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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AUSTRALIA
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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“Ja sam svoju glavu dao čestiti care za svoju reč...” – Miloš Obilić (1389, Boj na Kosovu)
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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.
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<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 A</td>
<td>Apoprotein</td>
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<td>A&lt;sub&gt;s&lt;/sub&gt;</td>
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<td>ASBT</td>
<td>Sodium-dependant bile acid transporter</td>
</tr>
<tr>
<td>A&lt;sub&gt;std&lt;/sub&gt;</td>
<td>Absorbance of the standard</td>
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<td>AUC</td>
<td>Area under the curve</td>
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<td>BMI</td>
<td>Body Mass Index</td>
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<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>CA</td>
<td>Cholic acid</td>
</tr>
<tr>
<td>C&lt;sub&gt;av&lt;/sub&gt;</td>
<td>Average concentration of EGCG in plasma</td>
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<td>CD36</td>
<td>Cluster of differentiation-36 protein</td>
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<tr>
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<td>Cheno-deoxycholic acid</td>
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<td>CETP</td>
<td>Cholesterol ester transfer protein</td>
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<tr>
<td>CH</td>
<td>Control High Sample</td>
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<td>CHD</td>
<td>Coronary Heart Disease</td>
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<td>CL</td>
<td>Control Low Sample</td>
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<tr>
<td>C&lt;sub&gt;min&lt;/sub&gt;</td>
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<td>Chylomicron remnants</td>
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<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
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<tr>
<td>C&lt;sub&gt;std&lt;/sub&gt;</td>
<td>Concentration of the standard</td>
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<td>Cardiovascular Disease</td>
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<td>CYP7A1</td>
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<td>Cys-Gly</td>
<td>Cysteinyl-Glycine</td>
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<td>d</td>
<td>Density</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode array detector</td>
</tr>
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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>Docosahexaenoic acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DI-water</td>
<td>Deionised water</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dyne/cm²</td>
<td>Dyne per Centimetre Square</td>
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<td>EC</td>
<td>(-)-epicatechin</td>
</tr>
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<td>ECG</td>
<td>(-)-epicatechin 3-gallate</td>
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<td>EDRF</td>
<td>Endothelial-derived relaxation factor</td>
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<td>EDTA</td>
<td>Disodium ethylenediamine tetra acetate</td>
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<td>EGC</td>
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<td>Fatty Acids</td>
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<td>FH</td>
<td>Familial Hypercholesterolaemia</td>
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<td>FID</td>
<td>Flame ionisation detector</td>
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<td>FPP</td>
<td>Farnesyl Pyrophosphate</td>
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<td>Gravitational force</td>
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<td>Glutathione</td>
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<td>Green tea</td>
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<td>h</td>
<td>Hour</td>
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<td>HepG2</td>
<td>Cultured human hepatoma cells</td>
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<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl CoA</td>
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<td>HPLC</td>
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<td>IDDM</td>
<td>Insulin Dependent Diabetes Mellitus</td>
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<tr>
<td>IDL</td>
<td>Intermediate Density Lipoproteins</td>
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<td>IgG</td>
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<td>LCA</td>
<td>Lithocholic acid</td>
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<td>Lecithin cholesterol acyl transferase</td>
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<td>Lp(a)</td>
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<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>
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<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>-----------</td>
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<tr>
<td>MG</td>
<td>Monoglyceride</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of Mercury</td>
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<td>MRFIT</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>Multi-drug associated protein 2</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometer detector</td>
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<td>MTTP</td>
<td>Microsomal triglyceride transfer protein</td>
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<td>Monounsaturated Fatty Acids</td>
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<td>Mega Ohm centimetre</td>
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<td>Omega-3</td>
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<tr>
<td>n-6</td>
<td>Omega-6</td>
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<td>NADPH</td>
<td>Nicotine adenine dinucleotide phosphate</td>
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<td>NIDDM</td>
<td>Non-Insulin Dependent Diabetes Mellitus</td>
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<td>NO⁺</td>
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<td>Niemann-Pick C1 Like 1 protein</td>
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<td>O₂⁻</td>
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<td>Peroxynitrite</td>
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<tr>
<td>oxLDL</td>
<td>Oxidised low density lipoprotein</td>
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<tr>
<td>p</td>
<td>Probability value</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
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<td>Photo-Diode Array detector</td>
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<td>Polyethylene glycol 6000</td>
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<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
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<td>PPAR-γ</td>
<td>Peroxysome proliferator activated receptor-γ</td>
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<tr>
<td>PROCAM</td>
<td>Prospective Cardiovascular Muster Study</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acids</td>
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<tr>
<td>RHR</td>
<td>Resting Heart Rate</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis water</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to noise ratio</td>
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<tr>
<td>SAH</td>
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</tr>
<tr>
<td>SAM</td>
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<tr>
<td>SBA</td>
<td>Secondary bile acids</td>
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<td>SE</td>
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<td>Simvastatin and Ezetimibe Aortic Stenosis trial</td>
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<td>SFA</td>
<td>Saturated Fatty Acids</td>
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<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<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(_{max})</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>
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<td>WHHL</td>
<td>Watanabe heritable hyperlipidaemic</td>
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Publications arising from this thesis


**Naumovski N, Roach PD,** ‘Effects of ingestion conditions on the oral bioavailability of epigallocatechin gallate (EGCG) after single-dose administration in healthy humans’ *The Proceedings of the Joint New Zealand and Australian Nutrition societies meeting and scientific conference*, Newcastle, Australia (2009)


**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).

Roach PD, **Naumovski N**, Shipman K, Thorne R, ‘Liver low density lipoprotein receptor and CD36 protein are increased in hypercholesterolaemic rabbits by epigallocatechin gallate’, *Program & Abstracts Australian Atherosclerosis Society*, Sydney, NSW, Australia (2008)


**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)


Roach PD, Vu HL, **Naumovski N**, Blades BL, ‘Despite its instability, epigallocatechin gallate effectively lowers serum cholesterol in the hypercholesterolaemic rabbit’, *Atherosclerosis Supplements*, Rome, Italy (2006)

**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)

Chapter 1. Introduction

1.1 Cardiovascular disease

The term ‘cardiovascular disease’ (CVD) is used as an “umbrella term” for different types of illnesses closely associated with the degeneration and inflammation of blood vessels and organs of the cardio-vascular system. This broad definition includes coronary heart disease (CHD), which originates as a consequence of an insufficient supply of blood to the heart muscle. The obstruction of blood flow is fundamentally due to narrowing of the internal lumen of the arteries and this is primarily caused by the deposition of atherosclerotic plaque. If progression of atherogenesis is not acted upon, buildup of plaques can be followed by thrombosis, consequently leading to myocardial infarction and sudden cardiac death [1-3].

1.1.1 Global burden of CVD

Despite a dramatic decline in the rates of CVD in developed countries over the past few decades, this type of disease is still, and is likely to remain, the first cause of death and disability in the world. It was estimated that in 2000, over 48% of all deaths were caused by CVD and that this figure will only be slightly reduced (46.4%) by the year 2020 [4]. Although CVD is not regarded as a leading cause of death and disability in developing countries, the increasing rate of this type of illness in such countries is still a driving force for the global incidence of CVD. Furthermore, CVD has been proposed to soon surpass infectious diseases as a leading cause of death and disability in the developing world, primarily due to improved public health measures successfully targeting mortality rates from infectious diseases [5].

It has been predicted that, by 2020, CVD will account for nearly a third of all deaths in developing countries [4-6]. This trend in the increasing burden of CVD in
developing countries was noted over a decade ago with the incidence reaching levels, in parts of populous countries like China, India, Indonesia and the Philippines, which are similar to those in developed countries such as Singapore, Hong-Kong and Australia [7]. However, even with the increase in CVD mortality in developing countries, the epidemiological data indicates that populations from developing Asian countries still have lower overall mortality rates than those in the developed Western world [4, 6, 7].

1.1.2 CVD in Australia

The mortality rate from CVD has steadily and dramatically declined in Australia since the 1960s. However, CVD is still one of the principal causes of death in the country [1]. The main components of CVD, CHD (which includes heart attacks and related diseases) and cerebrovascular disease (primarily stroke), were the two leading causes of death in 2004 for both genders. These diseases accounted for over 25% of all deaths that year and if the ‘other heart diseases’ category was added, the number of deaths from these three categories accounted for nearly 33% of all deaths for both genders [8].

1.1.3 Atherosclerosis

In CHD, the cause is a narrowing of the blood vessels that supply the heart with nutrients and oxygen. It usually results from the deposition of fatty plaques on the internal lumen of the arteries, contributing to their increased rigidity, a condition commonly called atherosclerosis [9]. Atherosclerosis is a multifactorial degenerative disease with relatively slow development, progressing without manifestation of any symptoms over a long period of time [10]. However, the fundamental pathophysiologic
characteristic of its development is the atherosclerotic lesions called plaques which accumulate (Figure 1.1) over several years [11].

![Figure 1.1](https://example.com) Development of atherosclerosis. Adopted from Harkreader[12].

It is widely accepted that there are five principle stages in the systemic degenerative process of atherogenesis with each phase possessing characteristic lesions and symptoms. The initial phase (Phase 1 in Figure 1.1) is characterised by the development of fatty streaks with lipid accumulation, macrophage recruitment and smooth muscle cell proliferation [9-11]. This phase is also called an asymptomatic phase, due to the lack of any atherosclerotic clinical symptoms and it is commonly seen in individuals younger than 30 years of age [9]. It is important to note that, at this point in the physiological development of atherosclerosis, not all fatty streaks will develop into advanced atherosclerotic lesions.
The extracellular lipid accumulation can progress into more advanced lipid rich lesions (Phase 2 in Figure 1.1), which establishes the pathogenic basis for the occurrence of plaque rupture and formation of thrombus. Small elongated plaque ruptures and the formation of mural thrombus have been associated with the rapid and clinically asymptomatic progression of the lesions to Phase 3 of atherogenesis, which is characterised by advanced plaques (Phase 3 in Figure 1.1).

The clinically acute set of coronary symptoms, fundamentally associated with angina and myocardial infarction, commonly occur in Phase 4 of the atherosclerotic development. This phase is particularly characterised with ruptures of the plaques and the formation of large blood clots or thrombus. Alternatively, domination of smooth muscle cell proliferation and collagen matrix synthesis may occur in Phase 3 and the atherosclerotic lesions may then progress into advanced stable sclerotic lesions (Phase 5), with similar clinical manifestations to those of Phase 4 [9-11, 13]. It is important to note that the progression through these degeneration phases of atherogenesis is strongly influenced and accelerated by a number of environmental and genetic CVD risk factors [14].

1.1.4 Theories of atherosclerosis

The atherosclerotic lesions mainly contain three components: 1) cholesterol, in the form of cholesterol esters and some oxidised lipids; 2) cells, consisting predominately of macrophages and smooth muscle cells; 3) connective tissue, composed of collagen, elastin and glycosaminoglycans [3, 15, 16]. Therefore, numerous explanations for the complexity of the pathological atherosclerotic process have been proposed based on the components of the atherosclerotic plaques. These and other...
explanations have also been based on known risk factors associated with the development of the atherosclerotic plaques (Section 1.1.5).

The theories of atherosclerosis include: the lipid theory, the hemodynamic theory, the fibrin incrustation theory, the response to injury hypothesis, and the oxidative modification theory, among others [3].

**The lipid theory of atherosclerosis**

The lipid theory of atherosclerosis is primarily based on cholesterol being a predominant component of the human atherosclerotic plaque. The first two phases of the atherosclerotic lesions (Figure 1.1) are characterised by the formation of fatty streaks and the assemblage of intracellular cholesteryl esters within macrophage foam cells [10]. This theory has been supported by studies using cholesterol and lipid rich diets in animal models, which have shown that these diets can result in the accumulation of cholesterol in the arterial intima [17, 18].

As part of the lipid theory of atherosclerosis, it has also been proposed that the accumulation of cholesterol in the arterial intima is due to an increased plasma cholesterol concentration causing an increased encroachment and retention of cholesterol-rich lipoproteins in the arterial intima [10, 17, 18].

**The oxidative modification theory of atherogenesis**

Although elevated serum cholesterol is an important risk factor for atherogenesis, the incidence of atherosclerosis is known to vary among and between populations with the same serum cholesterol level. Therefore, there are other factors that have an impact on the lipoprotein-arterial wall interaction and accelerate or reduce the atherogenic process [19].
In an attempt to further develop the lipid theory of atherosclerosis, the oxidative modification theory was proposed in the late 1980s [20] based on observations that oxidation of lipoproteins could provide a biological modification which could lead to a rise in the formation of foam cells. Therefore, it was hypothesised that the most significant event in the formation of early lesions and the progression of atherosclerosis is lipid oxidation. At the centre of this theory of atherosclerosis [20], the oxidation of low density lipoproteins (LDL), played the crucial role in the initiation and development of this disease (Section 1.5).

**The hemodynamic theory of atherosclerosis**

The hemodynamic theory of atherosclerosis is based on the observation that plaques preferentially form at the outer edges of arterial bifurcations. These areas are thought to be predisposed to atherosclerosis because the hemodynamic shear stress, the frictional force which the endothelial cell surface of the arteries are subjected to as the blood quickly flows by, is weaker (<4 dyne/cm²) in these regions than in other parts of the arteries which appear to be protected from developing plaques [3, 21, 22].

This low shear stress is associated with cellular changes in the arterial wall which are atherogenic. These areas may also accumulate cholesterol-carrying lipoproteins and other atherogenic blood components, in a fashion similar to the accumulation of sand or snow in areas of lower wind speed, and it is thought that the resulting prolonged exposure of the endothelium to the presence of atherogenic particles causes an increased encroachment and retention of cholesterol in the arterial intima and the development of fatty streaks [3, 21, 22].

This theory is also linked with hypertension being a predisposing risk factor for the development of atheroma (Section 1.1.5.1), particularly at arterial branching sites [3,
However, the exact link between high blood pressure and the low shear stress arterial zones is not completely clear. Also unknown, are the reasons why not all fatty streaks progress to form plaques [21].

**The response-to-injury hypothesis of atherogenesis**

The response-to-injury hypothesis of atherogenesis proposes that the initial step in atherogenesis is due to damage to the endothelial cells lining the arteries in a process referred to as endothelial denudation. The denudation or wearing away of the surface of the arterial endothelium, a notion supported by several authors [23, 24], is then postulated to cause the adherence of platelets and monocytes to the denuded area.

Therefore, atherogenesis was initiated by the endothelial dysfunction arising from injury to the arterial endothelium and the adhesion of monocytes to the areas of endothelial injury and progressed when the monocytes migrated into the arterial intima, converted into macrophages and scavenged cholesterol and other lipids and turned into foam cells.

The healthy endothelium maintains the balance between vasodilation and vasoconstriction, but also the inhibition and stimulation of smooth muscle cell proliferation and migration [25-28]. Any disturbances to this balance causes a dysfunction of the endothelium and as a consequence results in damage caused to the arterial wall [27]. The endothelial dysfunction is considered an early marker of atherosclerosis and the damage to the endothelium disturbs the balance between vasoconstriction and vasodilation, initiating a number of processes that promote atherosclerosis. The results of this endothelial dysfunction include increased endothelial permeability, platelet aggregation, leukocyte adhesion and generation of cytokines [25-31]. Therefore, the integral role of the endothelium in vascular health has generated
considerable interest in the potential for reversal of endothelial dysfunction using lipid lowering therapies.

It was proposed that the atherosclerotic process was also promoted by platelets aggregating on the denuded endothelium, releasing growth factors and cytokines, which stimulated further inflammatory responses and caused the migration of smooth muscle cells into the arterial intima thus leading to the formation of atherosclerotic. The latter proposal that platelet-derived growth factors could play an important role in atherogenesis was based on the observation that these factors promoted the growth of arterial smooth muscle cells in culture [32, 33].

A wide array of physical and chemical stimuli could cause endothelial denudation and the subsequent monocyte and platelet adherence [3]. Among these are the well-established risk factors of hypertension and smoking (Section 1.1.5.1).

The fibrin incrustation of atherosclerosis

According to Tegos et al.[3], the fibrin incrustation theory of atherosclerosis was proposed by the nineteenth century pathologist, Rokitansky. The theory postulates that fibrinogen is converted to fibrin on the luminal surface of the arterial wall forming a clot or thrombus, which becomes a tissue-like structure and is incorporated into a developing lesion [2, 29]. Therefore, this theory does not relate to the initiation phase of atherosclerosis but to the subsequent phases when the size of the plaque gradually increases.

The incrustation of fibrin is thought to lead to plaque instability and possible rupture consequently producing one of the final outcomes of the atherogenic process, an occluding blood clot [2, 29]. This theory is therefore linked with a prothrombotic condition being a predisposing risk factor for the development of atheroma (Section
1.1.5.1) and importantly for the formation of a thrombus which can occlude affected arteries [2, 29].

1.1.5 Risk factors for atherosclerosis

Atherosclerosis is a multifactorial disease and, as such, there are a number of risk factors associated with its aetiology. The characterisation of the risk factors was initially defined by Kannel et al.[34] based on the findings of the “Framingham Heart Study”, which helped to change the practice of modern medicine relative to CVD. Since then, the term ‘risk factor’ is usually defined as a measurable characteristic usually associated with an increased disease frequency. However, these risk factors often co-exist and can act synergistically in such a manner that it has not been possible to single out one of the risk factors as the one single cause of atherosclerosis [35].

There are several risk factors associated with the development of atherosclerosis and these are classified in a number of different ways [35, 36]. Most commonly, they are listed under three broad categories: established risk factors such as hypercholesterolaemia (including genetic disorders), hypertension, cigarette smoking, low high density lipoprotein (HDL) cholesterol, hypertriglyceridemia, diabetes and prothrombotic conditions; other factors such as age, gender, inadequate nutrition, obesity, physical inactivity; and emerging non-traditional risk factors such as elevated homocysteine, inflammatory markers and psychosocial indicators [34-36].

1.1.5.1 Established risk factors for atherosclerosis

Hypercholesterolaemia

An elevated plasma or serum cholesterol level is one of the most important major risk factors for the development of atherosclerosis. This is fundamentally due to
the deposition of cholesterol in the internal lumen of the arteries, which has been identified as a crucial occurrence in the formation of atherosclerotic plaques (Figure 1.1). Elevated levels of serum cholesterol were identified as a strong independent risk factor for the development of CHD in several studies, including the landmark Framingham study [37-40], the Multiple Risk Factor Intervention Trial (MRFIT) [41, 42] and the Prospective Cardiovascular Munster (PROCAM) study [43]. All these studies had similar findings and they conclusively linked elevated blood cholesterol levels with an increase in mortality from CHD. The positive relationship between plasma cholesterol and mortality rates from CHD found in the MRFIT study [41, 42] is shown in Figure 1.2.

There are also a number of genetic disorders, which result in very high blood cholesterol levels and CVD at a very early age, including familial hypercholesterolaemia, which is further discussed in Section 1.4.2.

Clinical trials using pharmacological therapies (predominately statins) to lower serum cholesterol have also shown a reduction in the incidence of CHD [44-46]. The findings from population studies and cholesterol-lowering trials, not only conclusively linked elevated blood cholesterol levels with an increase in mortality from CHD, they also consistently indicated that lowering cholesterol levels could substantially reduce the incidence of and mortality from CHD. From these studies, the development of national and international guidelines and reference values for blood cholesterol were initiated [47, 48]. Nonetheless, although important, hypercholesterolaemia is not the only risk factor for atherosclerosis; it has been shown to have positive associations with other CHD risk factors such as hypertension [38], high triglyceride levels [49] and hyperhomocysteinaemia [50, 51].
Hypertension

Hypertension, or high blood pressure, is defined as a systolic blood pressure higher than 140 millimetres of Mercury (mmHg) and/or a diastolic blood pressure in excess of 90mmHg [52]. Chronically elevated blood pressure, like hypercholesterolaemia, is a major risk factor for CVD. It is believed to be associated with causing atherosclerosis by damaging the arterial endothelium [53] and thus, contributing to plaque development [54-57]. The relationship between an increased blood pressure and an elevation in CVD incidence has also been conclusively established and it has been shown that the CVD risk doubles for every 20 and 10mmHg incremental increase in systolic and diastolic blood pressure, respectively. Furthermore, the prevalence of hypertension is very high; individuals have a 90% lifetime risk of developing hypertension [52, 58].

Cigarette smoking

Smoking, usually cigarette smoking, along with hypercholesterolaemia and hypertension, is the third major risk factor for the development of atherosclerosis [59],
causing both the formation of new plaque lesions and accelerating plaque progression [60]. A multitude of studies have indicated that there is a higher incidence of myocardial infarction in individuals who smoke than in their non-smoking counterparts [61-63]. These adverse effects are primarily related to the large amount of toxic and pro-oxidant substances in cigarette smoke but tobacco used for chewing or inhaling as snuff also has detrimental effects [63]. Blood cholesterol levels have also been found to be elevated in smokers and therefore, these individuals have a further increased risk of developing atherosclerosis and CVD [64-67]. A cessation of smoking is now widely advised as a powerful way of lowering the risk of developing atherosclerosis and preventing CVD [68].

**Reduced High Density Lipoprotein cholesterol**

A low level of high density lipoprotein (HDL) cholesterol in blood is also a well-established risk factor for CHD and atherosclerosis. Several epidemiological studies [69-72] have indicated that there is an inverse and independent relationship between circulating HDL cholesterol and the severity of CHD. It has also been estimated that for every 1mg/dl (0.026mmol/l) increase in HDL cholesterol there is a 2-3% decrease in cardiovascular risk [73].

The atheroprotective function of HDL has been ascribed to its role in reverse cholesterol transport, which involves clearing excess cholesterol from peripheral tissues, including the arteries, and its transport to the liver for further processing [74]. More specifically, it has been shown to be effective at removing cholesterol from lipid-laden macrophages, which deposit in atherosclerotic lesions and, in doing so, HDL prevents the accumulation of excess cholesterol in the arterial wall and thereby suppresses the progression of atherosclerosis [75, 76].
Apart from its main role in reverse cholesterol transport, HDL has another beneficial function, which could contribute to the prevention of atherogenesis. This is related to its antioxidant capacity, a property predominately due to the presence of apolipoprotein A-I, the main protein component of HDL [77]. The lipoprotein can prevent the synthesis of lipid hydroperoxides and can remove them from LDL particles and therefore, it can modulate the oxidation of LDL, a risk factor related to the oxidation theory of atherosclerosis (Section 1.1.4). Furthermore, HDL also carries a number of antioxidant enzymes (paraoxonase and platelet-activating factor acetylhydrolase) which can remove and degrade proinflammatory lipid molecules [78-82]. It has also been found to enhance the bioavailability of endothelial nitric oxide and to stimulate prostacyclin synthesis thus inducing smooth muscle cell relaxation [80]. Finally, HDL is also capable of opposing insulin resistance by stimulating the uptake of glucose and fatty acids by cells for oxidation and therefore, it can oppose insulin resistance, one of the major risk factors for atherosclerosis in patients with type II diabetes [76, 83].

**Hypertriglyceridermia**

Whether hypertriglyceridaemia is an independent risk factor for CHD or whether it works through being related to low HDL cholesterol, has been the focus of debate for several years [36, 84]. However, the findings of a meta-analysis of 17 prospective trials in 1996 [84] did indicate that hypertriglyceridermia was an independent risk factor for CVD and this finding was supported in a more recent meta-analysis of 29 prospective studies in 2007 [85]. These finding also supported the results from population based studies such as the PROCAM study, in which it was found that hypertriglyceridermia was a risk factor for major coronary events [86]. Similar findings were reported in the
“Caerphilly Heart Study”, which found that serum triglycerides were independently predictive of ischemic heart disease at the 10-year follow up of the participants [87]. The “Cardiovascular Study in the Elderly” also reported serum triglycerides to be an independent predictor of CHD morbidity in this elderly cohort [88]. Based on the findings from these prospective and population based studies, it is evident that high triglyceride levels are an independent risk factor for development of CVD as well as a predictor of morbidity.

Diabetes mellitus

In the past several years, the prevalence of obesity coupled with diabetes mellitus (DM) has increased globally and in Australia [8]. It has been established that children and adolescents with DM are at an increased risk of developing CVD in later life [89-92]. Furthermore, childhood onset of insulin dependent diabetes mellitus (IDDM) has been shown to lead to an earlier occurrence of atherosclerosis in adulthood [93, 94], a higher rate of cardiac failure [95] and a shorter survival rate [96-98] compared to the general population. Similarly, as the prevalence of obesity and non-insulin depended diabetes mellitus (NIDDM) has grown in the past few years, there is emerging evidence indicating an increased risk of CVD in NIDDM with consequences potentially similar to those in individuals with IDDM [99].

Although the exact mechanism of action still remains unclear, there are several conditions in diabetes that have been proposed to accelerate atherosclerosis: hyperglycaemia [100], increased oxidative stress [101], hypertriglyceridaemia [102] and a whole scope of genetic variables such as hepatocyte nuclear factor-1 alpha gene, the ob gene, ACE/ID and APOC3 3175C/G polymorphisms [103]. Furthermore, in NIDDM, hyperinsulinaemia may also have a role [103].
**Prothrombotic risk factors**

Deposition of atherosclerotic plaques is a gradual process over decades (Figure 1.1) and the disease can be clinically silent until there is a plaque rupture and the coagulation system is activated, an event that can be fatal in minutes [104, 105]. The coagulation system, which can generate the fatal thrombus, is a cascade of events that is traditionally represented as two relatively independent pathways, an intrinsic pathway involving clotting factors VIII, IX, XI and XII and an extrinsic pathway activated by tissue injury [104, 106]. Some conditions can predispose the system to being activated and these are commonly referred to as prothrombotic risk factors.

Platelet numbers (count) and how predisposed they are to aggregate (aggregability) are thought to be important predictors of atherothrombotic events [107-109], as platelets play an essential role in thrombus formation. The platelets respond to vascular injury by adhering to the intima when it is exposed by damage to the endothelial cell layer. The adhesion is triggered by different collagen types and adhesive proteins such as Willebrand factor [110-112], fibronectin [113, 114], laminin [115] and thrombospondin [116, 117].

Furthermore, circulating platelets are continually and actively involved in all phases of atherosclerosis development, from plaque formation to plaque rupture and thrombus formation [108]. During the atherogenic process, platelet reactivity is increased by a number of factors including reactive oxygen species [118], reduction of endothelial antithrombotic properties [119] and increased availability of proinflammatory mediators such as cytokines [120]. Soluble markers of inflammation such as serum C-reactive protein (CRP) and interleukin 6 (IL-6) have been positively correlated with indexes of the prothrombotic state [106]. However, whether these markers are related to a cause or a consequence of atherothrombosis is yet to be determined.
Another prothrombotic risk factor is lipoprotein(a) (Lp(a)). An increase in serum concentrations of Lp(a) has been suggested to play a predictor role for arterial stenosis in patients with ischemic stroke [121] and concentrations higher than 30mg/dl are correlated with the development of atherosclerosis [105]. The Lp(a) has also been associated with increased atherothrombotic events, suggesting that this particle is prothrombotic [122]. The precise mechanisms for the prothrombotic properties of Lp(a) still remain unknown but it has been suggested that the apoprotein (apo) associated with the lipoprotein, called apo(a), is the part of the lipoprotein involved in the pathogenesis of atherothrombosis [105, 123-125]. The Lp(a) is a ‘transporter’ of plasma cholesterol, which is similar in composition to LDL, as it consists of protein (apoB100), cholesterol, phospholipid and triglyceride but contains an additional protein, apo(a), linked to apoB100 by a single disulfide bond.

Apart from the above stated prothrombotic risk factors, several of the other traditional risk biomarkers have been identified as playing a significant role in the development of thrombus; these include cholesterol (Section 1.2), homocysteine (Section 1.1.5.3) and diabetes mellitus (Section 1.1.5.1), among others and together they form the basis for the fibrin incrustation theory of atherosclerosis (Section 1.1.4).

1.1.5.2 Other risk factors of atherosclerosis

Age

Age is one of the most important risk factors associated with the incidence of CVD [14]. The most characteristic finding to support this notion can be found in the Framingham Heart Study risk score used to predict the likelihood of a CVD event in the next 10 years; in this predictive tool, age accounts for up to 7 points on the 14 point increasing risk score scale [126].
The concept of age being one of the important risk factors is clearly seen from the increasing mortality rate observed as age increases. For example, in Australia (Figure 1.3), each 20 year increase in age is related to an average increase in the percentage of deaths caused by CVD of around 10%, irrespective of gender except for the 45-64 female age group [8].

Figure 1.3 Percent of deaths by CVD based on the gender and age groups. Adapted from Australian Institute of Health and Welfare 2006 [8].

Gender

Despite the fact that most of the CVD studies have historically focused more on men rather than females [127], observational studies have indicated that women develop CVD later than men and are treated clinically less ‘aggressively’ after the CVD event [128]. Due to the longer life span of women compared to men, the overall prevalence of CVD is actually higher in females [129]. However, this does not mean that women are at greater risk than men of developing CVD, especially in younger age groups. For
example, the incidence of CVD is considerably lower in females than in males up to the middle of the sixth decade (Figure 1.3). On the other hand, after the fifth decade, age-related CVD increases more sharply in women than in men [129-131]. This increase of CVD in females after the age of menopause has been proposed to be due to the loss of oestrogen, the primary female sex hormone [130-132]. However, studies on the effect of oestrogen replacement therapy on CVD after menopause have indicated that there was no cardiovascular protection [133, 134].

**Inadequate nutrition**

The high prevalence of atherosclerosis and CVD is predominately attributable to the contemporary lifestyle often exhibited in Western societies, which includes diets high in saturated fats [135] and sugars [136] and low in polyunsaturated fatty acids (PUFA), fruits, vegetables and fibre [137-139].

As early as 1957, it was well established that increasing saturated fat, as a percentage of total energy in the diet, increased blood cholesterol and that similarly increasing omega-6 (n-6) PUFA had the opposite effect. However, the n-6 PUFA were only half as potent in lowering blood cholesterol as saturated fat was at increasing it. An equation predicting these changes, referred to as the “*Keys equation*”, was developed then and it has passed the test of time over the past 63 years [140]. By the 1990, it was also widely accepted that monounsaturated fatty acids also effectively lowered blood cholesterol and had other beneficial effects on CVD risk [141].

Since the 1980s, the long chain omega-3 (n-3) fatty acids have also been studied in relation to CVD. Significant dietary sources of n-3 PUFA include fish oils rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and plants that are rich in α-linolenic acid. Results of epidemiological [142-145] and clinical studies [146-148]
have revealed that supplementation with EPA and DHA plays a significant role in the prevention of CHD and suppression of its clinical symptoms. There are multiple mechanisms by which n-3 PUFA exhibit their cardioprotective effects; the two most prominent ways appear to be related to their anti-inflammatory [149, 150] and anti-thrombotic [151, 152] properties. Other observed effects of high intakes of n-3 PUFA have included anti-arrhythmia [153], an hypotriglyceridaemic effect [154, 155], a reduction in blood pressure [155] and an improvement of the endothelial function in subjects predisposed to diabetes and vascular complications [156].

A consistent CVD protective effect has also been found for dietary fibre. The results of the Nurses’ Health Study [157] indicated that females in the highest quintile of fibre intake had an age-adjusted relative risk for major coronary events nearly 50% lower than females in the lowest quintile. Epidemiological studies, as reviewed in [158], have also demonstrated an inverse association between dietary fibre and levels of CRP a clinical indicator of inflammation [159].

The most consistent finding has been that types of soluble fibre including β-glucan from oat and barley bran, pectin from fruits, gums such as guar gum and fibre from psyllium husks can lower total blood and LDL cholesterol [160]. As a result of these studies, the United States Food and Drug Administration (USFDA) has approved the use of health claims on packaging for soluble fibre and coronary heart disease [161].

**Physical inactivity**

One of the major consequences of physical inactivity is a systemic increase in resting heart rate (RHR). Several epidemiological studies [162-166] have identified that a faster RHR is associated with an increased risk of mortality from CVD in a variety of different population subgroups, including the general population [162-164] and those
with established atherosclerosis [165]. Furthermore, results from the recent National FINRISK study [166], which analysed the effects of RHR in over 20000 people, demonstrated a strong, graded and consistent relationship between RHR and the incidence of CVD in healthy men and women.

In the past few decades, there has been an increase in the confirmation of the health benefits of physical activity, in particular on the effects and type of exercise on longevity and cardiovascular health [167-170]. The importance of physical activity on increasing lifetime is evident in the results from the Helsinki Businessmen Study [168], a 34-year follow up study of healthy men, which reported that leisure-time physical activity in midlife was associated with a reduction in mortality, independently of CVD risk, glucose and body mass index. However, Holtermann et al. [170] reported, in the results of the 30 year follow up Copenhagen Male Study of 274 males who had a history of pre-existing CVD, that a high level of physical fitness was associated with a reduced risk of ischaemic heart disease and all cause mortality in this cohort.

The beneficial effects of exercise have also been evident in studies looking at some of the biological markers for CVD; these studies have found effects such as an increase in the activity of post-heparin lipoprotein lipase (LPL) [171-173] and an increase in insulin sensitivity [171]. In a study by Taskinen et al. [173], the post-heparin LPL was increased by up to 20% after exercise. Additionally, the pre-heparin serum LPL was also increased after physical activity in overweight and obese males [174]. Although the pre-heparin enzyme is reported to be catalytically inactive and its physiological role is not completely defined, its blood levels have been reported to be considerably lower in participants diagnosed with NIDDM [175] and hyperlipidaemia [176].
Aerobic and strength based training have indicated effective mechanisms for improving cardiovascular protection with positive adaptations observed for total, LDL and HDL cholesterol, high sensitivity CRP and waist circumference [177-180]. The results of a study by Martins et al.[178] revealed that a 16-week training regime in elderly subjects reduced plasma triglyceride by 11%, total cholesterol by 6%, LDL cholesterol by 13% and high sensitivity CRP by 26% and increased HDL cholesterol by 5%. Furthermore, a study by Mediano et al.[179] identified that an increase in home based exercise combined with slight dietary energy restriction promoted a greater weight reduction in the first 6 months of the study and increased HDL cholesterol by 18.3mg/dl after 1 year.

Therefore, based on the results of these studies, it is evident that even a slight increase in exercise patterns, irrespective of the age group or gender, has a positive impact on the reduction of risk factors for cardiovascular disease and the disease itself.

**Obesity**

One of the consequences of inadequate nutrition and/or inadequate physical activity is obesity. In many countries around the world, the pattern of inadequate nutrition and/or physical activity has increased and it has lead to what has been referred to as an obesogenic environment.

Primarily due to this obesogenic environment, obesity has reached global epidemic proportions in developed and developing countries and the known relationship between obesity and CVD has become a considerable concern [89, 181]. Excess body weight, predominately seen as adipose tissue around the midsection of the body, has been widely accepted as a risk factor for the incidence of CVD [66, 90, 182]. Obesity has also been reported to be one of the most prevalent abnormalities in already
diagnosed CVD sufferers [49]. The exact mechanism of action of this phenomenon still remains unclear. However, a combination of other known CVD risk factors coexist with obesity including hypertension, diabetes mellitus, elevated total cholesterol and triglyceride levels and low HDL cholesterol and various combinations of these are known as features of the metabolic syndrome, which is linked to the development of CVD [49, 90]. Encouragingly, weight loss in overweight and obese men with features of metabolic syndrome has been shown to significantly improve heart rate recovery [183].

1.1.5.3 Non-traditional risk factors of atherosclerosis

Hyperhomocysteinaemia

Homocysteine (Hcy) is an amino acid that is associated with many different types of illnesses [184] and results from animal, epidemiological and clinical studies have identified it as an independent risk factor for the development of CVD [185-188]. The involvement of Hcy in many different diseases is primarily due to the crucial position of the Hcy in intermediary metabolism. It is located at the nexus of two metabolic pathways, methylation and transsulfuration (Figure 1.4).

Although our understanding of the pathology of Hcy is still emerging [189, 190], the metabolism of this amino acid is well documented, primarily due to its role as an intermediate in the regeneration of the essential amino acid, methionine [189-193].

Methionine metabolism involves activation to S-adenosylmethionine (SAM) by the enzyme SAM synthetase (Figure 1.4), which allows SAM to become the major methyl donor for a significant number of transmethylation reactions. After transfer of its methyl group, SAM becomes S-adenosylhomocysteine (SAH), which is further hydrolysed to adenosine and Hcy (Figure 1.4) and, in turn, Hcy can be methylated to
reform methionine or sulfurred to form another amino acid cysteine [190]. The methylation pathway through SAM is influenced by the vitamins folate and B₁₂. Homocysteine acquires a methyl group from 5-methyl-tetrahydrofolate to form methionine, a reaction which is dependent on vitamin B₁₂ (Reaction 1, Figure 1.4) [189]. Therefore, deficiencies in folate and vitamin B₁₂ are associated with higher Hcy levels [184, 189, 194, 195]. Furthermore, when one or more of the Hcy metabolising pathways is impaired due to enzymatic defects or vitamin deficiencies, Hcy accumulates and is reflected as an elevation of Hcy in plasma [196].

Figure 1.4 Homocysteine metabolism, adapted from [197, 198]
Despite Hcy being identified as an independent risk factor for the development of CVD, the exact cause for the formation of atherosclerotic plaques in patients with elevated Hcy levels still remains unclear. However, several mechanisms of actions have been proposed including increases in: endothelial toxicity [199, 200], proliferation of smooth muscle cells [199], free radical-mediated cellular damage [201], platelet activation [202] and thrombosis [203]. High plasma Hcy is also associated with other CVD risk factors such as gender, age, smoking, hypertension an elevated cholesterol levels, which further indicates the complexity of the atherosclerotic development process [204].

**Inflammatory markers**

There are several inflammatory markers that are associated with an increased risk of CVD and atherosclerosis including white blood cell count [205, 206], highly sensitive CRP [207], circulating pro-inflammatory cytokines [208, 209], cellular adhesion molecules [210, 211] and lipoprotein associated phospholipase A2 [212-214], among others. However, one of the main problems with using these markers as measures of atherosclerotic risks is that reliable and cost-effective assays for the majority of these markers are not yet available in routine pathology laboratory practice [215].

**Psycho-social indicators**

The morbidity and mortality rates of CVD in developed countries are much higher in socio-economically disadvantaged groups [216-219]. Therefore, it has been postulated that socioeconomic position may influence the development of CVD in a number of ways including poor maternal nutrition and poor childhood nutrition and
later in life, health behaviours and psychosocial conditions such as depression may have an impact [216, 218].

Depression is categorised as a mood disorder because it affects our mood state [220] and major depressive disorders (MDD) involve the occurrence of a very sad mood over a prolonged period of time (at least 2 years). Depression has been shown to be a risk factor for the onset of CVD [221-224] and MDD has been identified as an important psychosocial risk factor in the incidence of CVD for initially healthy males and females with a relative risk between 1.5-2, independent of traditional CVD risk factors [222, 225]. Also, depression not only contributes to the onset of CVD but it further heightens its severity by increasing the risk of mortality after acute myocardial infarction [221].

However, although prognostic studies have indicated that depression may be a cause, they have also shown that it can be a consequence of CVD. Therefore, there is evidence supporting a bidirectional relationship between depression and CVD [222].

1.2 Cholesterol

As described above (Section 1.1.5.1), it is well established that cholesterol plays a fundamental role in the development of atheroclerosis (Figure 1.1); plasma cholesterol is a major risk factor for CVD (Figure 1.2), it is at the heart of the lipid theory of atherosclerosis (Section 1.1.4) and it is a major component of the atherosclerotic plaque. According to Goodman [226], the identification of cholesterol dates back nearly two centuries ago, when it was identified as the waxy white component of gallstones after they were dissolved in organic solvents. Its original name was changed from “cholesterine”, a word derived from the Greek words chole (bile) and steros (solid), to cholesterol after the discovery of its reactive hydroxyl group [226, 227]. A significant
amount of research was conducted on determining the structure of cholesterol in the first century after its discovery but other early findings also linked cholesterol with its involvement in atherosclerosis.

It is believed [226] that Aschoff was the first person to point out the occurrence of high amounts of cholesterol in human atheromatous aortas in 1906 and that it was later identified by Windaus, in 1910, to be present primarily as cholesteryl esters. At the time, these observations in humans were supported by experiments in rabbits by Ignatowski and later Anitschkow who showed that atherosclerosis could be produced in rabbits fed a high cholesterol diet.

Cholesterol is an organic alicyclic compound, whose structure includes the perhydrocyclopentanophenanthrene nucleus, which consists of four fused rings (Figure 1.5). It has a single hydroxyl group at the C-3 position, an unsaturated centre between C-5 and C-6, a branched hydrocarbon chain attached to the D-ring at position 17 and two methyl groups, one designated C-19 attached at position 10 and the other designated C-18 attached at position 13 [228].

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Figure 1.5 Structure of cholesterol (top) and cyclopentanophenanthrene ring (bottom). Adopted from [228]
Cholesterol is a waxy substance that can be synthesised de novo (Figures 1.6-1.9), but it can also be supplied through the diet containing animal products such as meat, poultry, fish and dairy products but it is not a component of the plant foods in our diet [228]. Functionally, cholesterol is needed in the body for nerve insulation, production of cell membranes and the synthesis of bile acids and certain hormones, including sex hormones. Importantly, it is present in every cell in the human body; it is an important indispensable lipid as a structural component for all of our cell membrane structures. Cholesterol synthesis can play an important part during the active growth phases of our lives, especially if dietary intake is limited. Therefore cholesterol is not an essential nutrient and otherwise well nourished vegan people, who do not have any animal foods in their diets, are without evidence of ill health [229].

1.2.1 Cholesterol Synthesis

As previously stated, all cells in the body require cholesterol and it can be acquired from two sources; absorbed from ingested animal foods across the gastrointestinal mucosa or synthesized de novo from acetyl coenzyme A (acetyl CoA). For the latter, the greatest synthesis capacity is found in organs such as liver, intestines, adrenal cortex and reproductive tissues [228].

The de novo biosynthetic pathway for cholesterol was first demonstrated by Schoenheimer and Breusch [230, 231] and it involves a series of over 30 biochemical reactions that is often divided into three major stages. The first stage of cholesterol biosynthesis (Figure 1.6) is initiated by the conversion of acetyl CoA to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), which is further reduced to mevalonate, a reaction catalysed by HMG-CoA reductase. Mevalonate can then be used to continue the
The HMG-CoA reductase is a microsomal enzyme considered to catalyse the rate limiting step in the pathway for cholesterol synthesis and, because of its importance, it is subject to multiple regulatory mechanisms [232]. These complex regulatory controls include regulation at several levels including gene transcription, messenger ribonucleic acid (mRNA) translation, protein degradation and modulation of enzymatic activity [233].

Figure 1.6 First group of reactions in cholesterol biosynthesis. Adapted from [234]

Transcriptional regulation of the HMG-CoA reductase gene occurs via feedback regulation by cellular sterols mediated through members of the sterol regulatory element binding protein (SREBP) family [232]. However, regulation of HMG-CoA reductase at
the translation and protein stability levels appears to be controlled by the cells’ demand for non-sterol isoprenoids. This is seen when a reduction in mevalonate production is caused by inhibiting the HMG-CoA reductase activity using statins. It this case HMG-CoA reductase mRNA is translated efficiently and the enzyme is stable even in the presence of sterols. However, when non-sterol requirements are fulfilled by the addition of mevalonate to bypass the inhibited HMG-CoA reductase, the mRNA translation rate is rapidly reduced and degradation of the enzyme is accelerated [235]. The catalytic activity of HMG-CoA reductase can also be reduced by phosphorylation of the enzyme by the adenosine monophosphate (AMP) dependent kinase [236].

The inhibition of the HMG-CoA reductase enzyme by statins is predominately due to the HMG-like moiety of the statin molecules occupying the HMG binding site of the enzyme in a competitive inhibitor fashion. Additionally, a study by Istvan [237] has indicated differences in the type and number of binding interactions between different statins and the HMG binding site, which are associated with the structural differences of the statins. The type 1 statins, such as simvastatin, interact with the HMG binding site via their decalin ring structure. In contrast, the type 2 statins, such as rosuvastatin, interact via their fluorophenyl group. These differences in the interaction with the HMG binding site by the two types of statins may contribute to the differences in their potency for inhibiting the HMG-CoA reductase enzyme [237].

In the second stage of cholesterol biosynthesis, mevalonate is converted to squalene after a series of condensation reactions (See figure 1.7). Mevalonate is first phosphorylated using two adenosine triphosphates (ATP) to form a pyrophosphate intermediate and then decarboxylated to form the isoprenoid unit isopentenyl pyrophosphate (IPP). The condensation of three molecules of IPP then forms farnesyl pyrophosphate (FPP) and subsequently, the condensation of two molecules of FPP form
squalene pyrophosphate. Finally, in a reaction that is catalysed by the enzyme squalene synthase (Figure 1.8), the reduction of squalene pyrophosphate, using the reduced form of nicotine adenine dinucleotide phosphate (NADPH), leads to the formation of squalene [238].

Figure 1.7 Stage 2 of the cholesterol biosynthetic pathway - the conversion of mevalonate to squalene. Adapted from [234]
**Figure 1.8** Formation of lanosterol from FPP. Adapted from [239]

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**Figure 1.9** Stage 3 of the cholesterol biosynthetic pathway - reactions from lanosterol to cholesterol. Adapted from [240].
In the third stage of cholesterol biosynthesis, squalene is first used to make the steroidal ring which eventually is used to produce cholesterol. Squalene is first converted to 2,3-oxidosqualene (Figure 1.8) by the enzyme squalene epoxidase (SE). This is followed by cyclisation of 2,3-oxidosqualene to form the first intermediate sterol, lanosterol, a reaction catalysed by oxidosqualene cyclase [239]. The SE step has also been considered to be a rate limiting enzyme reaction in cholesterol synthesis (Section 1.2.1.1) and green tea polyphenols (Section 1.6.6) have recently been identified as potent inhibitors of this enzyme [241, 242].

Lanosterol differs from cholesterol by having three extra methyl groups and two double bonds. Therefore, the next reactions in the cholesterol biosynthetic pathway remove the methyl groups, transfer one double bond to carbon position five and saturate the other double bond. As seen in Figure 1.9, there are multiple pathways which ultimately result in the final product, cholesterol.

1.2.1.1 Regulation of cholesterol synthesis – negative feedback

As total body cholesterol increases, the rate of cholesterol synthesis has a tendency to decrease. This effect is primarily due to the negative feedback regulation of the HMG-CoA reductase enzyme (Figure 1.10). However, other enzymes in the pathway, including squalene epoxidase, are also affected [243].

Inhibition of the HMG-CoA reductase results in a decreased synthesis of cholesterol and other products in the pathway that are formed after mevalonate. It is well established that the class of therapeutic inhibitors of this enzyme called the ‘statins’ effectively reduce cholesterol synthesis [237]. All statins are reversible competitive inhibitors, which function similarly by binding to the active site of the HMG-CoA reductase. Once a statin is bound to the enzyme, the substrate HMG-CoA is sterically
prevented from binding to it and its conversion to mevalonate is prevented [237]. In response to a lowered cholesterol synthesis rate, tissues such as the liver, which require high amounts of the sterol, increase their importation of cholesterol from the blood circulation through a process involving the low-density lipoprotein receptor (Section 1.4.1). Therefore, the ‘statins’ have been shown to reduce the levels of circulating blood cholesterol by both reducing cholesterol synthesis and increasing the low-density lipoprotein receptor in the liver.

