The Effects of (-)-Epigallocatechin-3-O-gallate (EGCG), a Green Tea Catechin, on Blood Cholesterol

Thesis submitted as fulfilment of the requirement for the degree of
Doctor of Food Science

By
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August, 2011

School of Environmental and Life Sciences
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Declaration of Authorship

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other university or institution

_________________________________________________________

Nenad Naumovski

Acknowledgment of Collaboration

I hereby certify that the part of the work done on the scavenger receptor cluster of differentiation 36 (CD36) in rabbit liver samples was done in collaboration with Dr Rick Thorne and Dr Kristy Shipman of the Cancer Research unit of the University of Newcastle.

_________________________________________________________

Nenad Naumovski
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If this thesis is ever a success, it is not my achievement alone, it is the achievement of all those people around me, named and the ones that I have mistakenly forgotten, who have guided and helped me. My appreciation goes to everyone who has contributed to this work, to my degree and to the benefit of Humankind and World Peace above all.

“Ja sam svoju glavu dao čestiti care za svoju reč...” – Miloš Obilić (1389, Boj na Kosovu)
Table of Contents

Declaration of Authorship / Acknowledgment of Collaboration........................................... ii
Acknowledgments................................................................................................................................... iii
Table of Contents...................................................................................................................................... iv
Abstract...................................................................................................................................................... xiv
Abbreviations............................................................................................................................................. xvii
List of Tables................................................................................................................................................. xxii
List of Figures................................................................................................................................................ xlv
Publications arising from this thesis............................................................................................................ xxx

Chapter 1. Introduction............................................................................................................................... 1

1.1 Cardiovascular disease......................................................................................................................... 1
  1.1.1 Global burden of CVD.................................................................................................................... 1
  1.1.2 CVD in Australia.............................................................................................................................. 2
  1.1.3 Atherosclerosis............................................................................................................................... 2
  1.1.4 Theories of atherosclerosis............................................................................................................. 4
  1.1.5 Risk factors for atherosclerosis........................................................................................................ 9
    1.1.5.1 Established risk factors for atherosclerosis........................................................................... 9
    1.1.5.2 Other risk factors of atherosclerosis......................................................................................... 16
    1.1.5.3 Non-traditional risk factors of atherosclerosis.......................................................................... 22
  1.2 Cholesterol........................................................................................................................................... 25
    1.2.1 Cholesterol Synthesis.................................................................................................................... 27
    1.2.1.1 Regulation of cholesterol synthesis – negative feedback...................................................... 32
    1.2.2 Absorption of dietary cholesterol................................................................................................ 34
    1.2.3 Cholesterol esterification............................................................................................................... 35
    1.2.4 Bile acid synthesis – cholesterol metabolism............................................................................... 37
  1.3 Lipids and lipoproteins......................................................................................................................... 39
    1.3.1 Lipoprotein metabolism................................................................................................................ 41
      1.3.1.1 Chylomicrons............................................................................................................................ 41
      1.3.1.2 Chylomicron remnants............................................................................................................. 42
Chapter 2. General Methods ................................................................. 77

2.1 Introduction ............................................................................. 77

2.2 Ethics approvals ................................................................. 78

2.3 Chemicals and Reagents ....................................................... 78

2.4 Purity of the EGCG used in the rabbit and human studies ............... 80

2.4.1 Preparation of working standards for the EGCG standard curve .......... 80

2.4.2 Equipment and chromatographic conditions ............................... 81

2.4.3 Preparation of sample for determining the EGCG purity ................. 82

2.4.4 Identification of the standards on the HPLC chromatogram ................ 82

2.4.5 Preparation of the standard curve ........................................... 82

2.4.6 Calculation of the purity of the DSM Nutritionals EGCG .......... 84

2.5 Serum and plasma analysis in rabbit and human studies .................... 84

2.5.1 Triglycerides, total cholesterol, HDL cholesterol and glucose analysis using commercially available enzyme kits ......................................................... 85

2.5.2 Estimation of non-HDL, VLDL and LDL cholesterol ....................... 86

2.5.3 Analysis of serum sterols using gas chromatography (GC). ................ 87

2.5.4 Serum squalene by High Pressure Liquid Chromatography ............ 87

2.5.5 Serum thiol analysis by High Pressure Liquid Chromatography ........ 87

2.5.6 Determination of EGCG in human plasma ................................. 88

2.6 Rabbit faecal samples ............................................................ 88

2.6.1 Total faecal fat content ......................................................... 88

2.6.2 Faecal neutral sterols using Gas Chromatography ......................... 88

2.6.3 Faecal bile acids using Gas Chromatography ................................ 89

2.6.4 Faecal EGCG by High Pressure Liquid Chromatography ............. 89

2.7 Liver Samples ........................................................................ 89

2.7.1 Collection of liver samples .................................................... 89

2.7.2 Preparation of soluble rabbit liver membrane proteins ................. 90

2.7.3 Cholesterol and triglyceride in liver homogenates and membranes ....... 90

2.7.4 Analysis of LDL-receptor and CD36 in liver homogenates and solubilised liver membranes ................................................................. 90
Chapter 3. Systemic effects of EGCG in the cholesterol-fed rabbit model of hypercholesterolaemia

