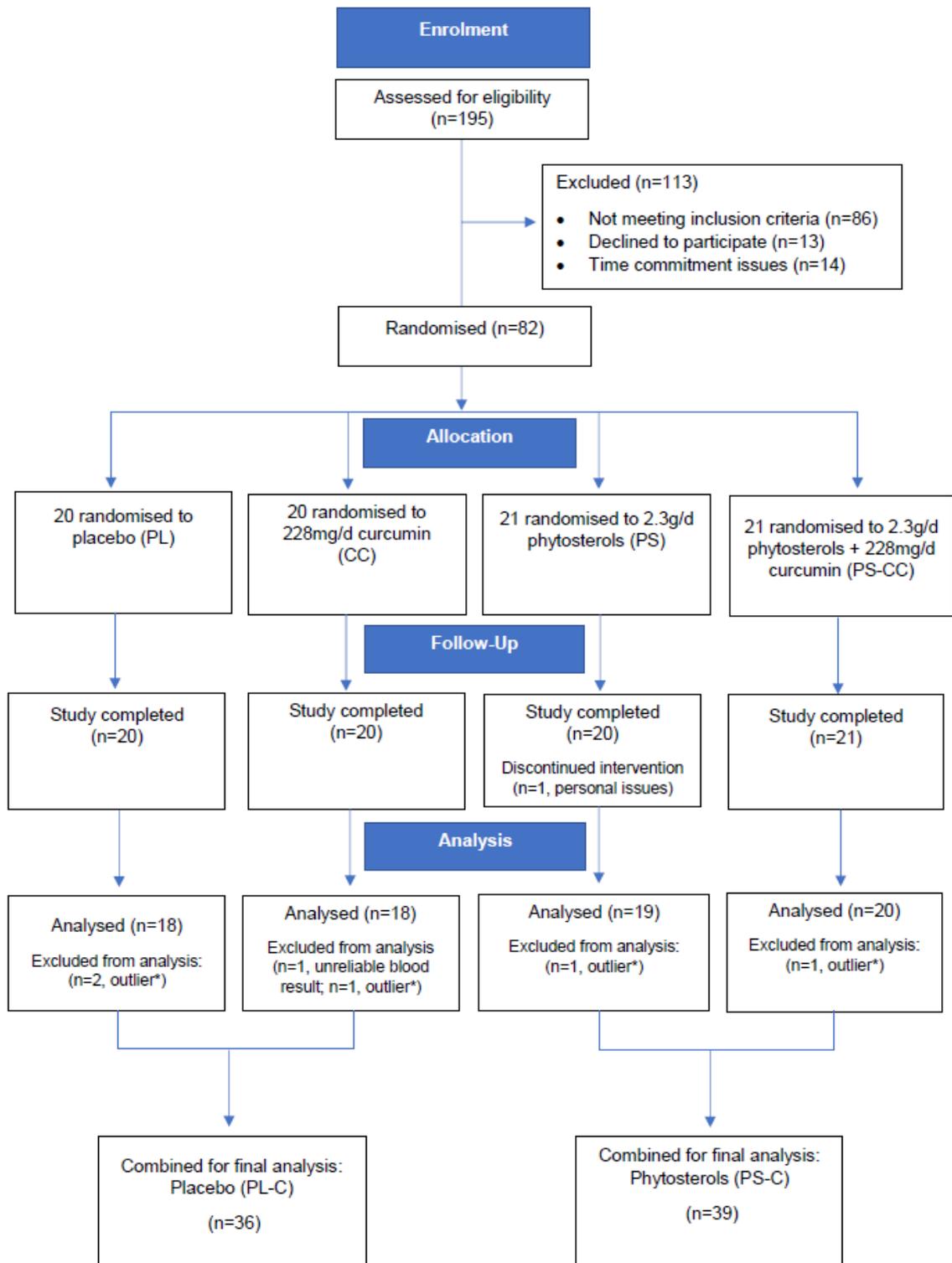


Figure 5.1: CONSORT schematic of participant recruitment, screening and assessment.

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

Table 5.2: 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)
Other²	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/m²)	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.

Table 5.3: 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)	<i>p</i> ^
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.

5.4.2 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 2) 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%).

5.4.3 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 5.3, Figure 5.2). 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 5.4). 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.

5.4.4 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 ($p<0.01$).

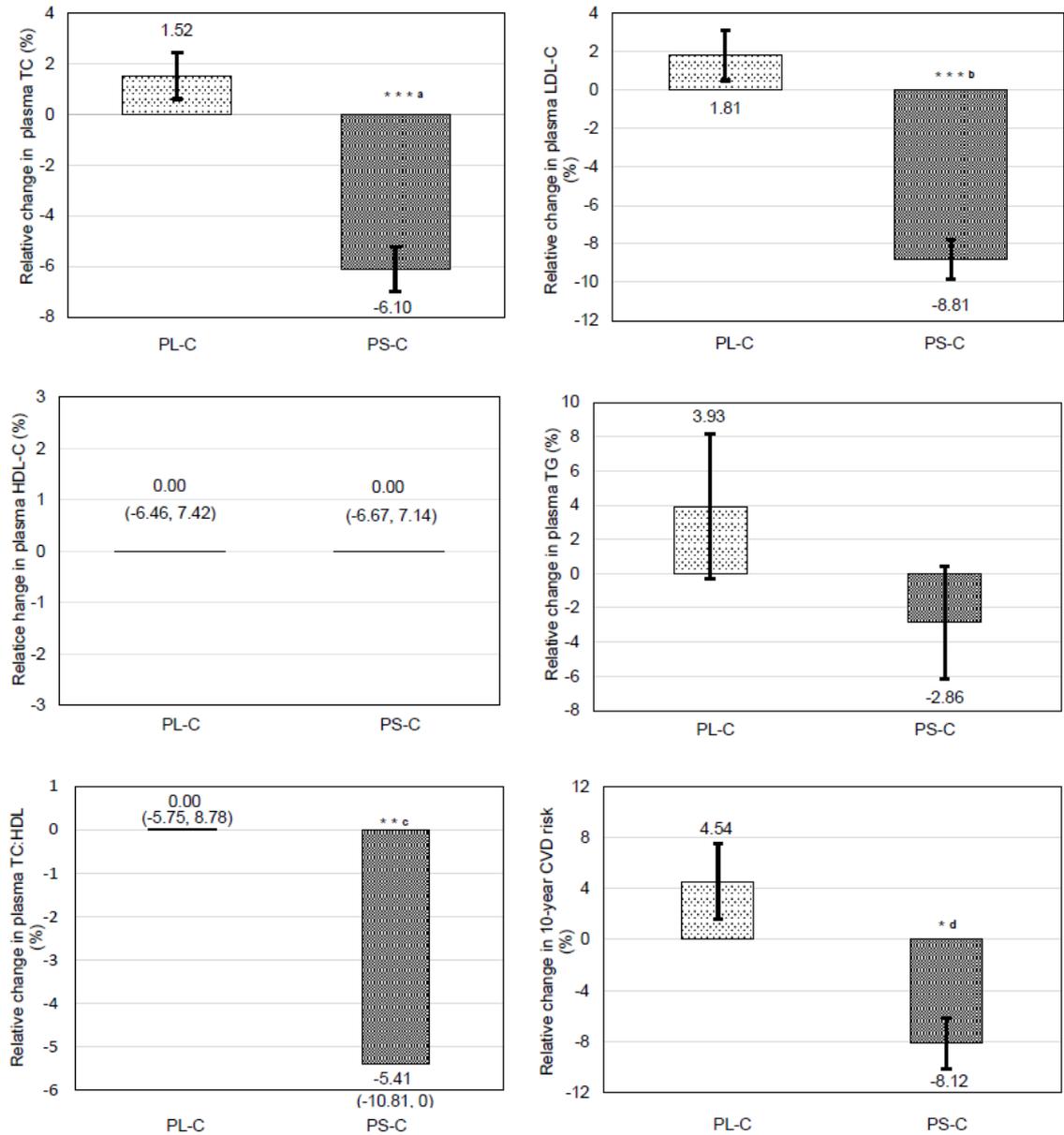


Figure 5.2: Percent change in TC, LDL-C, HDL-C, TC:HDL ratio, TG and 10-year CVD risk from baseline to post-intervention in hypercholesterolaemic individuals who consumed PL-C or PS-C for 4 weeks.

Data represent mean \pm SEM or median (25th and 75th 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.

Table 5.4: Lipoprotein profiles (total particle concentration and particle size) at baseline and post-intervention measured using nuclear magnetic resonance spectroscopy.

	PL-C	PS-C	<i>p</i> ^
<i>Total lipoprotein particle concentration</i>			
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
<i>Lipoprotein particle size (nm)</i>			
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 \pm 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.

5.5 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 [94]. 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 [318], 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 [94, 276], 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) [245]; 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 [94]. 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 [322] 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 [90], 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 [128, 323]. In addition, PS and curcumin have been reported to potentially activate and upregulate (respectively) expression of liver X receptor alpha (LXR α), a key regulator of cholesterol transporters (ABCA1, ABCG5 and ABCG8) and mediator

for free sterol efflux from enterocytes [56, 102, 309]. 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) [56]; reduced cholesterol esterification via indirect inhibition of Niemann Pick C1-Like 1 (NPC1L1) expression [129]; increased LDL-C uptake, receptor level and activity via down-regulation of proprotein convertase subtilisin /kexin type 9 (PCSK9) gene expression [136]; and stimulation of bile secretion via enhanced gene expression of CYP7A mRNA [133, 134].

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 [318]. 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 [311]. In this formulation, curcumin is on the surface of the phytosome and possibly less protected and unstable at higher temperatures (170 °C), 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 °C, to avoid thermal decomposition [324]. 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 (SLCPTM) formulation where curcumin is encapsulated into a lipid core, would be more robust for future food preparations involving heat exposure [119].

In recent years, increased attention has been drawn to measuring the number and size of LDL-P as these are more predictive of CVD risk than LDL-C concentrations [24, 325]. It has been shown in the Framingham offspring study [27] and Multi-Ethnic Study of Atherosclerosis (MESA) [34] that LDL-C and LDL-P are equally associated with coronary artery disease risk when LDL-C and LDL-P were concordant, however, if discordant (occurs in patients with metabolic syndrome and type 2 diabetes); the LDL-attributable atherosclerotic risk is better indicated by LDL-P. In the present study, individuals not only had elevated LDL-C concentrations, but they also had a high LDL-P level of ~1910 nmol/L (1600-2000 nmol/L); indicating a higher risk of CVD [34, 321]. LDL-P was significantly reduced following PS supplementation in our study and to the best of our knowledge there is only one other study which has measured LDL-P via NMR following PS supplementation, and they reported similar findings [326]. In this study, Matvienko et al reported

a borderline significant reduction in particle number ($p=0.058$) following consumption of ground beef enriched with 2.7 g/day PS in young mildly hypercholesterolaemic males [326]. Participants did, however, have a much lower baseline LDL-C (4.02 mmol/L) compared to our participants (4.55 mmol/L), which we have showed predicts change in LDL-P number. Moreover, their LDL-P at follow-up was much lower (1467 nmol/L, borderline-high risk) than ours (1863 nmol/L, high risk), suggesting they had a lower baseline LDL-P (baseline LDL-P was not reported) [326]. The amount of cholesterol per LDL-P varies depending on the size, with smaller LDL-P carrying less. Increased atherogenic potential of small LDL-P is suggested by increased binding ability to arterial proteoglycans [18], greater propensity for uptake by arterial tissue suggestive of enhanced transendothelial transport [19], reduced receptor-mediated uptake [20], and increased susceptibility to oxidative modification [21, 22]. Overall findings are inconsistent with respect to particle size modulation by PS, with studies reporting reductions in the concentration of small LDL-P [327] and studies reporting no effect on subclass concentrations [246] or shifts in subclass size [326]. 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 [33, 325], 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 [328]. The longest trial duration administering PS is 85 weeks [329], 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 [314]. There is some evidence for anti-inflammatory and immune-modulatory effects of PS in preclinical [330] and human studies [331, 332], however, the consensus on this remains inconsistent [333]. 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 [36, 109], 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 [328] and curcumin [334]; 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.

5.6 Conclusions

Findings from this study provides evidence for the use of bread as a safe, efficacious and compliant 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 [108] and mild hypoglycaemic effects [318, 335]. 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.

Chapter 6

High molecular weight oat β -glucan enhances lipid-lowering effects of phytosterols. A randomized controlled trial

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Statement of authorship is attached as Appendix 14

6.1 Abstract

Background & Aims: Oat β -glucan (OBG) and phytosterols (PS) are known to lower blood cholesterol levels via different mechanisms. Combination of high molecular weight (MW) OBG and PS in a single functional food could have complementary and/or synergistic effects for optimising heart health. The aim of this study was to investigate the effects of dietary supplementation with high-MW OBG with or without PS on plasma lipids in hypercholesterolaemic individuals.

Methods: In a double-blinded, placebo-controlled, 2x2 factorial trial, participants were randomised to receive biscuits fortified with either no PS or OBG (PL, n=18) or 2g PS (PS, n=18), 3g OBG (OBG, n=18), or combination of 2g PS and 3g OBG (PS-OBG, n=18) per day for 6 weeks. Primary outcome was fasting plasma total cholesterol (TC) and secondary outcomes were LDL-cholesterol, LDL-C; HDL-cholesterol, HDL-C; triglycerides, TG and TC to HDL-cholesterol (TC:HDL) ratio.

Results: TC and LDL-C were significantly lowered following PS (-4.6% and -7.6% respectively; $p<0.05$), OBG (-5.7% and -8.6%; $p<0.01$) and PS-OBG (-11.5% and -13.9%; $p<0.0001$) administration. The reduction in TC in the PS-OBG group was significantly greater compared to PL ($p<0.001$) and PS ($p<0.05$). PS-OBG group had a significantly greater reduction in LDL-C compared to PL ($p<0.01$) but not in comparison to PS or OBG groups. TC:HDL ratio was significantly reduced following PS-OBG (-8.9%; $p<0.01$) only, and there was no significant difference found between groups. Plasma TG reduced by 8.4% following PS-OBG, however, this was statistically non-significant. Plasma HDL-C remained unchanged across all groups.

Conclusions: Dietary supplementation with high-MW OBG and PS in a single functional food enhances their lipid-lowering potential. Blood cholesterol lowering by PS and OBG is additive. Delivery of these two bioactive nutrients in a single food allows optimisation of their lipid-lowering effects and may provide added heart health benefits with enhanced compliance.

Keywords: total cholesterol, LDL cholesterol, triglyceride, phytosterols, β -glucan

6.2 Introduction

Dyslipidaemia is prevalent in over 63% of the Australian adult population and remains a key modifiable risk factor for cardiovascular disease (CVD) [14]. One or more lipid abnormalities such as elevated total cholesterol (TC) or LDL-cholesterol (LDL-C) or triglycerides (TG) or reduced HDL-cholesterol (HDL-C) or a combination of two or more of these lipid profiles comprise dyslipidaemia [14]. A suite of diet and lifestyle changes are used for the management of dyslipidaemia and have been shown to modestly lower LDL-C. Long-term adherence, complexity of adopted diet/lifestyle changes, poor patient motivation, lack of clinical follow-up and food aversions can impede the ability to achieve and sustain target blood lipid levels [336]. Subsequently, pharmacological interventions are often indicated, however, cost, adverse effects and intolerance, lack of effectiveness, patient perceived concern of long-term side effects and complex drug regimens are barriers for long-term compliance [315]. Consequently, nutraceuticals such as phytosterols (PS) and soluble fibres (along with other bioactives) have been recognised as adjunct and/or alternative lipid-modulating therapies for optimising dyslipidaemia control as they are safe, effective and easily compliant for individuals with dyslipidaemia [87, 198, 316].

On average, 25-30g of dietary fibre is recommended for adults for optimal heart and gut health [151]. The LDL-C lowering effects of dietary fibre are primarily attributed by the viscous (soluble) fibres such as those found in oat products. The ATP III recommends a multifaceted lifestyle approach including regular consumption of dietary fibres involving 10-25g/d derived from soluble fibres [316]. Oat β -glucan (OBG) is the main soluble fibre found in oats comprising linear glucose polymers with mixed $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ linkages [154]. Consumption of ≥ 3 g/d OBG has been shown to lower TC and LDL-C concentrations with no significant changes in HDL-C or TG [161]. The key physiological property of OBG is viscosity and solubility which is determined by its molecular weight (MW) [154, 159]. High MW OBG has greater ability to increase viscosity of upper digestive tract contents and therefore influence bile acid metabolism; reduce intestinal bile acid reabsorption [158, 337] and increase bile acid synthesis [176], resulting in lower circulating cholesterol concentrations. There are discrepancies in the literature around the cholesterol-lowering ability of oat products pertaining to OBG, as many studies do not report the MW of the β -glucan used and external factors such as processing and storage can also alter this property [160].