![Diagram](image)

**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. Adapted from [243]

The SE has also been considered as a rate-limiting enzyme in the cholesterol synthesis pathway [241]. Therefore, inhibitors of SE have also been the focus of research to develop new therapeutic treatments for lowering blood cholesterol. Several inhibitors of SE have been developed including chemically synthesized squalene analogs [241, 244, 245] and allylamine derivatives [246] but there is also a rising
interest in naturally occurring inhibitors of SE such as the green tea (Section 1.6.6) polyphenols [247].

1.2.2 Absorption of dietary cholesterol

The other source of cholesterol is from ingested animal foods. In the mouth and stomach, the food is mixed with enzymes (salivary amylase, lingual and gastric lipase, and gastric pepsin) and stomach hydrochloric acid to produce the denaturation of protein, the partial digestion of carbohydrates, protein and fats and the emulsification of the food into chyme but cholesterol is not affected in any way.

The gastric chyme is then delivered from the stomach into the duodenum. The dietary cholesterol is delivered to the duodenum mixed with triglycerides and phospholipids in the form of a lipid emulsion but, in the duodenum, the lipid emulsion is mixed with bile and pancreatic juice. Generally, most of the cholesterol in food exists in unesterified forms [248] but any cholesteryl fatty acid esters is de-esterified by pancreatic cholesterol esterase, which is very important for it to be able to be absorbed [249].

The triglycerides in the chime are emulsified by the bile and then digested by the pancreatic lipase into free FA and monoglyceride (MG) mainly. This digestion of the lipid complex is necessary for the liberation of cholesterol and its incorporation into phospholipid and bile acid micelles. Cholesterol is poorly soluble in water; it depends on the detergent-like solubilisation properties of the phospholipids and bile acids [250-252] for its micellar solubilisation and its transport through the water layer at the brush border of the small intestinal wall, in order for it to come in contact with the enterocytes. Essentially, the bile micelles serve as the cholesterol transport vehicle.
through the water-layer towards the brush border and provide easier uptake of the monomeric cholesterol by the intestinal cells.

The intestinal sterol uptake and transport processes have been proposed to be mainly controlled by two enzymes; acyl-coenzyme A:cholesterol acyltransferase type 2 (ACAT-2), which is responsible for the intracellular esterification of cholesterol (Section 1.2.3) in the enterocytes, [253] and microsomal triglyceride transfer protein (MTTP), which is involved in the intra-intestinal assembly of the dietary lipid carrying lipoproteins called chylomicrons [249, 254]. Chylomicron structure and metabolism is further explained in Section 1.3.1.1.

The bile is made by the liver, kept in the gallbladder and excreted in the duodenum, along with the pancreatic juices, through the common duct when needed for fat digestion. After bile has completed its function in the intestine, the bile acids are mostly absorbed from the intestinal lumen of the ileum and transported via the portal vein back to the liver in a process called the enterohepatic circulation of bile acids [249, 255]. Bile acid synthesis is further explained in Section 1.2.4.

1.2.3 Cholesterol esterification

Excess intracellular cholesterol that is not needed for functional purposes, such as bile acid synthesis or membrane formation, can be stored within cells as cholesteryl esters after esterification by an ACAT enzyme. Esterification of cholesterol plays an important role in the regulation of intracellular unesterified cholesterol levels. Unesterified cholesterol is potentially cytotoxic and therefore, its esterification into metabolically inert cholesterol esters protects the cells [253].

Recently, two different ACAT esterifying enzymes have been characterised, ACAT-1 and ACAT-2, and these appear to have separate physiological functions. In
most cells of the periphery, ACAT-1 converts cellular cholesterol into cholesteryl esters as a response to excess intracellular unesterified cholesterol. However, the ACAT-2 enzyme is only expressed in enterocytes and hepatocytes, where ACAT-1 is silent, and it appears to provide cholesteryl esters for the assembly of lipoproteins in these cells [253]. In enterocytes and hepatocytes, either newly synthesised cholesterol or cholesterol absorbed into these cells from the intestinal lumen or the circulation, respectively, is esterified by ACAT-2 and incorporated into chylomicrons or very low density lipoproteins (VLDL), respectively. These lipoproteins are then excreted from the cells to transport the cholesteryl esters through the body, along with other lipids [256].

In knockout mice studies, deletion of the ACAT-1 enzyme showed problematic consequences; the deletion caused extensive tissue cholesterol deposition and stimulated the development of atherosclerotic lesions [257, 258]. In contrast, deletion of ACAT-2, lead to a depletion of cholesterol esters in apoB-containing lipoproteins and a significant reduction in total plasma cholesterol levels, despite an increase in the lecithin cholesterol acyl transferase (LCAT) enzyme which esterifies cholesterol in HDL [259, 260].

This has led to speculation that inhibiting ACAT-2 may be a novel and effective way of lowering blood cholesterol in humans. However, the beneficial effects of inhibiting ACAT-2 are yet to be shown because safe specific ACAT-2 inhibitors are still not characterised. The beneficial depletion of cholesterol esters in apoB-containing lipoproteins like LDL is also less likely to be seen in humans because the cholesterol esters produced by LCAT in HDL are likely to be transferred to LDL by cholesterol ester transfer protein (CETP) [260], which is present in humans but not in mice.
1.2.4 Bile acid synthesis – cholesterol metabolism

The synthesis of bile acids from cholesterol provides a direct means by which cholesterol can be metabolised and essentially disposed off. The synthesis of bile acids is exclusively found in liver and it involves a number of cytoplasmic and mitochondrial enzymes [261, 262]. The major synthetic pathway is initiated by the endoplasmic reticulum enzyme, 7α-hydroxylase but the mitochondrial enzyme, sterol 27-hydroxylase, can initiate an alternative pathway.

Once cholesterol has been hydroxylated at the 7th carbon position in the 7α-hydroxylase-initiated pathway, two separate pathways lead to the formation of the two primary bile acids, cholic acid and chenodeoxycholic acid, depending on whether or not 7-hydroxycholesterol is acted upon by sterol 12α-hydroxylase, respectively. In the 27-hydroxylase pathway, the metabolites (27-hydroxycholesterol and 3β-hydroxy-5-cholestanoic acid) are primarily metabolised to chenodeoxycholic acid.

Cholic acid and chenodeoxycholic acid can in turn regulate the expression of the genes involved in their synthesis, especially 7α-hydroxylase, and thereby a feedback mechanism is created. This metabolic feedback process efficiently down-regulates the activity of the 7α-hydroxylase enzyme, causing it to be a rate-limiting step in the hepatic bile acid production [261-264].

After the bile acids are synthesized in the liver, they are secreted into the bile by an ATP-dependent transporter commonly referred to as the bile salt excretory pump [262], which is collected and stored in the gallbladder until need. The bile acids at this step are mainly excreted as conjugates with the amino acids glycine or taurine but a small amount may be present as sulphate esters [265, 266].

When needed in response to food intake, bile is pumped from the gallbladder into the duodenum through the bile and common ducts. Upon their arrival in the
intestine, the bile acids play a fundamental role in digestion by emulsifying the fats and fat-soluble vitamins and helping pancreatic lipase access and hydrolyse the fat. A small percentage of the bile acids are reabsorbed in the proximal intestine but most are reabsorbed in the distal ileum by a sodium-dependent apically-located bile acid transporter and transported to the liver via the portal vein.

Completion of the enterohepatic circulation occurs at the surface of the hepatocytes by a transport process mediated by the sodium-dependent bile acid transporter [262]. However, during the enterohepatic circulation, the primary bile acids cholic and chenodeoxycholic acid, originally produced in the liver from cholesterol, undergo dehydroxylation by the action of intestinal bacteria enzymes and return to the liver as the secondary bile acids deoxycholic and lithocholic acid [261-263].

The enterohepatic circulation of bile can be interrupted or inhibited by a variety of factors, including drugs such as cholestyramine and soluble dietary fibre such as β-glucan, which sequester the bile acids and increase their faecal excretion. Such interruptions of the enterohepatic circulation may lead to a smaller bile acid pool and an impairment in the absorption of fat and cholesterol due to a reduced micellar solubilisation capacity [267]. Inhibition of the enterohepatic circulation also increases the hepatic synthesis of bile acids from cholesterol, an attempt by the liver to replace the bile acids eliminated from the body by the increased faecal excretion. Since some of the cholesterol used to make new bile acids is obtained from the blood circulation, inhibiting the enterohepatic circulation results in a lowering of blood cholesterol. However, the cholesterol-lowering effect is usually limited to 10-15% because hepatic cholesterol synthesis eventually predominates as the source of the cholesterol used to replace the bile acids [267].
1.3 Lipids and lipoproteins

Lipid is a general term describing substances that are relatively water insoluble organic molecules that often contain long chain FA’s. They can be classified in three groups based on their chemical composition; hydrocarbons, simple lipids and complex lipids. The FA’s are needed within the body for a number of biological functions including as a source of energy. The major clinically important simple and complex lipids in human plasma are free and esterified forms of cholesterol, triglycerides, phospholipids and glycolipids and all are differentiate based on their chemical structure [228].

Lipids can be endogenously synthesized in most cells. However, dietary fat, the main exogenous source of lipids in the Western diet usually represents more than the recommended 30% of the total caloric intake per day [268]. The great majority of the lipid intake is in the form of triglycerides (Tg) with a small fraction consisting of cholesterol esters and phospholipids [269]. The Tg structure usually contains three long chain FA, in general 12 or more carbon atoms long, that can be classified based on the number of double bonds between the carbon atoms: saturated (no double bonds), monounsaturated (one double bond) or polyunsaturated (more than one double bonds) [132, 228, 268]. However, some short (less than 6 carbons) and medium (6 to 10 carbons) chain FA are also found in triglycerides.

Although limited, the digestion of dietary Tg in humans begins in the mouth with lingual lipase, especially in newborn infants. Digestion continues in the stomach, where it is catalysed by lingual lipase and gastric lipase, an enzyme secreted by the gastric mucosa, which has its highest activity in the acidic stomach environment. The lingual and gastric lipases preferentially hydrolyse Tg containing short and medium chain FA into free fatty acids and diglycerides and the liberated FA can then be
absorbed through the stomach wall into the portal vein for transport to the liver [222, 237, 262].

However, most of the Tg are not hydrolysed until they leave the stomach for the upper part of the small intestine, the duodenum. This is the major site where emulsification of dietary Tg and other lipids by bile occurs. Successful emulsification is an important factor for the efficient hydrolysis of Tg by the enzyme pancreatic lipase, which is also secreted into the duodenum. This lipase hydrolyses Tg to free FA and MG as the food makes it way down the intestinal lumen. The FA and MG are absorbed at the bottom of the duodenum and in the jejunum by the intestinal enterocytes, where they are re-esterified into Tg and assembled into the Tg-rich lipoproteins called chylomicron particles. The fat soluble vitamins (A, D, E and K) are also assembled with the Tg and cholesterol esters into the nonpolar core of the chylomicrons and they are surrounded by a coat of phospholipid, unesterified cholesterol and one or several apolipoproteins [243, 268, 270, 271].

There are several types of lipoproteins that are differentiated by their molecular mass, size, composition, density and physiological role [272]. However, the five major classes of human lipoproteins are categorised based on their density as determined by ultracentrifugation; chylomicrons (d<0.95 g/ml), very-low density lipoproteins (d<1.006 g/ml), intermediate-density lipoproteins (1.006 <d<1.019 g/ml), low-density lipoproteins (1.019<d<1.063 g/ml) and high-density lipoproteins (1.063<d<1.21 g/ml) [273]. Lipoprotein density is strongly influenced by protein content; the greater the content of protein and by implication the lower the content of lipid in its structure, the denser the lipoprotein particle is [272, 273].
1.3.1 Lipoprotein metabolism

1.3.1.1 Chylomicrons

The term chylomicrons (CM) was originally proposed by Gage in 1920 (cited in [274]) to describe large particles seen in plasma after lipid digestion and absorption. In the postprandial state following the ingestion of fats, CM synthesis is induced specifically to transport the dietary fat and fat-soluble vitamins from the intestines to the rest of the body. The CM are large spherical shape particles [275] with the size predominately determined by the flux of triglycerides through the intestinal cells. The size of CM is small at the beginning of digestion but they progressively increase in size until the peak of lipid absorption is reached [274].

The lipid composition of CM has been shown to be relatively similar between humans and experimental animals [274], predominately consisting of triglycerides (85-92%), phospholipids (6-12%), cholesterol (1-3%) and proteins (1-2%). Of the lipoproteins, CM have the lowest content of protein and the highest content of Tg and therefore, they have the lowest density of the lipoproteins. They are also the primary Tg transport vehicles [276]. Although relatively protein-poor, the CM also have small amounts of several apolipoproteins; apoB_{48}, apoA-I and apoA-IV but the CMs also acquire apoE and apoC upon entering the plasma [274, 276].

However, the CM do not go directly into the blood circulation from the intestines; they first enter the lymphatic system and move up this system until they reach the thoracic duct above the heart where they enter the blood circulation via the right subclavian vein, go through the heart and are pumped through the rest of the systemic circulation [277].

Once in the blood circulation the CM bind to the enzyme LPL and they are metabolised very quickly and have a short half-life of around 6 min[278] (Figure 1.11).
The LPL enzyme hydrolyses triglycerides into free FA and MG, leading to the production of smaller particles called chylomicron remnants. Most of the free FA and MG are removed from plasma for storage in adipose tissue or for use as energy in muscle tissue. Also during this process, some of the phospholipids, unesterified cholesterol and apoC are transferred from CM to circulating HDL particles [277, 279].

### 1.3.1.2 Chylomicron remnants

After the hydrolysis of triglycerides in CM, the lipoproteins called chylomicron remnants (CMR) are formed. These lipoproteins (Figure 1.11), still have a high Tg content but they are relatively enriched in cholesteryl-esters, apoB_{48}, apoE, apoC-II and fat soluble vitamins if compared to the original CM [280, 281]. The majority of CMR are removed from the circulation by hepatic uptake through a number of processes such as directly by the LDL-receptor, binding to additional apoE and then removal by the LDL-receptor related protein or binding of the apoE of CMR to heparin sulphate proteoglycans [281, 282]. The CMR are rapidly cleared from plasma, have a high turnover rate and are capable of transporting large amounts of cholesterol daily [280, 283]. Most individuals spend the majority of their waking hours in the post-prandial state and therefore chylomicron remnants could significantly contribute to the deposition of cholesterol in the arteries and therefore, have been postulated to be atherogenic [284-286].

### 1.3.1.3 Very Low Density Lipoproteins

Very low density lipoproteins (VLDL) are synthesized in the liver and serve as primary transport particles for hepatic Tg through the body (Figure 1.11. pathway shown by green arrows) [287]. However, the fundamental difference between CM and
VLDL is that VLDL is somewhat smaller (25-100nm), contains less Tg but has more cholesterol, phospholipids and proteins. The proteins found in VLDL are apoB\textsubscript{100}, apoC and apoE.

Figure 1.11 Simplified scheme of lipoprotein metabolism. CM, chylomicrons; CMR, chylomicron remnants; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPL, lipoprotein lipase; HL, hepatic lipase; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; LCAT, lecithin: cholesterol acyl transferase; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; LDLR, LDL-receptor; LRP, LDL-receptor related protein; SR-B1; scavenger receptor class B type 1; SR-A, scavenger receptor class A. Blue arrows represent exogenous cholesterol transport; green arrows represent endogenous cholesterol transport; red arrows represent reverse cholesterol transport. Adopted from [288].

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Like those of CM, the triglycerides in VLDL are hydrolysed by the LPL enzyme to release free FA and MG. The outcome of this hydrolysis of triglycerides results in the conversion of VLDL to a VLDL-Remnant and further hydrolysis of triglycerides by LPL produces an intermediate density lipoprotein (IDL) (Figure 1.11; pathway indicated by green arrow) [288]. During the hydrolysis process, VLDL also exchanges Tg and apoC for cholesteryl esters from HDL, a process carried out by CETP. This exchange ultimately results in the transfer of extrahepatic tissue cholesterol to the liver (reverse cholesterol transport) when the IDL are taken up by the liver as are CMR.

1.3.1.4 Intermediate Density Lipoproteins

The intermediate density lipoprotein (IDL) particle can undergo two different pathways, either it is cleared directly by the liver via receptor mediated processes or its core Tg is further hydrolysed by LPL and hepatic lipase [289], which results in the formation of LDL [290, 291]. During the latter process, the IDL looses apoE protein and gets converted mainly by the action of hepatic lipase to the cholesterol-rich LDL [287, 291, 292].

Interestingly, the combination of cholesterol-enriched VLDL and IDL can significantly contribute to the development of coronary artery disease [293]. Furthermore, the IDL itself has been assigned as an independent risk factor for the development of aortic atherosclerosis in hemodialysis patients [294].

1.3.1.5 Low Density Lipoproteins

The main role of LDL is in transporting cholesterol to the peripheral tissues where the sterol may be required for the membrane synthesis, bile acid production or conversion to steroid hormones. The LDL particles are essentially composed of
cholesteryl esters with very small amounts of Tg. Human plasma LDL is a heterogenous collection of particles that vary in sizes, density, lipid and protein composition [295]. Due to this heterogeneity, LDL can be identified in three different subclasses as large, intermediate and small LDL [290].

The protein component is exclusively apoB (in humans apoB\(_{100}\)) that is critical for cholesterol transport [290]. The protein is also crucial for LDL clearance as it allows the LDL particle to be identified and bound to the LDL-receptor and cleared from the circulation via receptor mediated endocytosis. However, this is more important in humans, which carry most of their blood cholesterol in LDL, because other animals such as rats and rabbits predominately transport their blood cholesterol in HDL [296].

Being the main cholesterol-carrying lipoprotein in human plasma, LDL has been determined to be the most atherogenic of all the lipoproteins; several studies have indicated a strong association between elevated LDL levels and the development of CHD [38, 61, 297]. Efforts to reduce LDL cholesterol are also proved beneficial; for every 1% decrease in plasma LDL cholesterol it has been found that there is a 2-3% decrease in the incidence of clinical CVD events [231, 288].

### 1.3.1.6 High Density Lipoproteins

Of all the plasma lipoproteins, HDL is the smallest and the most dense [298]. In their mature form, they are spherical particles with a hydrophobic core surrounded by a molecular monolayer of phospholipids, unesterified cholesterol and apolipoproteins. Human HDL predominately has two apolipoproteins; apoA-I (70%) and apoA-II (20%). However, there are several minor apoproteins on the particles such as apoA-IV, apoC, apoD, apoE and apoJ. Similar to LDL, HDL is heterogenous in terms of structure, density, size, apolipoprotein composition and also surface charge. Despite these
differences, the major types in normal human plasma are α-migrating, spherical particles divided equally between the apoA-I HDL and apoA-I/apoA-II HDL [298, 299].

The HDL have their origins in the liver and intestine (Figure 1.11) [300]. However, the metabolism and regulation of HDL is governed by a complex set of intracellular factors and extracellular events that control not only the synthesis and cellular uptake of the lipoprotein but also its assembly and re-modelling. The synthesis of HDL is initiated by the entry of major HDL apolipoproteins in the plasma in a lipid-deprived form followed by lipidation. The initial addition of cholesterol to HDL occurs on cell membranes via the ATP-binding cassette transporter, which results in the generation of discoidal HDL containing apolipoproteins, phospholipids and unesterified cholesterol [301-303]. The discoidal HDL then acquires additional unesterified cholesterol from circulating plasma lipoproteins or cell membranes and cholesterol esterification by LCAT results in the formation of a spherical particle enriched in cholesteryl esters [299, 304].

The catabolism of HDL is also a complex mixture of regulatory steps where most of the HDL is metabolised to other entities and involved in the re-generation of other particles (Figure 1.11) [288]. The HDL cholesteryl esters can be transferred to VLDL and LDL by CETP or taken up by the liver through the scavenger receptor B1 (SR-B1). Furthermore, Tg and phospholipids can also be removed from HDL primarily by hepatic lipase [299].

In contrast to LDL, results from epidemiological studies have established that HDL cholesterol is an established inverse predictor of CVD [305]. For every 1mg/dl increase in plasma HDL cholesterol it has been found that there is a 2-3% decrease in the incidence of clinical CVD events. A low HDL cholesterol was also found to be the
strongest predictor of CHD clinical events in the Framingham study [306]. Furthermore, animal models have demonstrated that raising HDL cholesterol, by intravenous infusion of the lipoprotein, has the effect of reducing the development of atherosclerosis and could also possibly lead to regression of atherosclerotic plaques [307-309].

1.4 Lipoprotein Clearance

The removal of lipoproteins from the plasma circulation can be achieved via receptor-mediated processes in liver and peripheral tissues. There are many receptor mediated mechanisms such as: HDL-receptors [310], VLDL-receptors [311] and lipolysis-stimulated receptors [312].

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Figure 1.12 The structures of the LDL-receptor family. Adopted from [313].

However, the most documented is the low density lipoprotein receptor family. The members of the LDL-receptor family share similar structural characteristics (Figure 1.12) such as a single membrane anchor, complement type repeats that have ligand-
binding domains and epidermal growth factor precursor homology domains, which are required for the release of ligands in endosomes [313, 314].

1.4.1 Low density lipoprotein receptor

The LDL-receptor is a glycoprotein that is present on most cells chiefly dominating on the surface of liver cells [315, 316]. Structurally, it is a multi-zone protein (Figure 1.13), which consists of five distinct domains [315]. The first domain, located on the external side of the cell membrane, is termed “ligand binding domain” and consists of 292 amino acids composed of a sequence of 40 amino acids that are repeated seven times with some variation [317]. Interestingly, each of the amino acid repeats, contains six cysteine residues that are disulfide bonded, suggesting a tight cross-linked convoluted structure. This formation explains the extreme stability of the receptor binding domain, as long as the disulfide bonds remain intact. Furthermore, the clusters of negatively charged amino acids at one end of each repeat is proposed to be the binding site of the LDL-receptor to the positively charged sections of its ligands, apoB and apoE of LDL and remnant lipoproteins, respectively [315, 317].

The second domain consists of 411 amino acids and is 35% homologous to a portion of the extracellular domain of the precursor for epidermal growth factor [317]. Apart from being a region regulating the dissociation of the ligand from the receptor in the endosomes, it is also of importance for the proper recycling of the receptor to the cell surface. The third domain is positioned immediately external to the membrane-spaning domain and consists of 58 amino acids rich in serine and threonine residues. The fourth domain consists of 22 amino acids that are hydrophobic and span the plasma membrane thereby anchoring the LDL-receptor in the plasma membrane. Finally, the fifth domain is a cytoplasmic tail, which projects from the inside of the plasma
membrane into the cytoplasm and contains the protein’s carboxy terminus. This part of LDL-receptor plays an important role in the clustering of the receptor in coated pits via a direct interaction with the coated pit protein clathrin or via an interaction with a protein, on the cytoplasmic side of the membrane, which is associated with clathrin [315, 317].

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**Figure 1.13** The LDL-receptor structure with five characteristic domains numbered 1-5. Adopted from [315].

### 1.4.2 The importance of the LDL-receptor

The specific role of the LDL-receptor is to remove the cholesterol-carrying LDL from the plasma circulation via the LDL-receptor mediated pathway [313, 315, 316]. In hepatic cells, uptake of LDL can lead to excretion of cholesterol from the body. However, in extrahepatic cells, the LDL-receptor delivers the cholesterol rich LDL to cells that exhibit an increased demand for the sterol due to growth and proliferation or for the synthesis of other steroidal compounds [318, 319].
Increased levels of LDL in the blood can, in rare circumstances, be attributed to genetic defects in the apoB protein of the LDL, the ligand for the LDL-receptor. Hence, the disease where defective LDL-apoB occurs is known as Familial Defective apoB [320]. However, more often but still rare, an increased blood LDL is due to genetic defects in the structure of the LDL-receptor. The disease related to defects in the LDL-receptor is called Familial Hypercholesterolaemia (FH). It is transmitted in an autosomal dominant fashion where heterozygotes have around half the normal amount of receptors, while homozygotes have a complete or near complete absence of LDL-receptor activity compared to normal individuals. Consequently, in homozygotes, LDL levels can accumulate in blood reaching concentrations greater than 10 times higher than normal and these individuals develop fulminant athrosclerosis and are susceptible to its sequelae at a very young age [306].

The genetic disease FH can result from four distinct classes of mutations in the LDL-receptor gene: Class 1 mutations lead to an absence of LDL-receptor synthesis; Class 2 defects lead to a failure to transport the LDL-receptor precursor from the endoplasmic reticulum to the Golgi apparatus; Class 3 mutations result in a reduction in the ability of the LDL-receptor to bind LDL and Class 4 genetic lesions lead to an internalisation defect dominated by a failure of the receptor to cluster in coated pits [315].

The work in humans with FH [315] and with the animal model for FH, the Watanabe heritable hyperlipidaemic rabbit (WHHL) [321], has provided convincing evidence that an impaired LDL-receptor activity significantly increases blood LDL cholesterol. In the animal model of FH, the WHHL rabbits develop severe atherosclerosis followed by CHD only few months after birth, emphasising the importance of the LDL-receptor in regulating plasma LDL-cholesterol levels [321].
In the general population, it is now well recognised that a blood LDL-cholesterol level above 160mg/dl requires therapeutic treatment. Commonly, statins are given to inhibit the HMG-CoA reductase step of cholesterol synthesis. They also reduce the levels of cellular cholesterol and up-regulate the expression of the LDL-receptor, providing an increased LDL uptake and degradation through the LDL-receptor pathway and thus, a lowering of blood cholesterol [46, 237, 312, 320].

1.4.3 The LDL-receptor pathway and its regulation

The LDL-receptor pathway (Figure 1.14) was first described in cultured human fibroblast using $^{125}$I-labeled LDL, where it was noticed that LDL uptake by cells occurs in a sequential manner [322]. The LDL is first bound to its high-affinity receptor on the cell surface. It is then internalised bound to its receptor by the cell by the process of endocytosis. In the endosomes, the LDL dissociates from its receptor. The lipoprotein is then delivered to lysosomes where its cholesteryl esters are hydrolysed, providing free cholesterol for use by the cells [322, 323]. The LDL-receptor is recycled to the cell surface to bind more LDL.

Figure 1.14 Sequence of the LDL-receptor pathway in mammalian cells. Adopted from [324]

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The cholesterol that is derived from the internalised LDL plays a pivotal role in the regulation of the complex feedback system that stabilises the cellular cholesterol concentration. This homeostasis is maintained by three main regulatory feedback events that occur upon entry of the LDL particle in the cell and delivery of its unesterified cholesterol (Figure 1.14). Firstly, the incoming free cholesterol inhibits the activity of HMG-CoA reductase (Figure 1.10), an enzyme which catalyses a rate limiting step in cholesterol synthesis, and therefore, cholesterol synthesis is decreased [325]. Secondly, the incoming free cholesterol activates a cholesterol esterifying enzyme ACAT, leading to the re-esterification of any excess cholesterol and its storage in vacuoles as cholesteryl ester [256]. Finally, the incoming free cholesterol inhibits the synthesis of new LDL-receptors; this reduces the further entry of LDL into the cell thereby protecting it from over-accumulation of cholesterol [326]. Therefore, this system of regulation coordinates extracellular and intracellular sources of cholesterol to maintain a constant level of unesterified cholesterol within the cell, regardless of fluctuations in the external supply of lipoproteins [324].

1.5 Oxidatively modified low density lipoprotein

As previously discussed in Section 1.1.4, elevated plasma LDL levels are directly related to the development of atherosclerosis and CHD [37-40]. One of the theories briefly outlined in this section is ‘the oxidative modification theory’, which places the oxidation of LDL at the focal point of atherogenesis.

1.5.1 Oxidation

The oxidation process in biological system focuses on oxygen as an important acceptor of electrons, which leads to the formation of active oxygen and free radical
species. A free radical can be defined as any molecular species, containing one or more unpaired electrons, which is capable of independent existence [327]. There is a whole variety of free radicals that can be generated in biological systems and their role is dependent on the nature of the radical itself and on its molecular composition.

Some free radicals perform a range of biologically important functions. For example, nitric oxide (NO\(^\cdot\)), formed from intracellular L-arginine metabolism, has an important function as an endothelial-derived relaxation factor (EDRF) which causes smooth muscle cells to relax [14, 327]. Due to NO\(^\cdot\) having an unpaired electron in the highest orbital, it has the ability to reduce other molecules and can behave as a potential antioxidant. However, in the presence of another free radical, superoxide anion (O\(_2^\cdot\)), NO\(^\cdot\) is rapidly inactivated and forms peroxynitrite (ONOO\(^-\)), a potent inducer of lipid peroxidation. Furthermore, ONOO\(^-\) is a very strong oxidant species able to modify LDL into a form recognised by the scavenger receptors of macrophages, a process thought to be involved in the initiation and progression of atherosclerosis [328].

### 1.5.2 LDL Oxidation

Oxygen free radicals are attracted to carbon double bonds in organic compounds. The LDL particle contains an abundance of these double bonds in the phospholipid fatty acids present in its outer envelope [329]. The attack of free radicals on these phospholipid fatty acids can result in the formation of lipid peroxide products and through their propagation by auto-oxidation (Figure 1.15), this can lead to damage to apoB, the LDL protein which serves as the ligand for the LDL-receptor [330].

This can suppress the uptake of the oxidised LDL (oxLDL) particles through the normal LDL-receptor pathway because the LDL-receptor can no longer recognise and bind the modified LDL. Instead, the oxLDL can be taken up by the scavenger receptors
on monocyte-derived macrophages that are present in the arterial wall (Figure 1.15). The uptake of modified LDL via the scavenger receptor pathway can lead to the formation of foam cells which can cause massive cholesterol deposition within the arterial intima and the development of atherosclerosis [331-333].

One of these scavenger receptor proteins is the cluster of differentiation-36 (CD36). The CD36 is classified as a class B scavenger receptor protein with multifunctional involvement in immunity, metabolism and fatty acid transport [334, 335]. Furthermore, because CD36 has also been shown to bind native lipoproteins (LDL, HDL and VLDL), it may contribute to the regulation of lipid metabolism and to cholesterol lowering in a manner similar to the LDL-receptor [336].

The very complicated process of LDL oxidation is characterised by oxidation of both lipid and protein moieties and each class of lipid can be attacked. This does not produce a unique particle regarded as oxLDL but rather a whole spectrum of these types of particles. These events also do not occur in an orderly fashion but rather in a chaotic manner [337, 338]. Nevertheless, the various modified and oxLDL have the ability to promote the accumulation of cholesterol in macrophages [339] and provide the basis for a role of oxLDL in atherogenesis (Figure 1.15).

Studies on the oxidative modification of LDL have revealed that the concentration of oxLDL was significantly higher than normal in participants with acute coronary syndromes and acute angina [340], patients with transplant associated coronary artery disease [341], participants with CHD [342] and patients with acute coronary syndromes such as myocardial infarction, stable and unstable angina [297]. Furthermore, prospective nested case control studies have also indicated that the strongest predictor of CVD events, when compared with other traditional CVD risk factors, was elevated plasma oxLDL [343].
1.5.3 Inhibition of LDL oxidation

If LDL oxidation is accepted as a central feature of atherogenesis then inhibition of LDL oxidation should result in a reduction of atherosclerosis [14]. Among the factors that influence the oxidation of LDL, the fatty acid composition of LDL is of chief importance. Consistent with this, LDL with a high proportion of polyunsaturated fatty acids (PUFA) has been shown to have a higher susceptibility to oxidation \textit{in vitro} compared to LDL with a high proportion of monounsaturated fatty acids (MUFA) [344, 345]. The LDL particle also contains its own defensive mechanisms against oxidative damage, primarily in the form of fat-soluble antioxidants; ubiquinone, α- and β-tocopherols and β-carotene found in LDL can protect it against oxidative damage [14, 327].
Despite the identification of oxLDL as a risk factor for the development of CVD, the identification of the exact in vivo oxidising agents or oxidants responsible for the conversion of LDL into high uptake forms have not yet been substantiated. However, there is some evidence emphasizing the role of the heme enzymes and their associated oxidants [329].

1.5.4 Antioxidants and LDL oxidation

Antioxidants are defined as substances which, when present in much smaller quantities than substances that they protect, are effective at protecting against oxidative damage [327]. There is a whole array of substances that may prevent or delay the oxidation of other substances. Some antioxidants are able to neutralise free radicals by acting as scavengers (eg. Mannitol, superoxide dismutase, glutathione), as chain breaking antioxidants neutralising intermediate peroxyl radicals (eg. α-tocopherol, ascorbic acid) and as preventative antioxidants able to bind metal cations, preventing metal ion production of free radicals (eg. Haem, albumin) [14, 327].

Antioxidants can also be distinguished by their lipid solubility. Some are lipophilic and are soluble in the core of the LDL particle (α- and β-tocopherols and β-carotene) while others are hydrophilic and can be transported around the body in plasma as their free forms (ascorbic acid and catechins).

Similar to other biological oxidative processes, LDL oxidation also begins after the blood antioxidants have been consumed. Therefore, the susceptibility of LDL to oxidation is dependent on the antioxidant content in the LDL particle and in the surrounding blood. In LDL, α-tocopherol is by far the major antioxidant present on a molar basis but ubiquinol-10 has also been shown to be an important core antioxidant.
Dietary supplementation with these two antioxidants has resulted in reductions in the oxidation of LDL and therefore in a lowering of the oxLDL levels [346].

The phytochemicals referred to as flavonoids have also been shown to decrease the oxidation of LDL induced by the free radical ONOO⁻ and to reduce the modification of amino acids in the LDL protein, apoB [347-350]. Some of the most studied flavonoids are found in green tea [351-355].

1.6 Green tea

Tea is one of the most popular beverages worldwide and its world consumption ranks second only to water and well ahead of coffee, beer, wine and carbonated soft drinks [351-355]. Also, taking into consideration the traditional Chinese and Japanese belief that drinking tea promotes good health [352, 356], it is not surprising that the health promoting effects of tea have been intensively investigated during the past decade [352].

The tea that originates from the plant *Camellia sinensis* can be categorised into four types based on the fermentation level as; green (unfermented), oolong (partially fermented), black (completely fermented) and pu-erh (drastically fermented and aged) type of tea. Consumption of green tea is favoured in Oriental countries, black tea in Western counties while oolong and pu-erh tea are predominately consumed in China [352, 357, 358].

The manufacture of black tea involves the oxidative polymerisation of the monomeric flavan-3-ols by the enzyme polyphenol oxidase that further leads to the formation of bisflavanols, theaflavins, thearubigins and other oligomers. In contrast, during the production of green tea, freshly harvested leaves are quickly steamed or hot air-dried to inhibit the oxidising enzyme, polyphenol oxidase, which prevents
fermentation of the tea and yields a dry and stable product. The blanching of leaves caused by this exposure of tea to hot steam or air is also responsible for the production of its pronounced green colour [359].

The composition of green tea varies with the tea plant variety, harvest season, position of the plucked leaves, climate and horticultural practices [358]. However, all green teas are rich in polyphenols, including flavanols, flavadiols, flavonoids and phenolic acids. The beneficial health effects of green tea are primarily ascribed to the biologically active polyphenolic components especially the monomeric flavan-3-ols (or simply flavanols) called the catechins [352, 357, 358, 360].

1.6.1 Green tea catechins

The most prominent and biologically active compounds in green tea (GT) are the catechins, almost all of which can be extracted with hot water during the tea brewing process. These compounds also provide a slight astringent and bitter taste to GT infusions. The GT polyphenols are predominately comprised of four major catechins; in decreasing order of the amount present in tea they are: (-)-epigallocatechin 3-gallate (EGCG); (-)-epigallocatechin (EGC); (-)-epicatechin 3-gallate (ECG) and (-)-epicatechin (EC) [358]. The structure of these four catechins (Figure 1.16) is characterised by multiple hydroxyl groups on two benzene rings. The galloallocatechins (EGC and EGCG) have an extra hydroxyl group in the 5’ position of the B ring while the catechin gallates (ECG and EGCG) have an extra benzene ring with three hydroxyl groups [361].
Figure 1.16 Chemical structures of the four main GT catechins EC, ECG, EGC and EGCG. Adapted from [352].

The content of catechins in GT can reach up to 30% of the tea leaves’ dry weight [351] with EGCG being the most predominant catechin, accounting for up to 60% of the total catechin content [362]. However, in oolong, black and pu-erh teas, because these teas are allowed to ferment and oxidise, the catechins are converted to theaflavins and thearubigins, which consequently yields a lower catechin content [352].

1.6.2 Beneficial effects of green tea catechins

There is increasing evidence from epidemiological, clinical and experimental studies which suggest that the tea polyphenols have various biological activities such as anti-fungal [363], anti-inflammatory [364, 365] and antioxidative [353] properties. They have also been linked with possible protection against several types of cancer [366], a lower incidence of CVD [367] and a reduced mortality due to cardiovascular disease [368]. In agreement with the observed links with protection against CVD, an increased consumption of green tea has also been associated with a decrease in total serum
cholesterol [360] and with an attenuation of the postprandial increase in plasma triglycerides following a fat load [369]. However, although the GT catechins are strong antioxidants, it is still debatable whether its beneficial CHD reduction effects are due to prevention of the oxidation of LDL [370-372].

1.6.3 Absorption, distribution, metabolism and elimination of green tea catechins

After oral consumption of tea catechins, only a small amount is absorbed into the circulation from the small intestine with a large fraction eliminated primarily in the faeces and bile (Figure 1.17). This low catechin bioavailability was postulated to be due to their high molecular weights but also due to the ability of their phenolic hydroxyl groups to form large hydration shells [373, 374]. Furthermore, depending on the type of transport mechanism, absorption and biological activity of EGCG in particular may be affected by the form (purified or mixed GT extract) and type of administration [373].

Some of the absorbed catechins are modified and converted to glucuronides in the intestinal mucosa. Following their entry into the blood circulation, the catechins can also be converted to sulphates in the liver [375] or methylated by catechol-O-methyl transferase [376, 377]. After absorption, the EGCG in plasma is predominately found in a free unconjugated form while EGC and EC are mainly found in conjugated forms [378]. The catechins, particularly EGCG, EGC and EC, reach peak levels in human plasma at 1.5-2.5 h after their administration while ECG is often not detected [379].

Over 90% of the EGC and EC is excreted in the urine, mainly in their conjugated forms [380], while some of the EGCG is found in other tissues such as the liver, brain and small intestinal mucosa [381]. High levels of EGCG have also been found in faeces in their free and intact state when administered to rats [382].
Furthermore, catechins administered orally to pregnant rats have also been found in their placenta and foetuses [383].

Figure 1.17 Intestinal absorption and metabolism of the GT catechin EGCG. Adapted from [384].

1.6.4 Antioxidant properties of green tea catechins

Several *in vivo* studies have demonstrated an increase in plasma antioxidant activity after the consumption of tea or tea polyphenols [353]. The antioxidative activity of GT has been found to be due to the several types of polyphenols it contains but it possesses a higher antioxidant activity than black tea primarily due to its higher content of EGCG [385]. Of all the catechins, EGCG possesses the strongest antioxidant activity and it is a stronger antioxidant than other well known dietary antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E) and β-carotene (a vitamin A precursor) [386]. The number and arrangement of the phenolic groups of the catechins is a fundamental reason for these compounds exhibiting excellent electron donor
properties and providing efficient scavenging of free radicals such as $O_2^\cdot$, singlet oxygen, $NO^\cdot$ and ONOO$^{-}\$[347-350, 386].

*In vitro* studies have indicated that EGCG can prevent the oxidation of LDL [387-390], a proposed initiating step in the development of atherosclerotic plaques. Similarly, an *in vivo* experiment in humans has confirmed a decrease in plasma oxidised LDL after 4 weeks of consuming 600ml/day of GT [391]. In one study in rats, the administration of GT polyphenols successfully inhibited LDL oxidation and increased the level of HDL cholesterol thus providing an anti-atherosclerotic action [392]. On the other hand, one study indicated little or no increase in the plasma antioxidant potential after drinking one strong cup equal in strength to 4-6 regular strength green tea cups [377].

Metabolic studies have indicated that supplementation with EGCG can raise its plasma level to a maximum concentration of 4.4nmol/l, a concentration at which an increase in antioxidant activity would be exerted in the bloodstream [381, 393, 394]. However, even with high intakes of GT and its extracts, intracellular and plasma concentrations of catechins are most likely to be up to a 1000 times lower compared to other physiological antioxidants such as ascorbic acid and glutathione, [395].

### 1.6.5 Hypocholesterolaemic effects of green tea catechins

Several studies have identified an inverse association between GT consumption and serum cholesterol levels in humans [360, 396-398]. These studies have indicated that consuming five to ten cups of GT daily was significantly associated with lower serum cholesterol levels compared to those who did not drink GT. Furthermore, if heavy tea drinkers, who drank 10 cups or more/day, were excluded from the analysis, it was still found that consumption of one single cup of tea per day was associated with a
reduction in cholesterol levels of 0.015mmol/l [360]. However, the relationship between the consumption of GT and HDL levels is less clear as the results are more conflicting: some studies have indicated a positive association with HDL levels [396] while others have found no association with GT consumption [360, 391].

Animal models of hypercholesterolaemia have also shown positive results for the lowering of blood cholesterol levels through the consumption of GT or GT extracts [242, 399-404]. In these studies, hypercholesterolaemia was induced by feeding the animals either 1-2% (w/w) cholesterol and/or 15% (w/w) lard for a period of 1-4 weeks. The animals were then divided into two or more groups and fed freshly brewed tea or various GT extracts that were either mixed in their food or added to their drinking water for a period of 4-8 weeks. After the treatment period, plasma cholesterol concentrations were significantly reduced between 20–50% compared to controls and a significant improvement (-30%) was also found in the build-up of arterial plaque in one study [400]. Although there were no changes in the HDL cholesterol in the majority of the studies, the ratio of non-HDL:HDL-cholesterol, as an indicator of atherosclerosis risk, was also positively affected (decreased) in the animals receiving GT treatment [402].

Some of the human intervention studies on the effect of GT consumption on plasma lipids have indicated no significant change in the plasma lipid profile even with a relatively high GT consumption of 900ml/day or close to 4 cups per day [405, 406]. Also, a recent study, where the GT consumption was 600ml/day or just over two cups/day, did not show a reduction in the cholesterol levels but did inhibit the oxidation of LDL [391]. However, it is important to note that one of the possible reasons for these results was that the subjects had normal cholesterol levels (less than 5.5mmol/l) to begin with. It may be, as suggested by Nantz et al.[407], that only subjects with an
increased baseline blood cholesterol (greater than 5.5 mmol/l) will respond to treatment with the GT or its catechins.

1.6.6 Plausible mechanisms for the lowering of plasma cholesterol by green tea catechins

To date there are three proposed mechanisms of action for GT, its extracts and its pure catechins, lowering blood cholesterol levels: inhibition of cholesterol absorption [408, 409], reduction in cholesterol synthesis [242, 247] and an increase in the LDL-receptor[242, 410-412].

1.6.6.1 Catechins and inhibition of cholesterol absorption

The inhibition of cholesterol absorption by GT and its catechins has been shown to be a potential mechanism of action for lowering blood cholesterol levels in animal models. This was proposed primarily due to an observed increase in the faecal excretion of cholesterol, bile acids and total lipids in rats and hamsters consuming GT extracts [402, 413-416]. These studies also provided evidence suggesting that EGCG, the main catechin in GT, could be responsible for the inhibition of cholesterol absorption [402] by interfering with the micellar solubilisation of cholesterol in the small intestine and thereby reducing the efficiency with which cholesterol is absorbed from the lumen of the digestive tract [414]. The micellar solubilisation of cholesterol and other lipids by bile in the small intestine is discovered in Sections 1.2.2 and 1.3.

Furthermore, a study by Ikeda et al.[408] in rats with canulated thoracic duct, indicated that EGCG lowered the cholesterol content in the chylomicrons coming out of the lymph at the thoracic duct. They also showed that EGCG was very effective at precipitating cholesterol from solution in a preparation of bile micelles. Therefore, the
hypocholesterolaemic effect of EGCG was ascribed to a reduction of cholesterol absorption from the small intestine due to a reduction in the solubility of cholesterol in the mixed micelles [408].

1.6.6.2 Catechins and reduction of cholesterol synthesis

As previously described in Section 1.2.1, cholesterol biosynthesis is a very complex pathway and modifications or inhibitions of any of the many metabolic steps can potentially result in a reduction in blood cholesterol levels. The therapeutic success of the statins, the pharmaceuticals currently used for the treatment of hypercholesterolaemia, has clearly established that inhibition of the de novo cholesterol synthesis pathway is an effective approach for lowering plasma LDL-cholesterol. The statins achieve this by partially inhibiting the rate-limiting enzyme, HMG-CoA reductase, which consequently leads to a reduction in mevalonate synthesis and, further down the pathway, to a decrease in cholesterol synthesis. One in vitro study has indicated that the activity of the HMG-CoA reductase remained undisturbed after treatment with GT catechins [404]. However, the in vitro measurements of HMG-CoA reductase activity do not always reflect the level of cholesterol synthesis in vivo. Measurements of cholesterol synthesis in vivo are more reliable but studies relating the effects of catechins on HMG-CoA reductase activity and in vivo cholesterol synthesis are crucially limited.

Inhibition of another rate-limiting enzyme in the de novo cholesterol synthesis pathway, squalene epoxidase (Section 1.2.1.1), has also been proposed as a mechanism of action by which GT catechins could reduce blood cholesterol levels. Consistent with this possibility, an in vitro study has shown that GT catechins could directly inhibit SE activity [247]. In this study the inhibition kinetics indicated that EGCG, like other
galloyl esters [241, 245], potently inhibited SE in a noncompetitive and non-time-dependant manner. These results also strengthened the case for developing clinically safe inhibitors of SE, as this rate-limiting enzyme appears to be somewhat undervalued as a target for the development of hypocholesterolaemic treatments [239].

In the only *in vivo* study which looked at the effect of green tea catechins on cholesterol synthesis, Bursill *et al.*[242] showed that a reduction in the plasma lathosterol:cholesterol ratio, an index of cholesterol biosynthesis, by a GT catechin extract could have contributed to the marked cholesterol lowering effect the extract had in the cholesterol-fed hypercholesterolaemic rabbit model. However, in this study, the enzyme(s) affected or whether enzyme inhibition was involved was not determined.

1.6.6.3 Green tea catechins and up-regulation of the LDL-receptor

An increase in the LDL-receptor is another mechanism by which cholesterol levels can be lowered (Section 1.4) because the LDL-receptor is the main mechanism by which cholesterol can be removed from the blood circulation [334]. This was proposed as a possibility of action for GT catechins in studies with cultured human hepatoma (HepG2) cells, which showed that freshly brewed GT, a catechin-enriched GT extract and individual pure catechins could all up-regulate the LDL-receptor[410-412]. Furthermore, these studies provided evidence indicating that EGCG may be the main active catechin constituent in the cholesterol lowering effect of green tea [411].