3.1 Introduction

3.1.1 Green tea extracts and inhibition of cholesterol synthesis in animal models

3.1.2 Green tea and up-regulation of the LDL-receptor

3.2 Main hypothesis and aims

3.3 Secondary aims

3.3.1 Hepatic CD36

3.3.2 Plasma homocysteine

3.4 Methods

3.4.1 Ethics approvals

3.4.2 Food preparation

3.4.3 Animals and the experimental design

3.4.4 Serum analysis

3.4.4.1 Serum lipids using commercially available enzyme kits

3.4.4.2 Analysis of serum glucose

3.4.4.3 Analysis of serum sterols using Gas Chromatography

3.4.4.4 Serum squalene by high pressure liquid chromatography
4.2.8.5 Identification of the bile acids on GC chromatogram..........................166
4.2.8.6 The standard curve and calculation of bile acid concentrations..............166
4.2.8.7 Intra- and inter-assay coefficients of variation for bile acids .................169
4.2.8.8 Determination of faecal bile acids to dietary cholesterol ratios .............169
4.2.8.9 Determination of faecal neutral and acidic sterol ratios.......................170
4.2.8.10 Determination of sterol balance..................................................171
4.2.9 Faecal EGCG by High Pressure Liquid Chromatography..........................171
4.2.9.1 Preparation of standards for the standard curve.................................171
4.2.9.2 Selection of a quality control sample.............................................171
4.2.9.3 Extraction of free and conjugated EGCG in faecal samples..................172
4.2.9.4 Equipment and chromatographic conditions.....................................173
4.2.9.5 Identification of EGCG on the HPLC chromatogram for faeces .............173
4.2.9.6 The standard curve and calculation of EGCG concentrations.................174
4.2.9.7 Intra- and inter-assay coefficients of variations for faecal EGCG ............175
4.2.10 Statistical Analysis...............................................................................175
4.3 Results......................................................................................................176
4.3.1 Rabbits’ appearance and body weight..................................................176
4.3.2 Faecal weight, pH, moisture and total fat content....................................177
4.3.3 Faecal neutral and acidic sterols............................................................178
4.3.4 Cholesterol intake and absorption and sterol balance.............................183
4.3.5 Amounts of faecal sterols relative to dietary cholesterol intake................186
4.3.6 Amounts of faecal cholesterol relative to faecal acidic sterols...............189
4.3.7 Recovery of free and conjugated EGCG in faecal samples.......................192
4.4 Discussion...............................................................................................194

Chapter 5. The Effect of Food on the Systemic Absorption of Epigallocatechin Gallate in Humans..........................................................201
5.1 Introduction..............................................................................................201
5.1.1 Stability of EGCG in pure form and in food matrices............................202
5.1.2 Absorption, metabolism and bioavailability of EGCG.........................203
5.1.3 Pharmacokinetic studies of EGCG in healthy human volunteers............204
5.1.4 Aims and Hypothesis ................................................................. 206
5.2 Methods ..................................................................................... 207
5.2.1 Preparation of EGCG delivery products ........................................... 207
5.2.1.1 Capsule preparation .............................................................. 207
5.2.1.2 Strawberry sorbet preparation ................................................. 207
5.2.1.2.1 Recovery of EGCG from strawberry sorbet ......................... 209
5.2.1.2.2 HPLC setting and identification of internal standard and EGCG on the HPLC chromatogram ......................................................... 210
5.2.1.2.3 The standard curve and calculation of the EGCG concentration ........ 210
5.2.2 Participants .............................................................................. 211
5.2.2.1 Ethics approval ................................................................. 211
5.2.2.2 Recruitment and selection criteria ........................................... 211
5.2.2.3 Study clinics ......................................................................... 212
5.2.2.3.1 Breakfast and lunch composition ........................................ 213
5.2.2.3.2 Blood collection and handling ............................................. 214
5.2.2.4 Determination of EGCG in plasma samples ............................. 215
5.2.2.4.1 Chemicals and reagents ...................................................... 216
5.2.2.4.2 Preparation of stock and standard solutions .......................... 216
5.2.2.4.3 Preparation of quality control samples ................................. 217
5.2.2.4.4 Preparation of participants’ plasma samples ......................... 218
5.2.2.4.5 Extraction of EGCG from plasma and HPLC analysis ............ 218
5.2.2.4.6 Equipment and chromatographic conditions ......................... 219
5.2.2.4.6.1 Tuning and setting parameters of the mass spectrometer .... 220
5.2.2.4.6.2 Linearity, limit of detection and limit of quantification .......... 221
5.2.2.4.7 Plasma extraction efficiency and assay variation ..................... 221
5.2.2.5 Statistical and pharmacokinetic analysis ................................... 222
5.3 Results ....................................................................................... 224
5.3.1 Measurement of EGCG in strawberry sorbet ................................ 224
5.3.2 Measurement of EGCG in human plasma .................................. 226
5.3.2.1 Linearity, limit of detection and limit of quantification ............... 226
5.3.2.2 Plasma extraction efficiency and assay variation……………………………………227
5.3.3 Participants’ anthropometric and blood pressure data……………………………229
5.3.4 Plasma EGCG concentration-time results…………………………………………231
5.3.5 Pharmacokinetic parameters of plasma EGCG……………………………………233
5.3.6 Breakfast and lunch composition analysis…………………………………………235
5.3.7 Correlations between AUC and macronutrients…………………………………237
5.4 Discussion………………………………………………………………………………………238