PS are naturally found in a variety of plant-based foods such as fruit, nuts, seeds, vegetables and vegetable/nut oils. It has been established that 2g/d of PS from enriched products lowers circulating LDL-C by 8-10% in four weeks with no effects on HDL-C and no or modest TG lowering effects [94, 95, 317, 318]. The most widely accepted mechanism of action by PS for

cholesterol-lowering is micellar displacement of dietary and biliary cholesterol in the gut, leading to reduced cholesterol absorption and subsequent excretion [90].

Since the mechanism by which PS and OBG lower non-HDL cholesterol are different, their combination in an enriched food has the potential to produce complementary and/or synergistic reductions in LDL-C concentrations. Therefore, the primary aim of this study is to investigate the effects of concurrent consumption of PS and high-MW OBG delivered as a snack on fasting blood lipid profile in hypercholesterolaemic individuals.

6.3 Materials and Methods

6.3.1 Recruitment

Participants with hypercholesterolaemia were recruited from the Hunter region (NSW, Australia) via notice board flyers placed in the local community, word of mouth, newspaper articles and subjects who participated in earlier studies at our department were also invited to participate. Volunteers were assessed for eligibility by the lead investigator (JF) over the phone or in person and were deemed eligible if they 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 conditions, neurological conditions or untreated hypertension ($\geq 140/95$ mm Hg); not consuming PS- or OBG-enriched products or any other supplements known to influence the primary outcome (e.g. fish/krill/flaxseed oils, coenzyme Q10, fibre supplements); BMI < 40 kg/m²; not pregnant or lactating; non-smoker and no strong food aversion and/or intolerance or allergy to gluten or wheat. Eligible volunteers were provided with a detailed description of the study and written informed consent was mandatory for enrolment in the study. Participants were de-identified and assigned numeric codes. The study protocol was approved by the Human Research Ethics Committee, University of Newcastle (H-2017-0091) 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/> (ACTRN12618001455257).

6.3.2 Study design

This study was a six-week, double-blinded, randomised, placebo-controlled trial with a 2x2 factorial design in four parallel groups. The senior investigator (MG) allocated treatment groups using a computer-generated block randomisation method and participants were stratified by gender (Random Allocation Software version 1.0.0). Participants were de-identified and assigned numeric codes. Food product packets and storage boxes were labelled with colour-coded stickers

upon packaging by the manufacturer and therefore the treatment allocation could not be ascertained by the investigators nor the participants.

Participants were randomly allocated to one of four treatments for six weeks: eight small sweet biscuits/d containing either placebo (PL; no PS or OBG), phytosterol (PS; 2g/d PS), OBG (OBG; 3g/d OBG) or a combination of PS and OBG (PS-OBG; 2g/d PS + 3g/d OBG). All treatment biscuits were identical in sensory characteristics. Manufacturing and packaging of the biscuits were conducted by Sweethings Pasticceria Wholesale Bakery under GMP conditions. The biscuit dough was prepared and mixed in an automated planetary mixer for 5 minutes. Combined dough was placed in an automatic dropping machine (Maxidrop Plus, W&P Reedy) with customised dropping speed, conveyor height and wire-cutting speed to shape dough into uniform biscuits of identical size, shape and weight. The biscuits were baked in a commercial double rack fan-forced oven (Revent 620) at 170°C for 15 minutes. Each daily serve of biscuits contained 30g of fat derived from a vegetable fat spread. For the PS and PS-OBG biscuits, a commercially available PS-enriched vegetable fat spread (Logicol Original) was used and a vegetable fat spread equivalent in nutritional profile but devoid of PS (MeadowLea Gold'n Canola) was used for the PL and OBG biscuits. The primary source of PS esters used in Australian PS-enriched products is derived from soybean oil or tall (pine) oil obtained from trees during pulping [198]. The OBG and PS-OBG biscuits were enriched with 14 g of high-MW (2000-2500 kDa) [162] oat bran derived from Scandinavian oats (SWEOAT, Prorsum Healthcare AB, Sweden), providing 3g OBG per daily serve of biscuits. The energy content, carbohydrate, sugar, total fat and polyunsaturated, monounsaturated, and saturated fat content of the four experimental products were comparable (Table 6.1). Overall the biscuits were low in sugar (~10g per serve) and in addition to the test and placebo ingredients, they also contained wholemeal plain flour; vanilla extract and pastrycooks chocolate paste (flavourings); icing sugar and corn flour. The PL and PS biscuits contained 100% whey protein isolate powder as a source of protein, since protein in the OBG and PS-OBG biscuits was derived from the oat bran powder. Participants were instructed to replace one in-between meal (snack) with the study biscuits, all to be consumed at one time. To enhance viscosity of β -glucan, participants were encouraged to consume the biscuits with at least 250 mL fluid. To avoid weight gain, participants were instructed to consume the study biscuits instead of other foods at one snack time each day. Compliance was monitored by evaluation of the biscuit consumption log, biscuit packet count and analysis of habitual dietary intake pre- and post-intervention (analysed with FoodWorks, Xyris®, Professional Edition Version 8.0.3551).

Table 6.1: Nutrient composition of study biscuits and compliance.¹

Dietary component	PL	PS	OBG	PS-OBG
Energy (kJ)	1490.6	1567.1	1454.8	1531.3
Protein (g)	7.1	7	4.9	4.8
CHO (g)	33.1	33.1	30.0	30.0
Sugars (g)	9.9	9.9	9.7	9.7
Starch (g)	22.8	22.8	19.8	19.8
Total fat (g)	21.4	23.5	21.7	23.8
Saturated (g)	4.9	5.1	4.9	5.1
MUFAs (g)	10.3	11.1	10.1	11.0
PUFAs (g)	5.1	5.9	5.0	5.8
Phytosterols (g)	0	2.0	0	2.0
Oat β-glucan (g)	0	0	3.0	3.0
Dietary fibre (g)	2.9	2.9	8.0	8.0
Sodium (mg)	118.8	115.8	109.2	106.2
Compliance (%)	98.81 \pm 0.58	99.87 \pm 0.13 ^a	98.15 \pm 0.65	96.14 \pm 1.33 ^a

¹ Nutrient information is given for one serve (8 biscuits, ~80 g). Each participant had to consume one serving of biscuits per day. One-way ANOVA was used to compare compliance. Post-hoc analyses were used to compare differences between groups when significance was found. Values with common superscript in each row indicate statistically significant differences between corresponding groups, $p < 0.01$.

OBG, oat β -glucan; PL, placebo; PS, phytosterols; PS-OBG, phytosterol + oat β -glucan

6.3.3 Clinical assessments

Participants attended clinical trial facility of the Nutraceuticals Research Program, 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 and TG.

6.3.4 Anthropometry and body composition

Anthropometrics (height, weight, waist circumference, BMI) and body composition in all participants were measured wearing light clothing and participants were asked to remove shoes and all metal and/or electronic devices on their body for all measurements. Height (cm), waist circumference (cm) and weight (kg) were collected to the nearest 0.1 units. Height was measured using a wall-mounted stadiometer with a movable head piece (Seca 206 Bodymeter Wall Height Measure Ruler). Waist circumference was measured using a tensible tape measure positioned midway between the lower rib margin and the iliac crest (approximately in line with the belly-button) horizontally. BMI was calculated as $\text{weight}/\text{height}^2$ (kg/m^2). Weight and other body composition parameters (skeletal muscle mass, fat mass, total body water etc) were measured using bioelectrical impedance utilising two different frequencies (InBody230, Biospace Co.). Body composition was taken in the standing position following a ~10 hour fast and participants refrained from vigorous physical activity and alcohol consumption 24 hours prior to their appointments.

6.3.5 Medical history, dietary intake and physical activity

A self-administered medical history questionnaire was completed by all participants at baseline to collect information regarding past and present medical conditions; history of blood lipid profile, prescribed or over-the-counter medication(s), habitual supplement use and habitual consumption of alcohol, PS-enriched products, fibre, added sugars, fats and oils. Habitual diet and physical activity patterns at baseline and post-intervention were assessed by a 3-day food diary and physical activity questionnaire (International Physical Activity Questionnaire; IPAQ Long Last 7 Days Self-Administered Format, October 2002), respectively. Dietary data was evaluated using FoodWorks, Xyris. Professional Edition Version 8.0.3551. Physical activity data was interpreted as metabolic equivalent of task minutes per week (MET/week) to measure the energy cost of physical activities.

6.3.6 Blood sampling and serum lipid analyses

Fasted blood samples were collected at baseline and post-intervention via venepuncture into tubes pre-coated with EDTA by an experienced phlebotomist. Samples were centrifuged (Heraeus Biofuge Stratos) for 10 minutes at $3000 \times g$ at 4°C . Plasma and red blood cell fractions were aliquoted and stored at -80°C until further analysis. Blood parameters were measured on a VP auto analyser using standardized reagents by the Hunter Area Pathology Service. LDL-C concentration was determined using the Friedewald equation [191].

6.3.7 Statistical analysis

Statistical analysis was conducted using StataCorp 2015 (*Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP). All data are presented as means \pm SEM (standard error of the mean) except for baseline and post-intervention TG values which are presented as median (IQR). The significance level for all statistical tests was set at 0.05. 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 0.05 significance level for detection of a 0.50 mmol/L (~10%) reduction in TC whilst accounting for a 20% dropout rate. The Bonferroni correction was applied to account for the fact that there were multiple secondary outcome variables tested (LDL-C, HDL-C, TG, TC:HDL ratio) and the significance level was reduced accordingly. Normality was assessed via the Shapiro Wilk test and visual plots including histograms. Comparison of baseline characteristics across treatment groups for age, height, weight, BMI, 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. The chi-square test was used to compare gender and ethnicity between groups at baseline. Depending on the data being normally distributed, paired samples t-test or Wilcoxon Signed Rank test were performed for change from baseline to post-intervention within-treatment groups. One-way ANOVA was used to investigate the effect of each treatment on the absolute and percent change from baseline to post-intervention on the dependent variables between treatment groups. Two-way ANOVA was used to investigate whether there was a significant main effect for each treatment group (PS, OBG). An interaction effect between the two treatments [PS x OBG] was also tested to investigate their effect on the dependent variables. For any significant effects, Tukey's Honestly Significant Difference (HSD) was used to perform post hoc comparisons to test for complementarity and/or synergy between PS and OBG. Analysis of covariance was also used for each outcome variable (absolute and percent change in TC and LDL-C) with the inclusion of treatment group as a factor and the corresponding baseline values of the outcome variable as a covariate. Any explanatory variables which were statistically significantly related with the outcome variables from bivariate analyses were included in the model as additional covariates. The covariates considered included age and baseline data for: BMI, waist-to-hip ratio, fat mass, systolic blood pressure, exercise levels, dietary intake (energy, carbohydrates, trans fat, omega-6 polyunsaturated fatty acids, monounsaturated fatty acids, fibre, alcohol). 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. A backward stepwise regression model selection procedure was employed for each model to select the optimal set of predictors for each outcome variable.

6.4 Results

6.4.1 Baseline characteristics

Eighty participants were recruited during the period end-September 2017 to mid-August 2018. Five participants dropped out of the trial due to inability to comply with biscuit consumption (n=1), illness (n=2) and personal reasons (n=2). A further three participants were excluded from the trial due to poor compliance (n=1) and significant outliers to the data set (data for change in LDL-C greater than two standard deviations from the mean) (n=2). A total of 72 participants were included in the final analysis, resulting in eighteen participants in each group (Figure 6.1). Most participants were females (63%) and north-west European (78%) with a mean age of 55.07 ± 1.41 y, waist circumference of 92.42 ± 1.32 cm, waist-to-hip-ratio of 0.94 ± 0.01 , weight of 76.49 ± 1.79 kg, BMI of 26.79 ± 0.46 kg/m², skeletal muscle mass of 28.90 ± 0.94 kg and fat percentage of 32.20 ± 1.03 %. Participants were hypercholesterolaemic at baseline with TC 6.57 ± 0.11 mmol/L, LDL-C 4.39 ± 0.09 mmol/L, HDL-C 1.54 ± 0.04 mmol/L, TC:HDL ratio 4.47 ± 0.12 mmol/L and median (IQR) TG of 1.22 (0.82) mmol/L. Following randomisation, anthropometric measures (weight, waist circumference, BMI, fat mass) were significantly higher in PL group compared to PS-OBG group ($p < 0.05$). The effects of these baseline variables were explored further by adjusting for them by their inclusion in the multiple regression analyses. Groups were otherwise comparable for all other baseline characteristics with no other significant differences detected between treatment groups at baseline (Table 6.2 and Table 6.3). Weight significantly increased from baseline in the PL (0.63 ± 0.26 kg), PS (0.57 ± 0.24 kg) and PS-OBG (0.53 ± 0.14 kg) groups and BMI significantly increased in the PL (0.24 ± 0.08 kg/m²) and PS-OBG (0.19 ± 0.05 kg/m²) groups ($p < 0.05$). All other anthropometric measures and blood pressure did not significantly change within groups. Changes in anthropometric measures and blood pressure were not statistically significant between groups (data not shown).

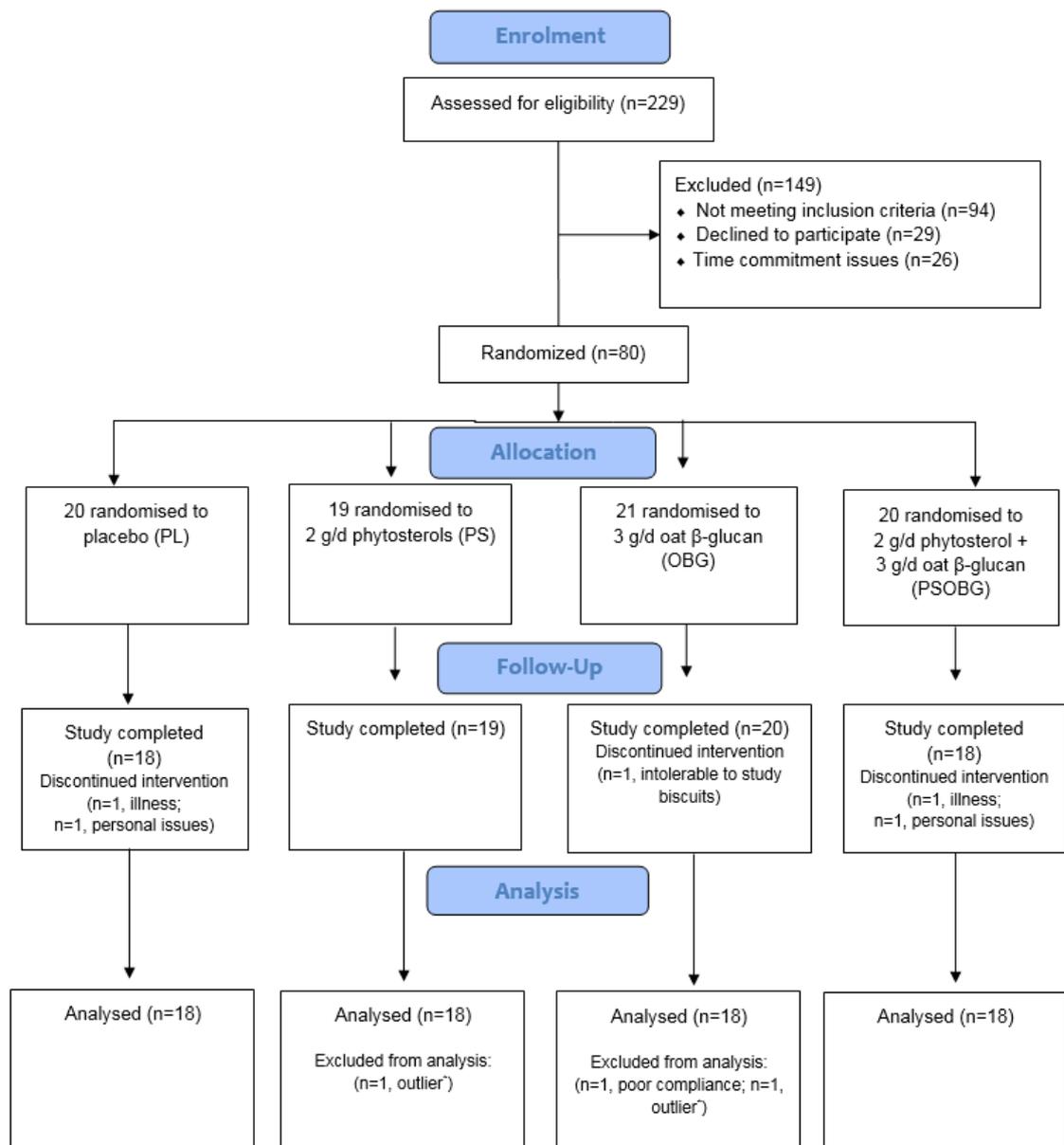


Figure 6.1: CONSORT schematic of participant recruitment, screening and assessment.