A study in the hypercholesterolaemic rabbit model also showed an increase in the hepatic LDL-receptor activity in rabbits treated with a crude GT catechin extract [242]. These results indicated that upregulation of the hepatic LDL-receptor may have been involved in the observed lowering of plasma, liver and aortic cholesterol by the GT catechin extract.
1.7 Epigallocatechin Gallate

Of the main four catechins (Figure 1.16), EGCG is the major compound found in green tea and it has been suggested to be responsible for the majority of the potential health benefits attributed to green tea consumption [362, 367, 417]. So far, EGCG has not been chemically synthesised and its only source has been purification of the catechin from GT, which is difficult and time-demanding. Up until recently, the high cost of purchasing purified EGCG has been prohibitive for conducting in vivo studies in animals and humans. However, a highly purified EGCG preparation from GT (TEAVIGO™) has now been available at an affordable price [418] for a few years for conducting such studies. The product is guaranteed to be at least 92% pure EGCG and it has the properties of being colourless, odourless and water soluble.

Therefore, there are emerging reports on the potential health benefits of EGCG from well-controlled and double-blind clinical studies [362]. Pharmacokinetic studies in humans have shown that physiologically relevant concentrations of EGCG may be difficult to achieve [373, 419, 420], indicating that high oral doses may be necessary to effect any clinical changes despite epidemiological studies showing a positive association between green tea drinking and significant health benefits [360, 368].

1.7.1 EGCG studies on CVD risk factors

The proposed beneficial effects of EGCG have included the attenuation of diet-induced obesity, which is a risk factor for CVD (Section 1.1.5.2). In one study [421], mice were fed a high fat diet (15% fat) for four weeks to induce obesity followed by randomisation to three groups with 0 (control), 0.5% and 1% w/w EGCG fed in their chow for the subsequent 4 weeks. The outcomes of this study indicated that dietary supplementation with EGCG dose-dependently reduced the mice’s body weight. In
another study, EGCG also prevented obesity in rodents by reducing their adipose tissue mass without affecting lean body tissue [422]. Other studies, which found that GT catechin extracts stimulated lipid catabolism in the liver [423] and had other anti-lipogenic effects [424] on rats fed high fat diets, have also supported EGCG as being an active ingredient in controlling obesity in rodents. The findings may be related to the interaction of EGCG with the leptin-independent appetite control pathway, as one study reported that rats in the EGCG treatment group consumed up to 50-60% less food than control [425]. Also, other studies have indicated a lower energy intake (5.6%) [423] and a decreased feeding efficiency and growth [424] in EGCG treatment groups compared to control.

Human trials with GT extracts [426] using up to 690mg/day of catechins have also showed positive effects on adiposity [427]. One human study has looked at the reduction of abdominal fat in obese subjects given 300mg of encapsulated EGCG daily for a period of 12 weeks [428]. However, this study found that there were no significant differences between the treatment and control groups in waist circumference, total body fat, abdominal fat and intra abdominal adipose tissue. Therefore, the findings of this study contrast with the results in the previous animal studies with EGCG [421, 429] and human studies with GT catechin extracts [426, 427]. This lack of effect may be due to the relatively low dose of EGCG used (300mg/day).

Elevated oxLDL (Section 1.5) is a recognised risk factor for the development of atherosclerosis [343]. Studies in vitro have indicated that EGCG can inhibit oxidation in LDL by a combination of multi-factorial interactions in the PUFA oxidation process, including a successful scavenging and reduction of copper anions, superoxide anions and hydroxyl radicals generated during the peroxidation of PUFAs [387, 430, 431].
However, studies on the effect of EGCG on in vivo oxidised LDL levels are scarce; to date there has not been any reports showing a reduction of oxLDL levels in plasma.

The effect of EGCG on the CVD risk factor, high blood cholesterol (Section 1.1.5.1) is the focus of this thesis and the experimental rationale is outlined in greater detail in the next section (Section 1.8). This focus is largely based on the in vitro finding that EGCG was the only one of the main four GT catechins, which was able to significantly increase the LDL-receptor in HepG2 cells [411]. Studies done in vivo on the effect on blood cholesterol in rodents have also indicated EGCG has hypocholesterolaemic properties [432, 433].

1.8 Experimental rationale

Based on previous research in various animal models, it is evident that the hypocholesterolaemic effect of GT catechins can be ascribed to three fundamental mechanisms: up-regulation of the LDL-receptor[242, 434], reduction of cholesterol synthesis [242] and inhibition of cholesterol absorption [402, 408, 413-416]. These promising beneficial effects of GT catechins, a potential new target for development as a therapeutic agent for hypercholesterolemia, have primarily been associated with EGCG, the most abundant catechin found in these polyphenolic complexes.

In vivo studies with pure EGCG as a cholesterol lowering agent are relatively scarce. Two studies have been conducted in rats where pure EGCG has also been shown to lower blood cholesterol and there was evidence that it may have done so by inhibiting cholesterol absorption [402, 415]. However, to date, no studies have been done on the in vivo effects of pure EGCG on the LDL-receptor or cholesterol synthesis.

A previous in vitro study in HepG2 cells [411] revealed that EGCG was the only catechin, of the four main ones in green tea, to increase the activity of the LDL-
receptor and to increase the relative amounts of the LDL-receptor protein, effects which had previously been reported for green tea and a crude catechin extract [410]. Based on this finding, it was postulated that pure EGCG was the only catechin likely to have a significant impact on the LDL-receptor. However, in vitro findings do not necessarily always predict the outcomes of in vivo studies.

A study in rats by Bursill et al.[434] did indicate that administration of a GT catechins crude extract produced effects on the LDL-receptor which were analogous to those previously observed in the in vitro studies with HepG2 cells [410, 411]. A further study in the hypercholesterolaemic rabbit model [242] also indicated that, upon the administration of a GT catechin extract at concentrations of 0, 0.5, 1 and 2% (w/w) in the rabbit chow, there was a dose dependant increase in the hepatic LDL-receptor activity (up to +80%) and the LDL-receptor protein (up to +70%). However, whether pure EGCG has a similar effect on the LDL-receptor in vivo, remains to be investigated.

In the study in hypercholesterolaemic rabbits [242], the GT catechin extract also had a dose-dependent inhibitory effect on whole-body cholesterol synthesis (up to -60%) as measured using the lathosterol/cholesterol index [435]. Again, it remains to be determined whether pure EGCG has a similar inhibitory effect on whole-body cholesterol synthesis in vivo.

However, in the rabbit study by Bursill et al.[242], possible effects of the GT catechin extract on cholesterol absorption were not investigated. Therefore, it is not known whether GT catechin or pure EGCG can inhibit cholesterol absorption in the cholesterol-fed hypercholesterolaemic rabbit model.

Human intervention studies with pure EGCG are also relatively scarce[428]; intervention trials have predominately been done using EGCG as part of various types of polyphenolic complexes, including a GT catechin extract [436] and a
theaflavin/catechin preparation [437]. The results from a GT catechin extract study by Nagao et al. [436] indicated that a catechin combination incorporated into a beverage could significantly reduce LDL cholesterol by 4% after administration of 583mg/day of catechins for 12 weeks. Even more promising, the study by Maron et al. [437] indicated that a daily capsule of a green tea extract containing 150mg of GT catechins, 150mg of other tea polyphenols and 75mg of theaflavins, significantly reduced total cholesterol by 11.3% and LDL cholesterol by 16.4%. The 240 participants in the Maron et al. [437] study had mild to moderate hypercholesterolaemia (LDL cholesterol 3.4-4.9mmol/l) and the study was conducted for 12 weeks in a double-blind, randomised, placebo-controlled, parallel-group intervention trial fashion.

In another study conducted over 12 weeks in subjects with normal cholesterol levels (4.5 mmol/l), Erba et al. [438] reported an 11% lowering of LDL cholesterol when they used a green tea extract which gave 320mg/day of catechins and 180 mg/day of EGCG. In a shorter study done over 6 weeks, Nantz et al. [407] used a green tea extract which gave 250mg/day of catechins and 170mg/day of EGCG. Of interest, in this study, there was a 7% lowering of LDL cholesterol in subjects with baseline LDL cholesterol levels greater than 2.5mmol/l but the extract had no effect in subjects with baseline LDL cholesterol less than 2.5mmol/l.

However, the few human intervention trials with pure EGCG available so far have been less promising. A 12 week human intervention trial by Hill et al. [428] indicated that pure EGCG (300mg/day) did not result in a significant change in cholesterol levels in overweight post-menopausal females despite reducing fasting plasma glucose levels and resting heart rate, but baseline cholesterol levels were not reported. Similarly, in a study by Brown et al. [439], in subjects with baseline
cholesterols averaging 5.1mmol/l, pure EGCG (800 mg/day) given for 8 weeks also did not affect cholesterol levels despite lowering blood pressure.

The reason why the two human studies with pure EGCG did not result in any effect on cholesterol is unknown. It may be, as suggested by Nantz et al.[407], that only subjects with an increased baseline cholesterol will respond to treatment with the catechin. Therefore, for this reason, the effect of pure EGCG should be tested on individuals with at least moderately elevated cholesterol levels (5.5 – 7.5mmol/l).

Another possibility is that the amounts of pure EGCG given in these two studies did not result in enough of the catechin being absorbed for it to have systemic effects on cholesterol metabolism including effects on the LDL-receptor or on cholesterol synthesis. The study by Bursill et al.[411] in HepG2 cells indicated that the lowest concentration of pure EGCG tested, 10µmol/l (4.58µg/ml), significantly increased the LDL-receptor protein by 60% and that lower concentrations of EGCG may also have had significant effects, although they were not tested.

Concentrations of EGCG similar to this have been reported in plasma in human absorption studies. A concentration of 4µg/ml was achieved in human plasma in a study by Chow et al.[440] when a single dose of 1.2g of EGCG in a GT catechin extract was given after an overnight fast and the participants remained fasted for an additional 4 h after the extract was taken. Ullmann et al.[420] achieved an EGCG plasma concentration of 5.2µg/ml when a single dose of 1.6g of pure EGCG was given under the same conditions.

However, the study by Chow et al.[440] showed that there was a much lower systemic absorption of EGCG when the GT catechin extract was taken with a standardised breakfast. For example, the plasma concentration was less than 25% (0.87µg/ml) when the 1.2g of EGCG in a GT catechin extract was given with food
compared to without breakfast. Therefore, the impact of food on the absorption of EGCG appears to be considerable but it has not been directly investigated in humans using pure EGCG.

1.8.1 Hypotheses and aims

Findings in animal studies using various GT catechin extracts have indicated cholesterol lowering effects for these preparations. The most predominant catechin in GT preparations, EGCG, is proposed to be the main catechin responsible for the beneficial effects exhibited by GT and its preparations. However, studies on the effects of pure EGCG in animal models and humans are still relatively scarce.

Therefore, the working hypothesis for the present studies was that ‘pure EGCG will lower cholesterol in hypercholesterolaemic rabbits and humans’. The main aim was to determine the effect of pure EGCG on blood cholesterol in cholesterol-fed, hypercholesterolaemic rabbits and in humans with moderately elevated blood cholesterol.

Three major experiments were conducted, one in rabbit and two in humans. In the rabbit study, whether pure EGCG could lower cholesterol and the mechanisms by which it could do this were studied (Chapters 3 and 4). In humans, the first experiment studied the absorption of pure EGCG, when given in three different formats (Chapter 5). The format of EGCG administration that provided the best systemic absorption was then used in a pilot study to investigate the effect of EGCG on blood cholesterol in humans with moderate hypercholesterolaemia (Chapter 6).

For the experiment with cholesterol-fed hypercholesterolaemic rabbits, it was hypothesised that ‘pure EGCG will lower blood cholesterol levels by up-regulating the LDL-receptor (Chapter 3), reducing cholesterol synthesis (Chapter 3) and inhibiting
intestinal cholesterol absorption (Chapter 4). The main aims were to determine whether pure EGCG could lower blood cholesterol in this hypercholesterolaemic rabbit model and to determine the mechanisms by which pure EGCG could lower blood cholesterol.

For the experiment investigating the best way to administer pure EGCG in order to maximise its systemic absorption in healthy humans (Chapter 5), it was hypothesised that ‘pure EGCG will be absorbed better when given as a capsule without food and give the highest plasma EGCG concentrations when given in this format compared to when given as a capsule with a typical breakfast’. However, it was also hypothesised that ‘incorporating EGCG in a food product like a strawberry sorbet will improve the absorption of EGCG compared to taking the catechin in capsule form with a typical breakfast’. Therefore, the main aim of this study was to determine which delivery format was the best of the three methods tested for the oral delivery of pure EGCG in humans.

Lastly, for the pilot human experiment (Chapter 6), it was hypothesised that ‘pure EGCG will lower blood cholesterol in humans with moderately elevated cholesterol levels (5.5-7.5mmol/l). Therefore, the main aim of this pilot study was to determine if pure EGCG, given for four weeks, in the format which gave the highest plasma EGCG concentrations in the absorption study (Chapter 5), could lower blood cholesterol in humans with moderately elevated cholesterol.

1.8.2 Research Protocols

To determine whether pure EGCG could lower cholesterol in the rabbit model and determine its mechanisms of action, New Zealand white rabbits were first rendered hypercholesterolaemic by feeding 0.25% (w/w) cholesterol for 2 weeks before being divided into 2 groups, a control (n=6) and a 2% (w/w) EGCG (n=6) group for a 4-week
treatment period. Blood and faecal samples were collected at the start and at the end of the treatment while livers were collected at the end of the experiment.

In Chapter 3, the effects of pure EGCG on serum and liver lipids, on the hepatic LDL-receptor and on indices of whole-body cholesterol synthesis and capacity to absorb cholesterol from the intestines were determined.

In Chapter 4, the effect of pure EGCG on sterol faecal excretion was determined; this included measuring the faecal excretion of cholesterol, secondary sterols and bile acids. The faecal excretion of free and conjugated forms of EGCG was also determined.

In Chapter 5, the study investigated the pharmacokinetic properties of pure EGCG in healthy human volunteers after EGCG was given in three different formats after an overnight fast: provided as a powder 1) in capsule form without food for a further 4h 2) in capsule form with a typical standardised breakfast and 3) incorporated into a food matrix, strawberry sorbet. The appearance of EGCG in plasma was determined over 8 h after its ingestion as a representation of systemic absorption of EGCG.

In Chapter 6, a pilot clinical trial in 10 participants was conducted in order to determine the effect of pure EGCG on blood cholesterol in humans with moderately elevated cholesterol levels (5.5–7.5mmol/l), using the administration format which gave the best systemic absorption of the catechin in Chapter 5. This study was a 4-week, double blind, randomised, placebo-controlled, parallel design intervention trial with 1g EGCG/day. Fasting blood samples were collected at baseline and at the end of the study and total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides were determined.
1.8.3 Expected Outcomes

The main outcomes of this study were expected to be the findings that pure EGCG: 1) lowered blood cholesterol levels in the cholesterol-fed hypercholesterolaemic rabbit model via an upregulation of the LDL-receptor, a decrease in whole-body cholesterol synthesis and an inhibition of cholesterol absorption; 2) was absorbed best when administered in capsule form without food but that incorporating the EGCG in a strawberry sorbet would be better than giving the catechin in capsule form with a typical breakfast; 3) lowered cholesterol in humans with moderately elevated cholesterol levels.
Chapter 2. General Methods

2.1 Introduction

In this thesis four studies were presented in respective Chapters: “Systemic effects of EGCG in the cholesterol-fed rabbit model of hypercholesterolaemia” (Chapter 3), “Intestinal effects of EGCG in the cholesterol-fed rabbit model of hypercholesterolaemia” (Chapter 4), “The effect of food on the systemic absorption of epigallocatechin gallate in humans” (Chapter 5) and “The effects of epigallocatechin-3-gallate on blood lipids in humans with moderately elevated blood cholesterol levels – a pilot study” (Chapter 6).

Both Chapters of the rabbit studies (Chapters 3 and 4) investigated the hypocholesterolaemic effect of EGCG in this animal model of diet-induced hypercholesterolaemia. However, the main focus of the animal study in Chapter 3 was to describe the effect of EGCG on processes related to cholesterol homeostasis in blood and liver. The study described in Chapter 4 was primarily focused on describing the effect of EGCG on the clearance of cholesterol and other sterols through the gastrointestinal tract.

In Chapter 5, methods for the oral administration of EGCG to healthy humans were investigated in order to maximise its systemic absorption. Based on the findings in Chapter 5, an administration method was chosen and used in a pilot study (Chapter 6) to investigate the hypocholesterolaemic effectiveness of EGCG in humans with mildly elevated cholesterol levels.

The present Chapter 2 will focus on general methods for analyses in rabbit and human serum, rabbit liver and rabbit faecal samples. Some methods are only briefly described in this chapter because they are described in more detail in subsequent Chapters 3 to 6.
2.2 Ethics approvals

Ethics approval for the use of animals in the rabbit studies was obtained from the Animal Care and Ethics Committee of the University of Newcastle (approval #938 0306), NSW, Australia. Ethics approval for the experiments in humans for, the “EGCG absorption study” and the “EGCG and cholesterol lowering pilot study” was granted by the Human Research Ethics Committee of the University of Newcastle (approval #H-2008-0089), NSW, Australia.

2.3 Chemicals and Reagents

All the following chemicals and materials were purchased from Sigma Aldrich (Castle Hill, Australia): (4-aminosalicylic acid; tetrahydrofuran; glacial acetic acid; alumina for column chromatography; disodium ethylenediamine tetra acetate (EDTA); 7-fluorobenzo-2-oxa-1,3-diazone-4-sulfonic acid ammonium salt (SBD-F); DL-homocysteine; glutathione; L-cysteine hydrochloride monohydrate (98% TLC); cysteinylglycine (85% TLC min); N-(mercaptopropionyl)-glycine; methanol (HPLC grade); perchloric acid (70%); tris(2-carboxyethyl)phosphine (TCEP); potassium hydroxide; potassium tetraborate; sodium chloride (NaCl); potassium chloride; sodium phosphate; orto-phosphoric acid; sodium hydroxide; acetonitrile (HPLC grade); isopropanol; (+)-α-tocopherol acetate; hydrochloric acid; chloroform; absolute ethanol (HPLC grade); hexane (GC grade); epicoprostanol (5β-cholestan-3α-ol); cholesterol (95% purity GC); lathosterol (5α-cholest-7en-3βol); β-sitosterol; campesterol; squalene; α-tocopherol acetate; sodium sulphate; (+)-catechin, epigallocatechin gallate (EGCG); ascorbic acid; potassium phosphate; formic acid; ethyl acetate; sulphuric acid; 1-butanol; 4-dimethylaminopyridine (DMAP); sodium acetate; lithocholic acid; deoxycholic acid; 5β-cholanic acid; chenodeoxycholic acid; cholic acid and
ursodeoxycholic acid; potassium carbonate; acetic anhydride; choloylglycine hydrolase from *Clostridium perfringens*; bovine serum albumin; β-glucuronidase type X-A from *Escherichia coli*; sulfatase, type VIII from abalone entrails; Tris-HCl; calcium chloride (CaCl$_2$); phenylmethanesulfonylfuoride (PMSF); N-ethylmaleimide; Tris-maleate; sodium carbonate; sodium tartrate; sodium dodecylsulfate (SDS); copper-sulfate pentahydrate; Folin-Ciocalteu reagent; triton X100; bromophenol blue; Tris-base; amberlite®XAD 4; glycerol; glycine; 2-mercaptoethanol; horseradish peroxidise).

Acrylamide (99.9%), bis(N,N′-methylene-bis)-acrylamide, ammonium persulfate; and tetramethylethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Gladeswille, NSW, Australia).

Tri-Sil TBT (10x1ml ampoules, Pierce Chemicals) was purchased from Quantum Scientific (Paddington, QLD, Australia) and polyethylene glycol 6000 (PEG6000) was purchased from BioLab (Mulgrave, VIC, Australia).

Enzymatic kits for the analysis of total cholesterol, triglycerides and glucose in rabbit serum were purchased from Australian Scientific Enterprise (Asquith, NSW, Australia). Enzymatic kits for the analysis of total cholesterol, triglycerides and HDL cholesterol in human plasma samples were purchased from Wako (Wako Pure Chemicals, Osaka, Japan).

Enzymatic test strips for analysis of fingerprick cholesterol, triglycerides using the AccutrendPlus (Roche Diagnostics Australia) and glucose using AccuCheck (Roche Diagnostics Australia) were purchased from Roche (Roche Diagnostics Australia, Castle Hill, NSW).

Deionised (DI) water used in the preparation of the assay reagents and the HPLC mobile phases was prepared on the day with a Millipore Milli-Q water purification system with resistivity greater than 18 MΩ cm (Millipore Australia, North Ryde, NSW).
2.4 Purity of the EGCG used in the rabbit and human studies

The EGCG, used for all the studies in this thesis, was purchased from RejuvaCare (Sydney, NSW, Australia). It is a product of DSM Nutritionals (Ermington, Australia), purified from green tea and sold under the tradename of TEAVIGO at a price ($1/gram), which was affordable for the animal and human studies described in this thesis. It is claimed by the manufacturers to be of high purity (greater than 92%) and suitable for human and animal consumption and for the development of food products. However, before using this EGCG preparation, its purity was ascertained by High Pressure Liquid Chromatography with ultraviolet detection (HPLC-UV) using EGCG from Sigma Aldrich (Castle Hill, NSW, Australia) as a reference standard[358, 441]. Sigma Aldrich only guarantees their EGCG to be 95% pure but only one peak was observed by HPLC analysis and therefore, it was taken to be 100% pure. However, this EGCG is prohibitively expensive for animal and human studies; for example, it would have cost close to $750,000 for the studies in the rabbit.

2.4.1 Preparation of working standards for the EGCG standard curve

The EGCG from Sigma Aldrich (Castle Hill, NSW, Australia) was dissolved, to make a 2mmol/l stock standard, in methanol containing 100mmol/l 4-aminosalycilic acid as an internal standard. The other working external standards for development of the EGCG standard curve were then made by serial dilutions. The concentrations of EGCG for the working external standards ranged from 62.5μmol/l to 2mmol/l and they were injected for HPLC analysis on the same day they were prepared.
2.4.2 Equipment and chromatographic conditions

The HPLC analysis was performed using a Shimadzu VP series system (Shimadzu, Kyoto, Japan), equipped with one liquid chromatogram pump (LC10-ATvp) connected to a degassing unit (DGU-14A) and the samples were introduced with an auto-sampler (SCL-10Avp) fitted with a 20μl loop. Separation was performed using an analytical Prodigy ODS(3) 250x4.6mm 5 micron column, protected by an analytical-size guard column (Phenomenex, Pennant Hills, NSW) and kept at 26ºC in a column oven (CTO-10A). The ultraviolet light absorbance intensities were measured at 210nm and 280nm using a UV-Vis detector (SPD-10Avp). The detector signal, recorded as peak area, was quantified using Class-VP 6.14 software installed on a computer assigned as a remote control operating system. The communication between the HPLC hardware and the computer system was enabled via a control unit (SPD10Vvp) which allowed for full automation of the system.

The mobile phases consisted of (A) 0.2% (v/v) phosphoric acid: acetonitrile: tetrahydrofuran (86:12.5:1.5 v/v), effectively 12.5% acetonitrile, and (B) 0.2% phosphoric acid: acetonitrile: tetrahydrofuran (73.5:25:1.5 v/v), effectively 25% acetonitrile. The auto-injector needle wash solution consisted of acetonitrile:DI water (50:50 v/v). All solvents were filtered through a Millipore 0.45μm cellulose filter (Millipore Australia, North Ryde, NSW) and degassed prior to use.

At a flow rate of 1ml/min, 100% mobile phase A was run for the first 10 min. The system was then switched to gradually increase the concentration of mobile phase B up to 100% through the following 30 min and then was held at 100% mobile phase B for an additional 10 min. This was followed by a gradual decrease in the concentration of mobile phase B to 0% over the next 10 min. For the following 20 min, mobile phase A was run, allowing the column to re-equilibrate on solvent A.
2.4.3 Preparation of sample for determining the EGCG purity

The EGCG from DSM Nutritionals, (TEAVIGO, Ermington, NSW, Australia) was weighed (0.0229g exactly) and dissolved in 100ml of 100mmol/l 4-aminosalycilic acid in methanol, providing a concentration targeted to be 0.500mmol/l EGCG. Ten samples were prepared under reduced light (red light), filtered through a 0.45μm nylon syringe filter (Phenomenex, Pennant Hills, Australia) into a brown HPLC vial, stored in the fridge at 4°C until injection and injected onto the HPLC column (20μl) within 2hafter their preparation.

2.4.4 Identification of the standards on the HPLC chromatogram

The elution times for1) the internal standard 4-aminosalycilic acid and 2) theEGCG from Sigma Aldrich(Castle Hill, NSW, Australia) were first determined by injecting each standard on separate HPLC runs. The EGCG from DSM Nutritionals (TEAVIGO) was then identified on the HPLC chromatogram after injection of the preparation made up on its own in methanol. The EGCG from DSM Nutritionals (TEAVIGO) was found to havean identical elution time (18.97min)to that of the EGCG from Sigma Aldrich. A typical HPLC chromatogram identifying the 1) internal standard 4-aminosalycilic acid and 2) EGCG peaks is presented in Figure 2.1.

2.4.5 Preparation of the standard curve

A standard curve was determined by plotting the peak area ratios (peak area of EGCG divided by the peak area of internal standard) against the known concentration of EGCG (Sigma Aldrich, Castle Hill, NSW, Australia)in each working standard solution. The standard curve of pure EGCG is represented in the figure 2.2.
Figure 2.1 Typical HPLC chromatogram of 4-aminosalycilic acid and EGCG. The retention times for 1) the internal standard, 100mmol/l 4-aminosalycilic acid (8.817min) and 2) EGCG (18.967min) are analysed using HPLC-UV as described in Section 2.4.2. The sample was 0.5mmol/l EGCG from DSM Nutritionals (TEAVIGO) prepared in methanol containing 100mmol/l 4-aminosalycilic acid as described in Section 2.4.3. A small peak was also seen eluting at 31.275min. This was a contaminant in the EGCG preparation and was identified by co-elution on HPLC as likely to be another catechin, epicatechin gallate (ECG).

Figure 2.2 The standard curve of EGCG. The EGCG was purchased from Sigma Aldrich and prepared in methanol containing 100mmol/l of the internal standard (IS) 4-aminosalycilic acid and analysed using HPLC-UV as described in Section 2.4.2. The ratio of the peak area for EGCG to the peak area for the IS was then plotted against the EGCG concentration.

\[ y = 5.7736x - 0.0259 \]
\[ R^2 = 0.9999 \]
2.4.6 Calculation of the purity of the DSM Nutritionals EGCG

The purity of the DSM Nutritionals EGCG was determined after injection of the ten samples onto the HPLC. The concentration of the EGCG in the ten samples was determined by dividing the peak area ratio (peak area of EGCG divided by the peak area of internal standard) by the mean slope of the standard curve. The measured EGCG concentration of the individual samples was then divided by the theoretical concentration of EGCG (0.5mmol/l) and multiplied by 100, providing a percentage of the EGCG concentration in each sample and the final purity was expressed as the mean percentage for the 10 individual EGCG samples. As seen in Table 2.1, the purity of the EGCG from DSN Nutritionals was found to average 97%. Therefore, this EGCG preparation was taken to be very pure and it was used in the rabbit and human studies without correcting for its very slight impurity.

Table 2.1 Results of the DSM Nutritionals EGCG purity analysis

<table>
<thead>
<tr>
<th>Number of Samples</th>
<th>Target EGCG Concentration (mmol/l)</th>
<th>Measured EGCG Concentration (mmol/l)†</th>
<th>EGCG Purity (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.500</td>
<td>0.483±0.01</td>
<td>97±2</td>
</tr>
</tbody>
</table>

†Values are Mean±SD

2.5 Serum and plasma analysis in rabbit and human studies

Serum samples were collected in the rabbit study while plasma samples were collected in the human trials. On the day of each assay described below, serum/plasma samples were taken out of the freezer (-84°C), allowed to thaw in the fridge (4±2°C), vortex mixed for 1min and centrifuged at 10,000 × g for 10min (Sigma 4K 15 centrifuge) to pellet any fibrin clots.
All the measurements of serum analytes were done at the Nutrition Laboratories at the University of Newcastle Central Coast Campus in Ourimbah by the author using various methods. Most of these methods are only briefly described below but are described in more detail in the results Chapters (3-6) where they are mainly used.

Analysis of triglycerides, total and HDL cholesterol was done in rabbit serum and human plasma while glucose analysis was done only in rabbit serum by enzymatic analysis using commercially available kits. Rabbit serum was also analysed for concentrations of individual sterols (lathosterol, campesterol, and β-sitosterol) using gas chromatography (GC) but also for concentrations of squalene and thiols (homocysteine, cysteine, cysteinyl glycine and glutathione) using high pressure liquid chromatography (HPLC) with ultraviolet detection (UV) and fluorescence detection respectively. Furthermore, human plasma samples were analysed for EGCG using HPLC with diode array detection coupled with mass spectrometer detector (HPLC-DAD-MS).

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

Enzymatic kits for the analysis of total cholesterol, triglycerides and glucose in rabbit serum were purchased from Australian Scientific (Asquith, NSW, Australia) while kits for the analysis of cholesterol, HDL cholesterol and triglycerides in human plasma were purchased from Wako (Wako Pure Chemicals, Osaka, Japan). All enzyme solutions were prepared according to the manufacturers recommendations.

Analysis of total cholesterol, triglycerides and glucose in rabbit serum was performed using a CobasBio® centrifugal autoanalyser (Roche Diagnostics, Dee Why, NSW, Australia). The settings for the instrument were adapted from previously
developed methods for serum cholesterol [442], serum triglyceride [443] and serum glucose [444, 445] analysis.

The analysis of HDL cholesterol was done in rabbit serum samples after the precipitation of plasma lipoproteins using 12% (w/v) PEG6000 (BioLab, Mulgrave, VIC, Australia) [446]. Samples were then analysed using enzymatic kit for total cholesterol analysis (Australian Scientific, Asquith, NSW, Australia) on the CobasBio® centrifugal autoanalyser (Roche Diagnostics, Dee Why, NSW, Australia) with the settings for the instrument being the same as for total cholesterol analysis. Detailed information about these methods is reported in Chapter 3 (Section 3.4.4.1).

Analysis of total cholesterol, HDL cholesterol and triglycerides in human plasma was performed using a Varian Carry UV-Vis spectrophotometer (Varian Australia, Clayton, VIC) equipped with a multi-cell module and a water temperature control system set at 26°C and connected to a computer (Dell Pentium IV) assigned as a remote controlling system. Data was collected using the Carry Win UV software (version 3) set in absorbance mode at wavelength of 600nm for all three measurements. Detailed information about these methods is reported in Chapter 6 (Section 6.2.2.5).

2.5.2 Estimation of non-HDL, VLDL and LDL cholesterol

The non-HDL cholesterol was calculated by subtracting the measured HDL cholesterol from the measured total cholesterol value for each sample [447]. To estimate the VLDL cholesterol the triglyceride value was divided by 2.2 [447]. The LDL cholesterol was calculated using the Friedewald formula [447] below (Equation 2.1):
\[ \text{LDL [mmol/l]} = \text{TC [mmol/l]} - \text{HDL [mmol/l]} - \frac{\text{Tg}}{2.2} \text{ [mmol/l]} \]

**Equation 2.1** Friedewald formula for calculating LDL cholesterol. Note: LDL is LDL cholesterol; TC is total cholesterol; HDL is HDL cholesterol; Tg is triglycerides. VLDL cholesterol is estimated by dividing the Tg value by 2.2 [447]

2.5.3 **Analysis of serum sterols using gas chromatography (GC)**

The method used for the determination of serum sterols [435] assessed the concentrations of cholesterol, lathosterol (5α-cholest-7en-3βol), β-sitosterol and campesterol with the use of epicoprostanol (5β-cholestan-3α-ol) as an internal standard. In order to achieve this TriSil-TBT reagent (Thermo Scientific, Sydney, Australia) was used to convert the hydroxyl groups into trimethylsilyl derivatives [448-450]. This method is further detailed in Chapter 3 (Section 3.4.4.3).

2.5.4 **Serum squalene by High Pressure Liquid Chromatography**

The method used for the determination of serum squalene was developed using (+)-α-tocopherol acetate as an internal standard and extracting with isopropanol [451-453]. This method is further detailed in Chapter 3 (Section 3.4.4.4).

2.5.5 **Serum thiol analysis by High Pressure Liquid Chromatography**

The method used for determination of serum thiols uses the fluorogenic ammonium salt of 7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) to react with the free sulfhydryl groups of the thiols thus rendering them able to be seen by fluorescence detection [454, 455]. The original method used tri-n-butylphosphine in N,N – dimethylformamide [454, 455] but in this thesis, the much less dangerous reducing agent tris(2-carboxyethyl)phosphine (TCEP) was used for the reduction of the disulfides
and protein-bound thiols [456, 457]. This method is further detailed in Chapter 3 (Section 3.4.4.5).

### 2.5.6 Determination of EGCG in human plasma

For the determination of EGCG in human plasma, a highly specific method was developed and validated using an HPLC equipped with a DAD and MS detector. This method is further detailed in Chapter 5 (Section 5.3.2).

### 2.6 Rabbit faecal samples

All the measurements of faecal analytes were done at the Nutrition Laboratories at the University of Newcastle Central Coast Campus in Ourimbah by the author using various methods. These methods are only briefly described below but are described in more detail in results Chapter 4 where they are used.

#### 2.6.1 Total faecal fat content

Total faecal fat content was determined by gravimetric analysis [458] after extraction with chloroform:methanol solution (2:1 v/v) [459]. This method is further detailed in Chapter 4 (Section 4.2.6).

#### 2.6.2 Faecal neutral sterols using Gas Chromatography

Neutral sterols were determined in lyophilised faecal samples after extraction with chloroform:methanol solution (2:1v/v). The neutral sterols were derivatised using TriSil-TBT reagent (Thermo Scientific, Sydney, Australia) and the same procedure used for serum sterol analysis [435]. This method is further detailed in Chapter 4 (Section 4.2.7).
2.6.3 Faecal bile acids using Gas Chromatography

The extraction and derivatisation procedures for faecal bile acids were adopted from previously developed methods [460-464] with some modifications. This method is further detailed in Chapter 4 (Section 4.2.8).

2.6.4 Faecal EGCG by High Pressure Liquid Chromatography

The extraction and de-conjugation of faecal EGCG was adopted from previously developed methods [375, 393, 465] with some modifications. This method is further detailed in Chapter 4 (Section 4.2.9).

2.7 Liver Samples

Liver samples were analysed at the Nutrition Laboratories at the University of Newcastle Central Coast Campus in Ourimbah for cholesterol, triglyceride and the LDL-receptor by the author of this thesis. The liver homogenates samples for the analysis of CD36 was also prepared by the author but the electrophoresis and immunoblotting were done by staff at the School of Biomedical Sciences and Pharmacy at the University of Newcastle at Callaghan. The analysis of the data was done by the author of this thesis.

2.7.1 Collection of liver samples

Following euthanasia of the rabbits, liver samples were collected and stored as several aliquots in a freezer at -84°C. Aliquots were thawed when needed for the analysis for protein content, total, unesterified and esterified cholesterol, triglycerides, LDL-receptor and CD36 receptor protein.
2.7.2 Preparation of soluble rabbit liver membrane proteins

Rabbit liver homogenate and a solubilised rabbit liver membrane fraction were prepared as described previously [242, 466]. These method are further detailed in Chapter 3 (Section 3.4.5).

2.7.3 Cholesterol and triglyceride in liver homogenates and membranes

Total, unesterified and esterified cholesterol and triglycerides were determined in liver homogenates and solubilised liver membranes using the enzyme kits purchased from Australian Scientific (Asquith, NSW, Australia) and a CobasBio® centrifugal autoanalyser (Roche Diagnostics, Dee Why, NSW, Australia), essentially as described above (Section 2.5.1) for the measurements of these lipids in rabbit serum.

2.7.4 Analysis of LDL-receptor and CD36 in liver homogenates and solubilised liver membranes

The LDL-receptor analysis was performed by immunoblotting [242, 466] on solubilised rabbit liver membranes and this method is further detailed in Chapter 3 (Section 3.4.5.2).

Analysis of CD36 was also performed by immunoblotting [467] on rabbit liver homogenates prepared as described previously [242, 466]. The electrophoresis and immunoblotting of CD36 were performed by staff at the School of Biomedical Sciences and Pharmacy at the University of Newcastle at Callaghan. This method is further detailed in Chapter 3 (Section 3.4.5.5).
Chapter 3. Systemic effects of EGCG in the cholesterol-fed rabbit model of hypercholesterolaemia

3.1 Introduction

Cholesterol is an essential compound required for normal growth and development and it also serves as a functional component of cellular membranes. However, an elevated level of cholesterol in the blood is a primary risk factor (Section 1.1.5.1) for the development of cardiovascular disease [229]. Therefore, cholesterol lowering is still a leading and central focus for reducing the incidence and development of CVD.

Green tea (GT), made from the plant Camellia sinensis, (L.) O. Kuntze, is one of the most widely consumed beverages in the world. The intake of GT extracts has been shown to exhibit a significant cholesterol lowering effect in animal models of hypercholesterolaemia and these positive results have been primarily ascribed to the high concentration of catechins, particularly EGCG (Section 1.6.5 and 1.6.6). In the animal models, including in the cholesterol-fed hypercholesterolaemic rabbit model, the cholesterol lowering effects of the GT catechin extracts were achieved by 1) inhibiting cholesterol biosynthesis, 2) up-regulating the LDL-receptor or 3) increasing cholesterol and bile acid excretion in the faeces [242, 401, 424]. However, whether EGCG, the main GT catechin, has similar cholesterol lowering mechanisms of action is not known. In this chapter the effect, of pure EGCG, on the first two mechanisms of action were investigated in the cholesterol-fed hypercholesterolaemic rabbit model. The effect of EGCG on the third mechanism of action was investigated in Chapter 4.
3.1.1 Green tea extracts and inhibition of cholesterol synthesis in animal models

Cholesterol requirements can be met from two sources, absorption of dietary cholesterol (Section 1.2.2) and \textit{de novo} synthesis from acetyl-CoA (Section 1.2.1), with the greatest cholesterol production capacity being in the liver [228]. Therefore, inhibition of either cholesterol absorption or cholesterol biosynthesis or both can result in a decreased circulating blood cholesterol level [242, 402, 408, 409]. The effect of EGCG on cholesterol biosynthesis is addressed in this chapter and its effect on absorption in Chapter 4.

The effects of GT on cholesterol biosynthesis have been examined in different animal models of dietary induced hypercholesterolemia such as the rabbit [242], rat [401, 424, 468] and hamster [469] models. These studies have used green tea products such as tea infusions and crude extracts rather than pure EGCG. Therefore, the effect of the major GT catechin, EGCG, on cholesterol biosynthesis still remains relatively unknown.

The findings from animal studies using infusions and extracts have been inconsistent. Intervention studies in rats found no effect of GT extracts on the activity of HMG-CoA reductase [404, 469]. This enzyme catalyses a rate limiting step in cholesterol biosynthesis and its inhibition, by the class of pharmaceuticals referred to as statins (Section 1.2.1.1), has proven to be very effective at lowering blood cholesterol [237, 470-472]. Also in rats, a GT extract had no effects on the plasma lathosterol:cholesterol ratio [434], an index of whole body cholesterol synthesis [435]. However, in a rabbit study, there was a 60% reduction in cholesterol synthesis as measured using the plasma lathosterol:cholesterol ratio [242].

It has also been proposed that GT catechins can inhibit the \textit{de novo} cholesterol synthesis at the squalene epoxidase (SE) level (Sections 1.2.1.1 and 1.6.6.2) in a non-
competitive and non-time dependant manner [247]. This enzyme is known to be regulated at the transcriptional level in response to intracellular sterol concentrations, similar to HMG-CoA reductase, and to play a central role in the maintenance of cholesterol homeostasis [239, 452, 473]. However, in vitro studies have found that the inhibition of SE by EGCG and other galloyl esters [241, 245] occurred as a direct inhibition of the enzyme’s activity in a non-competitive manner rather than at the level of DNA transcription. These in vitro findings suggest that inhibition of cholesterol biosynthesis at the SE level should be investigated as a main target for the hypocholesterolaemic effects of EGCG in vivo.

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

The LDL-receptor activity in hepatic tissue is the main mechanism by which cholesterol is removed from the blood circulation [334]. The LDL-receptor plays a central role in a very complex system that stabilises the cellular cholesterol concentration (Section 1.4). Intracellular cholesterol levels are maintained by three regulatory cholesterol feedback systems; inhibition of HMG-CoA reductase activity, activation of the cholesterol esterifying enzyme ACAT and inhibition of the synthesis of LDL-receptors, with all three preventing the cell from over-accumulating cholesterol (Figure 1.14). Therefore, the functioning of these systems provides a coordination and control of the intracellular cholesterol concentration regardless of the fluctuations in the external levels of lipoprotein cholesterol in the blood circulation. However, this system, especially through the activity of the liver LDL-receptor, can also have an impact on the extracellular cholesterol circulating in the blood in LDL [256, 324-326].

In vitro studies with human hepatoma HepG2 cells have indicated that the LDL-receptor was up-regulated when the cells were incubated with a GT extract [410] or
with pure EGCG [411, 412], suggesting that EGCG could have a cholesterol lowering effect by increasing the uptake of circulating LDL cholesterol via an upregulated hepatic LDL-receptor. Furthermore, the study by Bursill et al. [411] demonstrated that EGCG was the only one of the four major GT catechins (Section 1.6.1, Figure 1.16) to significantly up-regulate the LDL-receptor \textit{in vitro} and as such it was most likely to be the main active constituent in GT extracts.

In animal intervention studies, a GT extract was also shown to increase the hepatic LDL-receptor activity in rabbits and rats [242, 434]. Therefore upregulation of the hepatic LDL-receptor was most likely involved in the observed lowering of the circulating blood cholesterol and in the lowering of the aortic and hepatic cholesterol levels also observed in these studies. However, whether pure EGCG has the same effect on the hepatic LDL-receptor activity in animals is not known and the present study aims to determine whether EGCG can up-regulate the hepatic LDL-receptor in the cholesterol-fed hypercholesterolaemic rabbit model.

\subsection*{3.2 Main hypothesis and aims}

Based on the previous \textit{in vitro} studies in HepG2 cells [410, 411] and the \textit{in vivo} animal model studies [242, 434], the cholesterol lowering effects of GT extracts were likely to have been contributed to by upregulation of the LDL-receptor and inhibition of cholesterol synthesis and it was proposed that pure EGCG would cause a significant lowering of serum cholesterol via the same mechanisms of action in the cholesterol-fed hypercholesterolaemic rabbit model.

Therefore, the main hypothesis for this chapter was that ‘pure EGCG will significantly lower serum cholesterol concentrations in the cholesterol-fed rabbit model of hypercholesterolaemia by decreasing cholesterol synthesis and upregulating the
hepatic LDL-receptor’. The main aims were to determine the effect of pure EGCG on serum cholesterol in this rabbit model and to determine the mechanisms of action for the cholesterol lowering properties of EGCG. The effect on EGCG on the hepatic cholesterol and triglyceride concentrations was also determined.

3.3 Secondary aims

3.3.1 Hepatic CD36

As part of this study, there was an opportunity to determine if the catechin had any effect on the class B scavenger receptor protein CD36 (Section 1.5.2). This was prompted by a recent study by Sakuari et al. [474], which found that low doses of EGCG (10µmol/l) induced the expression of genes related to insulin sensitivity and adipocyte differentiation, including CD36 in the early stages of differentiation of the 3T3-L1 cells in vitro. Human CD36 is thought to be a high affinity receptor for the native lipoproteins LDL, VLDL and HDL and its upregulation by EGCG could also contribute to the cholesterol lowering effect of the catechin [336]. Furthermore, the increases in the expression of the hepatic CD36 contributes to the dyslipidemia associated with the diet induced obesity [475].

Therefore, a secondary hypothesis for this chapter was that ‘pure EGCG will upregulate the hepatic CD36 in the cholesterol-fed hypercholesterolaemic rabbit model’ and a secondary aim was to determine whether pure EGCG had any effect on the CD36 in the liver of the rabbits.

3.3.2 Plasma homocysteine

As part of this study, there was also an opportunity to determine if the catechin had any effect on the plasma concentration of homocysteine (Hcy), an independent risk
factor for atherosclerosis and thrombosis [185-187] (Section 1.1.5.3). This was prompted by a study by Zulli et al.[476], which found that when Wistar Kyoto rats were fed 2% (w/w) cholesterol incorporated into their normal chow, their plasma Hcy levels were significantly increased (+47%) compared to the control group. Furthermore, when the rats were fed a combination of methionine and the cholesterol diet, the increase in plasma Hcy levels was even higher (+133%) compared to the control group. These findings suggested that dietary cholesterol could increase plasma Hcy, especially when methionine, a precursor for Hcy, was also included in the diet.

Conversely, hyperhomocysteinemia was also identified as inducing cholesterol accumulation in the plasma and liver of rats by increasing hepatic cholesterol biosynthesis via activation of the transcription factors SREBP-2, cAMP response element binding protein and nuclear factor Y in liver cells [477, 478].

Therefore, another secondary hypothesis for this chapter was that ‘pure EGCG will decrease plasma Hcy in the cholesterol-fed hypercholesterolaemic rabbit model’ and another secondary aim was to determine whether pure EGCG had any effect on the plasma Hcy concentration in the rabbits.
3.4 Methods

3.4.1 Ethics approvals

Ethics approval for use of animals in this study was granted by the Animal Care and Ethics Committee of the University of Newcastle (#938 0306), NSW, Australia.

3.4.2 Food preparation

Pelletised rabbit food containing 18% (w/w) protein, 4% (w/w) fat, 18% (w/w) fibre and 11.1MJ/kg digestible energy was purchased from Speciality Feeds (Perth, WA, Australia). The preparation of the cholesterol and EGCG enriched food mixes was done in the Food and Nutrition Laboratories of the School of Environmental and Life Sciences at Ourimbah (NSW). The dry pellets were firstly grounded in a Warring food processor (John Morris Scientific Pty Ltd, Chatswood, NSW, Australia) to form a powder, to which was added 0.25% (w/w) cholesterol (Sigma Aldrich, Sydney, NSW, Australia). To provide the food for the EGCG treatment group, 2% (w/w) EGCG was added to some of the powder along with the 0.25% (w/w) cholesterol. Water was then added to the powder mixes to form a paste, which was passed through a meat mince maker (Breville, Pyrmont, NSW, Australia) to re-pellet the diets. The pellets were then air dried overnight in a BBO forced air oven drier (G.T.D. Pty Ltd, Sydney, NSW, Australia) set at 30°C.

3.4.3 Animals and the experimental design

Twelve male (12 weeks old) New Zealand white rabbits (IMVS Animal Facility, Adelaide, SA, Australia) were housed in individual cages at the John Hunter Hospital Animal Holding Facility (Newcastle, NSW, Australia). The rabbits were monitored daily during the six weeks period of the study and their appearance, weight and food
consumption was recorded. The rabbits were housed in individual cages in order to be able to monitor the food consumption and faeces excretion for each rabbit.