Chapter 6. The effects of epigallocatechin-3-gallate on blood lipids in humans with moderately elevated blood cholesterol levels – a pilot study…………………………249
6.1 Introduction……………………………………………………………………………………249
6.1.1 Aims and hypothesis……………………………………………………………………251
6.2 Methods………………………………………………………………………………………253
6.2.1 Capsule preparation……………………………………………………………………253
6.2.2 Participants…………………………………………………………………………………253
6.2.2.1 Ethics approval………………………………………………………………………253
6.2.2.2 Recruitment and selection criteria………………………………………………253
6.2.2.3 Study clinics…………………………………………………………………………255
6.2.2.4 Blood collection and storage………………………………………………………258
6.2.2.5 Determination of plasma total cholesterol, HDL cholesterol and triglycerides.………………………………………………………………………………………258
6.2.2.6 Estimation of VLDL, LDL and non-HDL cholesterol……………………………260
6.2.2.7 Indices of cardiovascular risk………………………………………………………261
6.2.2.8 Nutrient analysis of dietary intake …………………………………………………261
6.2.2.9 Monitoring of adverse effects……………………………………………………261
6.2.2.10 Data and statistical analyses………………………………………………………261
6.3 Results…………………………………………………………………………………………263
6.3.1 Gender, age, anthropometric data and blood pressure……………………………263
6.3.2 Plasma lipid profiles and indices of CV risk………………………………………..263
6.3.3 Dietary intakes………………………………………………………………………………268
6.3.4 Correlations…………………………………………………………………………………270
6.3.5 Capsule consumption compliance and adverse effects
6.4 Discussion

Chapter 7. General Discussion and Conclusions

7.1 Mechanisms by which pure EGCG lowered blood cholesterol in the cholesterol-fed hypercholesterolaemic rabbit model
7.2 Maximising the systemic absorption of EGCG in humans
7.3 Pilot study with EGCG in humans with moderately elevated cholesterol
7.4 Study limitations
7.5 Future directions
7.6 Conclusions

8. References
Abstract

**Background:** The catechin, (-)-epigallocatechin-3-O-gallate (EGCG), the most abundant compound in green tea, has been linked to numerous beneficial health effects, including a reduction in blood cholesterol levels and protection against cardiovascular disease. Previous studies, mostly with extracts of green tea containing mixtures of catechins and other compounds, in animal models of hypercholesterolaemia including the cholesterol-fed rabbit, have shown that these preparations can lower blood cholesterol.

Three plausible mechanisms by which they could lower cholesterol have been postulated: 1) an increase in the LDL-receptor and 2) a reduction in cholesterol synthesis postulated from studies in the hypercholesterolaemic rabbit model and in cultured human HepG2 liver cells and 3) an inhibition of intestinal cholesterol absorption postulated from studies in mice, rats and hamsters.

However, it is not known whether EGCG, as a pure compound, can lower cholesterol in the rabbit model and in humans and whether it works through the three postulated mechanisms of action.

**Hypotheses and Aims:** The working hypothesis for this thesis was that ‘pure EGCG will lower cholesterol in hypercholesterolaemic rabbits and humans’. Therefore, the aim was to determine the effect of pure EGCG on cholesterol in hypercholesterolaemic rabbits and in humans with moderately elevated cholesterol. For an experiment with the hypercholesterolaemic rabbits, it was hypothesised that ‘pure EGCG will lower cholesterol by up-regulating the LDL-receptor, reducing cholesterol synthesis and inhibiting the intestinal cholesterol absorption’. Therefore, the aims were to determine
whether pure EGCG could lower cholesterol in this animal model and by which mechanisms of action.