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

Table 6.2: Participant characteristics at baseline in the placebo (PL), phytosterol (PS), oat β -glucan (OBG) and phytosterol + oat β -glucan (PS-OBG) groups.¹

	PL (n = 18)	PS (n = 18)	OBG (n = 18)	PS-OBG (n = 18)
Sex, n (%)				
Male	8 (44)	6 (33)	8 (44)	5 (28)
Female	10 (56)	12 (67)	10 (56)	13 (72)
Ethnicity, n (%)				
North-west European	14 (78)	15 (83)	15 (83)	12 (67)
South-east European	1 (6)	1 (6)	0 (0)	0 (0)
Asian	0 (0)	0 (0)	2 (11)	4 (22)
Other²	3 (17)	2 (11)	1 (6)	2 (11)
Age (y)	54.78 \pm 2.81	54.67 \pm 2.77	56.39 \pm 2.88	54.44 \pm 2.99
Height (cm)	171.28 \pm 2.03	166.92 \pm 2.49	168.59 \pm 2.65	166.85 \pm 2.70
Waist circumference (cm)	98.48 \pm 2.81 ^a	89.32 \pm 2.45	94.86 \pm 2.06	87.00 \pm 2.45 ^a
Waist-to-hip ratio	0.95 \pm 0.01	0.94 \pm 0.01	0.95 \pm 0.01	0.93 \pm 0.02
Weight (kg)	83.94 \pm 3.66 ^a	73.11 \pm 3.62	79.48 \pm 3.02	69.44 \pm 3.28 ^a
BMI (kg/m²)	28.49 \pm 1.04 ^a	26.01 \pm 0.76	27.81 \pm 0.67	24.84 \pm 0.93 ^a
Skeletal muscle mass (kg)	30.68 \pm 1.87	28.28 \pm 1.95	29.93 \pm 1.89	26.71 \pm 1.78
Body fat mass (g)	28.91 \pm 2.33 ^a	22.22 \pm 1.49	25.86 \pm 1.62	21.12 \pm 2.01 ^a
Body fat (%)	34.49 \pm 2.19	30.90 \pm 1.90	33.02 \pm 2.00	30.42 \pm 2.15
SBP (mmHg)	124.97 \pm 3.56	119.58 \pm 3.23	121.69 \pm 3.24	121.61 \pm 3.28
DBP (mmHg)	80.56 \pm 2.48	74.33 \pm 2.08	75.83 \pm 2.14	75.39 \pm 1.52
MET (mins/wk)	5188 \pm 1538	3851 \pm 740	3260 \pm 712	3109 \pm 717

¹ Values are reported as means \pm SEM for continuous measures and as n (%) for categorical measures.

² Other races include Oceanian; North African and Middle Eastern; Other (combination of races).

One-way ANOVA was used to compare baseline data for normally distributed data and Kruskal-Wallis for non-normally distributed data. Post-hoc analyses were used to compare differences in baseline data between groups when significance was found. Values with common superscript in each row indicate statistically significant differences between corresponding groups, $p < 0.05$.

DBP, diastolic blood pressure; MET, metabolic equivalent; OBG, oat β -glucan; PL, placebo; PS, phytosterols; PS-OBG, phytosterol + oat β -glucan; SBP, systolic blood pressure

Table 6.3: Change in plasma outcome measures in the placebo (PL), phytosterol (PS), oat β -glucan (OBG) and phytosterol + oat β -glucan (PS-OBG) groups from baseline to post-intervention.¹

	PL	PS	OBG	PS-OBG	p^{\wedge}
TC					
BL	6.34 \pm 0.19	6.31 \pm 0.22	6.91 \pm 0.24	6.71 \pm 0.20	
PI	6.36 \pm 0.23	5.98 \pm 0.16**	6.49 \pm 0.23**	5.91 \pm 0.16**	
Δ mmol/L²	0.02 \pm 0.13 ^a	-0.33 \pm 0.11 ^b	-0.41 \pm 0.12	-0.80 \pm 0.13 ^{ab}	<0.001
LDL-C					
BL	4.22 \pm 0.16	4.25 \pm 0.16	4.67 \pm 0.21	4.42 \pm 0.21	
PI	4.17 \pm 0.19	3.89 \pm 0.11**	4.24 \pm 0.20**	3.76 \pm 0.14**	
Δ mmol/L³	-0.05 \pm 0.12 ^a	-0.37 \pm 0.12	-0.42 \pm 0.11	-0.66 \pm 0.13 ^a	0.006
HDL-C					
BL	1.56 \pm 0.09	1.54 \pm 0.11	1.48 \pm 0.06	1.57 \pm 0.09	
PI	1.60 \pm 0.11	1.55 \pm 0.13	1.46 \pm 0.06	1.53 \pm 0.10	
Δ mmol/L	0.04 \pm 0.04	0.01 \pm 0.03	-0.03 \pm 0.03	-0.03 \pm 0.03	0.319
TC:HDL					
BL	4.27 \pm 0.24	4.29 \pm 0.20	4.84 \pm 0.29	4.47 \pm 0.24	
PI	4.16 \pm 0.22	4.16 \pm 0.23	4.63 \pm 0.30	4.05 \pm 0.22**	
Δ	-0.11 \pm 0.12	-0.13 \pm 0.11	-0.21 \pm 0.09	-0.42 \pm 0.10	0.140
TG					
BL	1.13 (0.73)	1.05 (0.58)	1.38 (1.1)	1.29 (0.79)	
PI	1.15 (0.93)	1.16 (0.44)	1.36 (1.23)	1.19 (0.86)	
Δ mmol/L	0.05 \pm 0.06	0.05 \pm 0.05	0.08 \pm 0.17	-0.24 \pm 0.14	0.098

¹ Values are reported as means \pm SEM for all plasma concentrations except triglycerides. Baseline and post-intervention triglyceride values are median (IQR) 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.

[^] One-way ANOVA was used to compare change in outcome parameters across treatment groups. P < 0.05 indicates statistically significant difference between 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. Values with common superscripts in each row indicate statistically significant differences between corresponding groups.

² TC significantly reduced in the PS-OBG group compared to the PL (Δ mmol/L, $p<0.001$) and the PS group (Δ mmol/L, $p=0.044$).

³ LDL-C significantly reduced in the PS-OBG group compared to the PL group (Δ mmol/L, $p=0.003$) only.

BL, baseline; CC, curcumin; HDL, HDL-cholesterol; LDL-C, LDL-cholesterol; PI, post-intervention; OBG, oat β -glucan; PL, placebo; PS, phytosterols; PS-OBG, phytosterol + oat β -glucan; TC, total cholesterol; TC:HDL ratio, total cholesterol-to-HDL ratio; TG, triglycerides.

6.4.2 Dietary intake, physical activity and compliance

There were no statistically significant differences in dietary intake at baseline across groups (Table 6.4) and nor were there statistically significant differences in the mean change of dietary parameters from baseline to post-intervention across groups. The study biscuits were well tolerated by participants with excellent compliance overall ($98.24\pm 0.42\%$). All groups were comparable for compliance except for the PS group which had significantly greater compliance than the PS-OBG group ($3.73\pm 1.13\%$, $p<0.01$) (Table 6.1). There was no statistically significant change in physical activity levels from baseline to post-intervention within- and between groups.

6.4.3 Effect of phytosterol and oat β -glucan intervention on plasma lipid profile

After 6 weeks of supplementation, absolute- and relative change in TC were significantly lower: -0.33 ± 0.11 mmol/L ($p=0.010$) and $-4.60\pm 1.66\%$ ($p=0.013$) in the PS group; -0.41 ± 0.12 mmol/L ($p=0.003$) and $-5.69\pm 1.70\%$ ($p=0.004$) in the OBG group and -0.80 ± 0.13 mmol/L ($p<0.0001$) and $-11.48\pm 1.79\%$ ($p<0.0001$) in the PS-OBG group (Table 6.3, Figure 6.2). Moreover, absolute- and relative change in LDL-C was significantly lower: -0.37 ± 0.12 mmol/L ($p=0.006$) and $-7.58\pm 2.50\%$ ($p = 0.008$) in the PS group; -0.42 ± 0.11 mmol/L ($p=0.001$) and $-8.63\pm 2.43\%$ ($p=0.002$) in the OBG group and -0.66 ± 0.13 mmol/L ($p=0.0001$) and $-13.88\pm 2.39\%$ ($p<0.0001$) in the PS-OBG group post-intervention. Absolute- and relative change in TC:HDL ratio was significantly lower in the PS-OBG group (-0.42 ± 0.10 mmol/L, $p=0.0008$; $-8.85\pm 2.41\%$, $p=0.002$) only. Absolute- and relative change in TG reduced by -0.24 ± 0.14 mmol/L ($p=0.022$) and $-8.44\pm 5.62\%$ ($p=0.048$) in the PS-OBG group only which was not statistically significant. HDL-C did not significantly change within any treatment group.

Table 6.4: Reported dietary intake of hypercholesterolaemic adults who consumed placebo (PL), phytosterol (PS), oat β -glucan (OBG) and phytosterol + oat β -glucan (PS-OBG) at baseline and mean change from baseline to post-intervention (Δ).¹

	PL (n=18)		PS (n=18)		OBG (n=18)		PS-OBG (n=18)	
	BL	Δ	BL	Δ	BL	Δ	BL	Δ
Energy (kJ)	9379 \pm 636	1259 \pm 538	9178 \pm 499	-46 \pm 433	8756 \pm 519	118 \pm 375	8398 \pm 456	1118 \pm 380
Protein (g)	94.49 \pm 6.71	8.14 \pm 4.78	105.62 \pm 5.16	-0.95 \pm 6.03	94.81 \pm 6.41	-1.97 \pm 5.46	94.68 \pm 5.93	4.23 \pm 6.14
CHO (g)	221.35 \pm 14.66	22.18 \pm 12.77	218.02 \pm 15.69	-2.02 \pm 16.09	210.60 \pm 18.23	-0.27 \pm 13.91	208.54 \pm 16.13	15.64 \pm 12.90
Sugars (g)	101.75 \pm 9.29	7.28 \pm 10.52	105.01 \pm 9.04	-11.27 \pm 9.24	90.22 \pm 11.60	-4.43 \pm 7.90	85.44 \pm 6.51	-3.33 \pm 7.79
Starch (g)	117.35 \pm 8.95	13.94 \pm 8.88	109.48 \pm 8.67	10.59 \pm 8.82	117.81 \pm 10.83	3.25 \pm 10.54	121.12 \pm 12.92	18.60 \pm 10.76
Total fat (g)	91.08 \pm 8.01	20.83 \pm 8.11	81.23 \pm 5.42	5.33 \pm 5.73	79.87 \pm 5.50	3.99 \pm 5.63	71.41 \pm 5.30	22.90 \pm 4.64
Saturated (g)	32.25 \pm 3.20	5.52 \pm 2.89	30.96 \pm 2.43	-1.24 \pm 3.26	30.08 \pm 2.88	-1.14 \pm 2.72	25.59 \pm 2.01	5.06 \pm 1.82
Trans (g)	1.48 \pm 0.17	0.30 \pm 0.17	1.53 \pm 0.13	-0.14 \pm 0.21	1.57 \pm 0.19	-0.07 \pm 0.19	1.11 \pm 0.13	0.16 \pm 0.08
MUFAs (g)	35.69 \pm 3.37	7.96 \pm 3.59	30.67 \pm 2.35	3.66 \pm 1.96	30.41 \pm 2.18	2.77 \pm 2.42	27.89 \pm 2.56	10.53 \pm 2.03
PUFAs (g)	15.96 \pm 1.56	5.90 \pm 1.82	12.45 \pm 1.27	2.74 \pm 1.30	12.16 \pm 1.18	1.96 \pm 0.93	11.38 \pm 0.98	5.83 \pm 1.03
Cholesterol (mg)	284 \pm 27	-23 \pm 29	316 \pm 32	37 \pm 41	337 \pm 59	-82 \pm 54	317 \pm 47	-11 \pm 42
Fibre (g)	26.05 \pm 1.84	1.19 \pm 1.54	30.90 \pm 2.35	-4.90 \pm 3.28	26.93 \pm 2.26	3.42 \pm 2.61	30.35 \pm 2.44	4.08 \pm 1.86
Alcohol (g)	15.95 \pm 5.92	-0.79 \pm 2.84	14.79 \pm 6.16	-5.01 \pm 3.77	13.98 \pm 3.80	-0.59 \pm 2.32	12.22 \pm 4.39	-3.39 \pm 2.45

¹ Values are reported as means \pm SEM.

BL, baseline; Δ , change from baseline to post-intervention; CHO, carbohydrates; OBG, oat β -glucan; PL, placebo; PS, phytosterols; PS-OBG, phytosterol + oat β -glucan.

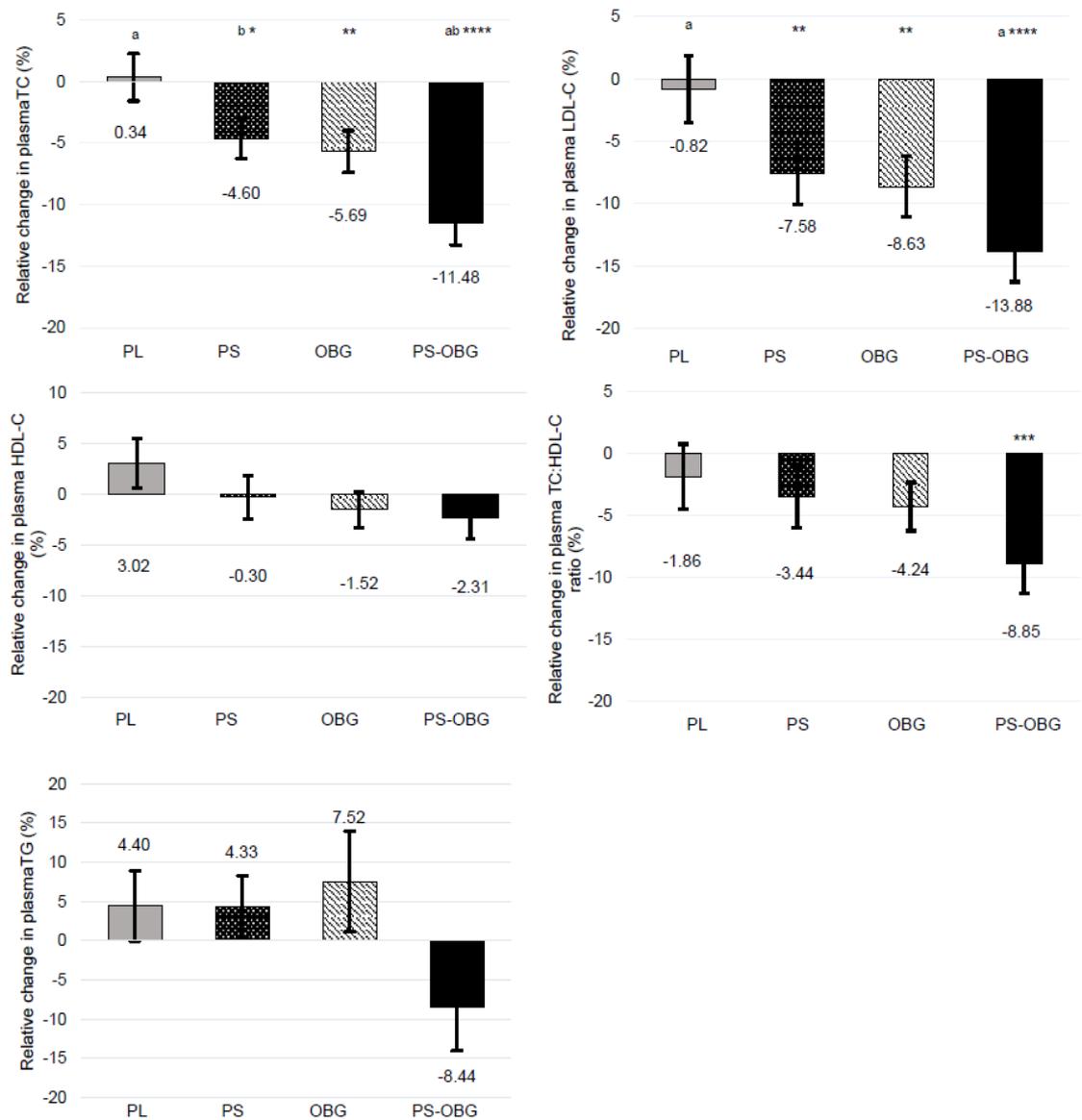


Figure 6.2: Percent change in TC, LDL-C, HDL-C, TC:HDL ratio and TG from baseline to post-intervention in hypercholesterolaemic individuals who consumed PL, PS, OBG or PS-OBG for 6 weeks.