Initially, all rabbits were fed a diet containing 0.25% (w/w) cholesterol incorporated into their rabbit pellets (Speciality Feeds, Perth, WA, Australia) for 2 weeks, in order to render them hypercholesterolaemic prior to the administration of the pure EGCG during the treatment phase. At the end of the 2 weeks on the 0.25% (w/w) cholesterol diet, all rabbits were fasted overnight and blood samples for plasma cholesterol analysis (Week 2) were collected from the marginal ear vein.

The next day, after their cholesterol analysis was done, the rabbits were allocated to one of two groups with equivalent average blood cholesterols, a control (n=6) and a treatment (n=6) group, based on their serum cholesterol levels. One group, the control group, continued for a further 4 weeks on the diet containing 0.25% (w/w) cholesterol only. The other group, the 2%EGCG group, was fed for 4 weeks on a diet containing 2% (w/w) EGCG (DSM Nutritional, Ermington, NSW Australia) mixed into their pellets along with 0.25% (w/w) cholesterol. This level of EGCG was based on previous observations in a study with a crude catechin extract, in which the extract lowered blood cholesterol in the same rabbit model of hypercholesterolaemia [242]. The rabbits were provided with exactly 300g of food every day and their daily consumption of the diet was determined by difference after weighing the amount of food left the next day. The rabbits were also weighed at the end of each week to monitor their growth.

At the end of the 4-week treatment period (end of week 6 for the whole experiment), the rabbits were anaesthetised by injecting 2mg/kg alphaxalone (Alfaxan CD, 10 mg/ml, Jurox Pty. Ltd., Rutherford NSW, Australia) through the ear vein. Once under deep anaesthesia, the rabbits were bled by cardiac puncture and euthanased using an intravenous injection of 150mg/kg pentobarbital (Jurox Pty. Ltd., Rutherford NSW,
Australia). Liver samples were then collected and the carcasses were disposed in accordance with ethical regulations. The blood and liver samples were transported on ice in containers to the nutrition laboratories of the University of Newcastle at Ourimbah, NSW, Australia.

All blood samples were allowed to clot at 4°C and the serum was separated from red blood cells by centrifugation at 5000 × g for 15mins at 4°C (Sigma 4K 15 centrifuge). The serum samples were aliquoted and stored at -84°C until analysis. The liver samples were also stored at -84°C until analysis.

3.4.4 Serum analysis

Only the needed amounts of serum samples were thawed out on the day of assay, ensuring that collected samples remained frozen for as long as possible. On the day of assay, designated samples were taken out of the freezer (-84°C), allowed to melt at the ambient room temperature (17±2°C) for 30minutes and centrifuged at 10,000 × g for 10min at 4°C (Sigma 4K 15 centrifuge) to pellet any fibrin clots.

3.4.4.1 Serum lipids using commercially available enzyme kits

Serum total cholesterol and triglyceride concentrations were measured on a CobasBio® centrifugal autoanalyser (Roche Diagnostics, Dee Why, NSW, Australia) using commercially available enzyme kits (Australian Scientific, Asquith, NSW, Australia) [242, 442, 443]. The HDL cholesterol was also determined using the CobasBio® and the cholesterol analysis enzymatic kit, after precipitation of plasma lipoproteins with 12% (w/v) PEG6000 (BioLab, Mulgrave, VIC, Australia) [446]. The settings for the CobasBio®instrument (Table 3.1) were adapted from previously developed methods for serum cholesterol [442], serum triglyceride [443] and serum
glucose [444, 445] analysis. Serum glucose analysis is further described in Section 3.4.4.2.

The concentration of non-HDL cholesterol was calculated by subtracting the HDL cholesterol concentration from the total cholesterol value. The concentration of VLDL and LDL cholesterol in the serum samples was calculated using the Friedewald formula [447] as described in Section 2.5.2 of the General Methods in Chapter 2.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cholesterol (value)</th>
<th>HDL cholesterol (value)</th>
<th>Triglycerides (value)</th>
<th>Glucose (value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units</td>
<td>mmol/l</td>
<td>mmol/l</td>
<td>mmol/l</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Calculation factor</td>
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<td>10.4</td>
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<td>1</td>
</tr>
<tr>
<td>Standard (1-3)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Limit</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>10</td>
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<td>30</td>
</tr>
<tr>
<td>Type of analysis</td>
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<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
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<td>500</td>
<td>500</td>
<td>340</td>
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<tr>
<td>Sample volume (μl)</td>
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<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Diluent volume (μl)</td>
<td>30</td>
<td>26</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Reagent volume (μl)</td>
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<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Incubation time (s)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Start reagent time</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Time of first reading</td>
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</tr>
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<td>Time interval (s)</td>
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<td>100</td>
</tr>
<tr>
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<tr>
<td>Printout mode</td>
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<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
3.4.4.2 Analysis of serum glucose

Serum glucose was also measured on the CobasBio® centrifugal autoanalyser (Roche Diagnostics, Dee Why, NSW, Australia) using commercially available enzyme kits (Australian Scientific, Asquith, NSW, Australia) [444, 445]. See Table 3.1 for the settings of the CobasBio® centrifugal autoanalyser for the glucose assay.

3.4.4.3 Analysis of serum sterols using Gas Chromatography

Previous studies have found that the ratio of serum lathosterol to total cholesterol correlates with whole body cholesterol synthesis [479] and that the ratio of the serum phytosterols, β-sitosterol and campesterol, to total cholesterol has been found to correlate with the intrinsic capacity to absorb dietary cholesterol [480]. Gas chromatography (GC) was therefore used to measure cholesterol, lathosterol, β-sitosterol and campesterol in the rabbit serum.

All measurements of serum sterols were done at the nutrition laboratories at the University of Newcastle in Ourimbah using GC equipped with a flame ionisation detector (FID). The method used for the determination of serum sterols [435] assessed the concentrations of cholesterol, lathosterol (5α-cholest-7en-3βol), β-sitosterol and campesterol with the use of epicoprostanol (5β-cholestan-3α-ol) as an internal standard. In order to achieve this, TriSil-TBT reagent (Thermo Scientific, Sydney, Australia) was used to convert the hydroxyl groups into trimethylsilyl derivatives [448-450].

3.4.4.3.1 Preparation of stock standard solutions

The stock standard solutions of 20mmol/l cholesterol, 1mmol/l lathosterol (5α-cholest-7en-3βol), 1mmol/l β-sitosterol and 1mmol/l campesterol were prepared in hexane (Sigma Aldrich, Australia). A stock solution of 2.5mmol/l epicoprostanol (5β-
cholestan-3α-ol) was also prepared in hexane and it was used as an internal standard in the analysis of the serum sterols. All solutions were stored in a freezer at -18°C until used.

3.4.4.3.2 Preparation of working standards for standard curves

Working standard solutions of 20mmol/l cholesterol, 20μmol/l lathosterol, 20μmol/l β-sitosterol and 1mmol/l campesterol were prepared from the stock solutions. An additional four working standards were prepared by serial dilutions of this standard solution with hexane to provide a concentration range of 1.25 – 20mmol/l for cholesterol, 25 – 1000μmol/l for campesterol and 1.25-20μmol/l for lathosterol and β-sitosterol. The concentration ranges of the different sterols were based on previous studies [479].

3.4.4.3.3 Preparation of quality control sample

One of the rabbit serum samples from week 6 was selected as a quality control sample and injected at the start and at the end of each chromatographic run. The obtained results were used to determine the intra- and inter-assay variation of this method.

3.4.4.3.4 Equipment and chromatographic conditions

The GC analysis was performed using a Varian Star 3400cx (Varian Australia, Clayton, VIC) equipped with a Varian 8200cx autosampler. The detector signal was recorded and the peak areas were quantified using Star Chromatography Workstation 6.0 installed on a computer. Samples were injected using the Varian 1177 split/splitless injector using a split ratio of 1:30 with a capillary column adaptor fitted. A non-polar
30mx0.32mm, ID 0.25μm Econo-cap (EC5) fused silica column (Grace-Alltech, Baulkham Hills, NSW, Australia) was used to separate the steroids. The carrier gas was a high purity (99.999%) helium (BOC Gasses Australia, West Gosford, NSW) set at flow rate of 3ml/min. The injector and detector temperatures were set at 300°IC and the chromatography was run for 20min at an isothermal oven temperature set at 290°IC.

3.4.4.3.5 Derivatisation of serum sterols

After thawing, the serum samples were centrifuged at 10,000 × g to separate any fibrin clot and 200μl of clear serum was transferred into Kimble® glass sample vials (Grace-Alltech, Baulkham Hills, NSW, Australia) containing 75nmol/l of the internal standard epicoprostanol (5β-cholestan-3α-ol). The Kimble® glass sample vials were pre-prepared by evaporating the hexane from 30μl of the 2.5mmol/l epicoprostanol in hexane solution under a stream of nitrogen to give an internal standard concentration of 1.5mmol/l epicoprastanol in all serum samples. All serum samples were analysed in duplicate.

For the development of standard curves, 200μl of the external standards prepared in hexane were added instead of the serum into Kimble® glass sample vials together with 30μl of the internal standard (IS), to give an internal standard concentration of 1.5mmol/l epicoprastanol, and the hexane was evaporated to dryness. The residue was then resuspended in phosphate buffered saline (PBS) pH 7.4 containing 10mmol/l Na₂HPO₄/KH₂PO₄, 137mmol/l NaCl and 2.7mmol/l KCl and the sterol derivatisation procedure was continued as for serum.

After the addition of serum or PBS, the glass vials were vigorously vortex-mixed for 5min (SMI Multi-tube Vortexer, Miami, FL, USA) followed by the addition of 100μl of 33% (w/v) potassium hydroxide solution and 2ml of ethanol. The samples
were then thoroughly vortex-mixed and placed in a shaking waterbath (Ratek Instruments, Boronia, Australia) set at 60°C for 30min. The solution was then allowed to cool to room temperature and 1ml of water was added followed by the addition of 2ml hexane. The hexane layer was removed into another glass vial and the hexane extraction was repeated twice more. All hexane extracts were combined together and evaporated to dryness under a stream of nitrogen gas on a dry block heater set at 60°C (Ratek Instruments, Boronia, Australia).

In order to generate trisilyl sterol derivatives, 100μl of Tri-Sil TBT (Thermo Scientific, Sydney, Australia) was added to the cooled evaporated residue and samples were incubated for 30min in a shaking waterbath (Ratek Instruments, Boronia, Australia) set at 80°C. After cooling, 4ml of 100mmol/l hydrochloric acid was added to the tubes followed by addition of 4ml of hexane. The tubes were then vortex-mixed for 3min (SMI Multi-tube Vortexer, Miami, FL, USA) and centrifuged at 2000 × g for 10min (Clements 2000 Centrifuge, Sydney, NSW, Australia) to separate the organic and aqueous layers. The aqueous layer was then removed and the organic layer was washed three times with 2ml DI water by vortex-mixing and centrifugation. The hexane layer was filtered through a Pasteur pipette containing sodium sulphate into borosilicate evaporation tubes, in order to remove any water residue from the hexane extract. The hexane was then evaporated to dryness under a stream of nitrogen gas on a dry block heater set at 60°C (Ratek Instruments, Boronia, Australia).

The residue in the evaporation tubes was resuspended in 50μl hexane and transferred into the GC cups having appropriate volume inserts and the samples were sequentially injected (1μl) onto the GC column for analysis of the sterols.
3.4.4.3.6 Identification of the sterols on the GC chromatogram

The serum sterols and the IS elution times were determined on the chromatogram after the individual preparation and injection of their respective pure standards. Furthermore, the identification of the sterols and the internal standard in the serum samples (Figure 3.1) was done by comparing serum samples individually spiked with known amounts of cholesterol, lathosterol, β-sitosterol, campesterol and IS to serum samples which were not spiked.

3.4.4.3.7 The standard curve and calculation of sterols concentrations

Standard curves were determined for each sterol by plotting the peak area ratios (peak area of each sterol divided by the peak area of the internal standard) against the known concentration of the sterols in each working standard solution (Figure 3.2).

The concentrations of the sterols in each duplicate of the rabbit serum samples were calculated by dividing the serum samples’ sterol peak area ratios by the standard curve slope value of the individual sterol. The final concentration of the sterols in the rabbit serum samples were derived as the mean value of the sample duplicates.
Figure 3.1 Typical GC-FID chromatogram of a rabbit serum sample containing internal standard after derivatisation and extraction as described in Section 3.4.4.3.5. The sterols, in order of elution, are (1) internal standard epicoprostanol (5β-cholestan-3α-ol) (8.306min), (2) cholesterol (10.087min), (3) lathosterol (5α-cholest-7en-3βol) (10.307min), (4) Un-identified (11.382min) and (5) β-sitosterol (13.060min).

3.4.4.3.8 Intra and inter assay coefficients of variation for the serum sterols

Intra assay and inter assay coefficients of variation for the individual serum sterols were determined using a quality control serum sample as shown in Table 3.2.
Figure 3.2 External standard calibration curves for sterols in spiked serum samples. The results for cholesterol (A), β-sitosterol (B), lathosterol (C) and campesterol (D) were used to derive linear equations by linear regression analysis of the sterol/internal standard chromatographic peak area ratios plotted versus the concentration of each sterol. The peak areas for the IS and the individual sterols were determined by GC-FID as described in Section 3.4.4.3.7.
Table 3.2 Intra and inter assay variation for serum sterols using a control serum sample

<table>
<thead>
<tr>
<th></th>
<th>Intra Assay Variation Analysis</th>
<th>Inter Assay Variation Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>8</td>
<td>8.38±0.25</td>
</tr>
<tr>
<td>Lathosterol (μmol/l)</td>
<td>8</td>
<td>2.78±0.15</td>
</tr>
<tr>
<td>Campesterol (μmol/l)</td>
<td>8</td>
<td>211.90±8.06</td>
</tr>
<tr>
<td>β-sitosterol (μmol/l)</td>
<td>8</td>
<td>16.92±0.42</td>
</tr>
</tbody>
</table>

*Note:* Values are expressed as Mean ± Standard Deviation (Mean±SD), n – number of replicates, CV – coefficient of variation calculated as (SD/Mean) x 100

3.4.4.4 Serum squalene by high pressure liquid chromatography

Based on *in vitro* studies [239, 245, 247, 452], which showed that EGCG was a potent inhibitor of squalene epoxidase, the production of cholesterol metabolites such as lathosterol relative to the amount of squalene metabolised at the SE step, the ratio of lathosterol to squalene was thought to be useful as an index of cholesterol synthesis. Therefore, squalene was measured in the rabbit serum samples.

All the measurements of serum squalene were done at the nutrition laboratories at the University of Newcastle in Ourimbah using HPLC with UV detection. The method used for the determination of serum squalene was developed using (+)-α-tocopherol acetate as an IS and extraction of squalene and internal standard was done using isopropanol [451-453]. A standard curve of pure squalene, made up in HPLC-grade acetonitrile was determined as described below and a control serum sample was used to standardise every assay run.
3.4.4.4.1 Cleaning of the glassware

As squalene is found on skin surfaces and commonly occurs in oils [481, 482], all glassware was meticulously cleaned first in 10% (v/v) Radacon solution (Fronine, Riverstone, NSW, Australia) followed by rinsing in tap water, distilled water, isopropanol:acetonitrile (2:1) solution and finally DI water. All glassware was allowed to air-dry and after drying all glassware and other equipment was handled wearing gloves free from latex powder.

3.4.4.4.2 Preparation of stock standard solutions

Stock solutions of 6.25μmol/l squalene and 10μmol/l (+)-α-tocopherol acetate were prepared in acetonitrile and stored at 4ºC. To prevent possible errors due to oxidation of the (+)-α-tocopherol acetate in acetonitrile, the (+)-α-tocopherol acetate IS stock solution was discarded and a new solution made every assay day. These stock solutions were used in the preparation of the working external standards.

3.4.4.4.3 Preparation of the working external standards for standard curves

Eight working standards and a blank were prepared by serial dilution in acetonitrile, of the stock 6.25μmol/l squalene standard, providing a squalene concentration range from 48.8nmol/l – 6.25μmol/l. This was followed by the addition of 25μl IS, 10μmol/l (+)-α-tocopherol acetate in acetonitrile, to account for possible losses during the extraction. The working standards were prepared and analysed by HPLC at the start and at the end of each HPLC sample run, to control for any drift during the HPLC runs.
3.4.4.4 Preparation of quality control serum samples

For the preparation of a control pooled sample, some of the rabbit serum samples collected from all the rabbits at week 6 were combined to provide enough serum to last for all assays. The quality control pooled serum samples were aliquoted (200μl) into cryo-vials and stored at -84°C until used.

3.4.4.5 Squalene absorption maxima wavelength

The absorption maxima wavelength for squalene was determined using the Varian Carry UV-Vis spectrophotometer (Varian Australia, Clayton, VIC) equipped with multi-cell module and temperature control system set at 23°C connected to a computer (Dell Pentium IV) assigned as a remote controlling system. Data was collected using the Carry Win UV software (version 3) set in “Scan mode” for wavelengths range between 100-600nm. Duplicates of a 0.5µmol/l squalene standard prepared in acetonitrile were placed into quartz cuvettes (Sigma Aldrich, Castle Hill, NSW, Australia) and the final wavelength for the maximum absorption was determined as the mean maximum wavelength for the duplicates.

3.4.4.6 Equipment and chromatographic conditions

The HPLC analysis was performed using a Shimadzu VP series system (Shimadzu, Kyoto, Japan) equipped with one liquid chromatogram pump (LC10-ATvp) connected to a degassing unit (DGU-14A) and the samples were introduced with an auto-sampler (SCL-10Avp) fitted with a 20μl loop. Separation was performed using an analytical Luna 5μ C18(2); 150x2mm 5μm column, protected by an analytical-size guard column (Phenomenex, Pennant Hills, NSW) stored at 23°C in a column oven (CTO-10Avp). The ultraviolet intensities were measured at 195nm using a UV detector.
The detector signal was recorded and peak areas were quantified using the Class-VP 6.14 software installed on the computer assigned as a remote control operating system. The communication between the HPLC hardware and the computer system was enabled via a control unit (SPD10Vvp), which allowed for full automation of the system.

The mobile phase consisted of 100% acetonitrile filtered through a Millipore 0.45μm cellulose filter (Millipore Australia, North Ryde, NSW) and degassed just prior to use by sonication (Soniclean-160T, Thebarton, SA, Australia). The isocratic flow rate for the mobile phase was 1.3ml/min and each run was for 20min in duration.

3.4.4.4.7 Serum squalene extraction

After thawing and centrifugation to clear any fibrin, 200μl serum samples were transferred into Kimble® glass sample vials (Grace-Alltech, Baulkham Hills, NSW, Australia) containing evaporated residue (25μl) of the IS, 10μmol/l (+)-α-tocopherol acetate in acetonitrile, to give a final concentration of 5μmol/l (+)-α-tocopherol acetate in all samples and external standards. Squalene and IS were carefully extracted into glass evaporation vials using 800μl of isopropanol and vortex-mixing for 10min (SMI Multi-tube Vortexer, Miami, FL, USA). To separate the organic layer, the tubes were centrifuged at 2000 × g (Clements 2000, Sydney, NSW, Australia). The bottom aqueous layer was subjected to isopropanol extraction twice more and all isopropanol extracts were combined and evaporated to dryness under a stream of nitrogen on a dry block heater set to 60ºC (Ratek, Vic, Australia). The residue in the evaporation vials was resuspended in 50μl acetonitrile and transferred into HPLC cups with appropriate volume inserts and 20μl of each sample sequentially injected onto the HPLC column.
3.4.4.8 Identification of squalene on the HPLC chromatogram

Squalene and the IS (+)-α-tocopherol acetate elution times were determined on the chromatogram after the preparation and injection of their respective pure standards (Figure 3.3). The identification of squalene and the IS in serum samples was done by comparing serum samples spiked with a known amount of squalene and IS to those that were not spiked.

![Typical HPLC-UV chromatogram (195nm) of pure standards of (1) IS (+)-α-tocopherol acetate (3.975min) and (2) squalene (6.108min) prepared in acetonitrile. Chromatographic conditions were as described in Section 3.4.4.4.6.](image)

**Figure 3.3**

3.4.4.9 The standard curve and calculation of squalene concentrations

To prepare a standard curve, 200μl of the squalene working standards and 25μl of the IS were evaporated to dryness at 60°C under a stream of nitrogen. The residue was dissolved in 50μl of acetonitrile and injected onto the HPLC column. The standard curve was developed by plotting the peak area ratio (peak area of squalene over the peak area of the IS) against the squalene concentration (Figure 3.4). The concentration of squalene in the rabbit serum samples was calculated by dividing the peak area ratio...
(squalene over IS) of the rabbit serum with the slope of the standard curve. All rabbit serum samples were done in duplicates and the final serum squalene concentration was the average concentration of the respective serum duplicates.

Figure 3.4 Calibration curve for squalene spiked in serum samples. Results were derived from linear regression analysis of the Squalene/IS chromatographic peak area ratio plotted versus the concentration of squalene. Peak areas for IS and squalene were determined by HPLC-UV as described in Section 3.4.4.4.6.

3.4.4.4.10 Recovery of squalene from rabbit serum

In order to determine the recovery of squalene from the serum samples, one rabbit serum sample was selected and analysed for its squalene. The same rabbit serum sample was then analysed after spiking with known concentrations of squalene prepared in acetonitrile at three different squalene concentration levels: Low (0.097µmol/l); Medium (1.56µmol/l) and High (6.25µmol/l) in ten replicates for each spiking concentration. To determine the recovery of squalene, the average value of squalene in the rabbit serum sample and the known spiked concentration were added to represent the total squalene. The extracted squalene concentration was then related to the total squalene and represented as a percentage of the total (Table 3.3). An intra-assay
coefficient of variation (CV) for each batch of 10 samples was also calculated (Table 3.3).

All the rabbit serum samples were analysed in a single HPLC run. A pooled rabbit serum sample (Section 3.4.4.4.4) was used as a control in duplicate at the start, middle point and at the end of the sample injection automation sequence. The coefficient of variation for serum squalene in this run was 2.05% (Mean±SD 2.44±0.05).

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Spiked concentration (μmol/l)</th>
<th>Total Squalene (μmol/l)</th>
<th>Extracted Squalene (μmol/l)</th>
<th>CV (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>10</td>
<td>0</td>
<td>2.37</td>
<td>2.37±0.20</td>
<td>8.6</td>
<td>100</td>
</tr>
<tr>
<td>Low</td>
<td>10</td>
<td>0.097</td>
<td>2.47</td>
<td>2.41±0.19</td>
<td>7.9</td>
<td>97.57</td>
</tr>
<tr>
<td>Medium</td>
<td>10</td>
<td>1.56</td>
<td>3.93</td>
<td>3.66±0.19</td>
<td>5.1</td>
<td>93.13</td>
</tr>
<tr>
<td>High</td>
<td>10</td>
<td>6.25</td>
<td>8.62</td>
<td>8.58±0.51</td>
<td>5.9</td>
<td>99.54</td>
</tr>
</tbody>
</table>

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

Note: Values are expressed as Mean±SD, n – Number of replicates, CV – Coefficient of variation calculated as (SD/ Mean) x 100, Recovery – Recovery percentage of squalene calculated as (Extracted squalene/Total squalene) x 100.

3.4.4.5 Serum thiols by high pressure liquid chromatography

All measurements of serum thiols were done at the nutrition laboratories at the University of Newcastle in Ourimbah using HPLC with fluorescence detection. The method used for determination of serum thiols uses the fluorogenic ammonium salt of 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) to react with the free sulfhydryl groups of thiols, thus making them able to be seen by florescence detection [454, 455].

The original method used tri-n-butylphosphine in N,N – dimethylformamide as a reducing agent [454, 455]. However, this chemical is no longer easily available due to
its very high flammability, so in this experiment, the much less dangerous reducing agent tris (2-carboxyethyl) phosphine (TCEP) was used for the reduction of disulfides and protein-bound thiol groups [456, 457].

A standard curve made up in a pooled rabbit serum sample and two quality control samples of human serum, one having a known low (CL) and the other a known high (CH) homocysteine concentration, were used to standardise every assay run.

### 3.4.4.5.1 Preparation of stock standard solutions

Stock standard solutions of 10mmol/l cysteine (Cys), 10mmol/l cysteinyl-glycine (Cys-Gly), 1mmol/l glutathione (Gsh) and 5mmol/l homocysteine (Hcy) were prepared in DI water with resistivity greater than 18MΩ cm (Millipore Australia, North Ryde, NSW). Furthermore, a stock standard solution of 10mmol/l N-(mercaptopropionyl)-glycine was also prepared in DI water and used as an internal standard. These solutions were aliquoted into cryogenic vials (0.5ml) and stored at -84ºC until used for making the working standards.

### 3.4.4.5.2 Preparation of the working standards for standard curves

Five working standard solutions and a blank (Working Standard Solutions 0 – 5, Table 3.4) were prepared by combining the thiol stock standard solutions (10mmol/l Cys, 10mmol/l Cys-Gly, 1mmol/l Gsh and 5mmol/l Hcy) and the stock solution of the internal standard (10mmol/l) and then further diluting in 100mmol/l potassium tetraborate/2mmol/l disodium EDTA buffer (pH9.5) to give the desired concentrations of each thiol and 0.1mmol/l of IS (Table 3.4). The working standard solutions (0 – 5) were stored in the fridge at 4ºC and used within 10 days of preparation.
Table 3.4 Thiol concentrations of the Working Standard Solutions

<table>
<thead>
<tr>
<th></th>
<th>Working Standard Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Cys (µmol/l)</td>
<td>0</td>
</tr>
<tr>
<td>Hcy (µmol/l)</td>
<td>0</td>
</tr>
<tr>
<td>Cys-Gly (µmol/l)</td>
<td>0</td>
</tr>
<tr>
<td>Gsh (µmol/l)</td>
<td>0</td>
</tr>
<tr>
<td>IS (mmol/l)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

3.4.4.5.3 Preparation of quality control and pooled plasma samples

Blood was collected from healthy human volunteers into 5 ml Vacutainer lithium heparin tubes containing a gel cell separator and, within 30min of blood collection, the tubes were centrifuged at 3000 × g for 10min (Sigma 4K 15 centrifuge). The plasma samples were stored on ice until analysed for plasma thiols.

After analysis of each individual’s plasma thiol concentration, the plasma samples with the lowest and highest Hcy concentrations were assigned as “Control Low” (CL) and “Control High” (CH) quality control samples, respectively.

For the preparation of a ‘pooled serum sample’, some serum from all rabbits taken at week 6 were combined into a glass tube and vortex-mixed for 5min, to provide enough serum for the completion of all thiol assays. The quality control and pooled plasma samples were aliquoted (200µl) into cryogenic vials and stored at -84°C until used.
3.4.4.5.4 Equipment and chromatographic conditions

The HPLC analysis was performed using a Varian ProStar System (Varian Australia, Clayton, VIC) with two pumps (ProStar 210) and samples were introduced with a Varian ProStar 430 autosampler fitted with a 100μl sample loop and a tray temperature controller set at 8°C. Separation of the thiols was performed using an analytical Sphereclone 5μ ODS (2) 250x4.60mm 5 micron column, protected by an analytical-size guard column (Phenomenex, Pennants Hills, NSW) and stored at 25°C in a Mistral column oven. The fluorescence intensities were measured with the excitation wavelength set at 385nm and the emission detection wavelength set at 515nm using the Varian ProStar 363 Fluorescence detector. The detector signal was recorded and the peak area was quantified using Star Chromatography Workstation 6.0 installed on a Dell Pentium IV computer assigned as a remote control operating system.

The mobile phases consisted of (A) 4% methanol in 0.1mol/l sodium acetate, (pH4.0) and (B) 15% methanol in 0.1mol/l sodium acetate, (pH4). The solvents were filtered through a Millipore 0.45μm cellulose filter (Millipore Australia, North Ryde, NSW) and degassed just prior to use. At a flow rate of 1ml/min, 100% mobile phase A was run for the first 3.6min. The system was then switched to 100% mobile phase B between 3.6 and 7.2min and then switched again to 100% mobile phase A for 10min before the next injection to allow the column to re-equilibrate on solvent A.

3.4.4.5.5 Thiol derivatization

After thawing and centrifugation, to pellet any fibrin clots, 60μl of the cleared rabbit serum supernatant samples were transferred into microcentrifuge tubes. Working Standard Solution 0, the blank, (20μl) was then added to the CL and CH human plasma quality control samples and to the rabbit serum samples. For the standard curve, 20μl of
each working standard sample (0 – 5, Table 3.4) were added, each to a different 60μl sample of the pooled rabbit serum.

To reduce the thiols, 8μl of 15% (w/v) TCEP in PBS was added to all rabbit serum, quality control and standard curve samples before incubating them for 30min in a water-bath set at 24°C [456]. This was followed by addition of 50μl of 0.6mol/l perchloric acid/1mmol/l EDTA solution to all samples, vigorously vortex-mixing and then standing at room temperature for 5min, in order to decouple the thiols from the plasma proteins. The denatured proteins were then pelleted by centrifugation at 10,000 x g at 4°C for 10min(Sigma 4K 15 centrifuge). The clear supernatant was then aliquoted (40μl) to new microcentrifuge tubes and mixed with 80μl of 0.5mol/l potassium-borate/5mmol/l EDTA solution (pH10.5). Then, 40μl of the SBD-F solution (4.25mmol/l 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt in 0.1mol/l potassium tetra borate/2mmol/ldisodium EDTA, pH9.5) was added to all tubes and the samples were incubated at 55±1°C for 1h. All samples were cooled on ice, mixed by vortex and then centrifugation at 10,000 x g at 4°C for 5min. Finally, 100μl of the samples were transferred into dark HPLC cups containing the appropriate inserts and the samples were sequentially injected (20μl) onto the HPLC column by an auto-injector.

A typical HPLC run consisted of two quality control samples (CL and CH), 6 working standard curve samples (0-5) in pooled rabbit serum and 12 rabbit study samples and lasted 16h or less. The standard curve samples were analysed at the beginning and at the end of each run and the quality control samples were run at the beginning, in the middle and at the end of each run, in order to control for any drift during the runs. Each rabbit sample was analysed in duplicate.
3.4.4.5.6 Identification of individual thiols on the chromatogram

The elution time of each plasma thiol was determined using the pooled rabbit serum samples spiked with known amounts of the pure thiols and IS (N-(mercaptopyrropropionyl)-glycine), before and after spiking. A typical HPLC chromatogram identifying the individual thiols and the IS is presented in Figure 3.5.

![Figure 3.5](image)

**Figure 3.5** Typical chromatogram of the plasma thiols and IS analysed by HPLC with fluorescence detection as described in Section 3.4.4.5.5. The thiols in order of elution are cysteine (1), homocysteine (2), cysteinyl-glycine (3) and glutathione (4) followed by N-(mercaptopyrropropionyl)-glycine acting as IS (5).

3.4.4.5.7 The standard curve and calculation of thiol concentrations

A standard curve was determined for each HPLC analysis run by plotting the peak area ratios (peak area of each thiol divided by the peak area of the IS) against the known concentration of the thiols in each working standard solution (Table 3.4). These were injected at the start and at the end of each run and the mean slope values were calculated.

The concentration of the thiols in each duplicate of the rabbit serum samples was calculated by dividing the serum samples’ thiol/IS peak area ratio by the mean of the
two standard curve slope values for each of the measured thiols in each HPLC run. The final concentration of the thiols in the rabbit serum samples were derived as the mean value for the sample duplicates.

The intra- and inter-assay coefficients of variation for the individual plasma thiols were also determined using the CL and CH quality control plasma samples (Table 3.5).

3.4.5 Western Blotting of the hepatic LDL-receptor

3.4.5.1 Preparation of soluble liver membrane proteins

Soluble rabbit liver membranes were prepared as described previously [242, 466]. Liver samples (about 5g) were thawed out overnight in a fridge at 4°C and homogenised in 20ml of homogenisation buffer (10mmol/l Tris-HCl, 0.154mol/l NaCl, 2mmol/l CaCl, 5mmol/l PMSF and 5mmol/l N-ethylmaleimide, pH7.5) using a Polytron PT10-35 homogenizer (Capitol Scientific Inc, Austin, Texas, USA) in four 10s pulses. The homogenate was then centrifuged (Beckman J2-MC centrifuge, Palo-Alto, California, USA) for 5min at 500 × g using a JA20 centrifuge rotor (Beckman, Palo-Alto, California, USA) and temperature set at 4°C, to remove nuclei and unbroken cell material. The supernatant was collected and centrifuged at 10,000 × g for 15min in a JA20 rotor (Beckman, Palo – Alto California, USA) to pellet the mitochondrial liver fraction. The supernatant was then ultra-centrifuged at 10^6 × g for 1h using an Optima L-100XP ultracentrifuge (Beckman Coulter, Paolo-Alto, California, USA) set at 4°C, to pellet the liver microsomal membranes.

The membrane pellets were then gently resuspended using a pasteur pipette in 2.5ml solubilisation buffer (250mmol/l Tris-maelate; 2mmol/l CaCl_2; 5mmol/l PMSF; 5mmol/l N-ethylmaleimide; pH6.0) and kept on ice. The membranes were then sonicated
Table 3.5 Mean thiol values for the low (CL) and high (CH) quality controls and intra assay coefficient of variations

<table>
<thead>
<tr>
<th>Control samples</th>
<th>n</th>
<th>Mean±SD (μmol/l)</th>
<th>CV (%)</th>
<th>n</th>
<th>Mean±SD (μmol/l)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys (CL)</td>
<td>2</td>
<td>164.40±3.78</td>
<td>2.29</td>
<td>10</td>
<td>180.31±10.13</td>
<td>5.61</td>
</tr>
<tr>
<td>Cys (CH)</td>
<td>2</td>
<td>191.00±6.91</td>
<td>3.61</td>
<td>10</td>
<td>219.34±13.96</td>
<td>6.36</td>
</tr>
<tr>
<td>Hcy (CL)</td>
<td>2</td>
<td>4.21±0.01</td>
<td>0.31</td>
<td>10</td>
<td>4.63±0.23</td>
<td>4.97</td>
</tr>
<tr>
<td>Hcy (CH)</td>
<td>2</td>
<td>11.07±0.61</td>
<td>5.49</td>
<td>10</td>
<td>12.22±0.74</td>
<td>6.01</td>
</tr>
<tr>
<td>Cys-Gly (CL)</td>
<td>2</td>
<td>21.66±0.27</td>
<td>1.28</td>
<td>10</td>
<td>24.32±1.43</td>
<td>5.88</td>
</tr>
<tr>
<td>Cys-Gly (CH)</td>
<td>2</td>
<td>19.89±1.12</td>
<td>5.61</td>
<td>10</td>
<td>22.28±1.31</td>
<td>5.86</td>
</tr>
<tr>
<td>Gsh (CL)</td>
<td>2</td>
<td>7.87±0.08</td>
<td>0.99</td>
<td>10</td>
<td>8.28±0.49</td>
<td>5.92</td>
</tr>
<tr>
<td>Gsh (CH)</td>
<td>2</td>
<td>5.40±0.25</td>
<td>4.66</td>
<td>10</td>
<td>5.60±0.29</td>
<td>5.18</td>
</tr>
</tbody>
</table>

Note: Values are expressed as Mean±SD, n – Number of replicates, CV – Coefficient of variation calculated as (SD/Mean) x 100
(MSE SoniPrep 150; Sanyo Co. Australia) using 3x20s pulses at amplitude 10.
Following the sonication, 2.5ml of 2% (w/v) Triton X-100 solubilisation solution
containing 2mmol/l of CaCl₂ was added to the membranes and agitated on a rotating
wheel (Rotator AG, Finepcr, Sydney, NSW, Australia) in a refrigerator at 4°C for
45min. The Triton X-100 was removed by adding thoroughly washed Amberlite®
XAD-4 (0.5g/ml) and agitating for a further 2 h on a rotating wheel in a refrigerator at
4°C. The Amberlite® XAD-4 was separated by allowing the solution to settle for 45min
at 4°C and the supernatant was collected and centrifuged (Beckman J2-MC centrifuge,
Palo-Alto, California, USA) at 10,000 × g for 15min at 4°C. The solubilised membranes
were then aliquoted and stored in a freezer at -18°C until analysis.

3.4.5.2 Electrophoresis and electro-transfer of LDL-receptor

The protein content of the solubilised liver membranes was performed using the
Folin-phenol reagent [483]. The same amount of protein (100µg) of each of the
solubilised liver membrane preparations were subjected to electrophoretic separation
(Bio-Rad laboratories, Gladesville, NSW, Australia) on 3-15% SDS polyacrylamide
gels [242, 466] along with RAINBOW™ molecular weight markers (GE Healthcare,
Rydalmere, NSW, Australia). Preparation of the molecular weight markers was done by
adding the 12µl of the markers, 4.5µl of 10% (w/v) SDS, 9µl of 0.5mol/l Tris-HCl
(pH6.8), 2.7µl glycerol and 1.6µl of β-mercaptoethanol and heating them at 95°C for
5min.

All samples and markers were loaded into the wells and initially run at 32mA
for 1.5h until reaching the running buffer. The current was then increased to 60mA for a
further 4.5h until the dye front eluted off the gel into the running buffer (3g/l Tris base
(w/v); 14.4g/l glycine; 1g/l SDS; pH8.3). After the complete elution of the dye front, the
gels were removed from the plates and incubated in transfer buffer (25mmol/l Tris base; 192mmol/l glycine and 20% (v/v) methanol) for 45min. Following the incubation, the separated proteins were electro-transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a transblot apparatus (Bio-Rad laboratories, Reagents Park, NSW, Australia) for 16 h with the power-supply set to a constant 45V. The transferred LDL-receptor was then detected by immunoblotting.

3.4.5.3 Immunoblotting of the LDL-receptor in liver membranes

The nitrocellulose membranes containing the transferred proteins were blocked by incubation in 10% (w/v) skim milk powder (Diploma, Fronterra Pty. Ltd, Mount Waverly, VIC, Australia) in buffer (10mmol/l Tris-HCl, 154mmol/l NaCl, 2mmol/l CaCl$_2$, pH7.4) for 1h at room temperature on a shaking water-bath (Ratek Instruments, Boronia, Australia) adapted to serve as platform rocker. The nitrocellulose membranes were then washed 4 times (10min each) with 1% (w/v) skim milk powder in buffer (5mmol/l Tris-HCl, 127 mmol/l NaCl, 1mmol/l CaCl$_2$, pH 7.4). This was followed by incubation of the membranes for 1 hour with a IgG polyclonal antibody, directed against the LDL-receptor, diluted 1:2000 in 1% (w/v) skim milk powder in buffer (10mmol/l Tris-HCl, 154mmol/l NaCl, 2mmol/l CaCl$_2$, pH 7.4) [484]. The nitrocellulose membranes were then washed 4 times (10min each) using 1% (w/v) of the skim milk powder in buffer (5mmol/l Tris-HCl, 127mmol/l NaCl, 1mmol/l CaCl$_2$, pH 7.4). The nitrocellulose membranes were then incubated for an additional 1h with an anti-IgG antibody conjugated to horseradish peroxidase (GE Healthcare, Rydalmere, NSW, Australia) diluted 1:5000 with 1% (w/v) skim milk powder in buffer (10mmol/l Tris-HCl, 154mmol/l NaCl, 2mmol/l CaCl$_2$, pH7.4). After this last incubation, the nitrocellulose membranes were gently washed four times
(10min each) in final wash buffer (10mmol/l Tris-HCl, 154mmol/l NaCl, 2mmol/l CaCl₂, pH7.4) and blotted dry between two layers of blotting paper.

The LDL-receptor was then detected on X-ray film (Hyperfilm-ECL, GE Healthcare, Rydalmere, NSW, Australia) using an enhanced chemiluminescence kit for horseradish peroxidise (GE Healthcare, Rydalmere, NSW, Australia). Scanning of the LDL-receptor protein bands on the X-ray film was then performed using the ImageQuant 300 (GE Healthcare, Rydalmere, NSW, Australia) and the results were quantified using the ImageQuantTL software (Amersham Bioscience, NSW, Australia). The results were expressed as the average volume area for the LDL-receptor bands determined from the densitometer scans.

3.4.5.4 Liver lipid determinations

Total cholesterol, unesterified cholesterol and triglycerides were measured on the liver homogenates and the solubilised liver membrane preparations. Both preparations (homogenate and membranes) were initially sonicated (MSE SoniPrep 150; Sanyo Co. Australia), then diluted 1:1 (v/v) with a 2% (v/v) Triton X-100 in 2mmol/l of CaCl₂ solution. The diluted preparations were agitated for 45min on a rotating wheel (Rotator AG, Finepcr, Sydney, NSW, Australia) in the refrigerator at 4°C and the protein content was then determined [483]. The lipid measurements were performed on a CobasBio® centrifugal autoanalyser (Roche Diagnostics, Dee Why, NSW, Australia) using commercially available enzymatic kits (Australian Scientific, Asquith, NSW, Australia) [442, 443] and the results were expressed relative to the protein concentration of each sample.
3.4.5.5 Analysis of CD36 in liver homogenates and solubilised liver membranes

The electrophoresis and immunoblotting for CD36 detection and measurement were performed at the School of Biomedical Sciences and Pharmacy at the University of Newcastle at Callaghan by immunoblotting of liver homogenates and solubilised liver membranes, essentially as described for the LDL-receptor above but using a monoclonal antibody against the CD36 protein [467]. The results were expressed as the average volume area for the CD36 bands determined from the densitometer scans.

3.4.6 Statistical Analyses

The data recording and analysis for the various plasma and liver measurements was performed using Microsoft Excel® 2003 (Microsoft Australia, North Ryde, NSW, Australia). The means, standard deviations and the Student t-test for comparing pre and post treatment values (paired t-tests) and for comparing differences (two sample assuming equal variances t-tests) between the groups (control and treatment) were also performed using this package. The correlation analyses using linear regression were performed using the Statistical Package for Social Sciences (PASW Statistics 17). Statistical significance was taken to be at the p≤0.05 level.
3.5. Results

3.5.1 Daily food consumption

The daily food intake for both treatments over the 6 weeks of the study was 188.6±22.3g/day per rabbit. For the first two weeks, the rabbits in the 2%EGCG group consumed 191.5±21.8g/day while rabbits in control group consumed 176.5±24.3g/day. For the following four week treatment period, the rabbits in the 2%EGCG group consumed 189.8±27.9g/day while the rabbits in the control group consumed 196.6±13.1g/day.

After the use of the Students’ t-test to compare the means between the two groups, there was no significant difference in the average daily food intake adjusted for the rabbits’ body weight between the control and the 2%EGCG group at the start (p=0.540) or at the end (p=0.489) of the treatment period. There was no significant change in the food intake in both groups, -2.36% for the control group (p=0.649) and -12.32% for the 2%EGCG group (p=0.113), during the treatment period. Furthermore, there was no significant difference in the change in the food intake between the two groups (p=0.304) at the end of the treatment period.

3.5.2 Serum lipids

The serum cholesterol levels after the initial 2 weeks of cholesterol feeding to render the rabbits hypercholesterolaemic, were matched between the control (8.6±5.7mmol/l) and 2%EGCG (8.6±5.7mmol/l) groups. Similarly, there were no significant differences between the control and 2%EGCG groups for HDL, LDL, VLDL, non-HDL cholesterol and triglycerides (Table 3.6).

In the treatment group, after the administration of 2% (w/w) pure EGCG along with 0.25% (w/w) cholesterol for 4 weeks, there was a significant decrease in the total
serum cholesterol (-85%; p<0.05), HDL cholesterol (-56%; p<0.01), LDL cholesterol (-92%; p<0.05) and non-HDL cholesterol levels (-91%; p<0.05) as highlighted in bold in Table 3.6. However, the concentrations of VLDL cholesterol and triglycerides remained unchanged in the 2%EGCG group during the intervention with EGCG.

In the control group, only the HDL cholesterol was significantly decreased (-40%; p<0.05) after the 4 weeks treatment phase of the study (Table 3.6).

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

<table>
<thead>
<tr>
<th>Serum Lipid</th>
<th>Control</th>
<th>2%EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>8.55±5.74</td>
<td>7.86±9.91</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/l)</td>
<td>1.16±0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70±0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/l)</td>
<td>7.23±5.33</td>
<td>7.04±9.67</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.82±0.60</td>
<td>0.61±0.43</td>
</tr>
<tr>
<td>VLDL Cholesterol (mmol/l)</td>
<td>0.16±0.12</td>
<td>0.12±0.09</td>
</tr>
<tr>
<td>Non-HDL Cholesterol (mmol/l)</td>
<td>7.39±5.39</td>
<td>7.16±9.73</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SD. Values, within a treatment group and for the same sterol, having the same letter superscript on the right hand side for week 2 and week 6 are significantly different for the two time points (p<0.05).

During the 4-week treatment period, there was also a significantly greater decrease in the total serum cholesterol (p<0.05) and LDL cholesterol (p=0.05) concentrations in the 2%EGCG group than in the control group (Figure 3.6). However, there were no significant changes in HDL cholesterol and triglycerides.
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. The (*) denotes a statistically significant (p≤0.05) greater reduction from the start to the end of the 4-week treatment period in the treatment group compared to the control group.

3.5.3 Liver lipids

The administration of pure EGCG resulted in a significantly lower content of total and esterified cholesterol in the liver homogenates (-44% and -63% respectively) of the 2%EGCG group compared to the control group (p<0.05). The unesterified cholesterol fraction accounted for about 36% of the total cholesterol in the control samples but at 60.1%, it accounted for a significantly higher percentage of the total cholesterol in the 2%EGCG group (p<0.05).

In the liver membrane fraction preparations, the administration of pure EGCG resulted in a significantly lower content of esterified cholesterol (-82%) in the 2%EGCG group compared to the control group (p<0.05). The esterified cholesterol consisted of about 36% of the total cholesterol in the control samples but at 9.5%, it was significantly lower (p<0.001) as a percentage of the total cholesterol in the 2%EGCG samples.
There were no significant differences in triglyceride concentrations in both liver fractions (homogenate and membranes) between the control and 2%EGCG groups (Table 3.7).

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

<table>
<thead>
<tr>
<th>Liver Fraction</th>
<th>Control</th>
<th>2%EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenate</td>
<td>Membranes</td>
</tr>
<tr>
<td>Total Cholesterol (µmol/g)</td>
<td>1313±577*</td>
<td>299±158</td>
</tr>
<tr>
<td>Unesterified Cholesterol (µmol/g)</td>
<td>455±172</td>
<td>185±91</td>
</tr>
<tr>
<td>Esterified Cholesterol (µmol/g)</td>
<td>859±412*</td>
<td>114±78*</td>
</tr>
<tr>
<td>Triglycerides (µmol/g)</td>
<td>2042±879</td>
<td>364±446</td>
</tr>
</tbody>
</table>

*Note:* Values are adjusted for grams of protein (g) content and expressed as Mean±SD. Values with the symbol (*) are significantly different between the two treatment groups (p<0.05).

### 3.5.4 Serum lathosterol, squalene and indexes of cholesterol synthesis

At the start of the treatment period (week 2) there was no significant difference in serum lathosterol between the control and 2%EGCG groups (p=0.373) (Table 3.8). During the treatment period, both groups had significant decreases in their lathosterol concentration (p<0.05). However, the 2%EGCG group had a significantly lower lathosterol concentration at week 6 than the control group (p<0.05).