An absorption study was then conducted in humans for which it was hypothesised that ‘pure EGCG will be absorbed better when given in capsule form without food compared to given in capsule form with a breakfast. It was also hypothesised that ‘incorporating the EGCG in a strawberry sorbet will improve the EGCG absorption compared to taking EGCG in capsule form with a breakfast’. Therefore, the aim was to determine, which of the three EGCG delivery formats, was the best for maximising the systemic absorption of the catechins in humans.

Finally, a pilot intervention study in humans was conducted for which it was hypothesised that ‘pure EGCG will lower cholesterol in mildly hypercholesterolaemic humans’. Therefore, the aim was to determine whether pure EGCG, given by the best of the three methods of delivery tested in the absorption study, could lower cholesterol in humans with moderately elevated cholesterol.

**Methodology:** For the animal model study, 12 New Zealand white rabbits were made hypercholesterolaemic by feeding with 0.25% (w/w) cholesterol for two weeks. Then, for four weeks, one group (6) was fed 0.25% (w/w) cholesterol and 2% (w/w) EGCG and the control group (6) was fed 0.25% (w/w) cholesterol only. Blood and faecal samples were collected prior to and at the end of the treatment period. Liver samples were also collected at the end of the study. Among other measurements, blood cholesterol, lathosterol, and squalene, LDL cholesterol, hepatic LDL receptor and CD36 protein and faecal neutral sterols and bile acids were determined.

For the absorption of EGCG study, 4 human subjects ingested on three separate occasions after fasting overnight and in random order, 500mg of EGCG taken either in
capsule form with 1) water only or 2) a breakfast cereal and milk or 3) incorporated in 200g of a strawberry sorbet. Venous blood samples were taken before ingestion and after 0.5, 1, 2, 3, 5 and 8 hours. The plasma concentration of EGCG was analysed by HPLC-MS and the area under the concentration-time curve (AUC) and other pharmacokinetic parameters were determined.

For the human pilot EGCG intervention study, 10 volunteers (6 males and 4 females) with moderate hypercholesterolaemia (5.5-7.5 mmol/l) were recruited for a placebo-controlled, double-blind, parallel design study. After a 2-week baseline period, the subjects were given EGCG or gelatine (placebo) for 4 weeks. Venous blood samples were collected on day 0 and day 28. Plasma cholesterol and triglycerides and HDL cholesterol were analysed using commercially available kits and LDL cholesterol was calculated using the Friedewald equation.

**Outcomes:** In the animal study, the hypotheses were mostly supported in that there was a 85% reduction in serum cholesterol and a 92% reduction in LDL cholesterol in the EGCG group at the end of the treatment period compared to control (p<0.05). The lathosterol to squalene ratio, an index of cholesterol synthesis, was also significantly lower (p=0.03) in the EGCG group (0.20±0.02) compared to control (0.62±0.16). After the 4-week treatment period, the hepatic LDL- receptor was significantly increased (+59%, p<0.001) as well as the hepatic CD36 protein (+62%, p=0.002) in the 2%EGCG group. However, there were no significant differences in faecal sterol excretion between the two groups.

In the EGCG absorption study, the first hypothesis was supported in that the plasma EGCG concentrations were markedly higher over the 8-hour period (AUC) when taken without food (174±68) than when EGCG capsules were taken with a
breakfast cereal and milk (64±53, p=0.044) or when the EGCG was taken incorporated in a strawberry sorbet (44±23, p=0.019). However, the second hypothesis was not supported in that there was no significant difference between the EGCG capsules taken with a breakfast or taken incorporated within a strawberry sorbet.

Therefore, for the pilot study, 1g/day EGCG was given in two doses of 500mg EGCG, one at least 30min before the morning meal and the other 30min before the evening meal. In this pilot study, the hypothesis was partially supported in that the LDL cholesterol was 25% lower (p=0.026) in the EGCG group than in the control group at the end of the 4-week treatment period, although the plasma total cholesterol was not different between the two groups.