Data represent mean \pm SEM. Note TG relative change is mean \pm SEM as this data is normally distributed. Symbols indicate significant changes from baseline as analysed by paired samples t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.00001$. One-way ANOVA and Tukey's HSD was used to perform post hoc comparisons of group means. Means with a common letter significantly differ to each other.
^a PL and PS-OBG significantly differ from each other for TC ($p < 0.0001$) and LDL-C ($p = 0.003$).
^b PS and PS-OBG significantly differ from each other for TC ($p = 0.037$)
 OBG, oat β -glucan; PL, placebo; PS, phytosterols; PS-OBG, phytosterol + oat β -glucan

As a result of the unexpected reduction in TG, the effect of baseline values was explored further by including them as covariates in the backward stepwise multiple regression. The associated model revealed baseline TG concentration as the only significant predictor ($p=0.043$) of the relative change in TG. To further explore the effect of baseline TG concentrations, TG data was dichotomised for baseline: $TG < 1.7$ mmol/L and $TG \geq 1.7$ mmol/L per treatment group. Those with the higher baseline TG concentrations had larger reductions in TG ($p=0.017$), however, this was only evident in the PS-OBG group and was not statistically significant due to the adjusted significance level of 0.0125 due to the Bonferroni correction (Figure 6.3). These additional analyses confirmed treatment to not be a statistically significant predictor of TG change after adjusting for baseline TG concentrations.

Blood lipid parameters did not significantly change from baseline in the PL group (Table 6.3). Comparison across treatment groups demonstrated a significant difference in absolute- and percent change in TC ($p<0.001$) and LDL-C ($p<0.01$) across groups. Post hoc analyses showed that PS-OBG had a significantly larger reduction in absolute- and relative change in TC compared to PL (-0.82 ± 0.18 mmol/L, $p<0.001$; $-11.82\pm 2.50\%$, $p<0.001$) and PS (-0.47 ± 0.18 mmol/L, $p=0.044$; $-6.88\pm 2.50\%$, $p=0.037$) only. As for LDL-C, the PS-OBG group had a significantly greater reduction in absolute (-0.61 ± 0.17 mmol/L, $p=0.003$) and percent change ($-13.06\pm 3.54\%$, $p=0.003$) compared to PL only. There were no statistically significant differences in TC:HDL ratio, HDL-C or TG concentrations between treatment groups.

Two-way ANOVA analysis revealed a significant main effect of PS and OBG for absolute- ($p=0.004$, $p<0.001$ respectively) and percent change ($p=0.003$, $p<0.001$ respectively) in TC. The main effect of PS did not reach statistical significance whereas OBG did for absolute- ($p=0.022$, $p=0.006$ respectively) and percent change ($p=0.019$, $p=0.006$ respectively) in LDL-C. There was no interaction between PS and OBG on any lipid measures.

6.4.4 The effects of baseline data on change in blood lipid profile

Baseline data including age, BMI, fat mass, waist-to-hip-ratio, systolic blood pressure, LDL-C concentration, exercise levels and dietary intake of energy, carbohydrates, trans fats, monounsaturated fatty acids, omega-6 polyunsaturated fatty acids, fibre and alcohol were assessed as potential confounders 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 statistically significant predictor of the change in TC and LDL-C.

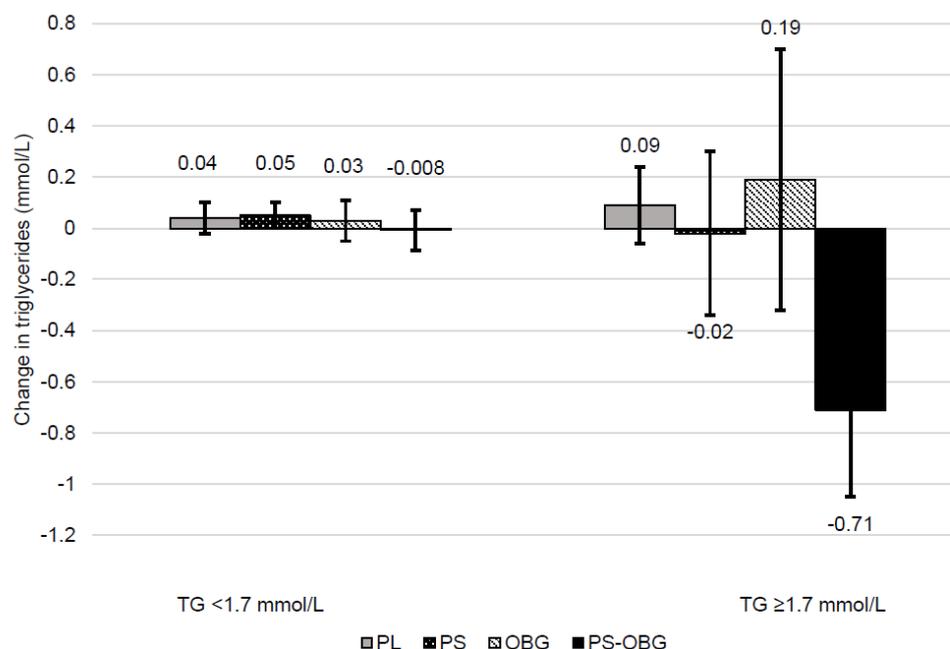


Figure 6.3: Absolute change in TG (mmol/L) when baseline TG <1.7 mmol/L or ≥1.7 mmol/L in hypercholesterolaemic individuals who consumed PL, PS, OBG or PS-OBG for 6 weeks.

A cut off of 1.7 mmol/L in TG was used as this is classified as normal under the ATP III guidelines [316]. Data represent mean ± SEM. Changes from baseline was analysed by Wilcoxon Signed Rank test and One-way ANOVA was used to compare group means.

TG <1.7 mmol/L: PL, n= 14; PS, n=16; OBG, n=12; PS-OBG, n=12

TG ≥1.7 mmol/L: PL, n= 4; PS, n=2; OBG, n=6; PS-OBG, n=6

OBG, oat β-glucan; PL, placebo; PS, phytosterols; PS-OBG, phytosterol + oat β-glucan; TG, triglycerides.

6.5 Discussion

The cholesterol-lowering ability of specific soluble fibres and PS have been recognised as key adjunct and alternative therapies that can be coupled with dietary changes and medications to enhance the efficacy of reaching blood cholesterol targets. Our findings show that daily dietary supplementation with high-MW OBG combined with PS for six weeks in a functional food, significantly lowered TC (~11.5%) and LDL-C (~14%), in free-living adults with hypercholesterolaemia. In addition, blood lipid profile was further improved as a marked reduction in TG concentration (8.4%) was also apparent in the PS-OBG group.

Consumption of ≥3g/d OBG has been shown to lower TC and LDL-C concentrations by 0.30 mmol/L and 0.25 mmol/L respectively, with no significant changes in HDL-C or TG [161]. An RCT reported that a high-MW (2210 kDa) and medium-MW (530 kDa) OBG induced a greater

LDL-C reduction compared to low-MW (210 kDa) and control [154]. Regardless of the MW, OBG has been shown to lower plasma cholesterol by reducing intestinal bile acid reabsorption [174, 175]. Once bile has facilitated the digestion and absorption of dietary fats, it is normally recovered in the distal end of the small intestine and then recycled [174]. OBG has a linear polymer structure to which the adjacent chains form cross-links, enabling it to swell as it absorbs water while passing through the length of the small intestine [175]. The OBG becomes highly viscous which traps bile, rendering it unable to be reabsorbed and thus excreted via stool [174]. This stimulates bile acid synthesis from cholesterol in the liver, which in turn is supplied by increased cholesterol synthesis, evidenced by raised concentrations of lathosterol and 7 α -hydroxy-4-cholesten-3-one (a marker of bile acid synthesis) [176-178] as well as upregulation of LDL receptor expression thus enhancing clearance of LDL-C from the blood to synthesise more bile acid [154, 174]. The degree of viscosity of OBG is determined by its MW [154, 175]. Native unprocessed oat kernels have a naturally high MW (~1730-2800 kDa) [161, 162], however, this can be reduced to as low as ≤ 100 kDa during food processing, extrusion and storage [162]. The efficacy of LDL-C lowering is reduced by 50% when the MW is 210 kDa, even when the same dose (3g/d) of OBG is administered [154].

Reductions in TC and LDL-C following OBG supplementation in the present study surpass that of previous studies administering the same dose [161], namely one that used the same high-MW (~2210 kDa) OBG [154]. Wolever et al [154] reported a significant 0.15 mmol/L or 5% (vs 0.42 mmol/L, 8.6% in our study) reduction in LDL-C following consumption of 3g/d high-MW OBG delivered via two servings of cereal per day. The lower baseline LDL-C (3.74 vs 4.67 mmol/L in our study) in the previous study's participants could account for the smaller reduction reported [154]. It is also possible that the degree of viscosity in the gut is limited or reduced when the daily dose of OBG is divided into two servings per day, thus minimising the efficacy for LDL-C-lowering. Given the mechanistic action of OBG, it has been suggested that optimal consumption is with meals to coincide with bile release [175], however, in the present study OBG was administered at snack times yet larger reductions in LDL-C were observed compared to previous findings. Further research into divided vs single dose and timing along with other potential confounders in study design or delivery are warranted to better understand how therapies can protect and capitalise on the physiochemical properties and thus hypocholesterolaemic effects of OBG.

The degree of cholesterol-lowering following supplementation with PS reported in this study is similar to previous studies of similar dose and duration, whereby an 8-9% (~0.32-0.35 mmol/L) reduction in LDL-C following 2g/d PS for at least four weeks is well documented [94, 276]. The magnitude of LDL-C lowering has been shown to be influenced by higher baseline LDL-C [94,

276]. In this study, baseline LDL-C and waist-to-hip ratio were predictors of the change in LDL-C, however, treatment remained the most significant predictor. There are several reported mechanisms by which PS lower plasma cholesterol levels, but the most widely accepted mechanism is displacement of cholesterol in the micelle [90]. This results in an overall net reduction of cholesterol absorption and only minimal uptake of PS into circulation, as the majority are selectively pumped back into the intestinal lumen via ATP-binding cassettes ABCG5 and ABCG8 where their fate is excretion [90].

The reduction in fasting TG observed in the PS-OBG group was unexpected as findings are inconsistent regarding the TG-lowering effects of PS. A meta-analysis [97] and pooled analysis of 12 RCTs [95] by Demonty et al concluded that 2g/d PS induced a modest but statistically significant reduction in TG (0.08-0.12 mmol/L or 4-6%) in hypercholesterolaemic individuals, and larger reductions were associated with higher baseline TG (~1.37-2.0 mmol/L). A more pronounced reduction (0.23 mmol/L or 27.5%) has been demonstrated in metabolic syndrome patients with baseline TG > 2.0 mmol/L [96]. In contrast to our study, Demonty et al [95] found this relationship to be statistically significant for absolute (mmol/L)- but not relative (%) change in TG. It is likely that this discrepancy of high inter-individual variation in TG concentrations and lack of statistical significance is because individual studies are primarily powered to assess effects on LDL-C, thus poorly powered to detect a meaningful change in TG [95]. In this study, baseline TG concentrations in the PS group were lower than the PS-OBG group (1.05 mmol/L vs 1.29 mmol/L) and had an increasing non-significant trend post-intervention (+0.05 vs -0.24 mmol/L, respectively). Further investigation from our additional dichotomous analysis revealed individuals with higher baseline TG (≥ 1.7 mmol/L) had greater reductions in TG post-intervention following PS-OBG supplementation. Since baseline TG concentrations appear to be explaining most of the variation in relative change in TG, it is acknowledged that this observation could possibly be attributed to the regression to the mean effect. The majority of human studies have reported slight but non-significant lowering of TG concentrations following OBG [161, 173] with few demonstrating significant reductions [338, 339]. A study in rats reported significant reductions in serum and hepatic TG concentrations and greater faecal bile acid excretion following partially hydrolysed OBG (MW 730 kDa) + cholesterol-rich diet [340]. The exact mechanism is unknown but since OBG increases the viscosity of the small intestine, it reduces efficiency of emulsification, which is likely to interfere with lipid digestion by reducing accessibility of fats by digestive enzymes. The potential TG-lowering effect by PS is likely due to lowered hepatic synthesis of large TG-rich very low-density lipoproteins (VLDL)-1 particles, since significant reductions in large and medium size VLDL particles have been reported in dyslipidaemic metabolic syndrome patients after plant stanol supplementation [96]. Some

limitations of our findings for the secondary measure, TG, are the small number of participants in each group after the measure was dichotomized as a secondary analysis and hence the limited associated statistical power to detect differences in TG as statistically significant. This study was not powered to detect changes in TG concentrations, and we were not aiming to recruit hypertriglyceridaemic individuals. These findings are exploratory, although prompts interest to investigate the individual action and interaction between PS and OBG in a larger study that is powered to assess change in TG in participants with elevated TG concentrations.

The combination of PS and OBG has been previously investigated in a muesli [176] and a series of food items (cereal, snack bar and beverage) [341], however, in the former study the saturated form of PS was used (plant stanols) at only 1.5g/d dose with 5g/d OBG for four weeks at main meal times via a moderate-fat food item (~15g). In the latter study, 1.8g/d PS with 2.8g/d OBG was delivered via a low-fat food (≤ 3 g per serve) across three separate timepoints including main meal and snack times. Neither of the trials specified MW of the OBG and this along with timing of consumption, multiple time points for consumption and low-moderate fat content of experimental food may contribute to the efficacy of OBG for modulating cholesterol concentrations [161]. Given the two distinct mechanistic roles of PS and OBG regarding cholesterol metabolism, they effectively complement each other for an amplified reduction in plasma LDL-C. The combination of PS and OBG proves to be efficacious by tackling cholesterol metabolism at the gut level from two angles: increased clearance of cholesterol from circulation via the inhibition of bile acid reabsorption and reduction in cholesterol absorption at the micellar level. Moreover, the addition of OBG to PS therapy potentially extends its health benefits beyond cholesterol-lowering as OBG has been shown to: significantly lower postprandial blood glucose concentrations by suppressing glucose uptake [164] and delaying gastric emptying [163]; increase postprandial fullness and satiety in healthy [165] and overweight/obese [166] individuals and promotes colonic fermentation by gut microbiota to produce short-chain fatty acids [167-169], which play various roles such as mediating calorie intake [171, 172] and inhibiting endogenous cholesterol synthesis [167, 170].

Findings from this study demonstrate a complementary action between high-MW OBG and PS for cholesterol-lowering in hypercholesterolaemic adults. These findings have the potential to provide a safe, efficacious and compliant adjunct therapy that is more efficacious than administering either bioactive alone for the management of dyslipidaemia. This combination may support the development of novel foods that could serve as a solution or adjunct therapy for individuals who are statin intolerant and/or have suffered adverse effects from pharmacological interventions. Further research into the additional health benefits of this dietary combination

relating to glycaemic parameters, short-chain fatty acids, calorie intake, weight management and long-term effects are warranted to establish optimal dose, duration and food delivery.

Chapter 7

Conclusions and Future Recommendations

7.1 Key findings

CVD remains a key burden to global health and is characterised by a vast array of risk factors. Dyslipidaemia is a major risk factor for atherosclerosis (a type of CVD) and is highly prevalent in the Australian adult population. Long-term sustainability of traditional interventions for combating dyslipidaemia such as diet, lifestyle, exercise and medications are difficult, with several factors impeding long-term adherence by individuals. Of concern is individuals who are intolerant to statins and/or have experienced side effects from statin or other medicinal therapies and the financial cost of these medicines to society. Nutraceuticals (also referred to as bioactives) have been shown to be an effective adjunct or alternative for these individuals, however, only modest results are achieved in comparison to medications, and, interventions whether they are diet, pharmacological or complementary medicine they often only target blood lipids; this can be a weakness when aiming to combat a multifaceted chronic condition such as CVD.

PS are a well-known and commonly advised non-pharmacological intervention for lowering blood cholesterol concentrations, however, only maximally reach LDL-C reductions of 8-10%. When PS are combined with a statin, an additional 10-15% reduction in LDL-C can be achieved, which is superior to doubling the statin dose (only ~6% additional lowering) [201, 203]. Moreover, PS combined with other bioactives such as LCn-3PUFA from fish oils synergistically lowered fasting plasma TC (-13%), LDL-C (-12.5%) and TG (-26%) concentrations more than either treatment alone in hyperlipidaemic adults [300]. These are prime examples of how bioactives in combination with one another or medications can assist with targeting health goals, empowering individuals to have more control over their risk markers and lower medication dosage and dependence. This thesis examined non-pharmacological strategies for enhancing the lipid-modulating ability of PS as well as trialling bioactive combinations for improving blood lipid profile with future implications to investigate additional heart health benefits obtained from such bioactive combinations.