The serum squalene concentration (Table 3.8) was not significantly different between the 2%EGCG and control groups at the start (p=0.437) or at the end of the 4-week treatment period (p=0.462). Furthermore, at the end of the treatment period (week 6) there was no significant change observed in the squalene concentration in either the control (p=0.454) or the 2%EGCG group (p=0.423).
Table 3.8 Serum lathosterol and squalene concentrations at the start and at the end of the treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2%EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
</tr>
<tr>
<td>Serum Lathosterol (µmol/l)</td>
<td>3.14±1.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>*0.93±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Squalene (µmol/l)</td>
<td>1.17±0.70</td>
<td>1.50±0.76</td>
</tr>
</tbody>
</table>

*Note:* Values are expressed as Mean±SD. Values, within a treatment group and for the same sterol, having the same letter superscript on the right hand side for week 2 and week 6 are significantly different for the two time points (p<0.05). Values with the symbol (*) on the left hand side are significantly different between the two treatment groups (p<0.05).

The lathosterol over cholesterol ratio (Figure 3.7), an index of cholesterol synthesis, was significantly lower in both groups (control and 2%EGCG) at the end of the treatment period (p<0.05). However, there were no significant differences between the two groups at the start of week 2 (p=0.218) or at the end of the treatment period at week 6 (p=0.848).

The lathosterol to squalene ratio (Figure 3.8), as a newly proposed index of cholesterol synthesis, was significantly lower (p<0.05) in both groups at the end (week 6) than at the start of the treatment period (week 2). At the start of the study (Week 2), there was no significant difference in lathosterol over squalene ratio between the control and 2%EGCG groups (p=0.068). However, at the end of the treatment period (Week 6), the 2%EGCG group had a significantly lower lathosterol to squalene ratio than the control group (p<0.05).
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. The values are Mean±SD and those with the same colour having the same letter superscript are significantly different (p<0.05).

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. The values are Mean±SD and those with the same colour having the same letter superscript are significantly different (p<0.05). Values with the symbol (*) are significantly different between the two treatment groups at week 6 (p<0.05).
3.5.5 Serum phytosterols and capacity to absorb dietary cholesterol

At the start of the treatment period (Week 2), there was no significant difference between the control and 2%EGCG group for β-sitosterol (p=0.572) and campesterol (p=0.835). Similarly, at the end of the treatment period (week 6) there was no significant difference between the two groups for β-sitosterol (p=0.331) and campesterol (p=0.174). However, at the end of the treatment period, serum β-sitosterol was significantly lower in both the control (p<0.05) and 2%EGCG (p<0.001) groups, than at the start of treatment. Furthermore, the serum campesterol was also significantly lowered in both the control (p<0.001) and 2%EGCG groups (p<0.05) (Table 3.9). However, there were no significant differences between the two groups at week 2 or week 6 for either β-sitosterol of campesterol.

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2%EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
</tr>
<tr>
<td>Serum β-sitosterol</td>
<td>34.45±21.08a</td>
<td>6.74±4.92a</td>
</tr>
<tr>
<td>(µmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Campesterol</td>
<td>409.02±282.91c</td>
<td>228.16±296.30c</td>
</tr>
<tr>
<td>(µmol/l)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Values are expressed as Mean±SD. Values, within a treatment groups and for the same sterol, having the same letter superscript for week 2 and week 6 are significantly different for the two time points (p<0.05).
The intrinsic capacity to absorb dietary cholesterol (Figure 3.9), measured as the sum of campesterol and \( \beta \)-sitosterol adjusted for the cholesterol concentration, was significantly lower in the control group (\( p<0.05 \)) but not in the 2\%EGCG group at the end of the treatment period. Furthermore, the control group also exhibited a significantly lower phytosterol to cholesterol ratio than the 2\%EGCG group at the end of week 6 (\( p<0.05 \)).

### 3.5.6 The hepatic LDL-receptor protein

The hepatic LDL-receptor protein was found to be significantly higher (\( p<0.001 \)) in the 2\%EGCG group (+59\%) than in the control group (Figure 3.10).
3.5.7 The hepatic CD36 protein

The hepatic CD36 protein was also found to be significantly higher (p<0.05) in the 2%EGCG group (+62%) than in the control group (Figure 3.11).

3.5.8 Other serum metabolites (thiols and glucose)

At the end of the 4-week treatment period, the control group had significantly increased serum concentrations of Cys (+26%), Hcy (+52%) and Cys-Gly (+35%) compared to week 2 (p<0.05).

![Figure 3.10](image.png)

**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. The symbol (*) denotes that the values are significantly different between the two treatment groups at week 6 (p<0.05).
Figure 3.11 The effect of EGCG on the hepatic CD36 protein in control and 2%EGCG group at the end of the treatment. The symbol (*) denotes that the values are significantly different between the two treatment groups at week 6 (p<0.05).

However, in the 2%EGCG group there were only significant increases in the serum concentrations of Cys-Gly (+19%) and Gsh (+13%) from week 2 to week 6 (p<0.05). Furthermore, there was no significant difference in serum metabolites between the two groups at the start and at the end of the 4-week treatment period (Table 3.10).

At the start of the treatment period, there were no significant differences in serum glucose between the control and 2%EGCG groups and this was also seen at the end of the 4-week treatment period (Table 3.10). However, the serum glucose concentrations were significantly decreased in both the control (28%) and the 2%EGCG (25%) groups at the end of the treatment period (p<0.05).
Table 3.10 Cysteine, homocysteine, cysteinyl-glycine, glutathione and glucose concentrations at start and at the end of the dietary treatments

<table>
<thead>
<tr>
<th>Serum metabolites</th>
<th>Control</th>
<th>2%EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
</tr>
<tr>
<td>Cys (mmol/l)</td>
<td>158.8±15.4a</td>
<td>197.7±23.2a</td>
</tr>
<tr>
<td>Hcy (mmol/l)</td>
<td>9.2±2.2b</td>
<td>13.4±2.5b</td>
</tr>
<tr>
<td>Cys-Gly (mmol/l)</td>
<td>5.8±0.5c</td>
<td>7.8±1.7c</td>
</tr>
<tr>
<td>Gsh (mmol/l)</td>
<td>83.2±12.5</td>
<td>90.8±16.1</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.1±0.8f</td>
<td>5.8±0.8f</td>
</tr>
</tbody>
</table>

Note: Values are expressed as Mean±SD. Values for the same metabolite within a treatment group having the same letter superscript for week 2 and week 6 are significantly different for the two time points (p<0.05).

3.5.9 Correlations

Table 3.11 shows that serum total cholesterol was highly correlated to serum squalene (r=0.829, p<0.001), serum lathosterol (r=0.855, p<0.001), serum campesterol (r=0.997, p<0.001) and serum triglycerides (r=0.664, p=0.019). The index of cholesterol synthesis (lathosterol/squalene) was positively correlated with serum lathosterol (r=0.856, p<0.001), serum β-sitosterol (r=0.796, p=0.002), total cholesterol in liver membranes (r=0.589, p=0.044) and triglycerides in liver membranes (r=0.824, p=0.001). The abdominal absorption capacity (campesterol+β-sitosterol/cholesterol) was negatively correlated with esterified cholesterol in liver homogenates (r=-0.585, p=0.046) (Table 3.11).

The CD36 protein in liver membranes was positively correlated with the hepatic LDL-receptor (r=0.689, p=0.013) and negatively correlated with serum lathosterol (r=-0.598, p=0.019) and esterified cholesterol in liver membranes (r=-0.661, p=0.019). However, when partial correlations were performed controlling for the hepatic LDL
receptor, CD36 was not significantly related to serum lathosterol \((r= -0.383, p=0.245)\) or esterified cholesterol in liver membranes \((r= -0.526, p=0.097)\).

Although close to significance, serum total cholesterol was not correlated to the lathosterol to cholesterol ratio \((r= -0.506, p=0.093)\) and the lathosterol to squalene ratio \((r=0.510, p=0.090)\). Similarly, serum total cholesterol was not significantly correlated to cholesterol found in liver homogenates \((r=0.558, p=0.060)\) and esterified cholesterol in liver homogenates \((r=0.556, p=0.055)\).
Table 3.11 Correlations between the measured parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Total Cholesterol Vs Serum Triglycerides</td>
<td>0.664</td>
<td>0.019*</td>
</tr>
<tr>
<td>Serum Total Cholesterol Vs Serum Lathosterol</td>
<td>0.855</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Serum Total Cholesterol Vs Serum Campesterol</td>
<td>0.997</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Serum Total Cholesterol Vs Serum Squalene</td>
<td>0.829</td>
<td>0.001**</td>
</tr>
<tr>
<td>Serum Total Cholesterol Vs Lathosterol/Cholesterol ratio</td>
<td>-0.506</td>
<td>0.093</td>
</tr>
<tr>
<td>Serum Total Cholesterol Vs Lathosterol/Squalene ratio</td>
<td>0.510</td>
<td>0.090</td>
</tr>
<tr>
<td>Serum Total Cholesterol Vs Cholesterol in liver homogenates</td>
<td>0.558</td>
<td>0.060</td>
</tr>
<tr>
<td>Serum Total Cholesterol Vs Esterified cholesterol in liver homogenates</td>
<td>0.556</td>
<td>0.055</td>
</tr>
<tr>
<td>Lathosterol/Squalene ratio Vs Serum Lathosterol</td>
<td>0.856</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Lathosterol/Squalene ratio Vs Serum β-sitosterol</td>
<td>0.796</td>
<td>0.002**</td>
</tr>
<tr>
<td>Lathosterol/Squalene ratio Vs Triglycerides in liver membranes</td>
<td>0.824</td>
<td>0.001**</td>
</tr>
<tr>
<td>Lathosterol/Squalene ratio Vs Cholesterol in liver membranes</td>
<td>0.589</td>
<td>0.044*</td>
</tr>
<tr>
<td>Abdominal absorption capacity Vs Esterified cholesterol in liver homogenates</td>
<td>-0.585</td>
<td>0.046*</td>
</tr>
</tbody>
</table>

Note: Significance levels *p<0.05, **p<0.01
3.6 Discussion

The aims of this study were to determine the effect of EGCG on serum cholesterol in the rabbit model of orally induced hypercholesterolaemia and to determine the mechanisms of action of the EGCG cholesterol lowering properties. It was hypothesised that EGCG would lower serum cholesterol in this model by decreasing cholesterol synthesis and increasing the hepatic LDL-receptor activity.

In order to achieve the aims and test the hypothesis, twelve New Zealand white rabbits were rendered hypercholesterolaemic during 2 weeks of feeding with 0.25% (w/w) cholesterol incorporated into their food. The animals were then divided into two groups of six matched by their serum cholesterol levels. One group of rabbits continued on their cholesterol diet (control) while the other group (2%EGCG) continued on the cholesterol diet to which 2% (w/w) EGCG was added for four weeks.

The hypothesis that EGCG would lower cholesterol in the rabbit model was strongly supported; the serum total cholesterol was significantly lower in the 2%EGCG group at the end of the treatment (-85%, p<0.05) compared to baseline as well as LDL cholesterol (-92%; p<0.05), HDL cholesterol (-56%; p<0.01), and non-HDL cholesterol (-91%; p<0.05). In contrast, only the HDL cholesterol was significantly decreased (-40%; p<0.05) after the 4 weeks treatment period in the control group (Table 3.6). The decreases in serum total cholesterol (p<0.05) and LDL cholesterol (p=0.05) during the 4-week treatment period were also significantly greater in the treatment group compared to the control group (Figure 3.6).

These findings are consistent with previous studies investigating the effect of GT extracts in rabbits [242], rats [401, 424, 434, 468] and hamsters [469]. However, this is the first study to demonstrate the cholesterol lowering effect of pure EGCG in the cholesterol-fed hypercholesterolaemic rabbit model.
A previous study by Bursill et al.[242] found a similar outcome in this rabbit model of hypercholesterolaemia after feeding the rabbits a GT extract containing a combination of catechins, which included EGCG as its major catechin. In that study, the cholesterol lowering effect was shown to be dose-dependent with the highest percentage lowering (-60%) reached at a dose of 2% (w/w) GT extract incorporated in their food.

In the presented study, 2% (w/w) pure EGCG, incorporated in the rabbit’s food, was just as effective (if not more effective) than the catechin extract, giving a reduction in serum cholesterol of 85%. Additionally, in the presented study, serum cholesterol levels at the start of the treatment period were nearly twice higher than in the Bursill et al.[242] study, possibly making the cholesterol lowering harder to achieve. Nonetheless, at the end of the treatment period, the serum cholesterol levels were nearly twice lower in the present study than in the catechin extract study [242].

The daily food consumption per rabbit may have been a factor in the differences between the two studies; it was around 40% higher in the present study compared to the Bursill et al.[242] study. However, in the present study there was no significant difference in daily food consumption between the control and the 2%EGCG groups at the start or at the end of the treatment period. Therefore, the greater decrease in serum cholesterol with 2%EGCG compared to control group could not be ascribed to differences in food consumption between the two groups. The present findings therefore single out pure EGCG as an effective cholesterol lowering agent in its own right in the diet-induced hypercholesterolaemic rabbit and suggest it may have been an active constituent in the catechin extract used by Bursill et al.[242] in this animal model.

The HDL cholesterol levels were significantly decreased in both the control (40%; p<0.05) and 2%EGCG group (-56%; p<0.01). This finding is consistent with the previous studies in the rabbit model of dietary induced hypercholesterolaemia [485,
where decreases in HDL cholesterol were ascribed to the high dietary intake of cholesterol. In these studies, the animals were fed 1% (w/w) cholesterol in comparison to the present study where the rabbits were fed 2% (w/w) cholesterol. The higher dietary cholesterol intake in the present could also account for the reduction in HDL cholesterol as seen in both groups in the present study.

The hypothesis that EGCG would lower cholesterol in the rabbit model by upregulating the hepatic LDL-receptor was also strongly supported; the administration of pure EGCG significantly increased the hepatic LDL-receptor by 59% (Figure 3.10). It is well established that the LDL-receptor is a primary pathway for cholesterol removal from the systemic circulation, an activity regulated by intracellular cholesterol concentrations [256, 324-326, 334]. Consistent with the effect of EGCG on the LDL-receptor, the liver homogenates in the 2%EGCG group were found to have significantly lower total and esterified cholesterol compared to control (Table 3.7). This difference in liver cholesterol as well as in serum cholesterol could not be explained by differences between the two groups in their capacity to absorb dietary cholesterol; the index for the intrinsic capacity to absorb dietary cholesterol(campesterol+β-sitosterol/cholesterol) [480] was higher, not lower as would be expected, in the 2%EGCG group compared to the control group at the end of the 4-week treatment period (Figure 3.9).

The upregulation of the LDL-receptor observed in this study was also entirely consistent with the findings in the study by Bursill et al.[242] who showed an 80% increase in LDL-receptor binding activity and 70% increase in LDL-receptor protein with the catechin extract compared to control in the same rabbit model. Similarly, Bursill et al. [434] also found a 2.7-fold increase in hepatic LDL-receptor binding activity and a 3.4-fold increase in LDL-receptor protein in rats fed a catechin extract compared to control.
The increase in the hepatic LDL-receptor is also consistent with the findings in HepG2 cells in culture. These in vitro studies indicated that the LDL-receptor was upregulated when the cells were incubated with a GT extract [410] or with pure EGCG [411, 412]. Furthermore, Bursill et al.[411] demonstrated that EGCG was the only one of the four major GT catechins (Section 1.6.1, Figure 1.16) to significantly upregulate the LDL-receptor in the HepG2 in vitro system and was therefore likely to be the main active constituent in GT extracts.

Although less strongly, the hypothesis that EGCG would also lower cholesterol in the rabbit model by decreasing cholesterol synthesis was also supported; the lathosterol to squalene ratio, a proposed index of cholesterol synthesis was significantly lower in the EGCG treatment than in the control group at the end of the treatment period (Figure 3.8). This finding is consistent with the findings that cholesterol synthesis can be inhibited at the level of squalene epoxidase [239, 487] and that EGCG is a non-competitive inhibitor of this rate-limiting enzyme in the cholesterol synthesis pathway [247]. In contrast, the lathosterol to cholesterol ratio, which has previously been used as an index of cholesterol synthesis [479] was lowered in both groups at the end of the treatment but there was no difference between the two groups (Figure 3.7). This result also differed from the Bursill et al.[242] study which showed a 60% decrease in the lathosterol to cholesterol ratio.

The SE enzyme is found in abundance in liver tissue [239, 487] and, similar to HMG-CoA reductase, it is a rate limiting enzyme, and plays a pivotal role in cholesterol homeostasis [473]. The inhibition of the pig liver SE enzyme has been reported for squalene analogues which contain acetylene and allene [488] but EGCG has been shown to be an equally potent inhibitor in a non-time-dependant manner [18].
The concentration of squalene in the serum did not significantly change from baseline at the end of the treatment period in either group and there was also no difference between the control and 2%EGCG groups at weeks 2 and 6 (Table 3.8). However, serum lathosterol, which is a more immediate precursor for cholesterol than squalene and is produced further down the cholesterol biosynthethic pathway (Section 1.2.1, Figures 1.7 to 1.9), was significantly lower in the 2%EGCG group than in the control group (Table 3.8), despite decreasing in both groups during the treatment period. Therefore, because inhibition of the SE enzyme is likely to result in a lower production of lathosterol further down the cholesterol synthesis pathway, the lathosterol/squalene ratio was examined as a possible measure of the inhibition of this enzyme by EGCG. The results proved to be consistent with EGCG being a possible inhibitor of SE in vivo and provided valuable information on EGCG potentially being a target for the development of novel hypocholesterolaemic agents [18].

Relative to the secondary aims of this chapter, the hepatic CD36 protein was also found to be significantly higher in the 2%EGCG group (+62%) than in the control group (Figure 3.11). The CD36 has been referred to as a scavenger receptor protein and studies [489, 490] have shown its involvement in the endocytosis of oxidised LDL (ox-LDL). High intracellular cholesterol concentrations are known to downregulate LDL-receptor gene transcription but not scavenger receptors. Consistent with this, in a macrophage cell line, the exposure to ox-LDL resulted in a marked induction of CD36 mRNA expression which was accompanied by an increase in CD36 protein [491, 492]. Furthermore, the mechanism of induction of CD36 by ox-LDL was shown to be related to the ability of ox-LDL to activate the gene for the transcription factor, peroxysome proliferator activated receptor-γ (PPAR-γ) [493, 494].
A study by Staprans et al.[495] has reported increased fasting LDL oxysterol levels in New Zealand white rabbits fed a cholesterol diet for 12 weeks. Although they were not measured in the present study, the rabbits were fed a high cholesterol diet for a period of 6 weeks, which may have been sufficient to increased the LDL oxysterol levels. This could in turn have been expected to lead to an increase in the CD36 protein. However, several studies [387, 390] have identified that EGCG inhibits the oxidation of LDL in vitro and lower levels of ox-LDL in the 2%EGCG group compared to control would have been more likely to decrease the CD36 protein rather than the increase seen in the present study.

However, supplementation of EGCG in rats fed a 1.5% (w/w) cholesterol diet significantly upregulated the PPAR-γ gene [496], indicating that the consumption of EGCG could increase in the CD36 protein by acting directly through the transcription factor. This possibly can only be speculated on, as PPAR-γ assays were not performed in this study. Similarly, a recent study by Sakuari et al. [474] found that low doses of EGCG (10µmol/l) induced the expression of genes related to insulin sensitivity and adipocyte differentiation, including CD36 in the early stages of differentiation of the 3T3-L1 cells in vitro.

Although the exact mechanism of action responsible for the increase in CD36 protein by EGCG remains unknown, human CD36 is thought to be a high affinity receptor for the native lipoproteins LDL, VLDL and HDL and its upregulation by EGCG could also contribute to the cholesterol lowering effect of the catechin [336]. In the present study, the CD36 protein in liver membranes was positively correlated with the hepatic LDL-receptor (r=0.689, p=0.013), suggesting that EGCG concomitantly increased the LDL-receptor and CD36 and both proteins could therefore be involved in
the observed cholesterol lowering (Table 3.11). However, whether the CD36 can play a role in cholesterol lowering is still unclear.

Also relative to the secondary aims of this chapter, there were no significant differences between the two groups at week 2 or week 6 in serum thiols or glucose concentrations (Table 3.10). However, homocysteine, which has been identified as an independent risk factor for the development of atherosclerosis [185-187] was found to be significantly increased (+52%, p<0.05) in the control group at the end of the treatment period compared to baseline but the 2%EGCG group did not show any significant changes in Hcy levels (Table 3.10). The increase of Hcy in the control group is similar to the finding by Zulli et al.[476] in the cholesterol fed rat where plasma Hcy was synergistically increased by the consumption of dietary cholesterol. Furthermore, in the present study the Gsh levels were significantly increased (+13%) in the 2%EGCG group. This could have been due to the EGCG acting as a powerful antioxidant [353, 468] and reducing reactive oxygen species [497] and thus protecting and having a sparing effect on the antioxidant Gsh.

In conclusion, this study found that the administration of pure EGCG extracted from GT (Camellia sinensis (L.) O. Kuntze) reduced serum and liver cholesterol in the cholesterol fed hypercholesterolaemic rabbit. The pure EGCG was observed to increased the hepatic LDL-receptor. It also decreased cholesterol synthesis when measured as the serum lathosterol to squalene ratio consistent with EGCG inhibiting the SE enzyme. This study has also identified avenues for further research in relation to the lowering of serum cholesterol by EGCG, including more research on the effect of EGCG on the SE enzyme and its relative impact on serum cholesterol and on the CD36 protein and possible synergistic actions of CD36 with the LDL-receptor in the lowering of serum cholesterol.
Chapter 4. Intestinal effects of EGCG in the cholesterol-fed rabbit model of hypercholesterolaemia

4.1 Introduction

The maintenance of whole body cholesterol homeostasis, referred to as cholesterol balance, and by consequence blood cholesterol homeostasis, is achieved by balancing the intestinal absorption and the endogenous synthesis of cholesterol with the excretion of cholesterol, other sterols and bile acids in the faeces. Essentially, because there is a balancing act between intestinal absorption and the endogenous synthesis of cholesterol, whole body cholesterol balance is often maintained by matching cholesterol synthesis with faecal losses of sterols and bile acids. However, biliary cholesterol and bile acids are very effectively reabsorbed by the small intestine. For this reason, decreasing the absorption of dietary and biliary cholesterol or decreasing the reabsorption of bile acids, by the intestines, have been successfully targeted as mechanisms for lowering plasma cholesterol levels [249, 498].

The cholesterol lowering properties of plant sterols and stanols in animal models were demonstrated nearly 60 years ago [499]. The plant sterols and stanols, mainly β-sitosterol and β-sitostanol, are compounds that are structurally related to cholesterol and exhibit their hypocholesterolaemic effect primarily by reducing cholesterol absorption via competition with intestinal cholesterol for incorporation into mixed micelles prior to absorption by the intestinal microvilli [500, 501]. Therefore, the cholesterol lowering effect is achieved in part by increasing the absorption of the plant sterols and stanols and concomitantly decreasing the absorption of cholesterol. Furthermore, a study by Plat and Mensink [502] indicated that mixed micelles that were enriched in β-sitostanol or a combination of cholesterol and β-sitostanol were potent inducers of the ATP binding cassette transporter A1 (ABCA1) in caco-2 cells, which is known to mediate
the efflux of cholesterol from intestinal cells back into the intestinal lumen. This finding indicated that plant sterols and stanols could decrease cholesterol absorption by both interfering with the absorption of cholesterol in mixed micelles and by increasing the ABCA1-mediated efflux of cholesterol back into the intestinal lumen.

The inhibition of cholesterol absorption has also been a target for the development of pharmaceuticals that would assist in the reduction of circulating blood cholesterol. One of these pharmaceuticals, Ezetimibe, is the first marketed specific inhibitor of the intestinal cholesterol uptake transporter called the Niemann-Pick C1 Like 1 (NPC1 L1) protein [503]. Ezetimibe is a potent cholesterol uptake inhibitor and it is currently used for the treatment of hypercholesterolaemia, including familial hypercholesterolaemia [504]. It has been shown to successfully lower blood cholesterol in humans by up to 20% [505, 506] and co-administration with statins results in additional cholesterol reductions [507]. However, the exact ratio of statin to Ezetimibe is still debated. Furthermore, a randomised trial of a combination of Simvastatin and Ezetimibe called the Aortic Stenosis trial (SEAS) has put into question the role of Ezetimibe in lipid lowering therapy; the effects on atherosclerotic plaque regression were mixed and concerns were raised about an increased cancer risk [508]. However, the findings of the recent ‘Study of Heart and Renal Protection’ (SHARP) indicated that the daily intake of a combination of 20mg Simvastatin and 10mg Ezetimibe successfully reduced LDL cholesterol by 28% and decreased the incidence of major atherosclerotic events by 17% without any evidence of an increased risk of hepatitis, gallstones or cancer [509].

The β-glucans are nonstarch polysaccharides which have also received much attention for their lowering of LDL cholesterol in animal models [510] and in human trials [511]. Due to their hypocholesterolaemic properties, the US Foods and Drug
Administration allowed, in 1997, the health claim on food packaging that ‘a diet high in soluble fiber and low in saturated fat may reduce the risk of CVD’. The exact mechanism of action of how β-glucans lower cholesterol is still debated but there is evidence that the β-glucans decrease the reabsorption of bile acids from the intestines and therefore increase the synthesis of the bile acids by the liver, using cholesterol as the precursor, and thus promote the lowering of blood cholesterol [512].

The synthesis of bile acid from cholesterol is a direct route by which cholesterol can be metabolised and disposed off; it is exclusively done by the liver and involves a number of cytoplasmic and mitochondrial enzymes [261, 262] (Section 1.2.4). Therefore, for over 30 years now, interruption of bile acid reabsorption by the intestine has been a successful target mechanism of action for the pharmaceutical lowering of blood cholesterol, using bile acid sequestrants such as cholestyramine and cholestipol [513]. These agents directly bind bile acids and effectively sequester them from being reabsorbed by the intestine and as a result they are secreted in the faeces. Notably, cholestyramine was used in the Lipid Research Clinics Coronary Primary Prevention Trial [514], which was the first major study to show that lowering LDL cholesterol caused a decrease in CVD morbidity and mortality.

Therefore, the intestinal absorption of bile acids is a critical point in the maintenance of the homeostasis of bile acids and consequently cholesterol [515]. Active bile acid absorption occurs via the apical sodium-dependant bile acid transporter (ASBT) and it is a key step in the recirculation of bile acids from the intestine to the liver. Therefore, the inhibition of ASBT activity has been flagged as a new therapeutic target because this is expected to lead to a reduction in the recirculation of bile acids, a promotion of their synthesis by the liver and, as a consequence, a reduction in the circulating level of cholesterol [515].
The green tea (GT) catechins have also been shown to inhibit cholesterol, bile acids and lipid absorption in several animal studies including mice, rats and hamsters [401, 402, 432, 433, 469, 516, 517]. This conclusion was primarily based on an observed increase in the faecal excretion of cholesterol, bile acids and total lipids in mice, rats and hamsters consuming GT extracts [402, 413-416]. Furthermore, these studies also provided evidence suggesting that EGCG, the main catechin in GT, was the catechin responsible for the inhibition of intestinal absorption [402]; it was shown to be able to interfere with the micellar solubilisation of cholesterol in the small intestine and thereby reduce the efficiency with which cholesterol was absorbed from the lumen of the digestive tract into the lymphatic system[414].

A study by Ikeda et al.[408], in rats with their thoracic duct cannulated, indicated that EGCG lowered the cholesterol content of the chylomicrons coming out of the lymphatics into the systemic blood circulation at the thoracic duct. They also showed that EGCG was the most effective of the GT catechins at precipitating cholesterol from solution in an in vitro system using biliary micelles. Therefore, the hypocholesterolaemic effect of EGCG was ascribed to a reduction of cholesterol absorption from the small intestine due to a reduction in the solubility of cholesterol in the mixed micelles in a dose dependant manner[408]. The decrease in the lymphatic absorption of cholesterol was also observed in ovariectomized rats with cannulated lymph ducts when a GT catechin extract was infused directly in the duodenum [414], providing direct evidence that GT catechins had a profound inhibitory effect on intestinal cholesterol absorption.

Interestingly, in the in vitro study by Ikeda et al.[408] the bile acids in the mixed micelles appeared to remain unaffected by the catechins, even EGCG. Nonetheless, there is evidence in the animal studies [401, 402, 408, 413-416, 432, 433, 469, 516,
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517], particularly in the hamster [469] that bile acids faecal excretion is increased by GT catechins. This may be explained by the recent in vitro study by Annaba et al.[518], which indicated that EGCG significantly decreased the activity of ASBT, a key transporter protein involved in the recirculation of bile acids from the intestine to the liver.

4.1.1 Aims and hypothesis

Based on the findings of the previous studies in mice, rats and hamsters [401, 402, 408, 413-416, 432, 433, 469, 516, 517], which found that GT catechins reduced cholesterol and bile acid absorption, the question was raised whether similar effects would be seen with pure EGCG in the rabbit model of orally induced hypercholesterolaemia. To date, such studies have not been performed in the rabbit, an animal model that has a significantly longer gastrointestinal tract than mice, rats and hamsters.

In order to test this, New Zealand white rabbits were fed a diet high (0.25% w/w) in cholesterol to render them hypercholesterolaemic and then divided into two equal groups based on their plasma cholesterol levels. One group (control) remained on the same hypercholesterolaemic diet (0.25% w/w cholesterol) for 4 weeks while the other group was fed the same hypercholesterolaemic diet, with the addition of 2% (w/w) EGCG (2%EGCG) for the 4-week period.

As shown in Chapter 3, the plasma cholesterol results were significantly reduced in the treatment group (-85%) compared to the control group. To test whether the cholesterol reduction could be ascribed to an increase in the faecal excretion of cholesterol and related sterols and/or bile acids, faecal samples were collected for three days prior to the start and at the end of the 4-week treatment period and analysed for
neutral sterols (mainly cholesterol and coprostanol) and bile acids (mainly lithocholic and deoxycholic acid). Faecal pH, moisture, total fat content and conjugated and free EGCG content were also determined.

It was hypothesised that the plasma cholesterol lowering effect of pure EGCG presented in Chapter 3 was due to an increase in the faecal excretion of neutral and/or acidic sterols. Therefore, the main aim of the study was to determine the effect of the EGCG supplementation on the content of neutral and acidic sterols in the rabbit faeces. The faecal pH, moisture content and total fat content were also measured. Furthermore, the amount of EGCG excreted at the end of the treatment period was determined in the faeces of the rabbits treated with EGCG.
4.2 Methods

4.2.1 Ethics approvals

Ethics approval for use of animals in this study was granted by the Animal Care and Ethics Committee of the University of Newcastle (#938 0306), NSW, Australia.

4.2.2 Food preparation

Pelletised rabbit food containing 18\% (w/w) protein, 4\% (w/w) fat, 18\% (w/w) fibre and 11.1MJ/kg digestible energy was purchased from Speciality Feeds (Perth, WA, Australia). The preparation of the cholesterol and EGCG enriched food mixes was done in the Food and Nutrition Laboratories of the School of Environmental and Life Sciences at Ourimbah (NSW). The dry pellets were firstly grounded in a Warring food processor (John Morris Scientific Pty, Chatswood, NSW, Australia) to form a powder, to which was added 0.25\% (w/w) cholesterol (Sigma Aldrich, Sydney, NSW, Australia). To provide the food for the EGCG treatment group, 2\% (w/w) EGCG was added to some of the powder along with the 0.25\% (w/w) cholesterol. Water was then added to the powder mixes to form a paste, which was passed through a meat mince maker (Breville, Pyrmont, NSW, Australia) to re-pellet the diets. The pellets were then air dried overnight in a forced air oven drier (G.T.D. P/L, Sydney, NSW, Australia) set at 30°C.

4.2.3 Animals and the experimental design

Twelve male (12 weeks old) New Zealand white rabbits (IMVS animal facility, Adelaide, SA, Australia) were housed in individual cages at John Hunter Animal Holding Facility (Newcastle, NSW, Australia). The rabbits were monitored daily during the six weeks period of the study and their appearance and food consumption was recorded. The rabbits were housed in individual cages in order to be able to monitor the
food consumption and faeces excretion for each rabbit. This enabled the cholesterol balance (amount of cholesterol, related sterols and bile acids excreted in the faeces subtracted from cholesterol intake) and the EGCG excretion rate to be determined.

Following their acclimatisation for one week in the animal holding facility, the rabbits were fed the diet containing 0.25% (w/w) cholesterol incorporated into their normal rabbit chow for two weeks in order to induce hypercholesterolaemia. Their cholesterol levels were then determined in blood obtained from the marginal ear vein and they were separated into the two groups, control (n=6) and treatment (n=6) that were matched for cholesterol levels.

For the following 4 weeks, the control group was fed the rabbit chow containing 0.25% (w/w) cholesterol and the treatment group (2% EGCG) was fed the rabbit chow containing 2% (w/w) EGCG in addition to 0.25% (w/w) cholesterol.

During the whole 6 week period each rabbit was provided with 300g of food daily, and the amount left in the feeder the next day was measured in order to determine each rabbit’s daily food intake. The rabbits were also weighed at the end of each week to monitor their growth and on the last day of each of the two 3-day faecal sample collection periods (Section 4.2.4) at the start and the end of the 4-week treatment period. The weights were reported as mean±SD for each treatment group.

4.2.4 Collection and storage of faeces

Faecal samples were collected for three days prior the commencement (week 2) and at the end (week 6) of the treatment period. Samples collected from each respective three day period for each rabbit were pooled, weighed and stored at -18°C in a freezer until analysis in the Nutrition Laboratory of the University of Newcastle at Ourimbah, NSW, Australia. The total wet faecal weights were adjusted for the rabbits’ body weight.
measured on the last day of each of the two 3-day faecal sample collection periods and were expressed as faecal weight per day per kilogram of body weight (g/day/kg) and reported as mean±SD for each treatment group.

4.2.5 Drying of faeces

The frozen faecal samples were lyophilised overnight using an FD3 Dynavac Engineering Freeze Drier set at approximately -40°C and connected to a Edwards vacuum pump set at 2x10^4 mbar (Rietschie Thomas, Seven Hills, NSW, Australia). The lyophilised faecal samples were then ground into a fine powder using an electric CG2B coffee grinder (Breville, Pyrmont, NSW, Australia) and any hair or other obvious contaminants were removed. The samples were stored in airtight, light-restricting containers and placed in desiccators kept at -18°C until used for analyses.

4.2.6 Determination of faecal pH, moisture and total fat content

The faecal pH levels were determined [519] after 1g of lyophilised sample was suspended in 5ml of deionised water (Millipore Australia, North Ryde, NSW) and sonicated using a Soniclean 500HT sonifier for 30min on maximum power (Soniclean P/L, Thebarton, SA). Samples were then centrifuged at 1500 × g for 5min using a Clements 2000 centrifuge (Clements, Sydney, NSW). The supernatant faecal water was transferred into glass vials and analysed in triplicate using a Toledo MP220 pH meter (Toledo, NSW, Australia) and the final pH values were reported as mean±SD for each treatment group.

Faecal moisture content was determined using the gravimetric method [520] by comparing the faecal weight prior to and post freeze drying. All samples were analysed
in duplicate and the results were reported as mean±SD percentage moisture for each treatment group.

The total faecal fat content was also determined by the gravimetric method [458] after extraction with organic solvents [459]. Briefly, to 0.5g of lyophilised faecal sample, 10ml of chloroform:methanol solution (2:1 v/v) was added and the sample sonicated using a water-bath sonicator (Soniclean P/L, Thebarton, SA) set on maximum power for 10min. Each sample was then filtered (Whatman Grade No1) under vacuum into a pre-weighed glass vial and dried under a stream of nitrogen in a dry block heater set at 50°C (Ratek Instruments, Boronia, Australia). After drying, the samples were placed into a dessicator and allowed to reach room temperature overnight.

The samples were then weighed and the total faecal fat was determined by subtracting the weight of the empty glass vial from the weight of the glass vial containing the fat extract. All samples were analysed in duplicate and the final weight of faecal fat was adjusted for the rabbits’ body weight measured on the last day of each of the two 3-day faecal sample collection periods and expressed in milligrams of faeces per day per kilogram (mg/day/kg) and reported as a mean±SD for each treatment group.

4.2.7 Faecal neutral sterols using gas chromatography

4.2.7.1 Preparation of standards for standard curves

A stock solution of 1mmol/l coprostanol, 20mmol/l cholesterol and 2.5mmol/l epicoprostanol (5β-Cholestan-3α-ol) as internal standard (IS) was prepared in hexane. The additional working standard solutions used for the determination of the standard curves were prepared by serial dilution in hexane containing 5mmol/l IS to provide concentrations ranging between 62.5 – 1000μmol/l for coprostanol and 1.25 – 20mmol/l for cholesterol and 5mmol/l of IS. The hexane from all of the working standards was
then evaporated dried under a stream of nitrogen on a dry block heater set at 50°C (Ratek Instruments, Boronia, Australia) prior to derivatisation.

4.2.7.2 Selection of a quality control sample

One lyophilised faecal sample, from the end of the treatment period (week 6) for a rabbit in the treatment group, was selected as the control sample and it was included in every set of samples done. It was injected at the start and at the end of each chromatographic run to provide a control for the possible variations in the extraction and derivatisation procedures.

4.2.7.3 Extraction and derivatisation of the neutral sterols

To a 0.5g aliquot of the lyophilised faecal samples, 100μl of 2.5mmol/l epicoprostanol prepared in hexane was added to give a final concentration of 5mmol/l IS. Ten ml of chloroform:methanol solution (2:1 (v/v)) was then added and the samples were sonicated using a water-bath sonicator (Soniclean P/L, Thebarton, SA) set on maximum power for 30min[459]. The samples were then filtered (Whatman Grade Number 1 filter paper) under vacuum and dried under a stream of nitrogen on a dry block heater set at 50°C (Ratek Instruments, Boronia, Australia).

The evaporated samples were then resuspended in 1ml of hexane three times and all the hexane extracts were pooled together into a 5ml V-shaped borosilicon evaporation vial (Grace-Alltech, Baulkham Hills, NSW, Australia). The hexane was then evaporated under a stream of nitrogen on a dry block heater set at 50°C (Ratek Instruments, Boronia, Australia). The extracted neutral sterols were then saponified and derivatised using the same procedure as described for the serum sterol analysis in Chapter 3 (Section 3.4.4.3.5).
To the evaporated residue, 200μl of phosphate buffered saline (pH7.4) was added and the samples were vigorously vortex-mixed for 10min (SMI Multi-tube Vortexer, Miami, FL, USA) followed by the addition of 100μl of 33% (w/v) potassium hydroxide solution and 2ml of ethanol. The samples were then thoroughly vortex-mixed and placed in a shaking waterbath (Ratek Instruments, Boronia, Australia) set at 60°C for 30min. The solution was then allowed to cool to room temperature and 1ml of water was added followed by the addition of 2ml hexane. The hexane layer was removed into another glass vial and the hexane extraction was repeated twice more. All hexane extracts were combined together and evaporated to dryness under a stream of nitrogen gas on a dry block heater set at 60°C (Ratek Instruments, Boronia, Australia).

In order to generate trisilyl sterol derivatives, 100μl of Tri-Sil TBT (Thermo Scientific, Sydney, Australia) was added to the evaporated residue from all of the working standards and samples before they were incubated for 30min in a shaking waterbath (Ratek Instruments, Boronia, Australia) set at 80°C. After cooling, 4ml of 100mmol/l hydrochloric acid was added to the tubes followed by addition of 4ml of hexane. The tubes were then vortex-mixed for 3min (SMI Multi-tube Vortexer, Miami, FL, USA) and centrifuged at 2000 × g for 10min (Clements 2000 Centrifuge, Sydney, NSW, Australia) to separate the organic and aqueous layers. The aqueous layer was then removed and the organic layer was washed three times with 2ml DI water by vortex-mixing and centrifugation. The hexane layer was filtered through a Pasteur pipette containing sodium sulphate into borosilicate evaporation tubes, in order to remove any water residue from the hexane extract. The hexane was then evaporated to dryness under a stream of nitrogen gas on a dry block heater set at 60°C (Ratek Instruments, Boronia, Australia).
The residue in the evaporation tubes was resuspended in 50μl hexane and transferred into the GC cups having appropriate volume inserts and the samples were sequentially injected (1μl) onto the GC column for analysis of the sterols.

4.2.7.4 Equipment and chromatographic conditions

The GC-FID equipment used was as described in Chapter 3 (Section 3.4.4.3.4). However, due to the nearly identical elution times of the IS epicoprostanol (5β-Cholestan-3α-ol) and coprostanol (5β-Cholestan-3β-ol), the GC chromatographic conditions were altered as follows. The carrier gas was high purity (99.999%) helium (BOC Gasses, Australia) set at flow rate of 3ml/min. The injector temperature was set at 305ºC while the detector temperature was set at 310ºC. The column oven temperature was initially set at 235ºC and it was kept there for 30min before it was increased at a rate of 2ºC/min until 260ºC was reached and the column was kept at this temperature for an additional 30min. The total chromatographic run time was 72.5min per sample injection. A typical chromatogram of the faecal neutral sterols is presented in Figure 4.1.

4.2.7.5 Identification of the neutral sterols on the GC chromatogram

Neutral sterols and internal standard elution times were determined on the chromatogram after the injection of pure standards prepared in hexane and derivatised using the method previously described (Section 3.4.4.3.5). Identification of the individual neutral sterols and internal standard in faecal samples (Figure 4.1) was done by comparing a sample spiked with the individual sterols to samples that were not spiked.
4.2.7.6 The standard curve and calculation of the sterol concentrations

Standard curves for the individual neutral sterols were determined by plotting the peak area ratios (peak area of each sterol divided by the peak area of the internal standard) against the known concentration of the sterol in each solution (Figure 4.2). The concentrations of the sterols in each duplicate of the faecal samples were calculated by dividing the sterol peak area ratio by the slope of the standard curve for each of the sterols. The final concentrations of the sterols in the faecal samples were derived as the mean values for each sample. Finally, the values were converted to mg of sterol and they were adjusted for the dry weight of the faecal samples and expressed per gram of dry faecal weight (mg/g).

The data for faecal neutral sterols are presented for the individual sterols: cholesterol and coprostanol and for the total neutral sterols, which is the sum of cholesterol and coprostanol.
Figure 4.2 Standard curves used for analysis of cholesterol (a) and coprostanol (b) in the faecal samples. The results were derived from linear regression analysis of Cholesterol/IS and Coprostanol/IS chromatographic peak area ratios plotted versus the concentration of the respective sterol. The peak areas for IS, cholesterol and coprostanol were determined by GC as described in Section 4.2.7.4.

4.2.7.7 Intra- and inter-assay coefficients of variations for neutral faecal sterols

The intra-assay variation was evaluated by doing ten separate extractions of neutral sterols using one control faecal sample. The GC-FID analysis using the standard
curves in Figure 4.2 showed that the intra-assay variation was excellent for cholesterol with a CV of <5% and less than 6% for coprostanol (Table 4.1).

The inter-assay variation was analysed over time using the same lyophilised faecal sample used in the intra-assay variation analysis. The inter assay CVs were also very good and were less than 6.2% for cholesterol and 7.8% for coprostanol.

**Table 4.1** Intra- and inter-assay variation for the neutral sterols in one lyophilised faecal sample analysed by gas chromatography as described in Section 4.2.7.4.

<table>
<thead>
<tr>
<th></th>
<th>Intra-Assay Analysis</th>
<th>Inter-Assay Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Cholesterol (mg/g)</td>
<td>10</td>
<td>1.13±0.05</td>
</tr>
<tr>
<td>Coprostanol (mg/g)</td>
<td>10</td>
<td>0.34±0.02</td>
</tr>
</tbody>
</table>

*Note:* The results are expressed as Mean±SD with ‘n’ representing the number of replicates, CV – coefficient of variation calculated as (SD/Mean) x 100

**4.2.7.8 Determination of cholesterol absorption**

Cholesterol absorption of each rabbit was expressed as a percentage of the cholesterol intake and it was calculated using equation 4.1 [521].

\[
\text{Cholesterol Absorption (\%)} = [1 - (\text{Faecal Cholesterol} / \text{Dietary Cholesterol})] \times 100
\]

*Equation 4.1* Formula used for the calculation of cholesterol absorption

**4.2.7.9 Determination of faecal to dietary neutral sterols ratio**

The faecal cholesterol to dietary cholesterol ratio for each rabbit was calculated by dividing the average daily value of faecal cholesterol excretion adjusted for the rabbits’ body weight by the average daily value of cholesterol intake also adjusted for the rabbits’ body weight.
The faecal coprostanol to dietary cholesterol ratio for each rabbit was calculated by dividing the average daily value of faecal coprostanol excretion adjusted for the rabbits’ body weight with the average daily value of cholesterol intake also adjusted for the rabbits’ body weight.

The faecal neutral sterols to dietary cholesterol ratio for each rabbit was calculated by dividing the sum of average daily values of faecal coprostanol and faecal cholesterol adjusted for the rabbits’ body weight, by the average daily value of cholesterol intake also adjusted for the rabbits body weight.

4.2.8 Faecal bile acids using gas chromatography

The extraction and derivatisation procedures of faecal bile acids were adopted from previously developed methods [460-464] with some modifications. All the measurements of faecal bile acids were done at the Nutrition Laboratories at the University of Newcastle in Ourimbah using GC-FID.

4.2.8.1 Preparation of standards for standard curves

A stock solution containing all the standards at 10mg/ml was prepared in methanol with lithocholic acid, deoxycholic acid, cheno-deoxycholic acid, cholic acid with 4mg/ml urso-deoxycholic acid used as the internal standard (IS) and stored in a freezer at -18°C until used. The working standard solutions were prepared by serial dilutions in methanol containing 10mg/ml urso-deoxycholic acid (IS) providing a concentration range for each external standard from 0.017 – 5mg/ml and 4mg/ml IS.
4.2.8.2 Selection of a quality control sample

One lyophilised rabbit faecal sample from the 2%EGCG group, at the end of the treatment period (week 6), was selected as the control sample and it was included in every set of samples done. This control sample was prepared in duplicate, treated as the other rabbit samples and injected at the start and at the end of each chromatographic run to provide a control for the possible variations in the extraction and derivatisation procedures.

4.2.8.3 Extraction and derivatisation of the bile acids

To a 0.25g aliquot of a lyophilised faecal sample in a boiling flask, 200µl of 10mg/ml ursodeoxycholic acid (IS) was added to give a final concentration of 4mg/ml. Additionally, 800µl of methanol and 3ml of 2.5mol/l sodium hydroxide were added and the content of the flask was boiled under reflux for at least 2h. Once the solution was cooled to room temperature, 1ml of 2.5mol/l sodium hydroxide was added on top of the condenser to wash off possible boiling splatter and condensation back into the boiling flask. The pH of the solution in the flask was then reduced to 1 using concentrated hydrochloric acid (around 1.5ml) and the content of the flask was transferred into a Kimble vial.