**Conclusions:** Pure EGCG exhibited strong cholesterol lowering properties in the cholesterol-fed rabbit model of hypercholesterolaemia by increasing the hepatic LDL-receptor and possibly by reducing cholesterol synthesis but not by increasing the faecal excretion of neutral or acidic sterols. The systemic absorption of EGCG in healthy human subjects was highest when the catechin was taken in capsule form without food on an empty stomach. Finally, in a 4-week pilot study in 10 subjects with moderate hypercholesterolaemia, 1g/day EGCG, given in capsule form without food, resulted in a 25% lower LDL cholesterol concentration compared to control. The human pilot study also showed that amount of EGCG given over the 4-week period was well tolerated, as no serious adverse effects were noted.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP binding cassette transporter A1</td>
</tr>
<tr>
<td>ABS</td>
<td>Australian Bureau of Statistics</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-coenzyme A:cholesterol acyltransferase</td>
</tr>
<tr>
<td>Acetyl CoA</td>
<td>Acetyl coenzyme A</td>
</tr>
<tr>
<td>AFS</td>
<td>All faecal sterols</td>
</tr>
<tr>
<td>AIHW</td>
<td>Australian Institute of Health and Welfare</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>apo</td>
<td>Apoprotein</td>
</tr>
<tr>
<td>As</td>
<td>Absorbance of the plasma sample</td>
</tr>
<tr>
<td>ASBT</td>
<td>Sodium-dependant bile acid transporter</td>
</tr>
<tr>
<td>Astd</td>
<td>Absorbance of the standard</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>CA</td>
<td>Cholic acid</td>
</tr>
<tr>
<td>Cav</td>
<td>Average concentration of EGCG in plasma</td>
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<tr>
<td>CD36</td>
<td>Cluster of differentiation-36 protein</td>
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<tr>
<td>CDOCA</td>
<td>Cheno-deoxycholic acid</td>
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<tr>
<td>CETP</td>
<td>Cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CH</td>
<td>Control High Sample</td>
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<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>CL</td>
<td>Control Low Sample</td>
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<tr>
<td>CM</td>
<td>Chylomicrons</td>
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<td>Cmax</td>
<td>Peak concentration of EGCG in plasma</td>
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<tr>
<td>Cmin</td>
<td>Concentration of EGCG in plasma at the end of the dosing</td>
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<td>Chylomicron remnants</td>
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<td>CRP</td>
<td>C-Reactive Protein</td>
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<td>Cstd</td>
<td>Concentration of the standard</td>
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<td>CVD</td>
<td>Cardiovascular Disease</td>
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<td>Cysteine</td>
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<td>Cys-Gly</td>
<td>Cysteinyl-Glycine</td>
</tr>
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<td>d</td>
<td>Density</td>
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<tr>
<td>DAD</td>
<td>Diode array detector</td>
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<td>DBP</td>
<td>Diastolic blood pressure</td>
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<tr>
<td>DCA</td>
<td>Deoxycholic Acid</td>
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<td>DF</td>
<td>Degree of Fluctuation</td>
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<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>DI-water</td>
<td>Deionised water</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dyne/cm²</td>
<td>Dyne per Centimetre Square</td>
</tr>
<tr>
<td>EC</td>
<td>(-)-epicatechin</td>
</tr>
<tr>
<td>ECG</td>
<td>(-)-epicatechin 3-gallate</td>
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<tr>
<td>EDRF</td>
<td>Endothelial-derived relaxation factor</td>
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<tr>
<td>EDTA</td>
<td>Disodium ethylenediamine tetra acetate</td>
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<td>EGC</td>
<td>Epigallocatechin</td>
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<td>EGCG</td>
<td>(-)-epigallocatechin 3-gallate</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation detector</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty Acids</td>
</tr>
<tr>
<td>FH</td>
<td>Familial Hypercholesterolaemia</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionisation detector</td>
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<tr>
<td>FPP</td>
<td>Farnesyl Pyrophosphate</td>
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<tr>
<td>g</td>
<td>Gravitational force</td>
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<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<td>Gsh</td>
<td>Glutathione</td>
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<td>GT</td>
<td>Green tea</td>
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<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Hcy</td>
<td>Homocysteine</td>
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<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<td>HDL-C</td>
<td>High Density Lipoprotein Cholesterol</td>
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<tr>
<td>HepG2</td>
<td>Cultured human hepatoma cells</td>
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<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl CoA</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure (Performance) Liquid Chromatography</td>
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<td>IDDM</td>
<td>Insulin Dependent Diabetes Mellitus</td>
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<td>IDL</td>
<td>Intermediate Density Lipoproteins</td>
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<td>Immunoglobulin G</td>
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<td>IL-6</td>
<td>Interleukin 6</td>
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<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
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<td>Internal Standard</td>
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<tr>
<td>LCA</td>
<td>Lithocholic acid</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin cholesterol acyl transferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low Density Lipoprotein Cholesterol</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>Lipoprotein(a)</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MDD</td>
<td>Major Depressive Disorder</td>
</tr>
</tbody>
</table>
MG Monoglyceride
mmHg Millimetres of Mercury
MRFIT Multiple Risk Factor Intervention Trial
mRNA Messenger ribonucleic acid
MRP2 Multi-drug associated protein 2
MS Mass Spectrometer detector
MTTP Microsomal triglyceride transfer protein
MUFA Monounsaturated Fatty Acids
MW cm Mega Ohm centimetre
n-3 Omega-3
n-6 Omega-6
NADPH Nicotine adenine dinucleotide phosphate
NIDDM Non-Insulin Dependent Diabetes Mellitus
NO' Nitric oxide
non-HDL-C Non-HDL Cholesterol
NPC1 L1 Niemann-Pick C1 Like 1 protein
O2' Superoxide anion
ONOO' Peroxynitrite
oxLDL Oxidised low density lipoprotein
p Probability value
PBS Phosphate buffered saline
PDA Photo-Diode Array detector
PEG6000 Polyethylene glycol 6000
P-gp P-glycoprotein
PMSF Phenylmethanesulfonylfluoride
PP Pooled plasma
PPAR-γ Peroxysome proliferator activated receptor-γ
PROCAM Prospective Cardiovascular Muster Study
PUFA Polyunsaturated Fatty Acids
RHR Resting Heart Rate
RO Reverse osmosis water
s Second
S/N Signal to noise ratio
SAH S-adenosylhomocysteine
SAM S-adenosylmethionine
SBA Secondary bile acids
SBD-F 7-fluorobenz-2-oxa-1,3-diaze-4-sulfonic acid ammonium salt
SBP Systolic blood pressure
SD Standard Deviation
SDS Sodium dodecylsulfate
SE Squalene Epoxidase
SEAS Simvastatin and Ezetimibe Aortic Stenosis trial
SFA Saturated Fatty Acids
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SHARP</td>
<td>Study of Heart and Renal Protection</td>
</tr>
<tr>
<td>SIM</td>
<td>Selective Ion Mode</td>
</tr>
<tr>
<td>SR-B1</td>
<td>Scavenger receptor B1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element Binding Protein</td>
</tr>
<tr>
<td>T1/2</td>
<td>Plasma EGCG elimination half-life</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tg</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Time required to reach peak concentration of EGCG in plasma</td>
</tr>
<tr>
<td>USFDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet detection</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VcEDTA</td>
<td>Vitamin C Disodium ethylenediamine tetra acetate solution</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoproteins</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>Very low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe heritable hyperlipidaemic</td>
</tr>
<tr>
<td>WHR</td>
<td>Waist to Hips Ratio</td>
</tr>
</tbody>
</table>
List of Tables