The most common PS enriched foods are vegetable fat spreads and dairy products such as butter, cheese, yoghurts, low-fat milk and fermented milk drinks. Although most of those delivery foods contain moderate-high amounts of fat, the type of fat varies from product to product and different dietary fats have known differential effects on circulating blood lipids. Therefore, the first aim of this thesis was to investigate whether the type of carrier fat used in these products could be influencing the cholesterol-lowering ability and thus overall efficacy of PS and plant stanols, as this could reveal novel insight into optimising lipid modulation of this type of intervention. The findings of the systematic review and meta-analysis presented in chapter 3 was the first to report that the carrier fats used in common PS enriched products (vegetable fat spreads and dairy foods) are a determinant of the cholesterol-lowering potential of PS [317]. We found that the major

carrier fats in common PS enriched products include: sunflower/soybean which are predominately rich in LA; rapeseed/canola which are predominately rich in oleic acid and contain higher amounts of ALA; and dairy fat which is rich in SFA with small amounts of PUFA or MUFA. Given these contrasting differences in carrier fats, we hypothesised that the fat type would influence the hypocholesterolaemic effect of PS and perhaps reveal an optimal carrier fat for enhancing cholesterol reduction.

A thorough review of the literature was undertaken totalling over 2500 publication titles and abstracts scanned for inclusion, resulting in 32 publications included in the meta-analysis after screening for inclusion criteria and methodological quality assessment. Most strata involved PS enriched products containing rapeseed/canola carrier fat, followed by sunflower/soybean and then dairy. The overall pooled means for reduction in TC (-0.38 mmol/L or -6.4%) and LDL-C (-0.34 mmol/L or -9.3%) agreed with previous studies [94, 276]. Our study demonstrated that the rapeseed/canola carrier fat lowered LDL-C by a statistically significant 0.1 mmol/L more than the sunflower/soybean group, which is clinically relevant since this is a further 29% decrease when using the pooled mean value (-0.34 mmol/L) reported in this study. It is noteworthy that compared to the rapeseed/canola and sunflower/soybean groups, the dairy fat group was more heterogenous with varying levels of SFA and a diverse range of products involved i.e. butter, cheese, yoghurt and low-fat milk; this may in part account for the lack of statistical significance when comparing this group to the other two fat types. The potential mechanistic interplay between PS and carrier fats is not entirely understood, however, as discussed in chapter 3, complementarity and/or synergy between PS and carrier fats could be explained by modulation of LDL-r activity by way of increasing affinity and overall LDL-r activity.

This review provides an in-depth understanding of how circulating cholesterol concentrations may be modulated using PS and certain carrier fats. These findings provide new evidence for optimising PS therapy in functional foods such that rapeseed/canola fats are a superior choice to other fats for optimum PS functionality. Rapeseed/canola fat would be a more favourable carrier fat in the context of heart health, since ALA favours anti-inflammatory pathways in the body and in small amounts leads to the formation of LCn-3PUFA (primarily EPA) which have well established heart health benefits. Moreover, the influence of dairy fat specific to types of PS enriched dairy products warrants further investigation to confirm the relationship between PS and dairy fat types.

As previously mentioned, the maximal reduction in LDL-C achieved with PS therapy is 8-10%, whereas statin monotherapy yields 21-55% [342] and ezetimibe monotherapy 18-20% [343]. A key barrier to statin therapy is the potentially harmful side effects, some of which are dose-dependent such as elevated liver function tests [344, 345]. It is evident that the general public is

slowly becoming aware of this as it has been reported that approximately 50% of patients discontinue statin therapy within the first year of commencing treatment, a trend that increases with subsequent years of treatment [346]. Most individuals discontinue statin therapy due to concerns about side effects and interactions with other medications/supplements, cost and uncertainty of obtaining actual benefits [347, 348]. It is evident that an effective but safe alternative and/or adjunct solution is required not only to individuals who are concerned about pharmacological management of blood cholesterol, but those who wish to prevent and lower their risk of CVD and/or have a family history of hyperlipidaemia. The following three studies presented in chapter 4, 5 and 6 aimed to investigate the lipidaemic effects of dietary intervention with PS in combination with curcumin, initially as dietary supplements (chapter 4, study 1), then via a functional food (chapter 5, study 2), and finally with OBG (chapter 6, study 3) in hypercholesterolaemic, community-dwelling adults.

Firstly, a combination of PS and curcumin delivered as dietary supplements to free-living hypercholesterolaemic adults led to a significant 0.74 mmol/L or 11.0% reduction in fasting TC and 0.63 mmol/L or 14.4% reduction in fasting LDL-C concentrations which was greater than PS or curcumin administered alone. To the best of our knowledge, these results presented in chapter 4 are the first to investigate this combination therapy. Interestingly, TC and LDL-C did not significantly change following curcumin-only treatment. Given the large magnitude of cholesterol-lowering achieved in study 1, it is possible that the two bioactives complemented the effects of each other. The curcumin-only group had a small non-significant trend of lowering TC and LDL-C but not enough for the reductions in the combined group to be simply just additive of the two separate bioactive treatment arms. In this study, PS and curcumin were not statistically synergistic, with our statistics indicating a significant main effect contribution from PS. The large variance observed within the curcumin group and small sample size is likely a contributing factor to the lack of statistical significance. Although not statistically significant for an interaction between PS and curcumin, the widespread of lipid-modulating mechanisms pertaining to PS and curcumin discussed in chapter 4 supports our findings and rationale for combining both bioactives. Therefore, the potential for complementarity and/or synergy based on our observations and possible mechanistic interplay cannot be entirely ruled out.

Our findings provide supporting evidence for the use of a novel bioactive combination to assist with combating high cholesterol in community-dwelling individuals with hypercholesterolaemia. The combination of PS and curcumin in this study yielded a ~14% reduction in LDL-C in only four weeks in free-living hypercholesterolaemic adults who did not alter their background diet or physical activity levels. Although not as powerful as statin monotherapy, our intervention could serve as an effective adjunct to pharmacological therapies thereby minimising dose dependence

and subsequent side effects. Or in some cases ~14% reduction in LDL-C may well be enough to normalise blood cholesterol concentration and therefore should be used as a therapy before a statin. Moreover, this intervention could serve as a safe, effective alternative strategy for individuals who are intolerant to statins, with the potential to enhance lipid management by coupling with diet/lifestyle changes and the addition of other bioactives.

In order to extend our findings, enhance practical adherence and potentially effectiveness of our combined dietary supplementation of curcumin tablets and PS enriched vegetable fat spread, a staple food (bread) enriched with both bioactives was developed and investigated in study 2 (chapter 5). Currently in Australia, the advised dose of 2g/day PS are consumed via 25g/day enriched vegetable fat spread, or 2-3 cups of enriched low-fat milk, or more recently 2 wheat biscuit cereals/day. Study 2 aimed to replicate the findings of study 1 by utilising a novel food, hypothesised to yield excellent compliance due to reduced daily consumption burden (only requiring 2 slices bread versus 25g vegetable fat spread plus 2 tablets) which could form part of one mealtime. This functional food would be in line with the Australian Guide to Healthy Eating as bread belongs to one of the five food groups to be consumed and is a staple food in Australia. We also hypothesised that the enriched bread would be more effective for modulating blood lipid concentrations, given excellent compliance and potentially enhanced synergy from co-consumption at the same time point. This study was the first to investigate the effects of PS in combination with curcumin in a functional food. In this study population, compliance was excellent across all groups; however, curcumin did not provide any additional cholesterol-lowering effects to PS therapy, since there was no significant difference in blood lipids when feeding PS in the presence or absence of curcumin. Therefore, as described in chapter 5, respective groups were pooled to reflect PS supplementation (PS group + PS-CC group) and no PS supplementation (PL group + CC group). Analyses of groups in this manner resulted in significantly lower fasting plasma TC (-0.44 mmol/L or -6.1%) and LDL-C (-0.44 mmol/L or -8.8%) concentrations in free-living hypercholesterolaemic adults consuming 2 slices of bread per day enriched with PS with or without curcumin as part of their habitual diet/lifestyle for four weeks (presented in chapter 5).

Furthermore, other risk factors of metabolic disease such as chronic inflammatory markers which are key contributors to the pathobiology of atherosclerosis, were not determined as part of this thesis. Curcumin is a potent anti-inflammatory, therefore, by adding curcumin to PS therapy, the impact on improving heart health is potentially augmented and multifaceted. More human studies administering bioavailability-enhanced formulations of curcumin in the context of dyslipidaemia and dysglycaemia are warranted to ascertain the optimal dose, regime and duration for favourably modulating circulating blood lipids and glycaemic parameters.

As discussed in chapter 5, it is likely that thermal exposure during baking may have degraded and/or destroyed the MERIVA® (curcumin-PHYTOSOME® complex) and/or curcumin, leading to limited bioavailability of curcumin in the final product. Unlike a liposome, the lipophilic guest (in this case, curcumin) in the PHYTOSOME® interacts with the polar heads of the phospholipid, situating it on the surface of the MERIVA® rather than dissolved in the central cavity if it were a liposome [311]. Curcumin in the MERIVA® technology is possibly more vulnerable to external factors whereas a SLCP™ formulation could have been more robust and heat resistant, as curcumin is encapsulated into a lipid core [119]. Moreover, the stability of the PHYTOSOME® technology is questionable, as thermal gravimetric analyses have shown that a large weight loss commences at 175-180°C due to degradation [311]. These are potential explanations for the lack of lipid-modulating findings in study 2, as we did hypothesise to at least replicate the findings of study 1 (chapter 4), whereby PS and curcumin were co-administered as separate dietary supplements using the same dose, duration and population type [318]. Future food preparations involving curcumin formulations need to consider the food processing methodology with respect to the formulation characteristics in order to maximise and protect the stability of both the curcumin and the delivery matrix.

Since the combination of PS with curcumin in a functional food was unsuccessful for enhanced lipid-lowering, the last study (study 3 presented in chapter 6) aimed to combine PS with a different bioactive (high-MW OBG), which has known lipid-modulating effects; and explore the combined effects in a functional food. OBG was selected due to its effective bile acid sequestering ability, leading to reduced gastrointestinal cholesterol absorption and subsequent lowering of circulating cholesterol concentrations. For this study, we developed chocolate biscuits enriched with PS and OBG to be consumed daily as a snack. After 6 weeks, plasma TC and LDL-C were significantly lower in all treatment groups except placebo, and the magnitude of reduction in the combined group for TC (-0.80 mmol/L or -11.5%) and LDL-C (-0.66 mmol/L or 13.9%) was significantly greater than the PS group and placebo group. As discussed in chapter 6, this combination has been previously investigated in two studies, one that administered both bioactives as an enriched muesli [176] and a series of enriched food items [341], however, neither trial specified the MW of OBG and this along with time of consumption, multiple timepoints of consumption and low-moderate fat content of experimental food, may contribute to the efficacy of OBG for modulating cholesterol concentrations. Interestingly, TG concentrations reduced by 8.4% following combined PS and OBG intervention, however, this did not reach statistical significance. This finding warrants further investigation in a trial powered to assess change in TG, as potential mechanisms for TG-lowering have been discussed with respect to PS and OBG.

Findings from this study provide novel insight into the combination of PS with a high-MW form of OBG, such that blood cholesterol-lowering by the two bioactives appears additive. The addition of high-MW OBG to PS in a single food enhances the lipid-lowering effects of PS, with the potential to provide additional heart health benefits such as improved gut health via promoting colonic fermentation to produce SCFAs, increased satiety and therefore a potential role in weight management and postprandial hypoglycaemic effects via delayed gastric emptying and reduced glucose uptake. Further investigation is warranted to confirm these additional health benefits potentially associated with combined dietary supplementation of PS and high-MW OBG.

Lipoprotein profile was analysed via NMR spectroscopy and was conducted for both functional food trials i.e. study 2 and 3 to explore further their influence on blood lipids in addition to cholesterol concentrations. In study 2, novel findings were reported such that LDL-P number significantly reduced following PS supplementation (chapter 5). The same was reported in study 3, however, only the OBG-only and combined PS and OBG groups had significantly lower LDL-P concentrations post-intervention (Appendix 15). This has also been shown in only one other study by Matvienko et al which used NMR technique following PS supplementation and they reported a similar trend [326]. Comparison of findings from our study and Matvienko's highlights the potential influence of baseline LDL-P in predicting change in LDL-P following intervention, since our participants had a much higher baseline LDL-P and had a greater reduction in LDL-P post-intervention. It is important to note some key differences between the two studies: in our study, regression analysis revealed baseline LDL-P was a significant predictor of the change in LDL-P; the food matrices are vastly different; and the study by Matvienko et al was only in young males. With regards to OBG, our findings are similar to a study in normocholesterolaemic overweight/obese men administered 5.5 g/day OBG cereal, whereby LDL-P concentration significantly reduced by 5% (versus our 10%) after 12 weeks [349]. The participants in the previous study were not hypercholesterolaemic and thus had lower baseline LDL-P concentrations than ours and the MW of the OBG was not reported; these factors are likely contributors to the milder effect on LDL-P compared to our study. LDL-P is emerging as a useful and more predictive tool for CVD risk than LDL-C concentrations, with a key relationship between LDL-C and LDL-P concordance and discordance more effectively indicating LDL-attributable atherosclerotic risk [34]. Additionally, TRL-P number significantly reduced in study 3 in the OBG and PS-OBG groups only. In addition to significant cholesterol-lowering and mild TG-lowering, this contributes additional cardiovascular benefit since higher TRL-P are associated with elevated CVD risk [350]. These findings highlight the usefulness of measuring lipoprotein particle concentrations in addition to traditional cholesterol concentrations to prevent underestimating the effect of interventions on overall lipoprotein profile. Our findings support the

need for future studies assessing the effects of bioactives on lipoprotein profile to provide increased precision for targeting CVD risk.

Some additional findings are noteworthy of discussion. In all studies, the TC:HDL ratio also significantly reduced in all bioactive combination groups, however, HDL-C and TG did not significantly alter from baseline in any studies. In study 1, the curcumin-only supplemented group had a significantly lower fasting plasma glucose concentration (-0.13 mmol/L) post-intervention. This observation was not evident in any other treatment group, and the magnitude of reduction was significant compared to the placebo group (-0.34 mmol/L) only. The curcumin group did have a statistically non-significant higher baseline plasma glucose compared to other treatment groups which could contribute to this result. The combined group had a much lower baseline plasma glucose level compared to the curcumin group (5.09 vs 5.33 mmol/L) which could be the reason why the combined group failed to replicate these findings. Nonetheless, the significant reduction in plasma glucose within the curcumin group warrants further exploration since a meta-analysis of 11 RCTs reported significant lowering of fasting glucose and glycated haemoglobin concentrations in trials conducted 4 or more weeks in duration [351]. In addition, a previous study conducted by our research group using the same formulation of curcumin, reported significant reductions in postprandial glucose following consumption of a standardised high carbohydrate and fat meal supplemented with a single dose of curcumin in healthy normoglycaemic adults [335]. The exact mechanism by which curcumin lowers fasting plasma glucose remains unclear, but possible mechanisms in diabetic models include reduced hepatic glucose production and glycogenolysis and increased glucose transporter gene expression leading to enhanced glucose uptake [352]. It must be noted that plasma glucose was a secondary outcome in our study, however, the preliminary findings from our study support the need for further exploration of the hypoglycaemic effects of curcumin in human trials powered to assess significant changes in glucose and possibly for a longer duration. True hypoglycaemic effects of combined dietary supplementation with PS and curcumin could not only effectively target dyslipidaemia, but also glycaemic indices to better target and prevent cardiometabolic diseases from multiple angles.

Lastly, the influence of PS therapy in combination with other bioactives presented in this thesis on 10-year CVD risk was evaluated via the Framingham Risk Algorithm to explore the relevance of the impact of blood cholesterol reduction (Table 7.1). It is noteworthy that only the combination strata (i.e. PS-CC, PS-OBG and PS-C) had significant reductions in 10-year CVD risk post-intervention. The two trials investigating PS and curcumin combination resulted in significant reductions in 10-year CVD risk compared to placebo, and the reductions reported in the PS-CC strata of study 1 were also significantly lower than the PS and CC groups. Secondary analyses of this finding revealed synergy between the two bioactives for both absolute- and relative change

in CVD risk ($p=0.002$), however, there was only a significant main effect from PS ($p<0.01$) for both absolute- and relative change. The main effect from curcumin was borderline significant ($p=0.08$ and $p=0.07$ for absolute- and relative change, respectively), suggestive of treatment with PS was the key driving force for synergistic interplay in this study. A closer evaluation of the individual risk measures that are used to calculate the risk score revealed that for all risk measures, groups were comparable at baseline and post-intervention as well as for mean changes from baseline. The only exception was systolic blood pressure significantly reduced by -8.25 ± 1.83 mmHg ($p=0.0009$) or $-5.84\pm 1.27\%$ ($p=0.0008$) in the PS-CC group only, and the mean changes were significantly different when compared to the CC group (-8.94 ± 3.00 mmHg or $-6.59\pm 2.26\%$, $p<0.05$) and the PS group (-10.87 ± 3.00 mmHg or $-8.01\pm 2.26\%$, $p<0.01$). The PS-CC group was mildly hypertensive (140-159 mmHg SBP) according to the current Australian National Heart Foundation Guidelines [353]. Although non-significant, the PS-CC group's systolic blood pressure was higher compared to the other treatment groups at baseline (PS-CC, 140.92 ± 5.28 mmHg; CC, 131 ± 4.92 mmHg; PS, 128.39 ± 3.38 mmHg; PL, 125.38 ± 4.31 mmHg), which could have promoted a greater response to dietary intervention. It is likely that the degree of systolic blood pressure lowering in the PS-CC group was a contributor to the observed 10-year CVD risk reduction, since systolic blood pressure is a variable in the algorithm. The effects of PS on blood pressure in humans remains unclear [354], however, some trials which have measured blood pressure as a secondary outcome have reported no change following PS supplementation [270, 355, 356], whereas few animal studies reported a mild raising effect in hypertensive animals, however, lack of adequately controlled study design in those animal studies prevents a clear inference from being drawn [354]. Some animal studies have reported hypotensive effects following curcumin supplementation in hypertensive rodents. A study reported tetrahydrocurcumin attenuated the elevation of blood pressure in a hypertensive rat model induced by nitric oxide synthase inhibition [357] and the same was observed in a mouse model of cadmium-induced hypertension whereby curcumin increased vascular responsiveness and normalised blood pressure levels [358]. Future human trials should conduct ambulatory blood pressure and central pressure (pulse wave velocity) as a better indicator of functional changes in blood pressure associated with bioactive intervention, as the majority have focused on seated, static blood pressure measurements [354].