To extract the lipids, 5ml of 2:1 chloroform:methanol solution (v/v) was added and the tube was vigorously vortex-mixed for 10min (SMI Multi-tube Vortexer, Miami, FL, USA). The tube was then centrifuged (Clements 2000, Sydney, NSW, Australia) at 1,500 × g for 20min and the chloroform layer was transferred into a v-shaped borosilicon evaporation vial (Grace-Alltech, Baulkham Hills, NSW, Australia). The lipid extraction was repeated twice more and all three extracts were combined together and dried under
a stream of nitrogen on a dry block heater set at 65°C (Ratek Instruments, Boronia, Australia).

To the extracted dried lipids, 2ml of 0.2N sodium acetate solution (pH5.6) and 1ml of 1.85%(w/v) EDTA were added and the tube was vigorously vortex-mixed. The solubilised extract was transferred into a Kimble vial and to deconjugate the bile acids, 1ml of choholylglycine hydrolase (15 units) from Clostridium perfringens was added and the pH was further adjusted to 5.6 by the addition of 5ml of 0.2N sodium acetate solution (pH5.6). The tube was then vortex-mixed for 10min and incubated in a shaking water bath (Ratek Instruments, Boronia, Australia) set at 37°C for 30min. The de-conjugation was stopped by the addition of 2.5ml of 2:1 chloroform:methanol solution (v/v). The Kimble vial was then vortex-mixed for 10min and centrifuged (Clements 2000, Sydney, NSW, Australia) at 2,000 × g and the chloroform layer was transferred into a clean v-shaped borosilicon evaporation vial (Grace-Alltech, Baulkham Hills, NSW, Australia). The chloroform:methanol extraction step was repeated twice more and all three extracts were combined together and evaporated under a stream of nitrogen on a dry block heater set at 65°C (Ratek Instruments, Boronia, Australia).

The butyl-ester derivatives of the extracted bile acids were prepared by the addition of 1ml of 6% sulphuric acid in n-butanol (v/v), vigorous vortex-mixing and incubation at 37°C for 2h in a shaking water bath (Ratek Instruments, Boronia, Australia). At this step, the working standards described above were also derivatised in order to develop the standard curves. After cooling, 5ml of chloroform was added and the solution was transferred into a clean Kimble vial, which was vortex-mixed for 5min and centrifuged at 1,500 × g for 20min. The aqueous layer was removed and the organic layer was washed three times with deionised water. After the final removal of the aqueous layer, the chloroform extract was filtered through a Pasteur pipette containing
1:1 sodium sulphate:potassium carbonate (w:w) into another Kimble vial and dried under a stream of nitrogen on a dry block heater set at 65°C.

The dry butyl-ester mixture was acetylated by adding 1ml of 3mg/ml 4-dimethylaminopyridine (DMAP) in chloroform and 0.5ml of acetic acid. The tube was then sealed under nitrogen and incubated for 16h in a shaking water bath (Ratek Instruments, Boronia, Australia) set at 37°C. The butyl ester acetates were extracted using 5ml of chloroform and the organic phase was washed off twice with 1ml of deionised water. The tube was then centrifuged at 2,000 × g (Clements 2000, Sydney, NSW, Australia) and the chloroform layer was then filtered through a Pasteur pipette containing 1:1 sodium sulphate:potassium carbonate (w:w) into an evaporation vial and dried under a stream of nitrogen on a dry block heater set at 65°C.

Finally, the residue in the evaporation tube was resuspended in 500µl of chloroform and transferred into a GC cup with an appropriate volume insert and the samples were sequentially manually injected (1µl) on the GC column.

4.2.8.4 Equipment and chromatographic conditions

The GC analysis was performed using a Shimadzu GC-17A (Shimadzu, Kyoto, Japan) and manual injection of the samples. The detector signal was recorded and the peak area was quantified using the Delta 5.5 software (DataworX P/L, Brisbane, QLD, Australia) installed on the data acquisition computer. A non-polar DB5-MS Phenyl Arylene polymer column (30mx0.25mm, ID 0.25µm) was used to separate the bile acids in the samples (Agilent, Santa Clara, CA, USA). The carrier gas was high purity (99.999%) helium (BOC Gasses, Australia) set at a flow rate of 2.2ml/min. The injector and detector temperature were both set at 300°C and the chromatograms were run for 60min. Injections were made 1min after the program was activated. The oven
temperature at injection was 50°C, where it was kept at for 5min and then increased to 200°C at a rate of 50°C/min, then to 285°C at a rate of 4°C/min and finally to 320°C at a rate of 2°C/min. The oven was held for a further 10min at 320°C to allow ample time for the compounds to be eluted from the column. Another injection was only made when the oven temperature had returned to 50°C, which took approximately 20min.

4.2.8.5 Identification of the bile acids on GC chromatogram

Bile acids and IS elution times were determined on the chromatogram (Figure 4.3) after the preparation and injection of each bile acid and IS as a pure standard as described in the method above (Section 4.2.8.3). This was further confirmed, by comparing a faecal sample spiked with known amounts of the individual bile acids to samples that were not spiked.

4.2.8.6 The standard curve and calculation of bile acid concentrations

A standard curve was determined for each individual bile acid by plotting the peak area ratio (peak area of each bile acid divided by the peak area of the internal standard) against the known concentration of the bile acid in each working standard solution (Figure 4.4).

The concentration of the bile acids in each duplicate of the rabbit faecal samples was calculated by dividing each bile acid peak area ratio by the slope of its standard curve. The final concentration of the bile acids in the faecal samples was derived as the mean value of the sample duplicates. Furthermore, the concentrations of the bile acids were adjusted for the dry weight of the faecal samples and expressed as mg/g.

The data for faecal acidic sterols are presented for the individual bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) and for the total acidic sterols (the
secondary bile acids), which is the sum of deoxycholic (DCA) and lithocholic (LCA) acids. Data are also presented for the total of all faecal sterols (AFS), which is the sum of the neutral sterols and the secondary bile acids.

**Figure 4.3** Typical chromatogram of the faecal bile acids; (1) lithocholic acid (46.5min); (2) deoxycholic acid (48.5min); (2) chenodeoxycholic acid (50.8min); (4) cholic acid (51.5min); (5) ursodeoxycholic acid (53.8min), the IS.
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)
4.2.8.7 Intra- and inter-assay coefficients of variation for bile acids

The intra-assay variation was evaluated using eight separate extractions of bile acids from one control faecal sample. The GC-FID analysis using the standard curves in Figure 4.4 showed that the intra-assay variation CV was less than 8.4% for lithocholic acid and less than 7.2% for deoxycholic acid. Cholic and cheno-deoxycholic acid were not detected in the control and other faecal samples (Table 4.2) and therefore, they were reported as being not detected (n.d.).

The inter-assay variation was analysed over time using the same lyophilised faecal sample used in the intra-assay variation analysis. The inter-assay CV’s were less than 10.2% for lithocholic and less than 9% for deoxycholic acids. Again, cheno-deoxycholic and cholic acid were not detected (n.d.) in the prepared faecal sample.

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>Intra-assay Variation</th>
<th>Inter-assay Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Lithocholic Acid</td>
<td>8</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>Deoxycholic Acid</td>
<td>8</td>
<td>0.80±0.06</td>
</tr>
<tr>
<td>Cheno-deoxycholic Acid</td>
<td>8</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cholic Acid</td>
<td>8</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Note: Values are presented in mg/g of dry weight for each faecal sample. Cheno-deoxycholic and cholic acids were not detected in the faecal samples (n.d.). CV – coefficient of variation calculated as (SD/Mean) x 100

4.2.8.8 Determination of faecal bile acids to dietary cholesterol ratios

The ratio of faecal lithocholic acid to dietary cholesterol was calculated by dividing the average daily excretion value for LCA adjusted for the rabbits’ body
weight by the average daily dietary cholesterol intake also adjusted for the rabbits’ body weight.

The ratio of faecal deoxycholic acid to dietary cholesterol was calculated by dividing the average daily excretion value for DCA adjusted for the rabbits’ body weight by the average daily dietary cholesterol intake also adjusted for the rabbits’ body weight.

The ratio of acidic sterols to dietary cholesterol was calculated as sum of the average daily excretion value adjusted for the rabbits’ body weight of LCA and DCA divided by the average daily cholesterol intake also adjusted for the rabbits’ body weight.

**4.2.8.9 Determination of faecal neutral and acidic sterol ratios**

The faecal cholesterol to LCA ratio was calculated by dividing the average daily faecal cholesterol value adjusted for the rabbits’ body weight by the average daily LCA value, also adjusted for the rabbits’ body weight.

The faecal cholesterol to DCA ratio was calculated by dividing the average daily faecal cholesterol value adjusted for the rabbits’ body weight by the average daily DCA value also adjusted for the rabbits’ body weight.

The ratio of faecal cholesterol to secondary bile acids was determined by dividing the average daily faecal cholesterol value adjusted for the rabbits’ body weight by the sum of average daily LCA and DCA values adjusted for the rabbits’ body weight.

The ratio of LCA to DCA was calculated as average daily LCA value adjusted for the rabbits’ body weight by the average daily DCA value also adjusted for the rabbits’ body weight.
4.2.8.10 Determination of sterol balance

Sterol balance was calculated as the difference between the sum of all faecal sterols (neutral and acidic) and the dietary cholesterol intake for each rabbit (Equation 4.2).

\[
\text{Sterol balance} = (\text{Acidic + Neutral Sterols}) - \text{Dietary Cholesterol}
\]

\textbf{Equation 4.2} Equation for determining the sterol balance

4.2.9 Faecal EGCG by High Pressure Liquid Chromatography

The extraction and de-conjugation of faecal EGCG was adapted from previously developed methods [375, 393, 465] with some modifications. All measurements of faecal EGCG were done at the Nutrition Laboratories at the University of Newcastle in Ourimbah using HPLC equipped with a UV detector.

4.2.9.1 Preparation of standards for the standard curve

The standards were prepared by serial dilution of 2mmol/l pure EGCG (Sigma Aldrich, Castle Hill, Australia) dissolved in methanol containing 50mmol/l 4-aminosalycilic acid as internal standard (Section 2.4.1).

4.2.9.2 Selection of a quality control sample

One lyophilised faecal sample of a rabbit from the treatment group at the end of the treatment period (week 6), was selected as the control sample and it was included in every set of samples done. This sample was prepared in duplicate, treated exactly as the other rabbit faecal samples and it was injected at the start and at the end of each chromatographic run to provide a control for possible variations in the extraction and deconjugation procedures.
4.2.9.3 Extraction of free and conjugated EGCG in faecal samples

To a 0.5g aliquot of a lyophilised faecal sample, 50µl of 100mmol/l of 4-aminosalycilic acidacing as internal standard was added to give a final concentration of 50mmol/l. Additionally, 5ml of cold (4°C) 114mmol/l ascorbic acid solution was added and the sample was sonicated in an ice water bath set on maximum power for 30min (Soniclean 500HT, Soniclean P/L, Thebarton SA, Australia). The samples was then transferred into a Kimble vial and centrifuged at 1,500 × g (Clements 200, Sydney, NSW, Australia) for 5min to separate solids from faecal water.

Into a clean Kimble vial, 100µl of faecal water from the previous step was added and free EGCG was extracted using 1ml of ethyl acetate and repeated three times. The three ethyl acetate extracts were combined together into a V-shaped borosilicon evaporation vial (Grace-Alltech, Baulkham Hills, NSW, Australia) and evaporated to dryness under a stream of nitrogen on a dry block heater set at 60°C (Ratek Instruments, Boronia, Australia).

The total EGCG content was determined by de-conjugating the EGCG in 100µl of faecal water with the addition of 10µl of an enzyme mixture containing 250 units β-glucuoronidase and 15 units of sulfatase dissolved in DI water. The sample was then incubated in a shaking water-bath (Ratek Instruments, Boronia, Australia) set at 37°C for 90min. The enzymatic reactions were stopped by adding 1ml of ethyl acetate and the extraction and drying procedure was identical as for the extraction of free EGCG extraction described above.
Finally, the residue in the evaporation tube were resuspended in 100µl of methanol and transferred into an HPLC brown vial with an appropriate volume insert and the samples were sequentially injected (20µl) onto the column.

4.2.9.4 Equipment and chromatographic conditions

The equipment and chromatographic conditions used for the analysis of faecal EGCG are identical to those described previously for the determination of EGCG purity (Section 2.4.2).

4.2.9.5 Identification of EGCG on the HPLC chromatogram for faeces

The elution times for the internal standard and EGCG were determined using the control faecal sample spiked with a known amount of pure EGCG and internal standard and compared to a sample which was not spiked. A typical chromatogram identifying the internal standard and EGCG is presented in Figure 4.5.

![Typical chromatogram at 280nm](image)

**Figure 4.5** Typical chromatogram at 280nm of (1) 4-aminosalicylic acid as internal standard (9.742min) and (2) EGCG (28.338min) after their extraction from a faecal sample as described in Section 4.2.9.3.
4.2.9.6 The standard curve and calculation of EGCG concentrations

A standard curve was developed for EGCG by plotting the peak area ratio (peak area for EGCG divided by the peak area of the internal standard) against the known concentrations of EGCG in each standard solution (Figure 4.6). The concentration of EGCG was calculated by dividing the EGCG peak area ratio by the slope of the standard curve. The final concentration of the EGCG in the faecal samples was derived as the mean value of duplicates. The values for free and conjugated EGCG were derived using the same standard curve and its slope value. Finally, the values for EGCG were converted to mg and adjusted for the dry faecal sample weight and expressed as mg/g of dry faecal weight.

![Standard curve](image)

**Figure 4.6** Standard curve used for analysis of EGCG in faecal samples. Results were derived from a linear regression analysis of EGCG/IS chromatographic peak area ratios plotted versus the known concentrations of EGCG. Peak areas of IS and EGCG were determined by HPLC as described in Section 4.2.9.3. Concentrations of free and conjugated EGCG were determined using this curve.
4.2.9.7 Intra- and inter-assay coefficients of variations for faecal EGCG

The intra-assay variation was determined using ten separate extractions of EGCG from one control faecal sample. The HPLC-UV analysis using the standard curve in Figure 4.6 indicated an excellent intra-assay variation with the CV less than 5% (Table 4.3). The inter-assay variation was analysed over time on a same lyophilised faecal sample used in the intra-assay variation analysis and the results revealed a CVs less than 5.4%.

<table>
<thead>
<tr>
<th>Intra-assay Variation</th>
<th>Inter-assay Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>EGCG (mg/g)</td>
<td>10</td>
</tr>
</tbody>
</table>

Note: The results are expressed as Mean±SD with ‘n’ representing the number of replicates, CV – coefficient of variation calculated as (SD/Mean) x 100

4.2.10 Statistical Analyses

The statistical analysis for the rabbits’ dietary intake, excretion of faeces and the concentrations of neutral sterols, bile acids and EGCG in the faecal samples was performed using Microsoft Excel® 2003 (Microsoft Australia, North Ryde, NSW, Australia). The means, standard deviations and statistical significance, by the Students $t$-test comparing pre and post changes (paired $t$-tests) or comparing differences (two sample assuming equal variances $t$-tests) between the two groups (control and treatment), were determined for all measurements.
4.3 Results

4.3.1 Rabbits’ appearance and body weight

All of the animals appeared healthy throughout the experiment and none exhibited hair loss, xanthomatosus cutaneous lesions or fatty deposits in the eyes, manifestations commonly associated with rabbits fed large quantities of cholesterol [521].

The average body weight of the rabbits (Figure 4.7) was significantly increased (p<0.001) in both groups with the controls increasing their weight by +16% and the 2%EGCG group by +14.1% during the 4-week treatment period. However, there was no significant difference in weight between the two groups at the start (p=0.285) or at the end (p=0.519) of the treatment period and there was also no difference in the growth of the rabbits between the two groups during the treatment period (p=0.380).

![Figure 4.7](image)

**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. The values are Mean±SD and the bars with the same colour having the same superscript letter are significantly different (p<0.001). There was no difference between the two groups at the start (p=0.285) or at the end (p=0.519) of the treatment period.
4.3.2 Faecal weight, pH, moisture and total fat content

The results for total faecal output (g/day/kg), faecal moisture content, faecal pH, total faecal fat content (mg/day/kg) and faecal fat content as a percentage of the total faecal output were determined as described in Sections 4.2.4 to 4.2.6 and are presented in Table 4.4. Faecal weight was determined immediately upon collection of the stool samples for three days prior to the start of the treatment period (Week 2) and three days prior to the rabbits being euthanised (Week 6). The moisture content was expressed as a percentage of the faecal weight lost after the samples were freeze dried. The pH and total fat content was determined on lyophilised samples as described in the methods (Section 4.2.6). Furthermore, as both groups of rabbits had increases in body weight during the treatment period (Figure 4.7), the results for faecal total output and faecal fat were adjusted for the rabbits’ body weights measured on the last day of each of the two 3-day faecal sample collection periods.

After the use of the Students’ t-test to compare the means between the two groups, there was no significant difference in the average daily faecal output adjusted for the rabbits’ body weight (Table 4.4) between the control and the 2%EGCG group at the start (p=0.588) or at the end (p=0.562) of the treatment period. There was no significant change in the faecal output in both groups, -14.3% for the control group (p=0.324) and -5.2% for the 2%EGCG group (p=0.285), during the treatment period. Furthermore, there was no significant difference in the change in faecal output between the two groups (p=0.714) at the end of the treatment period.

A similar pattern was also observed for the faecal moisture percentage (Table 4.4), as there was no significant difference between the control and the 2%EGCG group at the start (p=0.291) or at the end (p=0.169) of the treatment period. There was also no significant change in faecal moisture content in both groups, +2.99% for the control
group (p=0.455) and -1.26% for the 2%EGCG group (p=0.769), during the treatment period. Furthermore, there was no significant difference in the change of faecal moisture percentage between the two groups (0.457) at the end of the treatment period.

The faecal pH values (Table 4.4) did not significantly differ between the groups at the start (p=0.499) or at the end (p=0.509) of the treatment period. Additionally, there was no significant change within the control (p=0.102) or 2%EGCG group (p=0.703) during the treatment period. Furthermore, at the end of the treatment period, there was no difference in the change in faecal pH (p=0.307) between the two groups.

The total faecal fat (Table 4.4) was also not significantly different between the two groups at the start (p=0.297) or at the end (p=0.373) of the treatment period. Furthermore, there was no significant change in faecal fat excretion over the treatment period for the control (p=0.759) or the 2%EGCG (p=0.842) group and, at the end of the treatment period, there was no significant difference in the change in total faecal fat (p=0.877) between the two groups.

4.3.3 Faecal neutral and acidic sterols

The values for the faecal neutral sterols and the faecal bile acids are presented in milligrams per day normalised to the rabbits’ body weight (mg/day/kg) in Table 4.5. The faecal neutral sterols (cholesterol and coprostanol) and the secondary bile acids in the faecal samples were analysed by GC after extraction and derivatisation as described in Sections 4.2.7 and 4.2.8. The neutral sterols are presented as the sum of cholesterol and coprostanol, the secondary bile acids as the sum of lithocholic and deoxycholic acid and the total of all the measured faecal sterols as the sum of the neutral sterols plus the secondary bile acids. All the results were adjusted for
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>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th></th>
<th></th>
<th>2% EGCG</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2 (W2)</td>
<td>Week 6 (W6)</td>
<td>Δ (W6-W2)</td>
<td>Week 2</td>
<td>Week 6</td>
<td>Δ (W6-W2)</td>
</tr>
<tr>
<td>Faecal Weight (g/day/kg)</td>
<td>43.54±10.22</td>
<td>36.4±13.17</td>
<td>-7.14±15.99</td>
<td>39.39±14.99</td>
<td>32.06±11.87</td>
<td>-7.34±15.04</td>
</tr>
<tr>
<td>Moisture Content (%)</td>
<td>40.18±13.99</td>
<td>43.17±11.26</td>
<td>2.99±9.07</td>
<td>32.12±10.87</td>
<td>30.85±16.98</td>
<td>-1.26±10</td>
</tr>
<tr>
<td>pH</td>
<td>8.09±0.15</td>
<td>7.98±0.06</td>
<td>-0.12±0.14</td>
<td>8.04±0.09</td>
<td>8.02±0.14</td>
<td>-0.03±1.89</td>
</tr>
<tr>
<td>Total Fat Content (mg/day/kg)</td>
<td>230.11±153.89</td>
<td>209.76±105.02</td>
<td>-20.36±154.35</td>
<td>154.1±70.1</td>
<td>145.62±131.57</td>
<td>-8.48±98.8</td>
</tr>
<tr>
<td>Fat Content (%)</td>
<td>0.49±0.21</td>
<td>0.54±0.16</td>
<td>0.05±0.13</td>
<td>0.38±0.09</td>
<td>0.4±0.22</td>
<td>0.02±0.14</td>
</tr>
</tbody>
</table>

Values are Mean±SD. The symbol (Δ) denotes the difference in values between Week 2 and Week 6 (Week 6 minus Week 2). Values for ‘Faecal Weight’ and ‘Total Fat Content’ are adjusted for the rabbits’ body weight measured on the last day of each of the two 3-day faecal sample collection periods. There were no significant differences found between the two groups for any of the measurements.
the rabbits’ body weight recorded on the last day of each of the two 3-day faecal sample collection periods.

After the use of the Student t-test for comparison of the means between the two groups there was no significant difference in faecal cholesterol excretion (Table 4.5) between the control and 2%EGCG group at the start (p=0.792) or at the end of the treatment period (p=0.293). There was also no significant change in faecal cholesterol excretion in both groups, -28.1% for the control group (p=0.161) and +32.6% for the 2%EGCG group (p=0.444), during the treatment period. Furthermore, at the end of the treatment period, there was no difference in the change in faecal cholesterol excretion (p=0.166) between the two groups.

The excretion of faecal coprostanol (Table 4.5) had a pattern similar to the faecal cholesterol excretion. There was no significant difference between the control and 2%EGCG groups at the start (p=0.854) or at the end (p=0.098) of the treatment period. There was also no significant change in faecal coprostanol excretion in both groups, -49.9% for the control group (p=0.094) and +60.9% for the 2%EGCG group (p=0.373), during the treatment period. Furthermore, there was no statistically significant difference in these changes between the two groups (p=0.095) at the end of the treatment period.

The total faecal neutral sterol excretion (Table 4.5), calculated as the sum of cholesterol and coprostanol, followed a pattern similar to its constituents. There was no significant difference between the control and the 2%EGCG group at the start (p=0.832) or at the end (p=0.222) of the treatment period. There was also no significant change in neutral sterol excretion within the control (-31%, p=0.143) or the 2%EGCG group (+34.78%, p=0.396) during the treatment period. Furthermore, at the end of the
treatment period, the changes in faecal neutral sterol excretion were not significantly
different (p=0.106) between the two groups.

The levels of faecal lithocholic acid did not significantly differ between the two
groups at the start (p=0.179) or at the end (p=0.698) of the treatment period (Table 4.5).
There was also no significant change in the faecal LCA levels within the control (-
16.79%, p=0.271) or the 2%EGCG group (+20.8%, p=0.493) during the treatment
period. Furthermore, these changes were not significantly different between the two
groups (p=0.317) at the end of the treatment period.

The faecal deoxycholic acid followed a pattern similar to lithocholic acid. There
was no significant difference in its faecal excretion between the two groups at the start
(p=0.219) or at the end (p=0.471) of the treatment period (Table 4.5). The levels of
faecal DCA did not change significantly in the control (-29.07%; p=0.368) and the
2%EGCG (-7.96%; p=0.852) group during the treatment period. Furthermore, at the
end of the treatment period, these changes were not significantly different between the two
groups (p=0.722).

The levels of total faecal secondary bile acids (Table 4.5), calculated as the sum
of lithocholic and deoxycholic acid followed a pattern similar to its constituents. There
was no significant difference between the two groups at the start (p=0.204) or at the end
(p=0.512) of the treatment period. There was also no significant change in the faecal
secondary bile acids within the control (-32.00%, p=0.335) or the 2%EGCG group (-
1.54%, p=0.969) during the treatment period. Furthermore, at the end of the treatment
period, the changes in the total faecal secondary bile acids levels were not significantly
different between the two groups (p=0.833).
Table 4.5 The mean daily excretion values of faecal cholesterol, faecal coprostanol, faecal neutral sterols (sum of cholesterol and coprostanol), faecal lithochoic acid, faecal deoxychoic acid, faecal secondary bile acids (sum of lithochoic and deoxychoic acid) and all faecal sterols (sum of neutral sterols and secondary bile acids) adjusted for the rabbits’ body weight.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>2% EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>20.43±4.5</td>
<td>14.59±8.29</td>
</tr>
<tr>
<td>Coprostanol</td>
<td>3.54±1.49</td>
<td>1.96±2.66</td>
</tr>
<tr>
<td>Lithochoic Acid</td>
<td>3.52±3.55</td>
<td>2.1±1.49</td>
</tr>
<tr>
<td>Deoxychoic Acid</td>
<td>10.2±8.42</td>
<td>7.23±2.7</td>
</tr>
<tr>
<td>Secondary Bile Acids</td>
<td>13.72±11.92</td>
<td>9.33±4.06</td>
</tr>
<tr>
<td>All Faecal Sterols</td>
<td>37.69±16.42</td>
<td>25.88±11.98</td>
</tr>
</tbody>
</table>

Values are Mean±SD and are adjusted for the rabbits’ body weight (mg/day/kg) measured on the last day of each of the two 3-day faecal sample collection periods. The symbol (Δ) denotes the difference in values between Week 2 and Week 6 (Week 6 minus Week 2). There were no significant differences found between the two groups for any of the measurements.
The levels of all the faecal sterols, calculated as the sum of the neutral sterols plus the faecal secondary bile acids (Table 4.5), also followed a pattern similar to all its constituents: cholesterol, coprostanol, LCA and DCA. There was no significant difference in AFS between the two groups as the start (p=0.381) or at the end (p=0.346) of the treatment period. There was also no significant change in AFS within the control (-31.34%, p=0.160) or the 2%EGCG group (+23.04%, p=0.431) during the treatment period. Furthermore, at the end of the treatment period, the changes in AFS were not significantly different between the two groups (p=0.112).

4.3.4 Cholesterol intake and absorption and sterol balance

The values for the cholesterol intake, the percentage cholesterol absorption and the sterol balance are presented in Table 4.6. The percentage cholesterol absorption was calculated using Equation 4.1 (Section 4.2.7.8) and the daily sterol balance was calculated as the difference between the average daily excretion of all the sterols and the average daily intake of cholesterol as per Equation 4.2 (Section 4.2.8.10).

The average daily cholesterol intake (Table 4.6) did not differ between the two groups at the start (p=0.540) or at the end of the treatment (p=0.489). There was also no significant change in the daily cholesterol intake within the control (-2.36%, p=0.649) or the 2%EGCG group (-12.32%, p=0.113) during the treatment period. Furthermore, at the end of the treatment period, there was no significant difference in the changes in daily cholesterol intake between the two groups (p=0.304).

The percent of cholesterol absorption also did not differ between the two groups at the start (p=0.739) or at the end of the treatment (p=0.284). There was also no significant change in the percent of cholesterol absorption within the control (+3.35%, p=0.324) or the 2%EGCG group (-8.62%, p=0.349) during the treatment period.
Furthermore, at the end of the treatment period, there was no significant difference in the change in the percent of cholesterol absorption between the two groups (p=0.179).

The sterol balance did not differ between the two groups at the start (p=0.243) or at the end of the treatment (p=0.306). There was also no significant change in sterol balance within the control (-10.03%, p=0.579) or the 2%EGCG group (+20.00%, p=0.178) during the treatment period. Furthermore, there was no significant difference in the change in sterol balance between the two groups (p=0.142) at the end of the treatment period.
Table 4.6 The average daily cholesterol intake, cholesterol absorption and sterol balance

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>2%EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
</tr>
<tr>
<td>Cholesterol Intake (mg/day/kg)</td>
<td>140.15±14.39</td>
<td>135.82±17.62</td>
</tr>
<tr>
<td>Cholesterol Absorption (%)</td>
<td>85.42±2.93</td>
<td>88.77±6.91</td>
</tr>
<tr>
<td>Sterol Balance (mg/day/kg)</td>
<td>-102.45±15.95</td>
<td>-109.94±26.27</td>
</tr>
</tbody>
</table>

Values are Mean±SD and are adjusted for the rabbits’ body weight measured on the last day of each of the two 3-day faecal sample collection periods. The symbol (Δ) denotes the difference in values between Week 2 and Week 6 (Week 6 minus Week 2). There were no significant differences found between the two groups for any of the measurements.

§ Cholesterol Absorption (%) = [1 – (Faecal Cholesterol / Dietary Cholesterol)] x 100

§§ Sterol balance = (Acidic + Neutral Sterols) – Dietary Cholesterol
4.3.5 Amounts of faecal sterols relative to dietary cholesterol intake

The amounts of faecal sterols relative to the intake of dietary cholesterol were calculated by dividing the individual faecal sterols with the dietary intake of cholesterol and expressed as a percentage. The results of this analysis are presented in Table 4.7.

The average faecal cholesterol to dietary cholesterol ratio (Table 4.7) did not differ between the two groups at the start (p=0.739) or at the end of the treatment (p=0.284). There was also no significant change in the ratio within the control (-21.34%, p=0.324) or the 2%EGCG group (+59.86%, p=0.326) during the treatment period. Furthermore, there was no significant difference in the change in the faecal cholesterol to dietary cholesterol ratio between the two groups (p=0.088) at the end of the treatment period.

The faecal coprostanol to dietary cholesterol ratio (Table 4.7) did not differ between the two groups at the start (p=0.840) or at the end (p=0.064) of the treatment period. There was also no significant change in the ratio within the control (-43.1%, p=0.172) or the 2%EGCG group (+86.9%, p=0.288) during the treatment period. Furthermore, there was no significant difference in the change in the faecal coprostanol to dietary cholesterol ratio between the two groups (p=0.096) at the end of the treatment period.

The faecal neutral sterols to dietary cholesterol ratio, followed a pattern similar to the ratios of its constituents to dietary cholesterol. There was no significant difference between the two groups at the start (p=0.782) or at the end (p=0.220) of the treatment period. There was also no significant change in the ratio within the control (-24.2%, p=0.287) or the 2%EGCG group (+61.5%, p=0.300) during the treatment period. Furthermore, there was no significant difference in the change in the faecal neutral
sterols to dietary cholesterol ratio between the two groups (p=0.095) at the end of the treatment period.

The faecal LCA to dietary cholesterol ratio was not significantly different between the two groups at the start (p=0.172) or at the end (p=0.733) of the treatment period. There was also no significant change in the ratio within the control (-12.92%, p=0.364) or the 2%EGCG group (+40.02%, p=0.359) during the treatment period. Furthermore, there was no significant difference in the change in the faecal LCA to dietary cholesterol ratio between the two groups (p=0.236) at the end of the treatment period.

The DCA to dietary cholesterol ratio did not differ between the two groups at the start (p=0.203) or at the end (p=0.539) of the treatment period. There was also no significant change in the ratio within the control (+3.29%, p=0.503) or the 2%EGCG group (+2.09%, p=0.894) during the treatment period. Furthermore, there was no significant difference in the change in the faecal DCA to dietary cholesterol ratio between the two groups (p=0.982) at the end of the treatment period.

The faecal SBA to dietary cholesterol ratio did not differ between the two groups at the start (p=0.191) or at the end (p=0.571) of the treatment period. There was also no significant change in the ratio within the control (-0.75%, p=0.458) or the 2%EGCG group (+4.93%, p=0.774) during the treatment period. Furthermore, there was no significant difference in the change in the faecal SBA to dietary cholesterol ratio between the two groups (p=0.906) at the end of the treatment period.
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>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>2% EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
</tr>
<tr>
<td>Coprostanol/Dietary Cholesterol</td>
<td>2.49±0.93</td>
<td>1.43±1.88</td>
</tr>
<tr>
<td>Neutral Sterols/Dietary Cholesterol</td>
<td>17.07±2.82</td>
<td>12.66±8.24</td>
</tr>
<tr>
<td>LCA/Dietary Cholesterol</td>
<td>2.45±2.44</td>
<td>1.63±1.23</td>
</tr>
<tr>
<td>DCA/Dietary Cholesterol</td>
<td>7.14±5.81</td>
<td>5.58±2.57</td>
</tr>
<tr>
<td>SBA/Dietary Cholesterol</td>
<td>9.58±8.22</td>
<td>7.21±3.68</td>
</tr>
</tbody>
</table>

Values are Mean±SD. The symbol (Δ) denotes the difference in values between Week 2 and Week 6 (Week 6 minus Week 2). There were no significant differences found between the two groups for any of the measurements. LCA – Lithocholic acid, DCA – Deoxycholic acid, SBA – Secondary bile acids, AFS – All faecal sterols.
The AFS to dietary cholesterol ratio was also not significantly different between the groups at the start (p=0.345) or at the end (p=0.326) of the treatment period. There was also no significant change in the ratio within the control (-21.62%, p=0.289) or the 2%EGCG group (+47.15%, p=0.298) during the treatment period. Furthermore, there was no significant difference in the change in the faecal DCA to dietary cholesterol ratio between the two groups (p=0.096) at the end of the treatment period.

4.3.6 Amounts of faecal cholesterol relative to faecal acidic sterols

The ratios of faecal cholesterol to the individual bile acids in the faeces was determined as an index of the metabolism of cholesterol by the intestinal microflora. The ratios of faecal cholesterol relative to the faecal acidic sterols is presented in Table 4.8.

The faecal cholesterol to faecal coprostanol ratio (Table 4.8) was not significantly different between the two groups at the start (p=0.368) or at the end (p=0.107) of the treatment period. There was also no significant change in the ratio within the control (+180.83%, p=0.143) or the 2%EGCG group (-57.04%, p=0.670) during the treatment period. Furthermore, there was no significant difference in the change in the faecal cholesterol to faecal coprostanol ratio between the two groups (p=0.326) at the end of the treatment period.

The faecal cholesterol to faecal DCA ratio (Table 4.8) was not significantly different between the two groups at the start (p=0.914) but it was significantly different at the end (p=0.014) of the treatment period. Similarly, there was no significant change in the ratio in the control group (+11.68%, p=0.324) but, in the 2%EGCG group, there was a significant increase (+127.17%, p=0.049) during the treatment period. Furthermore, there was a significant difference between the two groups (p=0.043), in
the change in the ratio of faecal cholesterol to faecal DCA, at the end of the treatment period.

The faecal cholesterol to faecal LCA ratio (Table 4.8) was not significantly different at the start (p=0.425) or at the end (p=0.172) of the treatment period. There was also no significant change in the ratio within the control (-23.67%, p=0.821) or the 2%EGCG group (+65.79%, p=0.420) during the treatment period. Furthermore, there was no significant difference in the change in the faecal cholesterol to faecal LCA ratio between the two groups (p=0.599) at the end of the treatment period.

The faecal cholesterol to SBA ratio (Table 4.8) was not significantly different between the two groups at the start (p=0.814) but it was significantly different between the groups at the end (p=0.032) of the treatment. However, there was also no significant change in the ratio within the control (-5.94%, p=0.373) or the 2%EGCG group (+92.50%, p=0.084) during the treatment period. Nevertheless, at the end of the treatment, there was a significant difference in the change in the faecal cholesterol to SBA ratio between the two groups (p=0.042).
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>
<thead>
<tr>
<th>Ratio</th>
<th>Control</th>
<th>2% EGCG</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 6</td>
<td>Δ (W6-W2)</td>
<td>Week 2</td>
<td>Week 6</td>
<td>Δ (W6-W2)</td>
</tr>
<tr>
<td>Faecal Cholesterol/Coprostanol</td>
<td>6.92±3.74</td>
<td>19.79±18.93</td>
<td>12.87±18.12</td>
<td>5.22±2.35</td>
<td>5.93±3.23</td>
<td>0.71±3.85</td>
</tr>
<tr>
<td>Faecal Cholesterol/DCA</td>
<td>3.19±2.39</td>
<td>*2.08±0.89</td>
<td>*-1.11±2.48$</td>
<td>3.32±1.68a</td>
<td>*7.54±4.38a</td>
<td>4.22±4.01$</td>
</tr>
<tr>
<td>Faecal Cholesterol/LCA</td>
<td>10.73±7.49</td>
<td>9.99±5.89</td>
<td>-0.74±7.59</td>
<td>14.58±8.51</td>
<td>20.95±17.28</td>
<td>6.37±17.77</td>
</tr>
<tr>
<td>Faecal Cholesterol/SBA</td>
<td>2.44±1.8</td>
<td>*1.69±0.79</td>
<td>*-0.75±1.87$</td>
<td>2.66±1.38</td>
<td>*5.13±3.29</td>
<td>2.46±2.8$</td>
</tr>
<tr>
<td>LCA / DCA</td>
<td>0.32±0.08</td>
<td>0.26±0.12</td>
<td>-0.05±0.11</td>
<td>0.25±0.09</td>
<td>0.55±0.53</td>
<td>0.29±0.59</td>
</tr>
</tbody>
</table>

The symbol (Δ) denotes the difference in values between the Week 2 and Week 6 (Week 6 minus Week 2). The values are Mean±SD and those with the same letter superscript are significantly different between week 2 and week 6 (p<0.05). The symbol (*) denotes that the values are significantly different between the two treatment groups at the same time point (p<0.05). The symbol ($^\$$) denotes that the change (Δ) from week 2 to week 6 is significantly different between the two groups (p<0.05). LCA – Lithocholic acid, DCA – Deoxycholic acid, SBA – Secondary bile acids, AFS – All faecal sterols.
The faecal LCA/DCA ratio (Table 4.8) was also not significantly different between the two groups at the start (p=0.202) or at the end (p=0.231) of the treatment period. There was also no significant change in the ratio within the control (-16.42%, p=0.284) or the 2%EGCG group (+114.30%, p=0.281) during the treatment period. Furthermore, there was no significant difference in the change in the faecal LCA to DCA ratio between the two groups (p=0.219) at the end of the treatment period.

4.3.7 Recovery of free and conjugated EGCG in faecal samples

The faecal samples collected from the 2%EGCG group at the end of the treatment were analysed for total, free and conjugated EGCG using the methods described in Section 4.2.9. The free EGCG represents the amount of EGCG found in the faecal samples measured without the de-conjugation step while total EGCG represents the amount of EGCG in the samples when the de-conjugation step was applied as described in Section 4.2.9.3. Furthermore, conjugated EGCG was calculated by subtracting the values for the free EGCG from the values of the total EGCG. All values are presented in Table 4.9.

Table 4.9 The average values of total, free and conjugated EGCG measured in the faeces by HPLC-UV analysis as described in the Section 4.2.9.

<table>
<thead>
<tr>
<th>EGCG type</th>
<th>Daily Intake (mg/day/kg)</th>
<th>Daily Excretion (mg/day/kg)</th>
<th>Percentage of daily EGCG intake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1016.09±194.99</td>
<td>5.58±2.91</td>
<td>0.55±0.26</td>
</tr>
<tr>
<td>Free</td>
<td>NA</td>
<td>5.1±2.64</td>
<td>0.5±0.24</td>
</tr>
<tr>
<td>Conjugated</td>
<td>NA</td>
<td>0.48±0.42</td>
<td>0.05±0.04</td>
</tr>
</tbody>
</table>

Values are Mean±SD and are adjusted for the rabbits’ body weight measured on the last day of each of the two 3-day faecal sample collection periods. NA is ‘Not Applicable’.
The analysis of EGCG in the faecal samples from the rabbits fed 2% (w/w) EGCG in their diet for the 4-week treatment period (Table 4.9), revealed that the total faecal EGCG only represented about 0.6% of the total daily intake. Furthermore, the free EGCG accounted for 91.5±7.5% while the conjugated accounted for only 8.5±7.5% of the total EGCG found in the rabbit faecal samples.
4.4 Discussion

It was hypothesized that the plasma cholesterol lowering effect of pure EGCG presented in Chapter 3 was due to an increase in the faecal excretion of neutral and/or acidic sterols. Therefore, the main aim of the study was to determine the effect of the EGCG supplementation on the content of neutral and acidic sterols in the rabbit faeces. The faecal pH, moisture content and total fat content were also measured. Furthermore, the amount of EGCG excreted at the end of the treatment period was determined in the faeces of the rabbits treated with EGCG.

In order to test the hypothesis, twelve New Zealand white rabbits were rendered hypercholesterolaemic over 2 weeks by feeding them with 0.25% (w/w) cholesterol incorporated into their food. The animals were then divided into the two groups (control and 2% EGCG) matched by their serum cholesterol levels. One group of rabbits continued on their cholesterol diet (control) while the other group (2% EGCG) continued on the cholesterol diet to which 2% (w/w) EGCG was added for four weeks. The daily dietary intake of the rabbits was recorded and their body weight and appearance was also determined. Faecal samples were collected for three days prior the commencement and three days prior the end of the 4-week treatment period. The collected faecal samples were analysed for pH, moisture content and total faecal fat using gravimetric methods, neutral and acidic sterols using gas chromatography and excretion of faecal EGCG using HPLC-UV.

The aims of the study were achieved but the hypothesis that the plasma lowering effect of EGCG presented in Chapter 3 was due to an increase in the excretion of faecal neutral and/or acidic sterols, was not supported. The EGCG had no effect on the faecal excretion of these sterols; the faecal excretion of the neutral sterols (cholesterol and coprostanol), the acidic sterols (lithocholic and deoxycholic bile acids) and all the
measured faecal sterols (neutral plus acidic sterols) did not significantly differ between the two groups of rabbits in this study, whether the values were normalized to the intake of dietary cholesterol (Table 4.7) or not (Table 4.5). There was also no significant difference between the two groups in the excretion of total faecal fat (Table 4.4) at the end of the treatment as well as in faecal weight, % faecal moisture content and faecal pH. Furthermore, there was also no evidence that EGCG affected the percentage of dietary cholesterol absorbed or the rabbits’ whole body sterol balance (Table 4.6).

These findings are in contrast to the numerous studies [22-33], which have shown that the GT catechins increase the faecal excretion of cholesterol and bile acids in several animal models, including mice, rats and hamsters, by inhibiting their absorption from the intestinal lumen. In studies in rats, it was shown that the catechins, especially EGCG, were able to interfere with the micellar solubilisation of cholesterol in the small intestine and thereby reduce the efficiency with which cholesterol was absorbed from the lumen of the digestive tract into the lymphatic system [408, 414]. Therefore, the hypocholesterolaemic effect of EGCG was ascribed to a reduction of cholesterol absorption from the small intestine due to a reduction in the solubility of cholesterol in the mixed micelles in a dose dependant manner [402, 408].

One difference between the present study in rabbits and some of the previous studies in mice, rats and hamsters [402, 432, 469, 517] is the level of fat in the animals’ diets. In the present study, the dietary fat content was only 4%(Section 3.4.2) compared to fat levels as high as 15% [401] and 18% (w/w)[402] in previous studies. Some authors have suggested that GT catechins and EGCG interferes with fat absorption rather than with the cholesterol absorption per se. For example, Chan et al.[469] attributed the observed dose-dependant hypolipidaemic effect of a GT extract, in a group of hamsters fed a high fat diet, to a higher faecal excretion of total fatty acids as well as neutral
sterol and acidic sterols compared to the control group. Similarly, Bose et al.[517] attributed the observed reduction in body weight gain and attenuation of plasma cholesterol, in mice fed a high fat diet supplemented with 3.2g/kg EGCG for 16 weeks, to an increase in faecal lipids and therefore a decreased intestinal lipid absorption in the treatment group.

However, a decrease in intestinal fat absorption and an increase in faecal fat absorption caused by EGCG would be more likely to have an effect on body weight rather than on blood cholesterol. A study by Sogawa et al. [516], investigating the anti-obesity effect of Awa tea in Wistar rats, found that the consumption of catechins from this type of tea decreased whole body weight and fat tissue mass by increasing the daily amount of faecal lipid excretion. However, Sogawa et al. [516] used Wistar rats, which were already obese and mature, and placed on a weight loss low energy diet. In contrast, the New Zealand white rabbits in the present study were still in their growing phase. Therefore, it was not surprising that there was a significant increase in rabbits’ body weight after the four weeks of treatment (Figure 4.7). However, EGCG had no effect on their body weight; there was no significant difference in the increase in body weight between the control and 2%EGCG treated groups.

A study by Prasad et al. [485] also reported an increase in body weight in New Zealand white rabbits fed a similar 0.25% (w/w) cholesterol diet, in normal laboratory rabbit chow, over a period of four months. Similar to the present study, the rabbits’ weights progressively increased in all groups, with the values reaching over 150% of the starting body weights, but the increases were not significantly different between the groups. In the Prasad et al. study [485], the impact of food consumption could not be evaluated because there was no data presented on the rabbits’ daily consumption of food.
In the present study, the observed increase in body weight could not be ascribed to an increase in food intake during the treatment period (Section 3.5.1). Furthermore, because there were no significant differences in food intake between the groups, there was also no statistically significant difference in their cholesterol intake (Table 4.6). Therefore, the decrease in blood and LDL cholesterol observed in the 2%EGCG group at the end of the treatment, presented in Chapter 3 (Table 3.6), was not simply due to differences in food intake and hence cholesterol intake between the two rabbit treatment groups.

The cholesterol absorption, determined as the percentage of excreted cholesterol over the dietary cholesterol (Equation 4.1), was very efficient in both groups (up to 89% absorption) and did not significantly differ between the two groups at the start or at the end of the treatment period (Table 4.6). This high absorption of dietary cholesterol is consistent with but somewhat higher to the 75% absorption of dietary cholesterol observed in a previous study [521] in cholesterol fed New Zealand white rabbits using radioactive cholesterol. The lack of difference between the two groups in cholesterol absorption further suggests the involvement of other mechanisms of action for the lowering of blood and LDL cholesterol by EGCG.

Overturf et al. [521] found that a major mechanism for controlling plasma cholesterol levels in cholesterol-fed rabbits was the rate of conversion of cholesterol to bile acids and their subsequent excretion. Relevant to this, an in vitro study, in human hepatocarcinoma HepG2 cells not treated with cholesterol or bile acids [522], reported that EGCG directly increased the gene expression of the CYP7A1 cholesterol 7α-hydroxylase enzyme, which is responsible for the rate limiting biosynthesis of bile acids from cholesterol [523]. Therefore, this finding of a stimulatory effect of EGCG on CYP7A1 transcription is consistent with the catechin lowering serum and LDL
cholesterol in the cholesterol-fed rabbit model by increasing the conversion of cholesterol to bile acids which could lead to an increase in the faecal excretion of the acidic sterols as suggested by Overturf et al. [521].

However, in the present study, the excretion of the acidic sterols, the secondary bile acids lithocholic acid and deoxycholic acid, did not significantly differ between the two groups of rabbits whether the values were normalized to the intake of dietary cholesterol (Table 4.7) or not (Table 4.5). Furthermore, in a recent study, Han et al. [524] found that 0.5% (w/w) dietary catechin significantly lowered faecal lithocholic (-76%) and deoxycholic (-51%) bile acids in male Sprague-Dawley rats fed a high fat diet. Although catechin and not EGCG was used in this study [524], the finding raised the possibility that pure EGCG may be able to suppress rather than increase the biosynthesis of bile acid from cholesterol in vivo. This possibility was supported by the significant increase in the ratio of faecal cholesterol to secondary bile acids and the ratio of faecal cholesterol to deoxycholic acid seen in the 2%EGCG treatment group compared to the control group (Table 4.8), which may have been caused by a decrease in deoxycholic acid biosynthesis. However, there was no significant difference in the ratio of faecal cholesterol to lithocholic acid between the groups (Table 4.8). Therefore, it is clear that the effect of EGCG on bile acids biosynthesis requires further research.