Table 2.1 Results of the DSM Nutritionals EGCG purity analysis……………………………84

Table 3.1 Parameter settings of CobasBio® centrifugal autoanalyser for analysis of total cholesterol (Cholesterol), HDL cholesterol, triglycerides and glucose in rabbit serum……………………………………………………………………………………………………100

Table 3.2 Intra and inter assay variation for serum sterols using a control serum sample.................................................................................................................................................................108

Table 3.3 Concentrations of extracted squalene in rabbit serum before and after spiking with a known squalene concentration……………………………………………………………………………………………………114

Table 3.4 Thiol concentrations of the Working Standard Solutions…………………………116

Table 3.5 Mean thiol values for the low (CL) and high (CH) quality controls and intra assay coefficient of variations……………………………………………………………………………………………121

Table 3.6 Summary of the serum lipid values for the control and treatment groups before and after the 4-week treatment period……………………………………………………………………………………127

Table 3.7 Total, unesterified and esterified cholesterol and triglyceride hepatic concentrations in homogenates and membranes at the end of the dietary treatments……………………………………………………………………………………………129

Table 3.8 Serum lathosterol and squalene concentrations at the start and at the end of the treatment period……………………………………………………………………………………………………130

Table 3.9 Serum phytosterols (β-sitosterol and campesterol) at the start and at the end of the treatment period……………………………………………………………………………………………132

Table 3.10 Cysteine, homocysteine, cysteinyl-glycine, glutathione and glucose concentrations at start and at the end of the dietary treatments……………………………………………………………………………………………136

Table 3.11 Correlations between the measured parameters…………………………………138
Table 4.1 Intra- and inter-assay variation for the neutral sterols in one lyophilised faecal sample analysed by gas chromatography

Table 4.2 Intra- and inter-assay coefficients of variation for bile acids of one lyophilised faecal sample analysed by gas chromatography

Table 4.3 Intra- and inter-assay coefficients of variation (CV) for EGCG in faeces using one lyophilised faecal sample analysed by HPLC-UV

Table 4.4 The mean values for faecal weight, % moisture content, pH and total fat content for each group of rabbits (control and 2%EGCG) at the start and at the end of the 4-week treatment period

Table 4.5 The mean daily excretion values of faecal cholesterol, faecal coprostanol, faecal neutral sterols (sum of cholesterol and coprostanol), faecal lithocholic acid, faecal deoxycholic acid, faecal secondary bile acids (sum of lithocholic and deoxycholic acid) and all faecal sterols (sum of neutral sterols and secondary bile acids) adjusted for the rabbits’ body weight

Table 4.6 The average daily cholesterol intake, cholesterol absorption and sterol balance