Table 7.1: Summary of 10-year CVD risk for intervention trials at baseline and post-intervention¹

10-year CVD risk ²					
	Sample size	Baseline (%)	Post-intervention (%)	Absolute change	Relative change (%)
Study 1³					
	51				
PL	13	9.62 ± 2.96	9.06 ± 2.54	-0.56 ± 0.67 ^a	-0.77 ± 4.40 ^a
PS	13	12.88 ± 2.88	13.00 ± 2.94	0.12 ± 0.30 ^b	-1.59 ± 3.33 ^b
CC	13	10.34 ± 1.96	10.82 ± 1.94	0.48 ± 0.39 ^c	5.00 ± 4.54 ^c
PS-CC	12	12.21 ± 2.07	9.51 ± 1.76 ^{**}	-2.71 ± 0.55 ^{abc}	-22.76 ± 4.26 ^{abc}
Study 2					
	72				
PL	18	7.48 (3.41, 15.26)	7.14 (3.68, 16.29)	-0.49 ± 0.63	-0.42 ± 4.23
PS	18	5.58 (3.41, 12.49)	6.23 (3.26, 12.65)	-0.23 ± 0.27	-1.18 ± 3.13
OBG	18	8.59 (6.18, 11.73)	8.19 (6.11, 12.15)	-0.05 ± 0.46	-3.05 ± 3.68
PS-OBG	18	8.51 ± 1.23	7.66 ± 1.17 [*]	-0.85 ± 0.40	-6.99 ± 4.38
Study 3					
	75				
PL-C	36	8.43 (5.04, 13.63)	9.30 (4.99, 14.41)	0.46 (-0.31, 1.78) ^a	4.54 ± 2.93 ^a
PS-C	39	10.46 (5.04, 17.40)	9.96 (4.32, 16.42) [*]	-0.64 (-1.53, -0.09) ^a	-8.12 ± 2.00 ^a

¹ Data are presented as mean±SEM or median (25th percentile, 75th percentile) for normally and non-normally distributed data (respectively).

² 10-year CVD risk was calculated via the Framingham Risk Algorithm which encompasses: age; gender; status of smoking, type 2 diabetes diagnosis and treated or untreated systolic blood pressure; fasting total cholesterol and HDL-cholesterol concentrations.

³ Only 51 participants had available data for calculation of 10-year CVD risk via the Framingham Algorithm. Significant change from baseline, * $p < 0.05$, ** $p < 0.01$. Values with a common superscript letter in the same column significantly differ from each other ($p < 0.05$).

CC, curcumin; OBG, oat β -glucan; PL, placebo; PL-C, placebo + curcumin (pooled groups); PS-C, phytosterol + curcumin (pooled groups); PS-CC, phytosterol + curcumin; PS-OBG, phytosterol + oat β -glucan.

Although the findings presented in Table 7.1 are exploratory, it is encouraging to see a mild lowering of CVD-risk achieved in free-living adults who are already at risk of developing CVD in only 4–6 weeks of dietary supplementation with PS-bioactive combinations. It is likely that these findings could be replicated in larger trials, as the measure of precision (i.e. SEM) is smaller in study 3 by almost half that of SEM's in study 1 and 2 for CVD risk data. The implications of these findings provide insight into the broader impact on heart health that bioactive combinations could potentially pose. It must be noted that none of the intervention trials were powered to assess changes in 10-year CVD risk as the primary outcome, and therefore these findings are only

exploratory and prompt further investigation using larger trials that are adequately powered with a longer duration.

7.2 Strengths and limitations

7.2.1 Strengths

To the best of our knowledge, this is the first research to report that the carrier fat used in commercially available PS enriched products is a determinant of the cholesterol-lowering potential of PS. This research is also the first to investigate the effects of the bioactive combination: PS and curcumin, as well as to investigate this combination in a novel function food in humans. Furthermore, the combination of PS and high-MW OBG is also a first with respect to high-MW weight OBG being used as the form of OBG.

A strength of the work presented in this thesis is the rigorous research methodologies used. Firstly, a systematic review and meta-analysis which revealed that the rapeseed/canola carrier fat in common PS enriched products enhances the cholesterol-lowering potential of PS. Findings from this research are regarded as the highest level of evidence and could therefore be used to inform the food industry to consider the fat composition used in PS enriched products in order to yield optimum cholesterol-lowering targets in hypercholesterolaemic individuals. At present, this study has been referenced in a review of the evidence of dietary fats and CVD by the Sax Institute for the National Heart Foundation in 2017 [359]. Secondly, all intervention studies were of high quality: randomised, double-blinded, placebo-controlled in a 2x2 factorial design in order to eliminate any risk of bias and to effectively investigate the single- and combination effects of bioactives. In addition, study participants were recruited from the wider community, thus enabling generalisation of our results to hypercholesterolaemic adults. Thirdly, the proton NMR spectroscopy (LP4 NMR MetaboProfile™ Analysis) analyses using LipoProfile-3 algorithm is a more precise, reliable and effective method for evaluating lipoprotein particle size and number. It was also conducted by a team of lipoprotein metabolism scientists at the National Heart, Lung and Blood Institute at the National Institute of Health in the United States and the detailed methodology has been reported previously [33].

A key strength of the interventional studies conducted as part of this thesis was excellent compliance monitored via back-count of tablets and food products provided, weighing of distributed and returned interventional foods, inspection and collection of interventional food packaging, regular contact with participants during the study period and analysis of habitual food diaries. Compliance was excellent across all three studies, which is exceptional given individuals

were free-living, community-dwelling individuals who were not instructed to alter habitual diet/lifestyle regimes. Although the trials were short-term (4-6 weeks), long-term compliance would be useful to explore, since 50% of individuals self-cease statin therapy after one year [346]; the bioactive combination therapies explored in this research could be a helpful alternative or adjunct tool in these cases.

A high biologically available formulation of curcumin was used for both curcumin intervention trials which is a strength of this research. The curcumin-PHYTOSOME® complex is developed by Indena (Milan, Italy) using patented technology combining curcumin with soy phospholipids [311]. This formulation of curcumin is more bioavailable than the herbal extract due to its enhanced ability to cross the lipid-rich membranes in the body to eventually reach circulation [311]. The PHYTOSOME® utilises phospholipids such as phosphatidylcholine to chaperone polyphenolics such as curcumin through biological membranes [360]. The total curcuminoid absorption from Meriva® is 29-fold higher than unformulated curcuminoids [147, 361]. Unlike other formulations of curcumin, Meriva® is safe and does not bare any side effects [147].

The form of OBG used in the BETA-GAPs trial was of high-MW, which is a strength given the greater capacity to increase viscosity of intestinal contents, thereby exerting potent cholesterol-lowering effects as demonstrated in our trial. Other key strengths of this study include the 2x2 factorial design, intervention of a commonly consumed, portable and shelf-stable snack food, high compliance and the potential for other health benefits.

7.2.2 Limitations

Some limitations when interpreting this work need to be acknowledged. Firstly, the manufacturing of the interventional bread in study 2 which led to lack of findings associated with curcumin. It is still uncertain as to what happened to the curcumin (Meriva®) during the bread manufacturing process, but it is likely the PHYTOSOME® structure was destroyed or degraded during baking at high temperature. Future investigation is required via food kinetic studies to ascertain the stability of curcumin formulations such as Meriva® or SLCP™ whilst considering the food manufacturing process and treatment of ingredients.

Secondly, although compliance was excellent in all three intervention trials, it was self-reported and relied on participants returning the true amount of remaining interventional products. Perhaps using objective compliance measures such as circulating sterol content may provide insight to the few unexpected findings observed in our trials. However, given the 2x2 factorial study design including placebo employed in all three trials, choosing a cohesive collection of blood markers to track compliance to all bioactives makes it impractical.

Thirdly, despite blinding and randomly allocating individuals to treatment groups for all trials, some heterogeneity was evident at baseline in study 3 whereby anthropometric measures such as weight, waist circumference, BMI and fat mass were significantly higher in the PL group compared to the PS-OBG group. These along with other potential confounders were adjusted for and did not predict change in the primary outcome, however, it is noteworthy since higher weight, waist circumference, BMI and fat mass could influence the response to treatment and/or be associated with other chronic risk factors and diet/lifestyle behaviours.

Fourthly, polymorphisms could explain, at least in part, some of the variation, lack of response to intervention and unexpected findings reported in the intervention studies of this thesis. Polymorphisms in apoE are of particular relevance to blood lipids and are responsible for variations in lipoprotein metabolism, the onset of chronic disease and response to diet and lifestyle changes [362, 363]. When apoE genotype distribution was explored in the PAC-CVD Trial, the majority of individuals (71%) possessed the E3 allele and the rest E4 (Table 7.2). This is similar to previous findings as the E3 allele is the most prevalent [364]. Given the large imbalance between allele groups in our study, statistical comparison would have been inadequate to draw conclusions from such a small sample, however, this does highlight the fact that apoE genotype could have been a determinant of the lipid-lowering response observed in our studies. It is known that E4 carriers are at higher risk of chronic disease but are more responsive to dietary changes when compared to E3 and E2 carriers [365-367]. This warrants the need to include polymorphism data as routine measures when investigating nutrition interventions in order to improve understanding and interpretation of clinical outcomes and thus better target nutrition therapies.

Table 7.2: ApoE genotype in individuals in the placebo (PL), phytosterol (PS), curcumin (CC) and phytosterol + curcumin (PS-CC) groups from the PAC-CVD Trial (chapter 4).

	PL	PS	CC	PS-CC	Total
<i>ApoE genotype, n (%)</i> ¹					
ApoE3	11 (61)	15 (88)	13 (72)	11 (65)	50
ApoE4	7 (39)	2 (12)	5 (28)	6 (35)	20

¹ ApoE genotype was determined from purified genomic DNA extracted from buffy coats. Each sample was genotyped for the presence of ApoE variants based on TaqMan SNP genotyping assays as per manufactures instructions (AB Applied Biosystems by Life Technologies, Scoresby, VIC, Australia). The methodology employed for ApoE genotyping has been published previously [368].

ApoE3, apolipoprotein E variant E3; ApoE4, apolipoprotein E variant E4; CC, curcumin; PL, placebo; PS, phytosterols; PS-CC, phytosterol and curcumin.

Lastly, the duration of all the trials are short-term i.e. 4-6 weeks. Although these were substantial to investigate the effects of respective bioactives on the primary outcomes, longer trials are warranted to substantiate the long-term effects on blood lipids and other risk factors for CVD, practical adherence, interaction with lipid-modulating medications or supplements and exploration in other disease groups that could also benefit such as type 2 diabetes, pre-diabetes or hypertriglyceridaemic individuals.

7.3 Conclusions

The results presented in this thesis provide evidence for the combined therapy with bioactive compounds for reducing circulating lipids with translational capacity to the dyslipidaemic population. Findings presented in chapter 3 provide novel evidence for the influence of carrier fats used in PS enriched products that are readily available to consumers. Findings from this portion of work support the use of PS enriched products that are based on a rapeseed/canola fat which are rich in both MUFA and n-3PUFA for enhancing the cholesterol-lowering capacity of PS. This is highly relevant for the management of dyslipidaemia, particularly if an individual is managing existing risk factors through diet/lifestyle changes and/or in combination with medication. Choosing these products may allow the individual to optimise their intervention strategies and possibly better target their lipid goals, leading to an overall enhancement in self-efficacy which is a key driving force to individuals adhering to diet and lifestyle changes long-term.

The results presented in the later part of the thesis are the first to provide clinical evidence for the combination of PS and curcumin for the management of elevated blood cholesterol concentrations in free-living hypercholesterolaemic adults. Beyond the combination, the novelty lies in the effective cholesterol-lowering achieved which surpassed that of solo PS or CC therapy in only 4 weeks. Moreover, the combination of PS and a high-MW OBG provides effective cholesterol-lowering and mild TG-lowering in only 6 weeks, again which surpassed that of solo PS and OBG therapy. The significance of these findings is that these interventions are safe and effective, providing an adjunct and/or alternative solution that is more effective than solo PS therapy for individuals who are statin intolerant or require additional assistance to safely meet blood lipid targets. Findings from chapter 5 provide the first clinical evidence for a functional food enriched with both PS and curcumin and supports the use of bread as an effective food delivery mode for PS. The limitations surrounding the delivery of curcumin is manageable and requires further investigation into the optimal formulation of curcumin to use as well as food matrix for delivery.

Nonetheless, the enriched bread was highly compliant and enjoyed by study participants, supporting the use of fortified staple foods to combat cardiometabolic diseases.

Lastly, the above trials have raised several unresolved questions with great potential to positively impact health beyond blood lipids. The interventions explored in this thesis have potentially widespread beneficial implications for other cardiometabolic conditions such as type 2 diabetes, pre-diabetes, dysfunctional gut, overweight/obesity, postprandial lipaemia and glycaemia and healthy individuals who desire to prevent hereditary cardiometabolic conditions. Future research will involve exploring these bioactive combinations in new functional food formats, differing durations and across a wide range of cardiometabolic conditions in order to capitalise on the health benefits these bioactives have to offer. Bioactive interventions such as these will provide individuals with the self-efficacy to manage their CVD risk factors, empowering and motivating them to take better control of their heart health and move towards their health goals.

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v 1.1

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Confirmation Number: 11827119

Citation Information

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Appendix 3: Ethics approval notification for PAC-CVD Trial

HUMAN RESEARCH ETHICS COMMITTEE



Notification of Approval

To Chief Investigator or Project Supervisor:	Professor Manohar Garg
Cc Co-investigators / Research Students:	Mrs Jessica Ferguson Doctor Lesley MacDonald-Wicks
Re Protocol:	Complementary and/or synergistic effects of phytosterols and curcumin on cardiovascular disease risk factors in hyperlipidaemic individuals.
Date:	18-Jun-2015
Reference No:	H-2015-0162
Date of Initial Approval:	17-Jun-2015

Thank you for your **Initial Application** submission to the Human Research Ethics Committee (HREC) seeking approval in relation to the above protocol.

Your submission was considered under **L3 Full** review by the Committee.

I am pleased to advise that the decision on your submission is **Approved** effective **17-Jun-2015**.

In approving this protocol, the Human Research Ethics Committee (HREC) is of the opinion that the project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research, 2007*, and the requirements within this University relating to human research.

Approval will remain valid subject to the submission, and satisfactory assessment, of annual progress reports. *If the approval of an External HREC has been "noted" the approval period is as determined by that HREC.*

Your approval number is **H-2015-0162**. If the research requires the use of an Information Statement, ensure this number is inserted at the relevant point in the Complaints paragraph prior to distribution to potential participants.

*****Please note – In granting this approval, the Committee agreed that the researchers should still be required to respond to the follow:**

1. Source of recruitment.

The response at section 6.3.1 of the NEAF referred to sourcing participants from an 'existing data base of volunteers that have been identified as likely to meet the inclusion criteria'. It was unclear whether this was referring to the HMRI Volunteer Register or another source. Please clarify.

2. Managing incidental findings.

With any project involving health-based assessments, the researchers need to acknowledge the potential for incidental findings in relation to results and consider how these will be managed. In relation to this project, the Committee agreed that if any of the health-based assessment results indicated a need for further follow-up or referral, this should be presented by the researchers in a letter that the participant could provide to their GP or other health practitioner. This requirement also extended to blood results indicating elevated cholesterol at levels that should be medically monitored and managed.

Please amend the following participant documents as indicated.

3. Participant Information Statement.

a. Eligibility requirements.