Hepatic cholesterol is the source of primary bile acid synthesis. However, once excreted into the intestines, the primary bile acids are metabolised into secondary bile acids by the intestinal microflora. Therefore, another mechanism by which EGCG could impact on cholesterol and bile acid metabolism is by influencing the microbial flora of the gastro-intestinal tract. Such a change in microflora could explain the significant increase in the ratio of faecal cholesterol to secondary bile acids and the ratio of faecal cholesterol to deoxycholic acid observed with EGCG treatment in the present study.
(Table 4.8); a change in microflora could cause a decrease in the bacterial biosynthesis of deoxycholic acid.

In a human study [525], where elderly patients in a long term care facility were provided with a mixture of catechins (300mg) for six weeks, the faecal pH was significantly reduced at the end of the treatment period. This decrease in pH was ascribed to an increase in the lactic acid bacteria content of the stool samples collected at the end of the treatment period. Furthermore, once the supplementation with the catechin mixture was discontinued, the pH returned to the original values.

However, in the present rabbit study, the feeding of EGCG did not significantly change the faecal pH at the end of the treatment period (Table 4.4), suggesting that there was no change in the lactic acid bacteria. However, possibly unlike the elderly patients in the long term care facility [525] the rabbits in the present study were healthy without any symptoms of gastro-intestinal problems prior or during the experiment. Therefore, any change in faecal flora caused by EGCG supplementation was likely to be minimal, as seen by the lack of change in the faecal pH during the treatment period (Table 4.4).

To have an effect on the microbial flora in the rabbit, the EGCG most likely would need to be delivered past the small intestine into the large bowel where most of the bacteria reside in the rabbit [526]. In the present study, only a very small amount of the administered EGCG was recovered in the faeces; only 0.5% of the dietary EGCG was measured in the faecal samples of the rabbits treated with 2% (w/w) EGCG for 4 weeks (Table 4.9). This was similar to the findings in a rat study [527], where the average recovery of EGCG in the faeces was 0.74% over four doses. In the present study, most of the EGCG (91.5%) excreted in the faeces was found in the free unconjugated form (Table 4.9). A study by Kim et al. [382] also reported similar
findings in rats fed a GT extract where the majority of the catechins excreted in the faeces was unconjugated (free) EGCG.

Clearly, the possibility that EGCG decreased the serum and LDL cholesterol in the present rabbit study (Table 3.6) by increasing the faecal excretion of cholesterol and bile acids as was previously found in mice, rats and hamster [22-33], was not supported. The low number of rabbits in each group (6) may have been a factor, which prevented more significant effects to be seen. However, there are well known physiological differences between rabbits and their smaller animal counterparts which may be relevant to the different results observed in the present study. For example, the rabbit differs from rats in several respects including having a longer gastro-intestinal tract, a gallbladder, a higher bile flow/day, a sacculated colon, a more basic pH in lower parts of the small intestines and a more human-like distribution of bacteria[526]. Furthermore, in rabbits, the main bacteria are the bacteriodes while rats and mice have a much greater variety of bacteria and much more of them in the small intestines[526].

In conclusion, the proposed hypothesis, that the cholesterol lowering effect of pure EGCG presented in Chapter 3 was due to an increase in faecal cholesterol and/or bile acid excretion, was not supported. The faecal neutral and acidic sterols were not significantly different between the two groups of rabbits (control and 2%EGCG) and there was also no increase in the faecal fat excretion. Therefore, the systemic effects of EGCG reported in Chapter 3, an increase in the LDL-receptor and a decrease in cholesterol synthesis, are more likely to be the main mechanisms of action by which EGCG lowered the rabbits’ blood and LDL cholesterol in this study.
Chapter 5. The Effect of Food on the Systemic Absorption of Epigallocatechin Gallate in Humans

5.1 Introduction

Tea (*Camellia sinensis*) is one of the oldest beverages, which has been consumed for the past thousands of years. An increased tea consumption has been linked with a wide range of different health and pharmaceutical benefits such as antioxidative properties [438], a reduction in mortality rate from cardiovascular disease [368], a reduced development of coronary artery disease [367] and cholesterol lowering properties [360]. Furthermore, tea consumption’s excellent long term safety record influenced a significant rise in research on individual tea components, particularly its major component epigallocatechin gallate (EGCG), as attractive targets for development of nutraceuticals and functional foods [362].

As the most abundant green tea (GT) constituent, EGCG has been the focus of research in the past few years in relation to the reduction of morbidity and mortality, consequences of cardiovascular diseases. Several mechanisms of action have been proposed as to why EGCG may benefit health in humans, such as the reduction of fat absorption and the lowering of plasma cholesterol and triglycerides as seen in animal models [372] such as the cholesterol-fed hypercholesterolaemic rabbit (Chapters 3 and 4).

However, many of the experiments of this type in humans used hot/cold GT preparations or whole GT extracts in powder form. The GT extracts and GT preparations used in health effect studies in humans have varied considerably in their EGCG and other catechin content and also in other components naturally found in GT such as caffeine [242, 372, 407, 528, 529]. Therefore, despite the fact that EGCG is the predominant compound found in these preparations it is still uncertain whether pure
EGCG would exhibit the same health properties in humans as EGCG imbedded in a polyphenolic matrix.

The relatively recent availability of a pure and relatively affordable EGCG product (TEAVIGO, DSM Nutritionals, Ermington, Australia) has enabled the development of studies with stable and quantity-controlled amounts of EGCG [420, 530]. This means that hard gelatine capsules containing precisely measured amounts of EGCG can be utilised in controlled clinical trials and that measured amounts of the crystalline form of EGCG can be incorporated into food products for use as nutraceuticals or functional foods.

5.1.1 Stability of EGCG in pure form and in food matrices

Based on the manufacturers information (TEAVIGO, DSM Nutritionals, Ermington, Australia) and previous research (Chapters 3 and 4) conducted in our laboratories [531], EGCG in crystalline form has shown excellent stability with the use-by date well over one year from its production. However, incorporating EGCG into food products requires dissolving the crystalline form in various liquids which could affect the stability of EGCG and this could lead to its degradation [531].

The stability and degradation of solutions containing EGCG are significantly affected by exposure to temperatures between 25-100ºC, where simultaneous degradation and epimerization of (-)-EGCG can occur [532]. This effect can also occur at lower temperatures (below 25ºC) but to a much lesser extent [533, 534]. Apart from the temperature, the pH of the solution is a very influential factor in the stability of EGCG. Keeping the pH below four has been associated with a lower degradation of EGCG and, when combined with lower temperatures (4ºC), the stability of EGCG is markedly improved [534].
5.1.2 Absorption, metabolism and bioavailability of EGCG

The major site of EGCG absorption in humans is in the mucosa of the small intestine but only a small portion of orally ingested EGCG is absorbed and appears into the systemic blood circulation, while the rest is eliminated in the faeces or possibly via the bile during the first pass through the liver [384]. The EGCG absorbed into the blood is mainly found in free form [378]. This free form is metabolically unmodified EGCG that passes through the wall of the small intestine and eventually gets into the systemic circulatory system. It has an unmodified 3-O-galloyl moiety in its structure [380, 528] and accounts for up to 77% of the total EGCG found in plasma [380].

Some conjugated forms of EGCG are also found in plasma, such as glucuronides and sulphates which are formed by enzymatic reactions occurring in the liver [375] which convert EGCG to more hydrophilic compounds [535]. Methylation of EGCG can also occur in the liver [536] by the action of cytosolic catechol-O-methyltransferase which produces 4″-O-methyl-EGCG and 4′,4″-di-O-methyl-EGCG. Furthermore, microbial metabolism occurs in the large intestine [537] where EGCG and other flavonoids are converted into smaller phenolic acids that can be absorbed into the circulation from the colon.

Glucuronidation of EGCG with UDP-glucuronic acid is catalysed by microsomal UDP-glucuronosyltransferase and sulfatation of EGCG with 3′-phosphoadenosine 5′-phosphosulfate is catalysed by cytosolic sulfotransferases, while catechol-O-methyltransferases catalyse the methylation of EGCG tranferring the methyl group from S-adenosylmethionine (SAM). These three reactions are reported to occur at different sites of the EGCG molecule [538].

The low bioavailability of EGCG is thought to be caused by several different factors: it’s relatively high molecular weight, it’s structural composition of more than
five hydroxyl groups [539] and the presence of two gallate rings [540]. Furthermore, the high pH environments (up to 8.5) found in the upper two thirds of the small intestine, through which EGCG travels through prior to absorption [528] can further contribute to its poor stability because the catechin has been shown to rapidly oxidise in alkaline environments [541]. The extent and rate of EGCG absorption in the small intestine is dependent on gastric and intestinal transit time and intestinal membrane permeability [542] and the dose of EGCG orally consumed [420, 543]. Higher doses of orally ingested EGCG are reported to saturate the hepatic metabolic pathways for glucuronidation and O-methylation and therefore can increase the amount of free EGCG in the plasma relative to conjugated forms [420, 440, 543].

5.1.3 Pharmacokinetic studies of EGCG in healthy human volunteers

Information on the pharmacokinetic properties of pure EGCG in humans are limited [380, 420]. Studies have primarily focused on preparations where EGCG was imbedded in a polyphenolic complex, as part of GT extracts [380, 440, 528]. When EGCG was orally administered in pure form [420] at a dose of 1.6g, physiologically-relevant plasma EGCG concentrations (greater than 1μmol/l) capable of having beneficial health effects were achieved. There was variation between individuals; the peak EGCG concentration ($C_{\text{max}}=3392\text{ng/ml}$) was reached between 1.3 and 2.2h ($T_{\text{max}}$) and the mean elimination half-life ranged from 1.9 to 4.6h. In this study, only encapsulated EGCG was studied as the method of delivery in healthy human volunteers after a 10h fast [420].

To date, only one study in humans examined the effectiveness of food oral bioavailability of EGCG imbedded in GT extract [440]. In this study, participants were provided the extract with or without a light breakfast after an overnight fast. The extract
was provided to give different doses of EGCG (400, 800 or 1200mg). The findings of this study indicated that EGCG, when taken in the form of a polyphenolic complex, showed a greater systemic absorption when the extract was taken on an empty stomach after an overnight fast. The light breakfast of one or two 4-oz muffins reduced the systemic absorption of EGCG by 71% (400mg), 64% (800mg) and 56% (1200mg). The areas under the absorption curve (AUC) were 127±47 (400mg), 254±214 (800mg) and 686±394 (1200mg) without food but only 37±25 (400mg), 91±37 (800mg) and 299±286 with the muffin breakfast.

The doses of 400mg and 800mg of EGCG, were very well tolerated under the fasting conditions with only mild symptoms of nausea, dyspepsia, abdominal pain and headaches reported. However, the higher dose (1200mg) was less well tolerated; nausea, eructation, abdominal discomfort and the occurrence of a rash were reported by some participants [440].

The modification and use of foods in order to deliver beneficial health outcomes, commonly referred to as functional foods, is a fast growing area of research in the last few years [544]. Apart from the health benefits that these functional foods can bring, the convenience of delivering the functional component within the food will reduce the fasting time and also increase the convenience of consumption. A recent study by Hirun and Roach [545] found that EGCG imbedded in a strawberry sorbet had very good stability during storage at -20°C for 16 weeks and it also had a higher stability under simulated digestion conditions than EGCG in buffer only, a benefit primarily ascribed to its low pH (3.1 – 3.3). In another study by Greenet al. [546], the stability of EGCG was also enhanced under simulated digestion conditions, when GT was mixed with fruit juices. However, there are no reports on the effectiveness of EGCG once it is incorporated into a food product relative to any health outcomes in humans.
5.1.4 Aims and Hypotheses

Therefore, based on the findings of a previous study [440], which investigated the effect of dosing conditions on the oral bioavailability of EGCG imbedded in a polyphenolic complex, the question was raised whether similar systemic absorption effects would be seen with pure EGCG. In order to test this, a dose of 500mg of pure EGCG was selected as suitable in regards to being unlikely to cause unwanted effects such as gastro-intestinal problems. Furthermore, the ingestion conditions (with and without breakfast) were expanded to test whether EGCG imbedded in a food product, such as a strawberry sorbet, could enhance the absorption of pure EGCG primarily due to the food products low pH.

Based on previous findings [440], it was hypothesised that ‘pure EGCG will be absorbed better when given as a capsule without food and give the highest plasma EGCG concentrations when given in this format compared to when given as a capsule with a breakfast’. However, it was also hypothesised that ‘incorporating EGCG in a food product like a strawberry sorbet will improve the absorption of EGCG compared to taking the catechin in capsule form with a typical breakfast’. In other words, EGCG incorporated in a strawberry sorbet would be absorbed as well as EGCG given in capsule form without food and better absorbed than EGCG given in capsule form with food given as a light breakfast cereal after an overnight fast.

Therefore, the main aim of this study was to determine which delivery format was the best of the three methods tested for the oral delivery of pure EGCG in humans: 1) EGCG given by itself within capsules, 2) EGCG given in capsules taken with food in the form of a light breakfast or 3) EGCG given as a powder incorporated in a strawberry sorbet.
5.2 Methods

5.2.1 Preparation of EGCG delivery products

Two different delivery products containing EGCG were evaluated in this study, capsules (2 x 250mg/capsule) and strawberry sorbet, both giving a dose of 500mg of EGCG. All capsules and the strawberry sorbet were prepared in the food science laboratories at the Ourimbah campus of the University of Newcastle following the Australian Food Safety guidelines [547].

5.2.1.1 Capsule preparation

Clear gelatine capsule casings (Melbourne Food Ingredient Depot, Australia) size 00 were filled with EGCG using a capsule filler (Cap-M-Quick, Murrieta, USA). Each capsule was weighed prior to filling and post filling to determine the amount of EGCG they contained. The capsules averaged 250±5mg; capsules showing a variation greater than 2.5% from the target weight were discarded and not used in the study.

5.2.1.2 Strawberry sorbet preparation

Strawberry sorbet was prepared based on a recipe (Table 5.1) previously developed in our Food Science and Human Nutrition laboratories at the Ourimbah campus of the University of Newcastle by Hirun and Roach [545] except that a strawberry flavoured whey protein isolate (WPI) (Vital Strength Nutraceuticals, Marrickville, NSW, Australia) was used instead of plain WPI (Designer Physique, The Pines Elanoro, QLD, Australia).

The method of preparation was also modified. Briefly, 300g of frozen strawberries (Creative Gourmet Pty Ltd, Silverwater, NSW, Australia) were thawed overnight in a fridge and juiced (Philips HR2820, Sydney, NSW, Australia) the
following day. The strawberry juice was passed through a double-layered cheese cloth to remove any seeds or unprocessed residue, and the weight was adjusted with reverse osmosis (RO) water to 250g. The mixture was placed in a pot and heated until the temperature reached the boiling point and then 45g of caster sugar (CSR, Yarraville, VIC, Australia) was added. The mixture was removed from the heat and vigorously stirred using a whisk until all the sugar was dissolved. Once the sugar was dissolved, 30g of strawberry flavoured WPI (Vital Strength Nutraceuticals, Marrickville, NSW, Australia) was added to the mix followed by 0.4g of carboxyl methyl cellulose (Sigma Aldrich, Castle Hill, NSW, Australia).

**Table 5.1** Ingredients and percentages (w/w) used in the preparation of strawberry sorbet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight (g)</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strawberries (Creative Gourmet Pty Ltd, Silverwater, Australia)</td>
<td>250</td>
<td>76.63</td>
</tr>
<tr>
<td>Caster Sugar (CSR, Yarraville, Australia)</td>
<td>45</td>
<td>13.79</td>
</tr>
<tr>
<td>Strawberry Flavoured WPI (Vital Strength, Nutraceuticals, Marrickville,</td>
<td>30</td>
<td>9.20</td>
</tr>
<tr>
<td>NSW, Australia)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxyl methyl cellulose (Sigma Aldrich, Castle Hill, Australia)</td>
<td>0.40</td>
<td>0.12</td>
</tr>
<tr>
<td>EGCG (TEAVIGO, DSM Nutritionals, Ermington, Australia)</td>
<td>0.84*</td>
<td>0.25</td>
</tr>
<tr>
<td>Total Weight</td>
<td>326.24</td>
<td>100</td>
</tr>
</tbody>
</table>

*For the control strawberry sorbet without EGCG, 0.84g of RO water was added instead of EGCG.

Once all the ingredients were dissolved, the solution was placed in a fridge until the temperature reached 4°C, after which 0.84g of EGCG (TEAVIGO, DSM Nutritionals, Ermington, NSW, Australia) was added through a fine sieve. A control sample of strawberry sorbet was also prepared in which no EGCG was added. The
solution was then whisked vigorously over a bowl containing ice water and then it was poured into the pre-cooled turning chamber of the GC5000 ice cream maker (De'Longhi, Prestons, NSW, Australia). Once the mixture was completely frozen, 200g aliquots were transferred into plastic storage containers and stored in a freezer at -18°C until used.

5.2.1.2.1 Recovery of EGCG from strawberry sorbet

The preparation method for the strawberry sorbet required heating and exposure to light and as these conditions are not favourable for EGCG [532-534], a recovery study was conducted in order to determine the amount of EGCG left in the food product after preparation.

The extraction of EGCG was done on five samples of strawberry sorbet containing EGCG and on one sample of the control strawberry sorbet with no EGCG. To determine if there was a uniform distribution of EGCG through the frozen aliquots of strawberry sorbet two different sections of each storage container, the upper and the lower portion, were selected for sampling and the following procedure was applied for both portions. Briefly, 50g of frozen strawberry sorbet was placed in a large beaker and allowed to thaw completely in a fridge at 4°C. Once thawed, 100ml of 4-aminosalycilic acid solution (100mmol/l in methanol) was added to the beaker and samples were placed in a shaking water bath (Ratek Instruments, Boronia, Australia) at 60°C for 60min. The samples were then filtered through a Whatman No.1 filter paper under vacuum and the residue was returned to the beaker, washed twice more with 50ml of 4-aminosalycilic acid solution (100mmol/l in methanol) and the washes filtered through Whatman No.1 filter paper. All filtrates from each 50g strawberry sorbet sample were combined and filtered through a 0.45μm syringe nylon filter (Phenomenex, Pennant
Hills, Australia) into a brown HPLC vial and stored in a fridge at 4°C until injection (20µl) onto the HPLC system.

5.2.1.2.2 HPLC settings and identification of internal standard and EGCG on the HPLC chromatogram

Equipment and chromatographic conditions used for the analysis of faecal EGCG are identical as described previously in the determination of purity of TEAVIGO (Section 2.4.2).

5.2.1.2.3 The standard curve and calculation of the EGCG concentration

The elution times of EGCG and the internal standard (4-aminosalicylic acid) were identified by injecting each separately. No interfering peaks were found when extractions of control strawberry sorbet samples containing no added EGCG or extractions of control strawberry sorbet samples spiked with EGCG and the internal standard, were injected.

The samples for determination of the standard curve were prepared in methanol containing 100mmol/l of 4-aminosalicylic acid and EGCG at concentration range between 0 – 2mmol/l. The standard curve was developed by plotting the peak area ratios (peak area of EGCG divided by the peak area of the internal standard, EGCG/IS) against the known concentration of EGCG in each standard.

The concentration of EGCG in each injected strawberry sorbet sample was determined by dividing the peak area ratio (EGCG/IS) minus the y-intercept by the slope of the standard curve. The percentage recovery of EGCG was calculated by dividing the concentration of EGCG in the strawberry sorbet sample by the concentration at which it was prepared and multiplying by one hundred. The unpaired
student’s t-test was done using Microsoft Excel 2003 SP3 Package (Microsoft Australia, North Ryde, NSW) to test for any difference between the upper and lower sections of the strawberry sorbet.

5.2.2 Participants

5.2.2.1 Ethics approval

Ethics approval for this study was granted from the Human Ethics committee of the University of Newcastle (H-2008-0089), NSW, Australia. Informed written consent was also obtained from all participants prior the commencement of the study.

5.2.2.2 Recruitment and selection criteria

The participants for this study were staff and students of the School of Environmental and Life Sciences (SELS) at the Ourimbah campus of the University of Newcastle, Australia. All participants were recruited from responders to advertisement posters and generic emails. Each responder received an information statement letter, detailing the procedures, protocols and possible associated risks of participation in this study.

Initially, 18 responders showed interest in participating. However, after implementing the selection criteria, only 6 participants were selected for the study. The participants were selected for this study if they were healthy male or female aged between 18 – 65 years of age. Volunteers were excluded from a study if they were on any medication, dietary supplement or functional food to lower cholesterol or triglycerides prescribed by a health care provider. Exclusion criteria also included the following: baseline triglyceride levels of 4.0mmol/l or greater as determined using the finger-prick test and the Accutrend Plus hand-held instrument (Roche Diagnostic,
Australia), a history of coronary heart disease, a body mass index of 35 or higher (calculated as weight in kilograms divided by the square of height in meters), uncontrolled resting hypertension (greater or equal to 160/95 mmHg determined using a sphygmomanometer), any known active pulmonary, hematologic, hepatic, gastrointestinal or renal disease, pre-malignant or malignant disease, diabetes, thyroid dysfunction, or any pathology values known to be abnormal. All exclusion and inclusion criteria were developed based on the internationally accepted guidelines [548] and studies conducting similar research conducted in humans using pure EGCG and EGCG imbedded into the polyphenolic complex [420, 440, 549].

Volunteers who consumed dietary supplements or functional foods by choice such as fish oils, cholesterol lowering functional foods, niacin at doses higher than 400mg/day, soluble fibre supplements or antioxidants were asked not to consume these for at least 2 weeks before the start and during the duration of the study.

Of the 6 volunteers chosen to participate, four (3 males and 1 female) successfully completed the study. However, two participants withdrew from study at clinic 2 due to discomfort associated with the multiple blood collection times.

5.2.2.3 Study clinics

The participants attended three clinics in total, one for each of three EGCG methods of delivery with at least a one-week washout period between the clinics.

On each clinic visit, the participants’ height was measured using a stadiometer (S&M Supplies, Rose Park, SA, Australia) and weight using bathroom scales accurate to 0.1 kg (A&D Mercury, Australia). Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m$^2$). Resting blood pressure was determined using the mercury (Hg) sphygmomanometer (Livingstone
International, Roseberry, Australia) while participants were in a sitting position after they had been resting for at least 5min. Blood pressure (BP) was determined while the arm was rested on a desk by recording the height of the Hg in mm at the first and fifth Korotkoff sounds which were taken to represent the systolic blood pressure (SBP) and diastolic blood pressure (DBP) respectively, to the nearest 2 mmHg [550]. Three sets of readings were measured at each visit and the average value was taken as the participants’ resting blood pressure.

On each of the three clinics participants, after an overnight fast of at least 10h, ingested 500mg of EGCG given as two capsules containing 250mg EGCG without any breakfast, two capsules containing 250mg EGCG with a provided breakfast or 200g of strawberry sorbet containing 500mg of EGCG made as described in Section 5.2.1.2 and Table 5.1. The sequence for the three EGCG delivery methods was randomised for each participant. The two capsules of EGCG taken without breakfast were consumed with 100ml of distilled water. For the two capsules of EGCG taken with breakfast, they were consumed with 100ml of distilled water after at least half of the provided breakfast was eaten. The breakfast consisted of 50g of breakfast cereal (Special K, Kellogg’s, Australia) served with 200ml of full cream milk (Dairy Farmers, Australia).

All participants were also provided with the same standardised lunch and a drink after the third hour of blood collection. Furthermore, during the clinic day, participants did not consume any other food or beverage apart from water. Briefly, each participant was provided with two sandwiches and a glass (200ml) of orange juice (Daily Juice, Docklands, Australia). Each sandwich consisted of 2 slices of wholemeal bread (Bakers Delight, Tuggerah, Australia), 4 thin slices of ham (Hans foodservice, Blacktown, Australia), 1 slice of cheese (Kraft, Fishermans Bend, Australia), 0.5 of raw tomato
(Woolworths, Tuggerah, Australia) and 1 large leaf of lettuce (Woolworths, Tuggerah, Australia).

5.2.2.3.1 Breakfast and lunch composition

The macronutrient composition and the energy content of the breakfast, the strawberry sorbet and the lunch were analysed using FoodWorks 2007 (v5 SP1) (Xyris Software, Australia).

5.2.2.3.2 Blood collection and handling

Venous blood samples for measurement of EGCG were collected by a suitably qualified registered nurse after the insertion of an intravenous catheter (BD, North Ryde, Australia) in the median cubital vein of the antecubital fossa. The intravenous catheter was connected to a SmartSite® needle-free valve (Altaris Cardinal Health, Sydney, Australia), which allowed an access point for repetitive blood collection. Approximately 5ml of blood was collected into lithium heparin tubes (BD, North Ryde, Australia) using a luer adapter connected to a disposable luer holder (BD, North Ryde, Australia). In total, seven blood samples were collected at each clinic visit from each participant.

The first sample was collected before the ingestion of EGCG (time 0) and the other six samples were taken at 0.5, 1, 2, 3, 5, and 8h after the ingestion of EGCG was completed (once the capsules and/or the food provided was completely ingested), as indicated in figure 5.1.

Occasionally, the intravenous catheter became blocked and therefore did not allow for smooth blood collection. In this case, the catheter was purged by injecting 2.5ml of sterile isotonic 0.9% NaCl solution (AstraZeneca, North Ryde, Australia). The
A scheduled blood sample was then not collected for a further 10 min and the first blood sample collected (5 ml) was discarded. A second sample (5 ml) was then collected for analysis.

![Diagram showing blood collection times]

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

The blood samples were stored on ice in a dark thick-wall isolated container to protect from possible light and temperature degradation. Within 60 min of blood collection, the tubes were centrifuged at 3000 rpm for 15 min at 4°C (Sigma 4K 15 centrifuge). Plasma was siphoned off and aliquoted under red light and within 30 min of centrifugation, samples were stored at -84°C in cryogenic vials until assayed.

### 5.2.2.4 Determination of EGCG in plasma samples

For the determination of plasma EGCG, a highly specific and relatively easy method was developed and validated using a high pressure liquid chromatography
(HPLC) system equipped with a diode array detector (DAD) and a mass spectrometer (MS). All samples were analysed at the Food Science and Human Nutrition Laboratories of the University of Newcastle at Ourimbah.

5.2.2.4.1 Chemicals and reagents

All chemicals, (+)-catechin, (-)-epigallocatechin gallate, ascorbic acid, disodium ethylenediamine tetra acetate (EDTA), potassium dihydrogen phosphate (KH$_2$PO$_4$), formic acid, acetonitrile and ethyl acetate), were purchased from Sigma Aldrich (Castle Hill, NSW). The TEAVIGO was purchased from RejuvaCare (Sydney, Australia). Deionised (DI) water with resistivity greater than 18MΩcm, used in the preparation of the assay reagents and the HPLC mobile phases, was prepared on the day with a Millipore Milli-Q water purification system (Millipore Australia, North Ryde, NSW).

5.2.2.4.2 Preparation of stock and standard solutions

The vitamin C and EDTA solution (VcEDTA) was prepared by dissolving the KH$_2$PO$_4$ (1.36g), ascorbic acid (5g) and disodium EDTA (25mg) in 25ml of deionised water and adjusting the pH to 2 using the formic acid. The dissolution reagent was prepared by dissolving the ascorbic acid (20g) in 100ml of 15% (v/v) acetonitrile and the pH was adjusted to 2 using formic acid.

Each solution was filtered through a 0.45μm nylon syringe filter (Phenomenex, Pennant Hills, Australia), aliquoted in cryo-vials (0.5ml) and stored at -84°C until used to make working standards.
On the day prior to the preparation of the working standards, the EGCG (50μg/ml) and catechin (50μg/ml) stock solutions were thawed out overnight in a fridge at 4°C. The EGCG stock solution was diluted up to 5ml with the VcEDTA solution, providing the EGCG working standard with the highest concentration (1000ng/ml). Six additional working standard solutions were prepared by serial dilution with the VcEDTA solution, thus providing a concentration range from 15.63ng/ml to 1000ng/ml. The catechin stock solution was diluted in 50ml of the VcEDTA solution to provide an internal standard (IS) solution (100ng/ml) which was stored at 4°C and used within 5 days of preparation.

An EGCG standard curve was then developed using a pooled plasma (PP), which was prepared by combining fasting plasma samples taken before the ingestion of EGCG (time 0) from all the participants at each of the clinics. For the EGCG standard curve, different 200μl samples of PP were spiked with 200μl of each EGCG working standard and 200μl of the internal standard and the tubes were vortexed vigorously for 30s.

**5.2.2.4.3 Preparation of quality control samples**

On the days of sample analyses, an aliquot of PP was thawed out, centrifuged at 3000rpm at 4°C in the dark to pellet any fibrin clots, and the cleared plasma supernatant used to prepare control samples to monitor any inter-assay variation in the procedure. For preparation of the low EGCG concentration control samples (CL), 62.5ng/ml of EGCG (200μl) was added to 200μl of PP into 10ml Kimble vials kept on ice and for preparation of the high EGCG concentration control samples (CH), 500ng/ml of EGCG (200μl) was added. The internal standard (200μl) was also added and the tubes were vortexed vigorously for 30s.
5.2.2.4.4 Preparation of participants’ plasma samples

On the days of the assays, the participants’ plasma samples were thawed out and centrifuged at 3000rpm at 4°C in the dark to pellet any fibrin clots. Samples of the cleared plasma supernatant (200μl) were then transferred into 10ml Kimble vials kept on ice. The VcEDTA solution (200μl), not containing any catechins, was added to the 200μl of plasma. Then, 200μl of internal standard (100ng/ml) was added and the tubes were vortexed vigorously for 30s.

5.2.2.4.5 Extraction of EGCG from plasma and HPLC analysis

The extraction of EGCG was done using ethyl acetate (1ml) and the vials were vigorously shaken using a multi-tube vortex mixer for 5min[393]. The tubes were then centrifuged at 1500rpm for 10min at 4°C in the dark to separate the organic layer. The supernatant (900μl) was carefully transferred into a glass culture tube. The ethyl acetate extraction procedure was repeated twice more and all extracts were combined together and evaporated to dryness under a stream of nitrogen on a dry block heater set at 50°C (Ratek Instruments, Boronia, Australia).

The dried samples were redissolved in 200μl of 20% ascorbic acid (w/v) in 15% acetonitrile (v/v) pH2.0 and transferred into microcentrifuge tubes (Eppendorf, Riverstone, Australia). The tubes were then centrifuged at 10,000rpm at 4°C for 15min to pellet any undissolved material and 150μl of the supernatant was transferred into dark HPLC cups containing appropriate volume inserts and the samples were sequentially injected (50μl) onto the HPLC column using an auto-injector.

All extracted plasma samples were analysed by HPLC during analysis runs, which lasted less than 30h. A typical run consisted of the seven plasma samples collected from one participant during the absorption study (Section 5.2.2.3.2 and Figure...
5.1) and two control samples (CL and CH described in Section 5.2.2.4.3), which were injected before and after the participants’ samples. Each of the participants’ samples and both control samples (CL and CH) were analysed in duplicate.

A standard curve with EGCG concentrations between 15.63 and 1000ng/ml (Section 5.2.2.4.2) was then used to determine the concentration of EGCG in the participant’s samples. Samples of the working standard solutions were analysed by HPLC, in duplicate in one run that lasted less than 24h, to generate the standard curve.

**5.2.2.4.6 Equipment and chromatographic conditions**

The HPLC analysis was performed using a Finnigan Surveyor System (Thermo Fisher Electron Corporation, Sydney, Australia) equipped with a quaternary pump (Surveyor LC Pump 1.4) and samples, placed on a tray control temperature set at 4°C, were introduced with an autosampler (Surveyor AS1.4) fitted with a 100μl sample injection loop. Separation was performed using an analytical Prodigy ODS(3) 250x4.6mm 5 micron column, protected by an analytical-size guard column (Phenomenex, Pennant Hills, NSW), run at 26°C in a column oven. The elution times for the IS and EGCG were monitored using a Surveyor Photo-Diode array detector (Surveyor PDA 1.4) and UV-VIS absorption spectra was acquired over the range of 200 – 500nm. The outlet of the PDA detector was directly connected to a mass spectrometer (Surveyor MSQ Plus 1.4 SUR 1). The full portion of the effluent was delivered into the ion source of the mass spectrometer.

The mobile phases consisted of (A) 0.2%(v/v) formic acid (pH2) and (B) acetonitrile. The auto-injector and needle wash solution consisted of acetonitrile : DI water (50:50 v/v). All solvents were filtered through a 0.45μm Millipore cellulose filter (Millipore Australia, North Ryde, NSW) and degassed just prior to use.
The system was run at a flow rate of 1ml/min with 87.5% mobile phase A and 12.5% of mobile phase B for the first 10min after each injection. Then, for the following 30min, the system was switched to a linear gradient concentration increase of mobile phase B to 25%. Mobile phase B was held for an additional 10min at this concentration (25%) and then gradually decreased to 12.5% over the next 10min. For the following 20min, mobile phase A and B were run as described for the starting conditions (87.5% A and 12.5% B) to allow the column to re-equilibrate before the injection of the next sample.

The signals from the detectors (PDA and MS) were recorded and analysed using the ExcaliburTM (v1.4 SR1) software (Thermo Fisher Electron Corporation, Sydney, Australia) installed on the computer assigned as a remote control operating system.

5.2.2.4.6.1 Tuning and setting parameters of the mass spectrometer

The MS (Surveyor MSQ Plus 1.4 SUR 1), equipped with electrospray ionisation detector (ESI) set in full scan negative mode (100 – 1000 m/z), was initially used to optimise the analysis of EGCG. The tuning of the MS was optimised after the injection of 1μg/ml pure EGCG or (+)-catechin (as internal standard) in 0.2% formic acid (pH 2): acetonitrile (87.5 : 12.5 v/v) at a flow rate of 1 ml/min representing the chromatographic conditions prior the MS inlet. The ESI probe temperature was set to 629°C, the cone voltage to 75V and the needle source voltage to 4kV. Nitrogen was used as the nebulising gas at a flow rate of 50l/hr in order to create a fine spray of sample/solvent droplets.

From the optimisation procedure it was determined that the IS, (+)-catechin, would elute in the time range between 9-13min and EGCG between 19 – 26min. For subsequent analyses, the MS was operated in the selective ion monitoring
mode (SIM) with negative polarity to detect the IS and EGCG as they eluted from the HPLC column (See figure 5.3).

5.2.2.4.6.2 Linearity, limit of detection and limit of quantification

The EGCG standard curve was prepared over seven different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.63ng/ml) and five injections were made at each level. The peak area ratios of EGCG over IS were plotted against the corresponding concentration and linear regression was used to generate a standard response curve and a linear equation \(y=0.003x – 0.018; R^2=0.9947\).

The limit of detection (LOD) was set as the concentration of EGCG giving a signal to noise ratio of three to one (S/N = 3:1) [529, 551]. The limit of quantification (LOQ) was set as the EGCG concentration that gave a CV of 5% or less [551].

5.2.2.4.7 Plasma extraction efficiency and assay variation

The extraction efficiency of EGCG from plasma was evaluated for two EGCG concentration levels. This was done by spiking the blank plasma sample with 100ng/ml of internal standard and with the two concentrations of EGCG, a control low (CL) sample (62.5ng/ml) and control high (CH) sample (500ng/ml). Each sample (CL and CH respectively) were prepared in ten replicates and analysed on a same day of extraction using the method described above.

The intra-assay and inter-assay CV for the measurement of EGCG in plasma were also determined using the CL and CH quality control plasmas.
5.2.2.5 Statistical and pharmacokinetic analysis

The plasma EGCG concentration was calculated using Microsoft Excel 2003 SP3 Package (Microsoft Australia, North Ryde, NSW). The means, standard deviations and the intra and inter assay coefficients of variation for the assay quality control samples were calculated using this package as well.

Pharmacokinetic analysis was also performed using Microsoft Excel 2003 SP3 package (Microsoft Australia, North Ryde, NSW) in accordance with current industry guidance for orally administered drug products [548]. The maximum concentration of EGCG from time 0 to 8h was defined as $C_{\text{max}}$, with $T_{\text{max}}$ being a time required to reach the $C_{\text{max}}$. The concentration of plasma EGCG at the end of the dosing interval was defined as $C_{\text{min}}$ and mean concentration during the dosing interval was defined as $C_{\text{av}}$. Degree of fluctuation (DF) was also determined based on the formula $(C_{\text{max}} - C_{\text{min}})/C_{\text{av}}$ while swing of plasma EGCG was determined using $(C_{\text{max}} - C_{\text{min}})/C_{\text{min}}$. The plasma EGCG elimination half-life ($T_{1/2}$) was calculated based on the formula $T_{1/2} = 0.693/Ke$ where $Ke$ is the slope of the logarithmically transformed (ln) linear regression of plasma EGCG concentrations [420, 528]. Area-under-the-curve (AUC$_{0-8}$) analysis was determined using PK Functions Add-Ins for Microsoft Excel [552] using the linear trapezoidal rule from 0-8h.

The statistical analysis of pharmacokinetic variables and anthropometric measurements was performed using Statistical Package for Social Sciences (PASW Statistics 17) (SPSS Inc, Chicago IL). The one-way ANOVA and the Bonferroni post-hoc test were used to determine differences in values of anthropometric and pharmacokinetic variables between the three sampling conditions. The threshold for all statistical significances was set at $p<0.05$ level. All pharmacokinetic data was calculated and presented in accordance with internationally accepted and standardised methods.
The correlations were also analysed using linear regression analysis of the PASW Statistics 17.
5.3 Results

5.3.1 Measurement of EGCG in strawberry sorbet

The preparation of the strawberry sorbet exposed EGCG to conditions that could have affected its stability and possibly lead to its degradation in the food matrix [531]. Therefore, in order to ascertain that the amount of EGCG added in the sorbet was the amount that the participants would be receiving, EGCG was extracted from the strawberry sorbet after it had been prepared and frozen for at least one week at -20°C, as described in section 5.2.1.2.1. The extraction was done on five different storage containers of strawberry sorbet with EGCG and on one storage container of strawberry sorbet without EGCG (control).

There was no trace of EGCG detected on the HPLC chromatogram of the extract from the control sorbet sample and there were no other interfering peaks (Figure 5.2). As some strawberry sorbet containers appeared to have two horizontal sections with slightly different colouring, the two sections (upper and lower sections) were sampled for each sorbet storage container. The amount of EGCG extracted was measured and the recovery of EGCG from the strawberry sorbet was expressed as a percentage of the EGCG originally added during the preparation of the strawberry sorbet. The results, presented in Table 5.2., demonstrated that more than 97% of the EGCG originally added to the strawberry sorbet was extracted. There was also no significant difference between the concentration of EGCG in the upper and lower portions of the strawberry sorbet containers (p=0.999, unpaired Student’s t-test), the measurements and recovery for EGCG were virtually identical (Table 5.2).
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). In the control sample (A) there was no trace of internal standard or EGCG on the chromatogram at their respective retention times as indicated in chromatogram B. Chromatographic conditions were as described in Section 5.2.1.2.2.

Table 5.2 Results for the recovery of EGCG from strawberry sorbet.

<table>
<thead>
<tr>
<th>Section</th>
<th>n</th>
<th>EGCG added to strawberry sorbet (mg/g)</th>
<th>EGCG extracted from strawberry sorbet (mg/g) †</th>
<th>EGCG recovery (%) †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper</td>
<td>5</td>
<td>2.50</td>
<td>2.44±0.03</td>
<td>97.6±1.2</td>
</tr>
<tr>
<td>Lower</td>
<td>5</td>
<td>2.50</td>
<td>2.43±0.04</td>
<td>97.2±1.4</td>
</tr>
</tbody>
</table>

†Values for the recovery of EGCG represent the Mean±SD of five different sorbet containers.
5.3.2 Measurement of EGCG in human plasma

The methods used for the HPLC analysis of individual catechins in tea samples commonly use UV detection. However, the amounts observable in plasma are well below the detection limits of UV detectors. The use of a MS as a detector for HPLC offers a greater detection sensitivity and also greater specificity for the measurement of EGCG in biological liquids such as plasma. Therefore, in this section, the HPLC analysis of EGCG in human plasma using a MS as a detector was validated for specificity and sensitivity.

5.3.2.1 Linearity, limit of detection and limit of quantification

Figure 5.3 shows a typical HPLC chromatogram using the MS, set in SIM mode, as the detector. It shows that excellent chromatographic separation of the IS (11.28 min) and EGCG (22.25 min) was achieved without any interference from other plasma constituents.

To determine the linearity of response, the limit of detection (LOD) and the limit of quantification (LOQ), EGCG was added to plasma at seven different concentrations in quintuplicates (Table 5.3) and measured using the HPLC-MS. Figure 5.4 shows that the standard curve for EGCG was linear over the range of 15.63 to 1000 ng/ml and had a regression equation of $Y_{(\text{peak area ratio EGCG/IS})} = 0.0033X_{(\text{ng EGCG/ml})} - 0.0165$ with a correlation coefficient very close to perfect ($R^2=0.9952$) and the LOD was 15.63 ng/ml.

The LOQ was evaluated as the EGCG concentration that resulted in a CV of 5% or less; the results showed that, using this system, the lowest measurable concentration in terms of precision, with a CV of 4.78%, was 31.25 ng/ml.
Figure 5.3  Typical chromatogram of IS (100ng/ml) and EGCG (500ng/ml) analysed by HPLC-MS-ESI set in negative polarity SIM mode, as described in Section 5.2.2.4.6.1. When the EGCG peak in (A) was selected the molecular ion for EGCG (molecular mass -1) was clearly seen at 457 m/z (B).

5.3.2.2 Plasma extraction efficiency and assay variation

The plasma extraction efficiency and the intra-assay variation were evaluated by doing ten separate extractions of EGCG added to plasma at two concentrations, one CL and one CH. The HPLC-MS analysis using the standard curve in Figure 5.4 showed that the percentage recovery for CH was excellent and averaged 98% while the recovery for CL was very good at 87% but significantly lower (p<0.001) than for CH (Table 5.4). The intra-assay variation was excellent with a CV of less than 5% for CL and just over 2% for CH.
Table 5.3 Mean, standard deviation and coefficient of variation for seven different concentrations of EGCG.

<table>
<thead>
<tr>
<th>EGCG Concentration (ng/ml)</th>
<th>n</th>
<th>Mean Peak Area ratio (EGCG/IS)</th>
<th>SD Peak Area ratio</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>5</td>
<td>3.18</td>
<td>0.004</td>
<td>1.21</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
<td>1.79</td>
<td>0.041</td>
<td>2.32</td>
</tr>
<tr>
<td>250</td>
<td>5</td>
<td>0.88</td>
<td>0.023</td>
<td>2.67</td>
</tr>
<tr>
<td>125</td>
<td>5</td>
<td>0.35</td>
<td>0.014</td>
<td>4.01</td>
</tr>
<tr>
<td>62.5</td>
<td>5</td>
<td>0.11</td>
<td>0.003</td>
<td>2.82</td>
</tr>
<tr>
<td>31.25†</td>
<td>5</td>
<td>0.07</td>
<td>0.004</td>
<td>4.78</td>
</tr>
<tr>
<td>15.63†</td>
<td>5</td>
<td>0.03</td>
<td>0.003</td>
<td>8.77</td>
</tr>
</tbody>
</table>

†The lowest concentration of EGCG that had CV <5% indicating the LOQ; n – represents a number of replicates.

Figure 5.4 Calibration curve for EGCG spiked in plasma samples. Results were derived from linear regression analysis of the EGCG/internal standard chromatographic peak area ratios plotted versus the concentration of EGCG. Peak areas for IS and EGCG were determined by HPLC-MS-ESI set in negative polarity SIM mode, as described in section 5.2.2.4.6.1.
Table 5.4 Intra assay analysis of recovery for EGCG from plasma and HPLC-MS measurement variation.

<table>
<thead>
<tr>
<th>EGCG Added ng/ml</th>
<th>n</th>
<th>EGCG Measured ng/ml</th>
<th>CV (%)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5 (CL)</td>
<td>10</td>
<td>54.4 ± 2.7</td>
<td>4.88</td>
<td>87.0 ± 4.2</td>
</tr>
<tr>
<td>500 (CH)</td>
<td>10</td>
<td>489.9 ± 10.7</td>
<td>2.18</td>
<td>98.0 ± 2.1</td>
</tr>
</tbody>
</table>

Data for EGCG Measured and % Recovery are expressed as Mean ± SD with ‘n’ the number of replicates.

The inter assay variation was analysed over time using the same two plasma samples with added EGCG, CL and CH as quality controls with each HPLC-MS run (Table 5.5). The inter assay CVs were also very good at less than 4% for CH and less than 6.5% for CL.

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

<table>
<thead>
<tr>
<th>EGCG Added ng/ml</th>
<th>n</th>
<th>EGCG Measured ng/ml</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5 (CL)</td>
<td>24</td>
<td>59.5 ± 3.8</td>
<td>6.44</td>
</tr>
<tr>
<td>500 (CH)</td>
<td>24</td>
<td>474.5 ± 17.4</td>
<td>3.68</td>
</tr>
</tbody>
</table>

Data for Measured EGCG is expressed as Mean ± SD with ‘n’ the number of replicates.

5.3.3 Participants’ anthropometric and blood pressure data

Four subjects, three males and 1 female were recruited for the study. Their average age was 31.25±9.54 years (Males 27±5.3, Female 44).

The participants’ anthropometric data, standing height, weight, and their systolic and diastolic blood pressures were measured at the start of each clinic prior to the ingestion of product containing EGCG. These values and their BMI are listed in Table 5.6.
The subjects’ measurements were stable over the period of the study. Using the one-way ANOVA and the Bonferonni post-hoc test, it was found that there was no significant difference in BMI (p=0.999), systolic blood pressure (p=0.998), or diastolic blood pressure (p=0.999) between the three clinic visits (Table 5.6).

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

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Clinic 1</th>
<th>Clinic 2</th>
<th>Clinic 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n=3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>73.3±11.19</td>
<td>73.53±11.35</td>
<td>73.37±11.17</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.79±0.04</td>
<td>1.79±0.04</td>
<td>1.79±0.04</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>23.06±4.29</td>
<td>23.13±4.35</td>
<td>23.08±4.29</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>113±5.77</td>
<td>114±6.35</td>
<td>113±5.77</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>83±2.89</td>
<td>84±1.73</td>
<td>83±5.77</td>
</tr>
<tr>
<td>Female (n=1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>62</td>
<td>62.3</td>
<td>61.9</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.63</td>
<td>1.63</td>
<td>1.63</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>23.34</td>
<td>23.45</td>
<td>23.3</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>110</td>
<td>120</td>
<td>110</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>90</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>All (n=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.48±10.74</td>
<td>70.73±10.84</td>
<td>70.5±10.77</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.75±0.08</td>
<td>1.75±0.08</td>
<td>1.75±0.08</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>23.13±3.5</td>
<td>23.21±3.55</td>
<td>23.13±3.5</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>112.5±5</td>
<td>115.25±6.08</td>
<td>112.5±5</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>85±4.08</td>
<td>85.5±3.31</td>
<td>82.5±5</td>
</tr>
</tbody>
</table>

*BMI* – Body Mass Index; *SBP* – Systolic Blood Pressure; *DBP* – Diastolic Blood Pressure
### 5.3.4 Plasma EGCG concentration-time results

The plasma EGCG concentration was measured just before and over 8h after the administration of 500mg of EGCG delivered as two 250mg capsules without breakfast, two 250mg capsules with breakfast or 500mg in 200grams of strawberry sorbet. The EGCG concentration curves over this period of time for each delivery method and for each participant as well as the arithmetic mean EGCG concentration curves for each delivery method in all four participants are represented in figure 5.5.