Table 4.7 The average amounts of faecal cholesterol, faecal coprostanol, faecal lithocholic acid (LCA), faecal deoxycholic acid (DCA), faecal neutral sterols (sum of cholesterol and coprostanol), faecal secondary bile acids (SBA − sum of LCA and DCA) and all faecal sterols (AFS sum of neutral sterols and bile acids) relative to the intake of dietary cholesterol

Table 4.8 The average ratios of faecal cholesterol to coprostanol, lithocholic acid (LCA), deoxycholic acid (DCA) and secondary bile acids (SBA); average ratio of lithocholic acid to deoxycholic acid (LCA/DCA) is also represented

Table 4.9 The average values of total, free and conjugated EGCG measured in the faeces by HPLC-UV analysis

Table 5.1 Ingredients and percentages (w/w) used in the preparation of strawberry sorbet
Table 5.2 Results for the recovery of EGCG from strawberry sorbet ............... 225

Table 5.3 Mean, standard deviation and coefficient of variation for seven different concentrations of EGCG ................................................................. 228

Table 5.4 Intra assay analysis of recovery for EGCG from plasma and HPLC-MS measurement variation ............................................................... 229

Table 5.5 Inter assay analysis of HPLC-MS measurement variation ............... 229

Table 5.6 Mean values of anthropometric and blood pressure data at each clinic visit ................................................................. 230

Table 5.7 Plasma kinetic parameters of EGCG (results are represented as Mean±SD) after three different methods of EGCG ingestion ...................... 234

Table 5.8 Energy and macronutrient profile of the breakfast, strawberry sorbet and gelatine capsules ................................................................. 235

Table 5.9 Macronutrient profile of the lunch served to participants at each clinic .......... 236

Table 6.1 Anthropometric and blood pressure results ...................................... 264

Table 6.2 Plasma lipids and indices of cardiovascular risk .................................. 267

Table 6.3 Dietary intakes at the start and at the end of the treatment period .......... 269

Table 6.4 Some correlations between lipid parameters ................................. 270

Table 6.5 Some correlations between plasma lipid parameters and dietary intake values ................................................................. 271
List of Figures

Figure 1.1 Development of atherosclerosis ................................................................. 3

Figure 1.2 Serum cholesterol concentration and mortality from coronary heart disease (CHD) in 361,662 men in the MRFIT study ................................................................. 10

Figure 1.3 Percent of deaths by CVD based on the gender and age groups ............... 16

Figure 1.4 Homocysteine metabolism ........................................................................ 22

Figure 1.5 Structure of cholesterol (top) and cyclopentanophenanthrene ring (bottom) .................................................................................................................. 26

Figure 1.6 First group of reactions in cholesterol biosynthesis ............................... 28

Figure 1.7 Stage 2 of the cholesterol biosynthetic pathway – the conversion of mevalonate to squalene ................................................................. 30

Figure 1.8 Formation of lanosterol from FPP ......................................................... 31

Figure 1.9 Stage 3 of the cholesterol biosynthetic pathway – reactions from lanosterol to cholesterol ................................................................. 31

Figure 1.10 An overview of cholesterol synthesis in hepatocytes, indicating the negative feedback regulatory effect of cholesterol on the HMG-CoA reductase and squalene epoxidase rate limiting reactions ................................................. 32

Figure 1.11 Simplified scheme of lipoprotein metabolism ......................................... 42

Figure 1.12 The structures of the LDL receptor family ........................................... 47
Figure 1.13 The LDL receptor structure with five characteristic domains numbered 1-5.................................................................49

Figure 1.14 Sequence of the LDL-receptor pathway in mammalian cells...............51

Figure 1.15 “Probable sequence” of chemical events accompanying the oxidation of LDL.................................................................54

Figure 1.16 Chemical structures of the four main GT catechins EC, ECG, EGC and EGCG................................................................58

Figure 1.17 Intestinal absorption and metabolism of the GT catechin EGCG.........60

Figure 2.1 Typical HPLC chromatogram of 4-aminosalycilic acid and EGCG........83

Figure 2.2 The standard curve of EGCG. .........................................................83

Figure 3.1 Typical GC-FID chromatogram of a rabbit serum sample containing internal standard after derivatisation and extraction ...........................................106

Figure 3.2 External standard calibration curves for sterols in spiked serum samples.............................................................................107

Figure 3.3 Typical HPLC-UV chromatogram (195nm) of pure standards of (1) IS (+)-α-tocopherol acetate (3.975min) and (2) squalene (6.108min) prepared in acetonitrile.................................................................112

Figure 3.4 Calibration curve for squalene spiked in serum samples.................113

Figure 3.5 Typical chromatogram of the plasma thiols and IS analysed by HPLC with fluorescence detection..............................................119
Figure 3.6 Change in the concentration for total serum cholesterol, HDL cholesterol, LDL cholesterol and triglycerides from the start to the end of the 4-week treatment period in each treatment group.  