The response to section 6.2 of the NEAF indicated that the researchers were seeking to recruit participants who were 'Hyperlipidaemic (fasting total cholesterol \geq 5.5mmol/L)'. The Clinical Protocol also stated that the eligibility criteria required participants to have 'Total plasma cholesterol level $>$ 5.5mmol/L'. There was however no mention of this requirement in the Information Statement. Please clarify or amend.

b. Under Risks and Benefits, indicate the potential for incidental findings and how this will be managed (as per point 2 above).

c. The response to s6.6.1 of the NEAF states that participants will receive \$40 as compensation for time and expenses (pro-rata payments of \$20 per visit are available to those who withdraw prior to study completion).

Potential participants need to be informed of this via the Information Statement.

4. Recruitment Poster.

Please address the repetition in relation to the complaints information. The researchers may wish to note that the complaints statement is not required on Recruitment Posters. The first sentence identifying that the project has been ethically approved and quoting the HREC Approval number should remain.

Matters for noting and/or consideration.

5. Exclusions.

a. In section 6.1.1 of the NEAF the researchers had indicated that the project design specifically excluded 'People who may be involved in illegal activity'. The Committee wished to note to the researchers that as they were unlikely to know whether participants were involved in illegal activity, a more suitable response would be 'Probably coincidental recruitment'.

b. In this same section the researchers had also indicated that 'People whose primary language is other than English (LOTE)' were also excluded. The Committee wished to remind the researchers that even where English was not a person's primary language, it did not necessarily mean that they could not read or understand English to the required degree for participation in the project.

Please submit your response and a copy of any amended documents via email to Ruth.Gibbins@newcastle.edu.au.

Conditions of Approval

This approval has been granted subject to you complying with the requirements for *Monitoring of Progress*, *Reporting of Adverse Events*, and *Variations to the Approved Protocol* as [detailed below](#).

PLEASE NOTE:

In the case where the HREC has "noted" the approval of an External HREC, progress reports and reports of adverse events are to be submitted to the External HREC only. In the case of Variations to the approved protocol, or a Renewal of approval, you will apply to the External HREC for approval in the first instance and then Register that approval with the University's HREC.

- **Monitoring of Progress**

Other than above, the University is obliged to monitor the progress of research projects involving human participants to ensure that they are conducted according to the protocol as approved by the HREC. A progress report is required on an annual basis. Continuation of your HREC approval for this project is conditional upon receipt, and satisfactory assessment, of annual progress reports. You will be advised when a report is due.

- **Reporting of Adverse Events**

1. It is the responsibility of the person **first named on this Approval Advice** to report adverse events.
2. Adverse events, however minor, must be recorded by the investigator as observed by the investigator or as volunteered by a participant in the research. Full details are to be documented, whether or not the investigator, or his/her deputies, consider the event to be related to the research substance or procedure.
3. Serious or unforeseen adverse events that occur during the research or within six (6) months of completion of the research, must be reported by the person first named on the Approval Advice to the (HREC) by way of the Adverse Event Report form (via RIMS at <https://rims.newcastle.edu.au/login.asp>) within 72 hours of the occurrence of the event or the investigator receiving advice of the event.
4. Serious adverse events are defined as:
 - o Causing death, life threatening or serious disability.
 - o Causing or prolonging hospitalisation.
 - o Overdoses, cancers, congenital abnormalities, tissue damage, whether or not they are judged to be caused by the investigational agent or procedure.
 - o Causing psycho-social and/or financial harm. This covers everything from perceived invasion of privacy, breach of confidentiality, or the diminution of social reputation, to the creation of psychological fears and trauma.
 - o Any other event which might affect the continued ethical acceptability of the project.
5. Reports of adverse events must include:
 - o Participant's study identification number;
 - o date of birth;
 - o date of entry into the study;

- treatment arm (if applicable);
 - date of event;
 - details of event;
 - the investigator's opinion as to whether the event is related to the research procedures; and
 - action taken in response to the event.
6. Adverse events which do not fall within the definition of serious or unexpected, including those reported from other sites involved in the research, are to be reported in detail at the time of the annual progress report to the HREC.

- *Variations to approved protocol*

If you wish to change, or deviate from, the approved protocol, you will need to submit an *Application for Variation to Approved Human Research* (via RIMS at <https://rims.newcastle.edu.au/login.asp>). Variations may include, but are not limited to, changes or additions to investigators, study design, study population, number of participants, methods of recruitment, or participant information/consent documentation. **Variations must be approved by the (HREC) before they are implemented** except when Registering an approval of a variation from an external HREC which has been designated the lead HREC, in which case you may proceed as soon as you receive an acknowledgement of your Registration.

Linkage of ethics approval to a new Grant

HREC approvals cannot be assigned to a new grant or award (ie those that were not identified on the application for ethics approval) without confirmation of the approval from the Human Research Ethics Officer on behalf of the HREC.

Best wishes for a successful project.

Professor Allyson Holbrook
Chair, Human Research Ethics Committee

For communications and enquiries:
Human Research Ethics Administration

Research Services
Research Integrity Unit
The Chancellery
The University of Newcastle
Callaghan NSW 2308
T +61 2 492 17894
F +61 2 492 17164
Human-Ethics@newcastle.edu.au

RIMS website - <https://RIMS.newcastle.edu.au/login.asp>

Linked University of Newcastle administered funding:

Funding body	Funding project title	First named investigator	Grant Ref
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Appendix 4: Ethics approval notification for PAC-FOOD Trial

HUMAN RESEARCH ETHICS COMMITTEE



Notification of Expedited Approval

To Chief Investigator or Project Supervisor:	Professor Manohar Garg
Cc Co-investigators / Research Students:	Mrs Rebecca Muddle Mrs Jessica Ferguson Doctor Lesley MacDonald-Wicks Doctor Elizabeth Stojanovski Professor Harjinder Singh
Re Protocol:	Complementary and/or synergistic effects of phytosterols and curcumin on cardiovascular disease risk factors in hyperlipidaemic individuals.
Date:	22-Jan-2018
Reference No:	H-2015-0162

Thank you for your **Variation** submission to the Human Research Ethics Committee (HREC) seeking approval in relation to a variation to the above protocol.

Variation to:

1. Supplement phytosterols and/or curcumin in the form of foods (bread) rather than margarine and capsules as in the original protocol (phase 1). All other study details, protocol etc remain unchanged.
2. Recruit another 80 participants for this phase of the project. Participant description remains unchanged from the original protocol.
3. Abandon the last variation to deliver phytosterols and/or curcumin in the form of a hummus dip.
 - Clinical Protocol (v1.2, dated 7 December 2017)
 - Recruitment Flyer (v5, submitted 7 December 2017)
 - Information Statement (v1.3, dated 7 December 2017)
 - Consent Form (v1.3, dated 7 December 2017)
 - Participant Screening Criteria (v1.3, submitted 7 December 2017)
 - Medical History & Lifestyle Questionnaire (v1.3, submitted 7 December 2017)
 - Handout (v1.1, submitted 7 December 2017)
 - 3-Day Food Diary (v1.3, submitted 7 December 2017)
 - Log of Investigational Product Consumption (v1.3, submitted 7 December 2017)

Your submission was considered under **Expedited** review by the Chair/Deputy Chair.

I am pleased to advise that the decision on your submission is **Approved** effective **22-Jan-2018**.

The full Committee will be asked to ratify this decision at its next scheduled meeting. A formal *Certificate of Approval* will be available upon request.

Chair, Human Research Ethics Committee

For communications and enquiries:

Human Research Ethics Administration

Research & Innovation Services
Research Integrity Unit
The University of Newcastle
Callaghan NSW 2308
T +61 2 492 17894
Human-Ethics@newcastle.edu.au

RIMS website - <https://RIMS.newcastle.edu.au/login.asp>

Linked University of Newcastle administered funding:

Funding body	Funding project title	First named investigator	Grant Ref
BASF (Asia Pacific)/Newtrition Asia Research Grant(**)	Complementary and/or synergistic effects of Phytosterols and Curcumin for reducing Cardiovascular Disease Risk in Hyperlipidaemic Individuals	Garg, Manohar	G1601199
Hunter Medical Research Institute/Postgraduate Research Scholarship(**)	Optimising lipid-lowering ability of dietary phytosterols for reducing cardiovascular disease risk	Garg, Manohar	G1700528
Hunter Medical Research Institute/Greaves Family Postgraduate Scholarship in Medical Research(**)	Optimising lipid-lowering ability of dietary phytosterols for reducing cardiovascular disease risk	Garg, Manohar	G1701564

Appendix 5: Ethics approval notification for BETA-GAPs Trial

HUMAN RESEARCH ETHICS COMMITTEE



Notification of Expedited Approval

To Chief Investigator or Project Supervisor:	Professor Manohar Garg
Cc Co-investigators / Research Students:	Mrs Jessica Ferguson Doctor Lesley MacDonald-Wicks Doctor Elizabeth Stojanovski
Re Protocol:	β -Glucan and Phytosterols for Reducing Cardiovascular Disease Risk: A Randomised Controlled Trial (β -GAPS Trial)
Date:	21-Jul-2017
Reference No:	H-2017-0091
Date of Initial Approval:	21-Jul-2017

Thank you for your **Response to Conditional Approval (minor amendments)** submission to the Human Research Ethics Committee (HREC) seeking approval in relation to the above protocol.

Your submission was considered under **Expedited** review by the Ethics Administrator.

I am pleased to advise that the decision on your submission is **Approved** effective **21-Jul-2017**.

In approving this protocol, the Human Research Ethics Committee (HREC) is of the opinion that the project complies with the provisions contained in the National Statement on Ethical Conduct in Human Research, 2007, and the requirements within this University relating to human research.

Approval will remain valid subject to the submission, and satisfactory assessment, of annual progress reports. *If the approval of an External HREC has been "noted" the approval period is as determined by that HREC.*

The full Committee will be asked to ratify this decision at its next scheduled meeting. A formal *Certificate of Approval* will be available upon request. Your approval number is H-2017-0091.

If the research requires the use of an Information Statement, ensure this number is inserted at the relevant point in the Complaints paragraph prior to distribution to potential participants You may then proceed with the research.

Conditions of Approval

This approval has been granted subject to you complying with the requirements for *Monitoring of Progress, Reporting of Adverse Events, and Variations to the Approved Protocol* as detailed below.

PLEASE NOTE:

In the case where the HREC has "noted" the approval of an External HREC, progress reports and reports of adverse events are to be submitted to the External HREC only. In the case of Variations to the approved protocol, or a Renewal of approval, you will apply to the External HREC for approval in the first instance and then Register that approval with the University's HREC.

- *Monitoring of Progress*

Other than above, the University is obliged to monitor the progress of research projects involving human participants to ensure that they are conducted according to the protocol as approved by the HREC. A progress report is required on an annual basis. Continuation of your HREC approval for this project is conditional upon receipt, and satisfactory assessment, of annual progress reports. You will be advised when a report is due.

- **Reporting of Adverse Events**

1. It is the responsibility of the person first named on this Approval Advice to report adverse events.
2. Adverse events, however minor, must be recorded by the investigator as observed by the investigator or as volunteered by a participant in the research. Full details are to be documented, whether or not the investigator, or his/her deputies, consider the event to be related to the research substance or procedure.
3. Serious or unforeseen adverse events that occur during the research or within six (6) months of completion of the research, must be reported by the person first named on the Approval Advice to the (HREC) by way of the Adverse Event Report form (via RIMS at <https://rims.newcastle.edu.au/login.asp>) within 72 hours of the occurrence of the event or the investigator receiving advice of the event.
4. Serious adverse events are defined as:
 - o Causing death, life threatening or serious disability.
 - o Causing or prolonging hospitalisation.
 - o Overdoses, cancers, congenital abnormalities, tissue damage, whether or not they are judged to be caused by the investigational agent or procedure.
 - o Causing psycho-social and/or financial harm. This covers everything from perceived invasion of privacy, breach of confidentiality, or the diminution of social reputation, to the creation of psychological fears and trauma.
 - o Any other event which might affect the continued ethical acceptability of the project.
5. Reports of adverse events must include:
 - o Participant's study identification number;
 - o date of birth;
 - o date of entry into the study;
 - o treatment arm (if applicable);
 - o date of event;
 - o details of event;
 - o the investigator's opinion as to whether the event is related to the research procedures; and
 - o action taken in response to the event.
6. Adverse events which do not fall within the definition of serious or unexpected, including those reported from other sites involved in the research, are to be reported in detail at the time of the annual progress report to the HREC.

- **Variations to approved protocol**

If you wish to change, or deviate from, the approved protocol, you will need to submit an *Application for Variation to Approved Human Research* (via RIMS at <https://rims.newcastle.edu.au/login.asp>). Variations may include, but are not limited to, changes or additions to investigators, study design, study population, number of participants, methods of recruitment, or participant information/consent documentation. **Variations must be approved by the (HREC) before they are implemented** except when Registering an approval of a variation from an external HREC which has been designated the lead HREC, in which case you may proceed as soon as you receive an acknowledgement of your Registration.

Linkage of ethics approval to a new Grant

HREC approvals cannot be assigned to a new grant or award (ie those that were not identified on the application for ethics approval) without confirmation of the approval from the Human Research Ethics Officer on behalf of the HREC.

Best wishes for a successful project.

Associate Professor Helen Warren-Forward
Chair, Human Research Ethics Committee

For communications and enquiries:

Human Research Ethics Administration

Research & Innovation Services
Research Integrity Unit
NIER, Block C
The University of Newcastle
Callaghan NSW 2308
T +61 2 492 17894
Human-Ethics@newcastle.edu.au

RIMS website - <https://RIMS.newcastle.edu.au/login.asp>

Linked University of Newcastle administered funding:

Funding body	Funding project title	First named investigator	Grant Ref
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Appendix 6: General medical history questionnaire

ID _____



Professor Manohar Garg
Nutraceuticals Research Group
School of Biomedical Sciences and Pharmacy
Tel: (02) 4921 5647
Fax: (02) 4921 2028
manohar.garg@newcastle.edu.au

**MEDICAL HISTORY QUESTIONNAIRE FOR THE
STUDY:**

**Novel food enriched with phytosterols and curcumin for heart health
benefits
PAC-CVD (FOOD) Trial**

***Please ensure you complete this prior to your appointment and bring it
with you.**



Professor Manohar Garg
Nutraceuticals Research Group
School of Biomedical Sciences and Pharmacy
Tel: (02) 4921 5647
Fax: (02) 4921 2028
manohar.garg@newcastle.edu.au

MEDICAL HISTORY QUESTIONNAIRE FOR THE RESEARCH PROJECT:

Novel food enriched with phytosterols and curcumin for heart health benefits

PAC-CVD (FOOD) Trial

Participant ID Number: _____ Date: ___/___/___

Date of birth: ___/___/___ Sex: _____ Height: _____ cm Weight: _____ kg

Please write information or tick boxes where appropriate.

1. Which of the following ancestry do you most identify with? (only tick up to two)

- Aboriginal or Torres Strait Islander
- Northern European (English, Irish, Norwegian, Swedish, Finish, Dutch etc)
- Western European (German, French, Swiss etc)
- Asian (e.g. Chinese, Malaysian, Japanese, Indian, Sri Lankan)
- Mediterranean (Greek, Spanish, Italian, French)
- New Zealand
- Australian South Sea Islander
- Mixed / Other (*please specify*): _____

2. Please list all current medical conditions for which you are receiving treatment:

Condition: _____ Year diagnosed: _____
Condition: _____ Year diagnosed: _____
Condition: _____ Year diagnosed: _____

3. In the last 3 months, has a doctor told you:

- Your cholesterol levels were high? Yes No
- Your triglyceride levels were high? Yes No

Your blood pressure was high? Yes No

Comments: _____

4. In the last 3 months, have you experienced:

- Chronic or recurrent coughs
- Increased anxiety or depression
- Problems with recurrent fatigue, thirst, trouble sleeping or increased irritability
- Migraine or recurrent headaches
- Gastrointestinal problems e.g. recurrent heartburn, ulcers, constipation, diarrhoea, nausea
- Significant unexplained weight loss
- Lung and respiratory diseases
- Severe neurological diseases or seizures
- Under investigational drug 3 months prior to this study

5. Please list all medications that you are currently taking, as prescribed by a doctor:

Brand name: _____ Dose: _____ Frequency: _____

Brand name: _____ Dose: _____ Frequency: _____

Brand name: _____ Dose: _____ Frequency: _____

6. Please list all over-the-counter medications that you are currently taking regularly:

Brand name: _____ Dose: _____ Frequency: _____

Brand name: _____ Dose: _____ Frequency: _____

Brand name: _____ Dose: _____ Frequency: _____

7. Please list all vitamin, mineral, and/or herbal supplements that you are currently taking regularly:

Brand name: _____ Dose: _____ Frequency: _____

Brand name: _____ Dose: _____ Frequency: _____

Brand name: _____ Dose: _____ Frequency: _____

8. Please tick any of the following you have / have had before:

- | | |
|--|--|
| <input type="checkbox"/> Heart attack | <input type="checkbox"/> Pacemaker implant |
| <input type="checkbox"/> Stroke | <input type="checkbox"/> Myocardial infarction |
| <input type="checkbox"/> Angina | <input type="checkbox"/> Heart failure |
| <input type="checkbox"/> By-pass surgery | <input type="checkbox"/> Atherosclerosis |
| <input type="checkbox"/> Coronary artery disease | |

9. Please tick any of the following that apply to you:

- | | |
|--|--|
| <input type="checkbox"/> Gluten intolerance or sensitivity | <input type="checkbox"/> Pneumonia |
| <input type="checkbox"/> Wheat intolerance or sensitivity | <input type="checkbox"/> Epilepsy or seizures |
| <input type="checkbox"/> Diabetes or abnormal blood-sugar levels | <input type="checkbox"/> Other lung disease |
| <input type="checkbox"/> Hypertension | <input type="checkbox"/> Liver disease (i.e. cirrhosis, jaundice, fibrosis, hepatitis) |
| <input type="checkbox"/> Dizziness or fainting spells | <input type="checkbox"/> Kidney/renal disease or failure |
| <input type="checkbox"/> Asthma | |
| <input type="checkbox"/> Bronchitis | |
| <input type="checkbox"/> Cancer (<i>If yes, please specify</i> _____) | |

10. Women only: (Men skip to Q11)

Do you have menstrual problems? Yes No
(*If yes, please specify* _____)

Are you on hormone replacement therapy? Yes No
(*If yes, please specify* _____)

11. Have you ever smoked?

- Yes No

(If no, skip to Q12)

- a) If you currently smoke, how many cigarettes per day? _____
b) What age did you start smoking? _____
c) If you have quit smoking, how long ago did you quit? _____

12. How often do you have a drink containing alcohol (drink meaning 350mL can beer or 150mL wine or 1 shot of liquor?)

- Never 2-3 times / week
 Monthly or less 4 or more times / week
 2-4 times / month Everyday

13. Do you usually use oils or margarine in place of butter / lard / ghee?

- Yes No I do not use either

14. Do you regularly use any of the following: (see pictures below if unsure)

- Logicol margarine spread
 Flora Pro-activ margarine spreads
 Tablelands 'reduce cholesterol absorption' margarine spread
 Heart Active Milk (Dairy Farmers or Pura)



15. Do you regularly consume curcumin supplements and/or turmeric supplements?

- Yes No

16. How often would you normally eat bread?

- Never 1-2 days / week
 Monthly or less 3-4 days / week
 One day / week Everyday

17. Do you regularly (≥ 4 days/wk) consume fibre supplements e.g. psyllium husk, Metamucil, Benefibre etc?

- Yes No

18. Do you usually add sugar to your hot drinks (NOT including sweeteners)?

- Yes No

19. Do you usually add salt at the table?

- Yes No No, only when cooking

20. Do you eat differently on weekends compared to weekdays?

- Yes No

(If yes, would it be more or less? Please specify: _____)

Thank you for taking time to complete this questionnaire

Appendix 7: Three-day food and beverage diary

ID _____



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3 –DAY FOOD DIARY FOR THE STUDY:

**Novel foods enriched with phytosterols and curcumin for heart health
benefits**

PAC-CVD (FOOD) Trial

***Please ensure you complete this prior to your appointment and bring
it with you.**

2 weekdays + 1 weekend day



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3-DAY FOOD DIARY FOR THE RESEARCH PROJECT:

Novel foods enriched with phytosterols and curcumin for heart health benefits PAC-CVD (FOOD) Trial

Instructions for recording food and drink consumption

You are being asked to record everything you eat and drink for a period of 3 days. Choose consecutive days made up of 2 week days and 1 weekend day. The information you record is very important to the success of this study. These instructions will help you to fill in your food and drink consumption accurately.

Please complete the food diary prior to your appointment and be sure to bring it along with you.

Instructions:

- Write down **everything** you eat and drink (including water) for **3 consecutive days** (i.e. 2 week days and 1 weekend day).
- Include as much detail as possible.
- Provide the date and day for each entry.
- Start a new page each day (pages are provided):
 - **List the food and beverage and provide a description. Include food preparation and cooking methods** (e.g. grilled rump steak, deep fried fish fillets, grilled chicken breast marinated in soy sauce, fried lamb chops, fat trimmed)
 - **Include the food type** i.e. light, full-fat, fat-reduced (e.g. skim milk, light cream, full fat ice-cream)
 - **State the weight of the food or use a standard household measure** i.e. cup, tablespoon, teaspoon (e.g. 1 cup of milk, 1 tablespoon of butter, 100mL cream)
 - **Specify brand names of processed products where possible** (e.g. Yoplait yoghurt, Oak chocolate flavoured milk)
 - **Include supplements and any condiments used** such as sauces, salad dressings, dipping sauces, marinades etc.
- **Try to record each item when you eat or drink, so that nothing is forgotten.**
- **There is an extra piece of paper at the end in case you need more room – please label this clearly.**

Example

Time and Meal	Food / Drink	Description	Quantity
7 am (Breakfast)	Toast	Tip Top, Wholemeal sandwich	2
	Peanut butter	Mayvers, crunchy	2 tsp
	Black coffee + 1 teaspoon sugar	Nescafe Instant	1 cup
10am (Morning tea)	Banana		1 medium
	Greek Yoghurt	Farmer's Union, full fat	100g tub
	Water		1 cup
1 pm (Lunch)	1 Ham and salad sandwich	PAC-CVD FOOD study food	2 slices
	Baby spinach leaves		8-10
	Tomato		3 small slices
	Beetroot slices	Edgell's tinned	1 large slice
	Smoked ham	Coles Deli	2 slices
	Cheese	Coon regular sandwich slices	1 slice
	Sprite lemonade		355mL can
4pm (Afternoon tea)	Chocolate cream-filled biscuits	Arnott's	2
	Tea (with splash of milk) + 1 teaspoon sugar	Dairy Farmer's lite	1 cup
7:30pm (Dinner)	Chicken schnitzel		1 hand-sized breast
	Mashed potato		1 cup
	Butter		1 teaspoon
	Milk	Dairy Farmer's lite	100mL
	Steamed carrots		½ large
	Steamed broccoli		3 large florets
	Steamed corn		½ cup
	Water		3 regular glasses
9pm (Supper/dessert)	Mini apple pie	Nanna's	1
	Custard	Pauls double thick	½ cup
	Tea (made as above)		1 cup

Appendix 8: Self-Administered International Physical Activity Questionnaire (Long Form)

ID _____



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PHYSICAL ACTIVITY QUESTIONNAIRE FOR THE STUDY:

**Novel food enriched with phytosterols and curcumin for heart
health benefits
PAC-CVD (FOOD) Trial**

***Please ensure you complete this prior to your appointment and
bring it with you.**

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health-related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an *International Physical Activity Prevalence Study* is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

Yes

No →

Skip to PART 2: TRANSPORTATION

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ **days per week**

No vigorous job-related physical activity →

Skip to question 4

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ **hours per day**

_____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ **days per week**

No moderate job-related physical activity →

Skip to question 6

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

No job-related walking → **Skip to PART 2: TRANSPORTATION**

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

No traveling in a motor vehicle → **Skip to question 10**

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

No bicycling from place to place → **Skip to question 12**

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

No walking from place to place



***Skip to PART 3: HOUSEWORK,
HOUSE MAINTENANCE, AND
CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

No vigorous activity in garden or yard



Skip to question 16

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

No moderate activity in garden or yard



Skip to question 18

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

No moderate activity inside home



**Skip to PART 4: RECREATION,
SPORT AND LEISURE-TIME
PHYSICAL ACTIVITY**

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

No walking in leisure time



Skip to question 22

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

No vigorous activity in leisure time



Skip to question 24

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

No moderate activity in leisure time



Skip to PART 5: TIME SPENT SITTING

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Appendix 9: Statement of authorship

Statement of contribution and collaboration for chapter 3

I confirm that Research Higher Degree candidate Jessica JA Ferguson contributed to the following publication:

Ferguson JJA, Stojanovski E, MacDonald-Wicks L, Garg ML. Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs. *Progress in Lipid Research*. 2016, 64:16-29.

Jessica JA Ferguson designed the research, conducted the literature search and review, wrote the paper and had primary responsibility for the final manuscript. Dr Elizabeth Stojanovski provided statistical support, Dr Lesley MacDonald Wicks wrote the paper and Professor Manohar L Garg designed the research and wrote the paper within his capacity as primary supervisor. All authors read and approved the final manuscript.

Mrs Jessica JA Ferguson

Date: 06-02-2019

Dr Elizabeth Stojanovski

Date: 07-02-2019

Lesley MacDonald-Wicks

Date: 07-02-2019

Professor Manohar L Garg

Date: 07-02-2019

Signature of Faculty Assistant Dean (Research Training)

Date: 08-02-2019

Appendix 10: Statement of authorship

Statement of contribution and collaboration for chapter 4

I confirm that Research Higher Degree candidate Jessica JA Ferguson contributed to the following publication:

Ferguson JJA, Stojanovski E, MacDonald-Wicks L, Garg ML. Curcumin potentiates cholesterol-lowering effects of phytosterols in hypercholesterolaemic individuals. A randomized controlled trial. *Metabolism – Clinical and Experimental*. 2018, 82:22-35.

Jessica JA Ferguson designed the research, conducted the trial including recruitment of participants, conducted trial visits, collection and statistical analysis of data, wrote the paper and had primary responsibility for the final manuscript. Dr Elizabeth Stojanovski provided statistical support, Professor Manohar L Garg designed the research and wrote the paper within his capacity as primary supervisor. All authors read and approved the final manuscript.

_____ Date: 06-02-2019

Mrs Jessica JA Ferguson

_____ Date: 07-02-2019

Dr Elizabeth Stojanovski

_____ Date: 07-02-2019

Dr Lesley MacDonald-Wicks

_____ Date: 07-02-2019

Professor Manohar L Garg

_____ Date: 08-02-2019

Signature of Faculty Assistant Dean (Research Training)

Appendix 11: Supplementary Table 1 for Chapter 5

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 (25th and 75th 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.

Appendix 12: Supplementary Table 2 for Chapter 5

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	-0.44 ± 0.07	0.21 ± 0.05	-0.44 ± 0.12
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	-0.35 ± 0.06	0.18 ± 0.07	-0.52 ± 0.11
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	4.12 ± 0.21	4.73 ± 0.29	5.44 ± 0.38
PI	5.00 ± 0.34	3.95 ± 0.20	4.77 ± 0.29	5.06 ± 0.33***
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)	-0.50 (-1.42, -0.07)	-0.15 (-0.50, 1.63)	-0.95 (-2.21, -0.10)

¹ Values are reported as means ± SEM for all plasma concentrations except 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, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

Appendix 13: Supplementary Table 3 for Chapter 5

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

	Coefficient²	t	p
<i>Absolute change in TC (mmol/L)</i>			
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
<i>Relative change in TC (%)</i>			
TC	-2.40 ± 0.63	-3.84	<0.001
Treatment	-6.99 ± 1.18	-5.94	<0.001
<i>Absolute change in LDL-C (mmol/L)</i>			
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
<i>Relative change in LDL-C (%)</i>			
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
<i>Absolute change in LDL-P (nmol/L)</i>			
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
<i>Relative change in LDL-P (%)</i>			
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
<i>Absolute change in LDL-Z (nm)</i>			
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
<i>Relative change in LDL-Z (%)</i>			
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

Appendix 14: Statement of authorship

Statement of contribution and collaboration for chapter 6

I confirm that Research Higher Degree candidate Jessica JA Ferguson contributed to the following publication:

Ferguson JJA, Stojanovski E, MacDonald-Wicks L, Garg ML. High molecular weight oat β -glucan enhances lipid-lowering effects of phytosterols. A randomized controlled trial. *Clinical Nutrition*. 2019. Accepted for publication.

Jessica JA Ferguson designed the research, conducted the trial including recruitment of participants, conducted trial visits, collection and statistical analysis of data, wrote the paper and had primary responsibility for the final manuscript. Dr Elizabeth Stojanovski provided statistical support, Professor Manohar L Garg designed the research and wrote the paper within his capacity as primary supervisor. All authors read and approved the final manuscript.

Mrs Jessica JA Ferguson

Date: 06-02-2019

Dr Elizabeth Stojanovski

Date: 07-02-2019

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Date: 07-02-2019

Signature of Faculty Assistant Dean (Research Training)

Date: 08-02-2019

Appendix 15: Lipoprotein profiles (total particle concentration and particle size) at baseline and post-intervention measured using nuclear magnetic resonance spectroscopy.

	PL	PS	OBG	PS-OBG	<i>p</i> ^
<i>Total lipoprotein particle concentration</i>					
TRL-P (nmol/L)					
BL	157.24 ± 18.14	170.11 ± 15.35	204.70 ± 19.22	188.88 ± 15.95	0.232
PI	155.56 ± 22.25	142.02 ± 12.06	177.68 ± 20.21	153.85 ± 13.45	0.544
Δ nmol/L	-1.68 ± 12.44	-28.08 ± 12.54*	-27.02 ± 8.72**	-35.03 ± 6.50***	0.124
Δ %	-1.46 ± 7.44	-11.91 ± 6.57	-15.04 ± 4.76**	-18.25 ± 3.18***	0.194
LDL-P (nmol/L)					
BL	1869.67 ± 84.28	1757.83 ± 77.19	1929.89 ± 107.39	1874.50 ± 86.30	0.586
PI	1822.56 ± 78.46	1665.89 ± 71.19	1814.83 ± 102.99	1678.06 ± 76.32	0.379
Δ nmol/L	-47.11 ± 44.94	-91.94 ± 48.26	-115.06 ± 36.98**	-196.44 ± 36.80***	0.094
Δ %	-1.81 ± 2.20	-4.55 ± 2.71	-5.60 ± 1.83**	-10.08 ± 1.80***	0.065
HDL-P (μmol/L)					
BL	23.92 ± 0.73	22.86 ± 0.66	22.89 ± 0.63	23.33 ± 0.49	0.608
PI	23.77 ± 0.69	23.10 ± 0.59	22.85 ± 0.42	23.54 ± 0.64	0.688
Δ μmol/L	-0.15 ± 0.44	0.24 ± 0.32	-0.04 ± 0.41	0.21 ± 0.33	0.854
Δ %	-0.26 ± 1.83	1.37 ± 1.40	0.44 ± 1.78	0.81 ± 1.37	0.908
<i>Lipoprotein particle size (nm)</i>					
TRL-Z					

BL	47.05 (41.50, 50.30)	42.70 (37.90, 44.80)	45.80 (41.90, 51.30)	43.15 (39.80, 52.70)	0.265
PI	44.50 (42.50, 55.20)	43.70 (39.10, 46.80)	45.30 (43.50, 50.10)	47.20 (39.80, 49.40)	0.516
Δ nm	0.85 (-4.20, 3.30)	1.15 (0.10, 4.20)	-1.00 (-6.40, 3.60)	-0.80 (-3.70, 5.30)	0.724
Δ %	0.34 ± 2.96	2.70 ± 2.96	-2.04 ± 3.49	0.51 ± 4.03	0.806
LDL-Z					
BL	21.20 (21.00, 21.50)	21.35 (21.00, 21.80)	21.30 (20.90, 21.50)	21.35 (20.80, 21.50)	0.719
PI	21.25 (21.00, 21.60)	21.15 (20.90, 21.60)	21.25 (20.80, 21.40)	21.05 (20.70, 21.40)	0.760
Δ nm	0.00 (-0.20, 0.20)	-0.10 (-0.40, 0.10)	-0.10 (-0.30, 0.10)	-0.25 (-0.40, 0.10)	0.497
Δ %	-0.00 (-0.94, 0.96)	-0.46 (-1.83, 0.47)	-0.47 (-1.38, 0.48)	-1.17 (-1.90, 0.47)	0.540
HDL-Z					
BL	9.06 ± 0.10	9.10 ± 0.12	9.11 ± 0.09	9.17 ± 0.10	0.879
PI	9.08 ± 0.11	9.14 ± 0.13	9.09 ± 0.08	9.06 ± 0.11	0.963
Δ	0.03 ± 0.05	0.04 ± 0.04	-0.02 ± 0.04	-0.11 ± 0.05*	0.093
Δ %	0.31 ± 0.54	0.42 ± 0.48	-0.15 ± 0.42	-1.20 ± 0.55*	0.098

¹ Values are reported as means ± SEM or median (25th and 75th percentile) as per normality. Wilcoxon Signed Rank Test or Paired Samples t-test was used to investigate change from baseline within groups. Significant change from baseline, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

^ P -values for comparison between intervention groups was conducted using one-way analysis of variance by Kruskal-Wallis or one-way ANOVA.

BL, baseline; HDL-Z, high-density lipoprotein particle size; LDL-Z, low-density lipoprotein particle size; OBG, oat beta-glucan; PI, post-intervention; PL, placebo; PS, phytosterols; PS-OBG, phytosterol + oat beta-glucan; TRL-Z, triglyceride-rich lipoprotein particle size.