Figure 3.7 The index of cholesterol synthesis, lathosterol/cholesterol, at the start (week 2) and at the end (week 6) of the treatment period.

Figure 3.8 The index of cholesterol synthesis, lathosterol/squalene at the start (week 2) and at the end (week 6) of the treatment period.

Figure 3.9 Index of intestinal absorption (campesterol+β-sitosterol/cholesterol) in the control and 2%EGCG groups at the start and at the end of the 4-week treatment period.

Figure 3.10 The effect of EGCG on the hepatic LDL receptor in control and 2%EGCG groups at the end of the 4-week treatment period.

Figure 3.11 The effect of EGCG on the hepatic CD36 protein in control and 2%EGCG group at the end of the treatment.

Figure 4.1 Typical gas chromatogram of the unspiked faecal neutral sterols after the TriSil-TBT derivatisation.

Figure 4.2 Standard curves used for analysis of cholesterol (a) and coprostanol (b) in the faecal samples.

Figure 4.3 Typical chromatogram of the faecal bile acids.

Figure 4.4 Standard curves for individual faecal bile acids; lithocholic acid (LCA) (a), cheno-deoxycholic acid (CDOCA) (b), deoxycholic acid (DOCA) (c) and cholic acid (CA) (d).
Figure 4.5 Typical chromatogram at 280nm of (1) 4-aminosalycilic acid as internal standard (9.742min) and (2) EGCG (28.338min) after their extraction from a faecal sample………………………………………………………………………………………………………………173

Figure 4.6 Standard curve used for analysis of EGCG in faecal samples………….174

Figure 4.7 Bodyweight of the rabbits in the control and 2%EGCG groups on the last day at the start (Pre-treatment) and at the end (Post-treatment) of the 4-week treatment period………………………………………………………………………………………………………………176

Figure 5.1 Times for blood collection from participants used in all three clinics……215

Figure 5.2 Typical HPLC-UV chromatograms (280nm) of extracts from strawberry sorbet without (A) and with (B) the addition of EGCG (2.5mg/g) and IS (100mmol/l). ………………………………………………………………………………………………………………………………225

Figure 5.3 Typical chromatogram of IS (100ng/ml) and EGCG (500ng/ml) analysed by HPLC-MS-ESI set in negative polarity SIM mode……………………………………………………………………………………………………227

Figure 5.4 Calibration curve for EGCG spiked in plasma samples…………………228

Figure 5.5 Plasma EGCG concentration-time curves and arithmetic mean curve (broken green line) for the three different methods of EGCG oral delivery…………………232

Figure 5.6 Arithmetic mean ± SD of the plasma EGCG concentration-time curves for the three different methods of EGCG oral delivery: capsules without breakfast, capsules with breakfast and strawberry sorbet……………………………………………………………233

Figure 5.7 The relationship between the breakfast protein composition and the average AUC of each EGCG delivery method (p=0.014)…………………………………………………………237

Figure 6.1 The clinical protocols for the pilot study………………………………257
Figure 6.2 Standard curve for total cholesterol analysis in human plasma samples ...259

Figure 6.3 Standard curve for HDL cholesterol analysis in human plasma samples..259

Figure 6.4 Mean concentrations of LDL cholesterol at the start and at the end of the treatment period……………………………………………………………………….265

Figure 6.5 Mean concentrations of Non-HDL-Cholesterol at the start and at the end of the treatment period……………………………………………………………………….266

Figure 7.1 Proposed intra-luminal and systemic effects of EGCG on cholesterol absorption, excretion and synthesis……………………………………………………………284

Figure 7.2 Regulation of ECG uptake and efflux by specialised cellular transporters in intestinal cells………………………………………………………………………………293
Publications arising from this thesis


Naumovski N, Roach PD, ‘Epigallocatechin gallate lowers cholesterol in the cholesterol-fed rabbit by upregulating the LDL receptor and inhibiting cholesterol synthesis at the level of squalene epoxidase’ Atherosclerosis supplement (2009) Boston, MA, USA, (International Symposium on Atherosclerosis).


Naumovski N, Roach PD, ‘The serum lathosterol to squalene ratio is lowered by Epigallocatechin gallate in the hypercholesterolaemic rabbit model’, Program & Abstracts Australian Atherosclerosis Society, Freemantle, WA, Australia (2007)


Naumovski N, Blades BL, Roach PD, ‘Epigallocatechin gallate lowers cholesterol and lathosterol but not their ratio in the cholesterol-fed rabbit’, Program & Abstracts Australian Atherosclerosis Society, Couran Cove Island, Qld, Australia (2006)