It can be seen that the absorption of EGCG was higher when it was delivered as capsules without breakfast. This is more clearly seen in figure 5.6, which is a summary of the arithmetic means of the plasma EGCG concentration-time curves for the three delivery methods for the four participants. In this figure it can be clearly noted that taking the capsules of EGCG without breakfast resulted in a noticeably higher response than the other two treatments of capsules with breakfast or the strawberry sorbet. Moreover, figure 5.6 shows that the arithmetic mean concentration for plasma EGCG reached a maximum value at 1h ($T_{\text{max}}=60\text{min}$) for capsules taken without the breakfast, while the maximum concentrations for EGCG taken in capsules with breakfast and imbedded into the strawberry sorbet were reached at 2h ($T_{\text{max}}=120\text{min}$) after the subjects ingested the EGCG.
Figure 5.5 Plasma EGCG concentration-time curves and arithmetic mean curve (broken green line) for the three different methods of EGCG oral delivery: capsules without breakfast (A), capsules with breakfast (B) and strawberry sorbet (C). Blue lines indicate male participants while the pink line indicates the female participant.
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.

5.3.5 Pharmacokinetic parameters of plasma EGCG

After the one-way ANOVA analysis and the Bonferonni post-hoc test it was noted that the Area Under the Curve (AUC) for EGCG taken as capsules without breakfast was significantly higher than the AUC for EGCG taken as capsules with breakfast (p=0.044) and the AUC for EGCG taken incorporated in strawberry sorbet (p=0.019). However, the difference between the AUC for EGCG taken as capsules with breakfast and the AUC for EGCG taken in the strawberry sorbet was not statistically significant (p=1) (Table 5.7).

The maximum concentration ($C_{\text{max}}$) of EGCG in plasma (Table 5.7) after the ingestion of EGCG without breakfast was significantly higher than if EGCG was taken with breakfast (p<0.001) and it was also higher than if taken as strawberry sorbet
(p<0.001). However, there was no significant difference in maximum plasma EGCG concentrations between the EGCG taken with breakfast and strawberry sorbet intakes (p=1).

The average concentration of EGCG (C_{av}) in plasma during the treatment period was significantly higher for EGCG taken without the breakfast than with breakfast (p=0.004) and strawberry sorbet (p=0.002). However, there was no significant difference between EGCG taken with breakfast and strawberry sorbet (p=1) (Table 5.7).

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

<table>
<thead>
<tr>
<th></th>
<th>EGCG without breakfast</th>
<th>EGCG with breakfast</th>
<th>EGCG in Strawberry sorbet</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (μg/ml/8h)</td>
<td>173.79±67.61*</td>
<td>64.13±53.72</td>
<td>44.47±22.86</td>
</tr>
<tr>
<td>C_{max} (ng/ml)</td>
<td>824.15±75.07**</td>
<td>231.77±134.33</td>
<td>218.03±160.03</td>
</tr>
<tr>
<td>C_{min} (ng/ml)</td>
<td>86.85±75.23</td>
<td>49.27±24.64</td>
<td>25.06±5.76</td>
</tr>
<tr>
<td>C_{av} (ng/ml)</td>
<td>382.60±92.49*</td>
<td>91.95±45.98</td>
<td>87.60±42.97</td>
</tr>
<tr>
<td>DF</td>
<td>1.93±0.77</td>
<td>1.39±0.46</td>
<td>2.2±0.76</td>
</tr>
<tr>
<td>Swing</td>
<td>8.49±17.19</td>
<td>3.36±3.04</td>
<td>7.70±6.61</td>
</tr>
<tr>
<td>T_{max} (min)</td>
<td>60</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>T_{1/2} (min)</td>
<td>78.6±94.2</td>
<td>136.2±85.2</td>
<td>228.6±97.2</td>
</tr>
</tbody>
</table>

* Value is significantly different from values in the row at the level of p<0.05.
** Value is significantly different from values in the row at the level of p<0.001.

AUC – Area under the curve; C_{max} – maximal concentration; C_{min} – concentration at the end of the treatment; C_{av} – Average concentration; DF – degree of fluctuation; T_{max} – time required to reach the maximal concentration; T_{1/2} – half-life

From table 5.7 can be noted that the plasma EGCG concentration at the end of the treatment (C_{min}) was not significantly different between the three treatment groups.
(p>0.05) and a similar pattern was observed for the values for Swing (p>0.05), DF (p>0.05) and EGCG plasma half-life (p>0.05).

5.3.6 Breakfast and lunch composition analysis

To determine the energy and macronutrient profile of the breakfast and lunch, analysis was performed using FoodWorks 2007 (v5 SP1) (Xyris Software, Australia). The results, expressed per serve, are presented in table 5.8 for breakfast and 5.9 for lunch. For comparison, values for the gelatine capsules, based on the weight of two empty capsules, are also included in table 5.8.

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

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Breakfast</th>
<th>Strawberry Sorbet</th>
<th>2 Gelatine Capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>256.00</td>
<td>200.00</td>
<td>0.250</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>1367.32**</td>
<td>898.15</td>
<td>3.626</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>16.75</td>
<td>19.23**</td>
<td>0.212</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>8.93**</td>
<td>0.25</td>
<td>0.002</td>
</tr>
<tr>
<td>Saturated Fat (g)</td>
<td>5.30**</td>
<td>0.04</td>
<td>NA</td>
</tr>
<tr>
<td>Polyunsaturated Fat (g)</td>
<td>0.81</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Monounsaturated Fat (g)</td>
<td>2.36</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>26.78</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>44.38**</td>
<td>31.94</td>
<td>NA</td>
</tr>
<tr>
<td>Sugars (g)</td>
<td>17.23</td>
<td>31.90**</td>
<td>NA</td>
</tr>
<tr>
<td>Starch (g)</td>
<td>27.15</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Water (g)</td>
<td>181.66**</td>
<td>142.15</td>
<td>0.028</td>
</tr>
<tr>
<td>Dietary Fibre (g)</td>
<td>1.85</td>
<td>3.38**</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note: "NA" indicates that results are not available or not detectable; Breakfast composition includes analysis of 2 gelatine capsules; **Significant p<0.001
Using the Chi-square test to evaluate the distribution of the values for the major macronutrients and energy among the different EGCG intake methods, it was found that the breakfast had significantly higher values for energy, total fat, saturated fat, carbohydrate and water (p<0.001) than the other two ingestion methods. However, the strawberry sorbet had significantly higher values for protein, sugars and dietary fibre than the other two intake methods (p<0.001) (Table 5.8).

Three h after the ingestion of EGCG, the participants were provided with a standardised lunch and its macronutrient and energy profile is presented in table 5.9.

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

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Per Serve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>461.50</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>1368.39</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>27.67</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>6.78</td>
</tr>
<tr>
<td>Saturated Fat (g)</td>
<td>2.59</td>
</tr>
<tr>
<td>Polyunsaturated Fat (g)</td>
<td>0.73</td>
</tr>
<tr>
<td>Monounsaturated Fat (g)</td>
<td>2.35</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>51.87</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>35.53</td>
</tr>
<tr>
<td>Sugars (g)</td>
<td>18.44</td>
</tr>
<tr>
<td>Starch (g)</td>
<td>17.09</td>
</tr>
<tr>
<td>Water (g)</td>
<td>376.10</td>
</tr>
<tr>
<td>Dietary Fibre (g)</td>
<td>4.91</td>
</tr>
</tbody>
</table>
5.3.7 Correlations between AUC and macronutrients

The average values of AUC of plasma EGCG for each delivery method were correlated with corresponding macronutrient profile. The average AUC from the three EGCG delivery methods was significantly inversely correlated with the protein composition of the breakfast (p=0.014) (Figure 5.7). However, the AUC did not correlate with other macronutrients such as total fat (R²=0.1564), carbohydrate (R²=0.8362) and fibre (R²=0.8966).

![Figure 5.7](image)

**Figure 5.7** The relationship between the breakfast protein composition and the average AUC of each EGCG delivery method (p=0.014).
5.4 Discussion

In this chapter, it was hypothesised that ‘pure EGCG would be absorbed better when given as a capsule without food and give the highest plasma EGCG concentrations when given in this format compared to when given as a capsule with a breakfast’. However, it was also hypothesised that ‘incorporating EGCG in a food product like a strawberry sorbet would improve the absorption of EGCG compared to taking the catechin in capsule form with a breakfast’.

Therefore, the main aim in this chapter was to determine which delivery format was the best of the three methods tested for the oral delivery of pure EGCG in humans: 1) EGCG given by itself within capsules, 2) EGCG given in capsules taken with food in the form of a light breakfast or 3) EGCG given as a powder incorporated in a strawberry sorbet. It was anticipated that EGCG incorporated in the strawberry sorbet would be absorbed as well as EGCG given in capsule form without food and better absorbed than EGCG given incapsule form with food given as a light breakfast consisting of cereal and full cream milk.

In order to test the hypotheses, a strawberry sorbet was prepared[545]and the recovery of EGCG from the product was tested. For determination of the systemic absorption of EGCG, four participants were recruited and, on three separate occasions (days) at least one week apart, they were asked to ingest in random order either 1) 500mg of EGCG in capsules (2 x 250mg) taken with water only, 2) 500mg of EGCG in capsules (2 x 250mg) taken with a breakfast cereal and full cream milk or 3) 500mg of EGCG taken incorporated in 200g of strawberry sorbet. On each of these three occasions, venous blood samples were collected prior to the ingestion of EGCG and at selected times during the 8h period following the ingestion of the catechin. The plasma EGCG was analysed using high pressure liquid chromatography equipped with a mass
spectrometer. The data was analysed for pharmacokinetic properties, including the area under the curve for the absorption of EGCG over the 8h period post-ingestion, and the values were compared between the three delivery methods.

The aim of the study was achieved; the method of oral delivery for EGCG which gave the best systemic absorption was the capsules by themselves (Figures 5.5 and 5.6, Table 5.7). Therefore, the hypothesis that ‘pure EGCG would be absorbed better when given as a capsule without food and give the highest plasma EGCG concentrations’, was supported. This was true, not only compared to when it was given as a capsule with a light breakfast, but also compared to when it was given incorporated in a strawberry sorbet. However, the findings did not support the proposed hypothesis ‘that pure EGCG powder incorporated in a strawberry sorbet would be absorbed as well as EGCG given in capsule form without food and better absorbed than if given in capsules with a breakfast’ in healthy human volunteers.

The results for the concentration of free EGCG in plasma over the 8h period after the ingestion (Figures 5.5 and 5.6, Table 5.7) clearly revealed that EGCG taken in capsule form by itself without food gave higher plasma values; the AUC was 2.7 times higher than when EGCG was taken in capsules with a light breakfast (p=0.044) and 3.9 times higher than when EGCG was imbedded in the strawberry sorbet (p=0.019). Furthermore, there was no significant difference in the AUC values (Table 5.7) between the EGCG taken with a light breakfast or taken in the strawberry sorbet (p=1.000).

This pattern was also observed when the values for $C_{\text{max}}$ and $C_{\text{av}}$ were compared between the three ingestion conditions (Table 5.7). The EGCG taken on its own showed a 3.6 times higher plasma free EGCG maximum serum concentration than the EGCG taken with a light breakfast (p<0.001) and a 3.8 times higher $C_{\text{max}}$ than when it was taken in the strawberry sorbet (p<0.001) but there was no significant difference between
the $C_{\text{max}}$ for EGCG taken with the breakfast cereal and EGCG taken imbedded in the strawberry sorbet ($p=1.000$). Similarly, the average plasma free EGCG concentration levels, during the 8h post-treatment period (Table 5.7), for EGCG taken on its own was 4.2 times higher than for the EGCG taken with breakfast ($p=0.004$) and 4.4 times higher than for EGCG taken in the strawberry sorbet ($p=0.002$) but there was no significant difference between the $C_{\text{av}}$ for the EGCG taken with breakfast and the EGCG taken imbedded in the strawberry sorbet ($p=1.000$).

The amounts of EGCG measured in the plasma after its ingestion in the present study are consistent with those of the only previous study which used similar doses of administered pure EGCG, the study by Ullmann et al.[420]. When 500mg EGCG was given in capsule form without food in the present study, the plasma EGCG concentration curve (Figures 5.5A & 5.6) was very similar to the plasma EGCG values obtained when a similar dose of 400mg EGCG was given without food to humans by Ullmann et al.[420]. Furthermore, the $C_{\text{max}}$ and $C_{\text{av}}$ values for the 500mg EGCG dose in the present study (Table 5.7, 824.15ng/ml and 382.6ng/ml, respectively) were very comparable, relative to the dose given, to the $C_{\text{max}}$ and $C_{\text{av}}$ values for the 400mg EGCG dose (861.94ng/ml, and 568.12ng/ml, respectively) in the Ullmann et al. [420] study.

The results in the present study are also very consistent with the previous findings of Chow et al. [440], who showed that food intake also clearly interfered with the systemic absorption of EGCG when it was given as part of a GT extract. Similar to the present study (Table 5.7) Chow et al.[440] also reported markedly higher plasma EGCG AUC (3.5 times higher) and $C_{\text{max}}$ (5.7 times higher) values when the catechin extract was taken without food compared to when it was taken with a standardised breakfast consisting of muffins. This is also not entirely surprising because it is well known that the absorption of some pharmaceutical medications is decreased when they
are taken with various food products. This is predominately due to interactions between some medications and dietary components and disturbances in the gastric emptying rate, which can decrease the efficiency and/or rate of absorption [554]. As with the types of medications that are affected by food, it appears that EGCG should be taken without food in order to maximize its intestinal absorption [554].

With varied food types, such as muffins [554], a breakfast cereal plus full cream milk and a strawberry sorbet (Figures 5.5B and 5.5C, Table 4.7), all decreasing the plasma EGCG concentration measured after ingestion, it is evident that food is an important factor, which can affect the intestinal absorption and systemic levels of orally administered EGCG, whether it is in a GT extract or in pure form. It is not known how food gives rise to this effect or which particular food component plays a role in decreasing the absorption of EGCG. However, from what is known about the interactions of food with medications and plant bioactives [555, 556], several factors may influence the bioavailability of EGCG. These factors can be divided into three broad categories: 1) the effect of the vehicle in which the EGCG was administered, 2) the effect of the biological fluids on EGCG prior to it reaching its absorption site and 3) effects on EGCG due to physiological responses to the ingested food products.

Relative to the first category, ‘1) the effect of the vehicle in which the EGCG was administered’, it was hypothesised that ‘incorporating EGCG in a food product like a strawberry sorbet would improve the absorption of EGCG compared to taking the catechin in capsule form with a breakfast’. This was because it is well known that EGCG is relatively unstable and susceptible to degradation [531] at high temperatures [532] and at pH values above four [534]. Therefore, imbedding the EGCG in a food product like strawberry sorbet, which does not need exposure to high temperatures, is stored at -20°C and has a pH below 4, was a plausible possibility. This was supported
by the finding that the EGCG was very stable in this food product, as evidenced by the very high percentage (over 97%) observed for its recovery from the strawberry sorbet (Table 5.2). This finding indicated that the method used to prepare the strawberry sorbet and its acidic environment preserved the EGCG extremely well and therefore, the amount of chemically intact EGCG (500mg) ingested in the 200g of sorbet given to the human volunteers was the intended amount.

Evidently however, the fact that the EGCG was chemically intact and stable in the strawberry sorbet did not improve its bioavailability (Figures 5.5 and 5.6, Table 5.7). Obviously, similar to the breakfast cereal and the full cream milk in the present study and the muffins in the study by Chow et al. [440], the presence of other components in the strawberry sorbet posed an important role in reducing the bioavailability of EGCG and resulted in a significantly lower AUC for EGCG than when it was taken on its own without food. Of interest, although with a very limited number of values, was the correlation analysis looking at the different food components in the strawberry sorbet and the cereal and milk breakfast, especially the macronutrients, which found that the AUC for plasma EGCG was significantly (p=0.014) and inversely ($R^2=0.9995$) correlated with the protein content of the foods used (Figure 5.7). Furthermore, protein was the only food component for which any correlation was found.

Therefore, the protein content of the foods taken with EGCG may have been a factor in the reduction of the intestinal absorption of the catechin, compared to when it was taken without food. Similar effects of dietary protein on the pharmacokinetics of different pharmaceuticals have been observed when their ingestion with and without meals was compared [557-559]. One observation has been that there is a noted elevation of the gastric pH from the normal acidic range of 1.1-1.6 up to 5.8-6.7, 4 h after the
ingestion of a protein meal [557-559]. It is also known that bovine milk can increase the already high pH in the small intestine, particularly in the first 20 min after ingestion [560, 561].

Because of its sensitivity to pH above 4 [534], any increase in pH caused by the protein or other component of the food products used in the present study (strawberry sorbet, breakfast cereal and milk) may have lead to an accelerated degradation of EGCG and reduce the already low amounts of EGCG available for absorption, even when it is taken without food [420, 440, 465]. Relevant to this, *in vitro* studies [534, 541] have determined that only small amounts of intact EGCG remained after exposure to the pH values normally observed in the small intestine. Additionally, it was noted that the time needed for significant degradation of EGCG (5 min) was very much shorter than the transit time of foods (3-4 h) through the small intestine [556].

Another mechanism by which protein could reduce the absorption of EGCG, is the recognised property of polyphenols, such as EGCG which has a high degree of hydroxylation, having the capacity to directly bind to protein [562]. It is also documented that the GT catechins can interact with proteins to form a precipitate, which makes the tea solution look hazy, a process referred to as ‘tea cream’ formation [563]. Catechins can also interact with enzymes such as lipoxygenase, α-amylase, pepsin, trypsin and lipase to form precipitates and consequently inhibit the activity of these enzymes [564]. The catechins EGCG and ECG, which have an ester bonded gallate, also have a greater ability to form precipitates with these enzymes [564, 565].

Relative to the second category by which food may influence the bioavailability of EGCG, ‘the effect of the biological fluids on EGCG prior to it reaching its absorption site’, the principal biological fluids EGCG would come into contact with are the saliva, the gastric juice and the pancreatic/biliary juices. A study on the interaction of the GT
catechins and saliva [566] has indicated some conversion of EGCG to EGC can occur due to the activity of an esterase present in saliva. Interestingly, this study by Yang et al. [566] also observed that holding an EGCG solution in the oral cavity for a period of 5min without ingestion showed, not only that some of the EGCG was converted into EGC but that both catechins were absorbed through the oral mucosa and made their way into the systemic blood circulation and the urine from there.

If there had been a significant absorption of EGCG from the oral cavity in the present study, it would have been expected to be from the strawberry sorbet. This was the only vehicle which was held in the mouth for any length of time before swallowing; the capsules were quickly swallowed with water and therefore, they were in the mouth for a very brief time only. It took the participants longer than 5min to consume the full 200g of sorbet and they did not rinse their oral cavity with water.

Despite this, there was no indication that any significant absorption of EGCG took place from the oral cavity. If there had been uptake through the oral mucosa, the plasma EGCG levels at the first time point, 30min after the ingestion of the strawberry sorbet, could have been higher than for the other two EGCG delivery methods - capsules taken with or without food. Furthermore, based on the study by Yang et al. [566], the salivary esterase conversion of EGCG to EGC could have been expected to cause an increase in the amount of EGC measured in the plasma. There was no increase in the plasma EGCG levels detected at the first time point, 30min after the ingestion of the strawberry sorbet, compared to the other two ingestion methods.

Furthermore, the presence of EGC was not detected in the plasma samples analysed by HPLC-MS, indicating that there was little or no conversion of EGCG to EGC in the oral cavity or in the salivary mucosa during and after the consumption of the strawberry sorbet. Similarly, the EGCG taken in capsule form on its own or with
breakfast did not yield any traceable amounts of EGC in plasma, which is consistent with the findings by Yang et al. [566] when EGCG was given in capsule form and the capsules were not dissolved in the oral cavity and therefore, the EGCG was not exposed to the salivary esterase.

In the stomach, little degradation of EGCG is expected to occur because of the acidic nature of the gastric secretions. In effect, as shown by Record and Lane [567], EGCG was stable in acidic solutions (pH<3) made up to mimic those found in the fasting stomach environment (pH1.5 – 2) [557]. However, as discussed above, any increase in pH caused by protein or any other component of food could have lead to an accelerated degradation of EGCG and reduce its bioavailability [420, 440, 465].

In the small intestine, the acidic chyme that is pushed down from the stomach is quickly neutralised by the bicarbonate solution secreted by the pancreas into the duodenum [555, 568]. As shown by Record and Lane [567], EGCG is particularly unstable under conditions which mimic digestion fluids in the small intestines, with only 1% of the EGCG still measurable after an hour incubation. Therefore, this is where most of the EGCG is expected to be lost and taking the capsules without food is most likely to have allowed the EGCG to survive longer in the small intestines, therefore enhancing its chances of being absorbed.

The EGCG capsules by themselves would be expected to produce much less chyme than when taken along with food, either in the form of the strawberry sorbet or the breakfast cereal with full cream milk, and consequently elicit a less strong response from the pancreas to secrete bicarbonate to neutralise the chyme coming down from the stomach. Evidently, the possibility that the acidic nature of the strawberry sorbet could keep the pH of the small intestine less basic either did not occur or was not a factor in the bioavailability of the EGCG it contained.
Clearly, other ways of protecting the EGCG from the basic pH in the small intestines are needed. Several studies have reported different methods of preserving the EGCG such as encapsulation using oil-in-water sub-micrometer emulsions \[569]\(,\) liposomes \[570]\(\) and protein/polyphenols microparticles \[571]\(,\) but whether they can preserve the EGCG from degradation in the small intestine and increase its bioavailability remains to be determined.

Relative to the third category by which food may influence the bioavailability of EGCG, ‘effects on EGCG due to physiological responses to the ingested food products’, the small intestine is the primary absorption site for EGCG and the rate at which EGCG is presented into the upper portion of small intestine and travels down to the absorption site can determine the bioavailability of the catechin. It is known that the ingestion of food will delay the rate of gastric emptying \[556, 557]\(\) and that the rate of gastric emptying is one of the most important factors known to influence the absorption rate of orally administered pharmaceuticals from the gastrointestinal tract \[556]\(.
\)

In concurrence with this, EGCG taken with the breakfast and the strawberry sorbet showed a delay in the average time \(2h\) it took to reach its maximum concentration in plasma (Figure 5.6) compared to EGCG taken without food \(1h\). Therefore, a slower gastric emptying in the presence of food most likely prolonged the time needed for EGCG to travel into the upper portion of small intestine. However, given that the bioavailability of the EGCG was much lower when it was taken with food, some of the extra time is likely to have been spent transiting through the small intestine where exposure to a high pH for longer could have contributed to a greater degradation.

Another possibility, which could have explained the higher plasma EGCG concentrations observed when the catechin was taken without food, compared to with
food, was an increased clearance rate of EGCG from the plasma when it was taken with food. However, the mean elimination half-life, 78.6min for EGCG taken on its own, 136.2min for EGCG taken with breakfast cereal and milk and 228.6min for EGCG imbedded in the strawberry sorbet, was not significantly different between the three ingestion methods (p>0.05). Therefore, the ingestion of EGCG with food or incorporated in a food did not appear to significantly influence the clearance rate of free EGCG from the systemic circulation once it was absorbed. Furthermore, as discussed above, the significant differences in the $T_{\text{max}}$ values suggest that food significantly delayed the systemic absorption of EGCG rather than increase its clearance from the blood circulation.

The present findings illustrate the importance of determining the most appropriate method for the oral delivery of EGCG to maximise its systemic absorption and thereby reach levels capable of having physiological significance. However, it is not known what the EGCG concentrations need to be in humans in order for it to have systemic effects [420, 530]. Nonetheless, relative to cholesterol-lowering effects, in vitro studies with human liver HepG2 cells, have shown upregulation of the LDL-receptor with EGCG concentrations as low as 4.5μg/ml [410, 411]. In the present study, when 500mg EGCG was given as a single dose in capsule form without food, the highest average plasma concentration, $C_{\text{max}}=0.82μg/ml$ (Table 5.7), was lower but not very far from the value of 4.5μg/ml. When Ullmann et al.[420] gave a single dose of 1600mg EGCG without food, the $C_{\text{max}}$ was increased to 2.9μg/ml. Similarly, when they gave single doses of 400 and 800mg EGCG every day for 10 days, the $C_{\text{max}}$ values were 1.4 and 2.4, respectively [572]. Higher levels and therefore levels closer to those which have been seen to have upregulatory effects of the LDL-receptor in in vitro studies are also likely to be achieved if doses are given two or three times per day.
In conclusion, the hypothesis that ‘pure EGCG would be absorbed better when given as a capsule without food and give the highest plasma EGCG concentrations when given in this format compared to when given as a capsule with a breakfast’ was supported. However, the proposed hypothesis that ‘pure EGCG incorporated in a strawberry sorbet would be absorbed as well as if given in capsule form without food and better than if given in capsules with food’ was not supported. The AUC results as well as the $C_{\text{max}}$ values were significantly higher for EGCG taken in capsules without food after an overnight fast than with the other two ingestion methods. Based on the findings of the present study, ingesting EGCG capsules with water on an empty stomach is the most appropriate method for the oral delivery of EGCG in clinical trials where EGCG is to be investigated as a potential cholesterol lowering agent in humans.
Chapter 6. The effects of epigallocatechin-3-gallate on blood lipids in humans with moderately elevated blood cholesterol levels – a pilot study

6.1 Introduction

Over the last century, high blood cholesterol levels have gained a negative reputation due to the atherogenic properties of the sterol, which have made it a major risk factor for cardiovascular disease (Section 1.1.5.1). Therefore, the search for nutraceuticals and functional foods that may assist in cholesterol lowering is on the increase. Several food constituents such as dietary fibre [573], phytosterols [574], phytoestrogens [575] and grape polyphenols [576] have been shown to exhibit cholesterol lowering properties. Even red wine consumption has been shown to decrease fasting blood LDL cholesterol (LDL-C) by 8% and increase HDL cholesterol (HDL-C) by 17% in mildly hypercholesterolaemic postmenopausal women [576].

The GT (GT) consumption as a health promoting beverage dates back over 2000 years and more recently it has been associated with a variety of beneficial effects [353-355]. Epidemiological studies have consistently identified that drinking 5 to 10 cups of GT per day is associated with lower blood cholesterol levels, less coronary atherosclerosis, lower total mortality and an overall reduction in the risk for developing CHD [360, 367, 368, 577, 578]. In contrast, the findings are not as consistent, when the dietary intakes of black tea [579, 580] or flavonols from various sources [579, 580], are studied.

The catechins, the main constituents in GT, have been alleged to play a pivotal role in the beneficial health effects exhibited by the intake of GT. These beneficial health effects are ascribed to EGCG, the most abundant and pharmacologically active catechin found in GT [581]. In support to this observation, there are a number of in vitro studies which have documented beneficial effects of EGCG on various biomedically-
relevant molecular targets and disease-related cellular processes [362, 582-584]. Furthermore, numerous intervention trials in rats, mice, hamsters and rabbits have indicated that GT itself, GT extracts enriched in catechins and purified EGCG all exhibit blood cholesterol lowering properties [242, 401, 402, 404, 408, 413-416, 432, 433, 469, 516, 517, 585]. Similar findings have been observed in Chapter 3 of this thesis using pure EGCG in a rabbit model of orally-induced hypercholesterolaemia.

Although intervention trials investigating the effects of pure EGCG on plasma cholesterol in people with hypercholesterolaemia are scarce some studies, focused on weight loss in normocholesterolaemic subjects, have identified that the intake of GT extracts can lead to a reduction in LDL cholesterol[436] as well as in body fat, bodyweight (-4.6%) and in waist circumference (-4.48%) [427, 436, 586]. However, other studies [428, 587] found no statistically significant change in the plasma lipid profile of the participants or in their waist circumference, total body fat or abdominal fat.

As suggested by Hill et al.[428], one of the main reasons for the limited changes in the health parameters in some studies is likely to be the relatively low amounts of EGCG administered, which is usually no more than 300mg/day. Furthermore, participants are often advised to consume the capsules after a meal [587], which, as illustrated in Chapter 5, is likely to have lowered the systemic absorption of EGCG.

Undoubtedly, low bioavailability is one of the main issues associated with the use of EGCG and therefore, high doses of EGCG are required to achieve physiologically relevant plasma EGCG concentrations. However, high doses can also inherently cause unwanted non-beneficial health effects. The toxicity and tolerance levels for EGCG in humans have mainly been investigated with the oral administration of GT extracts, where EGCG was a functional component imbedded in a polyphenolic
matrix [419, 588, 589]. These studies showed excellent tolerance to the catechin extracts with only mild side effects such as gastrointestinal complaints (abdominal bloating, flatulence and nausea) and some central nervous system stimulation (agitation and restlessness), all of which could be due primarily to the high caffeine content (7%) of the preparations [589].

A study on the safety and tolerability of ascending doses of pure EGCG [420] has also reported that the catechin was well tolerated in healthy human volunteers at doses of up to 1.6g/day. This study reported that there were no cardiovascular variables, clinical chemistry (including urine chemistry) or physical findings, which were influenced by EGCG supplementation. Therefore, the intake of pure EGCG at concentrations higher than 300mg/day, as suggested by Hill et al. [428], appear to be safe and well tolerated in healthy individuals. Potential negative side effects can also be reduced by spreading the daily dose over more than one intake of EGCG/day as it is done for certain supplements [590, 591].

6.1.1 Aims and Hypotheses

Based on previous studies in animals [401, 402, 408, 413-416, 432, 433, 469, 516, 517], including the study in the cholesterol-fed hypercholesterolaemic rabbit presented in Chapter 3 and 4, and the epidemiological and other studies in humans [360, 396-398, 405, 406], it was hypothesised that ‘pure EGCG will lower blood cholesterol in humans with moderately elevated cholesterol levels, if given at a higher dose than 300mg/day, as suggested by Hill et al.[428].

Furthermore, the results of the EGCG absorption study in Chapter 5 indicated that the best method of EGCG delivery for maximising the systemic absorption of the catechin was in capsule form taken without food on an empty stomach.
Therefore, the main aim of the study was to determine if pure EGCG given for four weeks in the form of capsules taken without food on an empty stomach would lower blood cholesterol in humans with moderately elevated cholesterol (5.5-7.5mmol/l). As suggested by Hill et al., the dose of EGCG was chosen to be 1g/day. Furthermore, based on the results in Chapter 5, which indicated that 500mg EGCG was very well tolerated when taken all at once, the daily dose of 1g/day was split into two doses: 500mg EGCG taken without food before breakfast in the morning and the other 500mg EGCG taken without food before the evening meal.

A pilot study in 10 participants was conducted to test the hypothesis. The study was a 4-week, double blind, randomised, placebo-controlled, parallel design intervention trial with 1g EGCG/day given in two 500mg doses, one in the morning and the other in the evening. Fasting blood samples were collected at baseline and at the end of the study and total cholesterol (TC), LDL cholesterol, HDL cholesterol and triglycerides (Tg) were determined.
6.2 Methods

6.2.1 Capsule preparation

Based on the findings from the EGCG absorption study in Chapter 5, capsules filled with EGCG and taken without food, was selected as the best method to orally deliver EGCG to the participants. Size 00 clear gelatine capsule casings (Melbourne Food Ingredient Depot, Australia) were filled with 250mg of EGCG by “Visionary Health” compounding chemist (Hamilton, NSW, Australia). For the placebo, the clear gelatine capsule casings were filled with food grade gelatine powder (McKenzie’s, Altona, Vic. Australia) using a capsule filler (Cap-M-Quick, Murrieta, USA). The filled capsules (120/container) were packed in tamper-evident white high density polyethylene containers (Plasdene GlassPak Pty Ltd, Warabrook, NSW, Australia), which prevented the exposure of the EGCG or the placebo to light.

6.2.2 Participants

6.2.2.1 Ethics approval

Ethics approval for this study was granted by the Human Ethics committee of the University of Newcastle (H-2008-0089), NSW, Australia. Informed written consent was also obtained from all participants before they commenced the study.

6.2.2.2 Recruitment and selection criteria

The participants for this study were selected from the general population residing in the Central Coast area of NSW. All participants were recruited from responders to advertisements placed on the University of Newcastle website and in the local newspapers or to interviews on the local radio stations. Each responder received an
information statement letter, detailing the procedures, protocols and possible associated risks of participating in this study.

For the purposes of the pilot study, 10 participants (6 males; 4 females) were selected if they were male or female aged between 18-65 at the time of the initial assessment and had moderately high blood cholesterol levels (5.5-7.5mmol/l) as determined using a finger-prick test done with the Accutrend Plus hand-held instrument (Roche Diagnostic, Australia). Most of the participants also provided pathology laboratory reports, which showed they recently had moderately high blood cholesterol levels.

Responders were excluded from the study if they were on any medication, dietary supplement or functional food to lower cholesterol or triglycerides prescribed by a health care provider. Participants who consumed dietary supplements or functional foods by choice such as fish oils, cholesterol lowering functional foods, niacin at doses higher than 400mg/day, soluble fibre supplements or antioxidants were asked not to consume these for at least 2 weeks before the start and during the duration of the study.

The exclusion criteria also included the following: baseline triglyceride levels of 4.0mmol/l or greater as determined using the finger-prick test and the Accutrend Plus hand-held instrument (Roche Diagnostic, Australia), a history of coronary heart disease, a body mass index of 35 or higher (calculated as weight in kg divided by the square of height in meters), uncontrolled resting hypertension (greater or equal to 160/95mmHg determined using a sphygmomanometer), any known active pulmonary, hematologic, hepatic, gastrointestinal or renal disease, pre-malignant or malignant disease, diabetes, thyroid dysfunction, or any pathology values known to be abnormal. All exclusion and inclusion criteria were developed based on the internationally accepted guidelines.
and studies conducting similar research in humans using pure EGCG or EGCG imbedded into polyphenolic complexes[420, 440, 549].

All ten of the chosen volunteers successfully completed the study without any health complaints during the study period.

6.2.2.3 Study clinics

The participants attended three clinics in total; the screening clinic (Clinic 1) to determine their eligibility, the ‘start of treatment’ clinic (Clinic 2) and the ‘end of treatment’ clinic (Clinic 3) four weeks later (Figure 6.1).

Prior to attending each clinic, the participants were asked to fast overnight (at least 10h) and on each clinic visit, the participants’ height was measured using a stadiometer (S&M Supplies, Rose Park, SA, Australia) and weight using bathroom scales accurate to 0.1kg (A&D Mercury, Australia). Body mass index (BMI) was calculated as weight in kg divided by the square of the height in meters (kg/m²). The participants’ waist and hips circumference was also measured and their ratio was calculated by a previously standardised method [592]. Resting blood pressure was determined using the mercury sphygmomanometer (Livingstone International, Roseberry, Australia) while participants were in a sitting position after they had been resting for at least 5min. Blood pressure (BP) was determined while the arm was resting on a desk by recording the height of the Hg, to the nearest 2mm, at the first and fifth Korotkoff sounds which were taken to represent the Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP), respectively [550, 592]. Two sets of readings were measured at each visit and the average value was taken as the participants’ resting BP.

At Clinic 1, the screening clinic, the participants were informed about the study protocols and written informed consent was obtained. A fasting finger-prick blood
sample was collected and blood cholesterol and triglycerides were determined using the Accutrend Plus (Roche Diagnostic, Australia), while blood glucose levels were determined using the AccuCheck blood glucose meter (Roche Diagnostic, Australia). If the values were within the desired ranges, the participants were asked to sign a consent form enrolling them into the pilot intervention trial.

The enrolled subjects were then assigned to Group 1 or Group 2 in a randomised fashion using a sequence generated with Microsoft Excel 2003 SP3 package (Microsoft Australia, North Ryde, NSW). All the participants were also asked to refrain from taking GT or functional foods that could affect their blood cholesterol levels and not to start cholesterol-lowering medication during the duration of the trial. The participants were provided with a three-day food record diary and asked to complete it during the three days prior to the commencement of the study. Furthermore, the participants were also asked not to change their physical activity patterns during this time. Those participants with cholesterol or any other values outside the desired ranges were excluded from the study.

At Clinic 2, the participants were asked to provide a fasting venous blood sample and their anthropometric and blood pressure measurements were taken for baseline values. The completed baseline three day food diaries were collected and the participants were provided with another three day food diary to be completed during the last three days of the intervention period. The participants were also provided with a study diary to record any possible adverse effects that they believed were related to the intake of the capsules they were on. The capsules, with EGCG or placebo, were provided to the participants in a double-blind fashion. The participants were instructed to consume two capsules with water at least 30min prior to the first meal in the morning.
after an overnight fast and two capsules at least 30min prior to the evening meal after not eating for at least 4h and to do this daily for the 4-week duration of the study.

**Figure 6.1** The clinical protocols for the pilot study.
At Clinic 3, the participants were asked to provide a fasting venous blood sample and their anthropometric and BP measurements were collected for the ‘end of study’ values. The completed three day food record diaries for the last 3 days of the intervention trial and the study diaries for the whole intervention period were collected. Any unused capsules were also collected from the participants to determine whether they were likely to have complied with the instructions for taking their capsules.

6.2.2.4 Blood collection and storage

The venous blood samples for measurement of cholesterol and Tg were collected by a qualified phlebotomist from the medial cubital vein of the antecubital fossa into EDTA anticoagulated vacutainer tubes (BD, North Ryde, Australia) using a “butterfly needle” (BD, North Ryde, Australia). The blood samples were centrifuged at 3000 × g for 15min at 4°C (Sigma 4K 15 centrifuge) and the plasma was collected, aliquoted within 30min after centrifugation and stored at -84°C in cryogenic vials until assayed.

6.2.2.5 Determination of plasma total cholesterol, HDL cholesterol and triglycerides

Plasma total cholesterol, triglycerides and HDL cholesterol were determined using commercially available kits (Wako Pure Chemicals, Osaka, Japan). All enzyme solutions were prepared according to the manufacturers recommendations. Analysis was performed at 600nm using a Varian Carry UV-Vis spectrophotometer (Varian Australia, Clayton, Victoria) equipped with a multi-cell module and a water temperature control system set at 26°C. The data was collected using the Cary Win UV software (v3) (Varian Australia, Clayton, Victoria) installed on a Dell computer (Dell Pentium IV,
Dell Pty. Ltd, French Forrest, NSW, Australia) assigned as the remote controlling system for the spectrophotometer. Standard curves were developed, using serial dilutions in deionised water of the standards provided with the kit, to determine plasma total cholesterol (Figure 6.2) and HDL cholesterol (Figure 6.3).

**Figure 6.2** Standard curve for total cholesterol analysis in human plasma samples

**Figure 6.3** Standard curve for HDL cholesterol analysis in human plasma samples
The plasma total and the HDL cholesterol in the participant’s samples were calculated by dividing the plasma absorbance value by the slope of the respective standard curve. Each of the participants’ plasma samples were analysed in quadruplicate for plasma TC and in duplicate for HDL cholesterol. The values were converted from mg/dl into mmol/l by multiplying by 0.0259.

For plasma triglycerides a standard curve was not generated; as shown in Equation 6.1, the triglycerides concentrations (mg/dl) were determined by dividing the absorbance of the plasma sample ($A_S$) by the product of the absorbance ($A_{Std}$) and the concentration ($C_{Std}$) of the standard supplied with the plasma triglyceride measuring kit (Wako Pure Chemicals, Osaka, Japan). The standard and the participants’ plasma samples were analysed in triplicate. The values were converted from mg/dl into mmol/l by multiplying by 0.0113.

\[
\text{Triglycerides (mg/dl)} = \frac{A_S}{A_{Std} \times C_{Std}}
\]

**Equation 6.1** Equation for calculating the plasma triglyceride concentration in human plasma using the absorbance of the plasma sample ($A_S$), the absorbance of the standard ($A_{Std}$) and the concentration of the standard ($C_{Std}$).

### 6.2.2.6 Estimation of VLDL, LDL and non-HDL cholesterol

Estimates of the VLDL and LDL cholesterol concentrations were calculated using the Friedewald formula [447](Chapter 2 Equation 2.1) while non-HDL cholesterol (non-HDL-C) was calculated by subtracting the HDL-C from the TC value [447, 593].
6.2.2.7 Indices of cardiovascular risk

Several indices of cardiovascular risk were calculated: the LDL cholesterol to HDL cholesterol ratio (LDL-C/HDL-C) [594-597], the total cholesterol to HDL cholesterol ratio (TC/HDL-C) [598] and the triglycerides to HDL cholesterol ratio (Tg/HDL-C) [599].

6.2.2.8 Nutrient analysis of dietary intake

The three day food records collected from participants on two occasions, the last three days prior to the start (Clinic 2) and the last three days prior to the end (Clinic 3) of the treatment period, were analysed for the nutrient content of the participants’ diet using FoodWorks 2009 Professional (Xyris Software, Qld, Australia) and the mean daily values were determined. If a participant reported eating a food that was not in the database, a food with very similar ingredient composition was chosen. Nutrient information was also obtained from food labels or recipes obtained from the subjects.

6.2.2.9 Monitoring of adverse effects

The participants were asked to monitor the occurrence of adverse effects using the self reporting study diaries during the entire study treatment period. Furthermore, at the end of the treatment period, the participants were provided with an additional 7-day post study diary to monitor any adverse effects that the treatment (placebo or EGCG) may have on their overall health after the study.

6.2.2.10 Data and statistical analyses

At the end of the treatment period, the code used to allocate the participants to capsules containing EGCG or placebo was broken and the participants were allocated to
their respective treatment groups. The plasma lipids (TC, Tg, HDL-C, LDL-C, VLDL-C, non-HDL-C) were averaged for the two treatment groups using Microsoft Excel 2007 Package (Microsoft Australia, North Ryde, NSW, Australia). The unpaired Student t-test was used to examine for differences in the main outcomes between the group means. The paired Student t-test was used to examine for differences in the values within each group between clinic 2 and clinic 3. The different measurements were analysed for correlations between them using linear regression analysis with the Statistical Package for Social Sciences program (PASW Statistics 17). All p values were two tailed and the significance threshold was set at p<0.05.
6.3 Results

6.3.1 Gender, age, anthropometric data and blood pressure

There were no significant differences (p>0.05) observed between the control and EGCG groups in their gender, age and height. The 10 participants, 6 males and 4 females, were equally distributed between the two treatment groups; there were 3 males and 2 females in each group. The average age for all the participants was 51.5±5.3 years and was 53.6±2.5 years for the controls and 49.4±6.7 years for the EGCG group. The average height for all the participants was 1.71±0.08m and was 1.68±0.04m for the controls and 1.73±0.11m for the EGCG group. There were also no significant differences (p>0.05) observed between the control and EGCG groups in their other anthropometric data (weight, BMI, waist circumference, hip circumference and WHR) at the start or at the end of the treatment period and there were no significant changes during the 4-week treatment period (Table 6.1).

There were also no significant differences (p>0.05) between the control and EGCG groups in their DBP and SBP at the start or at the end of the treatment period and there were no significant changes during the treatment period (Table 6.1).

6.3.2 Plasma lipid profiles and indices of CV risk

At the start of the treatment period, the two groups had the same level of mild hypercholesterolaemia, within the targeted range of 5.5-7.5mmol/l; they had very similar values for plasma TC levels (Control 6.56±0.80 vs EGCG 6.53±0.43mmol/l, p=0.959), LDL-C (Control 4.41± 0.64 vs EGCG 3.89±0.78 mmol/l, p=0.282), HDL-C (Control 1.57±0.48 vs EGCG 1.64±0.39mmol/l, p=0.793) and non-HDL-C (Control 4.89±0.27 vs EGCG 4.99±0.34mmol/l, p=0.784).
**Table 6.1** Anthropometric and blood pressure results.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control group</th>
<th>EGCG group</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinic 2</td>
<td>Clinic 3</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>(Pre-treatment)</td>
<td>(Post-treatment)</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.20±8.62</td>
<td>69.08±8.33</td>
<td>0.810</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.52±1.88</td>
<td>24.49±1.81</td>
<td>0.826</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>87.20±11.58</td>
<td>85.86±11.69</td>
<td>0.335</td>
</tr>
<tr>
<td>Hips (cm)</td>
<td>100.26±5.11</td>
<td>100.10±5</td>
<td>0.761</td>
</tr>
<tr>
<td>WHR</td>
<td>0.87±0.09</td>
<td>0.86±0.10</td>
<td>0.261</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>121.80±8.56</td>
<td>130±4.85</td>
<td>0.077</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>71.4±7.6</td>
<td>74±3.16</td>
<td>0.429</td>
</tr>
</tbody>
</table>

Values for each clinic are Mean±SD and the p values are for differences between the two clinics in the control group or the EGCG group. *BMI* – Body Mass Index; *WHR* – Waist to Hip Ratio; *SBP* – Systolic Blood Pressure; *DBP* – Diastolic Blood Pressure.
Plasma triglycerides (Control 1.27±0.39 vs EGCG 2.20±1.36mmol/l, p=0.180) and VLDL cholesterol (Control 0.58±0.18 vs EGCG 1.01±0.62mmol/l, p=0.180) were also not significantly different at baseline.

As presented in Figure 6.4, analysis of the plasma lipids at the end of the 4-week treatment period revealed that there was a significant increase in plasma LDL-C (+15.9%) in the placebo group (p=0.016) but no significant change (+6.8%) in the EGCG group (p=0.365) over the treatment period. The average LDL-C was also higher (25%) in the controls than in the EGCG group (p=0.026) at the end of the treatment period. However, the change from the start to the end of the treatment period was not significantly different (p=0.84) between the two groups.

**Figure 6.4** Mean concentrations of LDL cholesterol at the start and at the end of the treatment period. There was a significant difference in LDL cholesterol in the control group between the pre- and post-treatment values (p=0.016). There was also a significant difference in LDL cholesterol between the control and EGCG group at the end of the treatment period (p=0.026). The values are Mean±SD and those, within the same group (same colour), having the same letter superscript are significantly different (p<0.05). The values with the asterisk symbol (*) are significantly different between the two treatment groups (p<0.05).

The non-HDL-C (Figure 6.5) also significantly increased (+13.4%) in the control group (p=0.038) while it remained unchanged (+2.1%) in the EGCG group.
(p=0.559) over the 4-week treatment period. However, the change from the start to the end of the treatment period was not significantly different (p=0.066) between the two groups. The mean non-HDL-C was also not significantly different between the control and the EGCG groups (p=0.114) at the end of the treatment period.

As shown in Table 6.2, there were no significant changes in the plasma TC concentrations in the control group (+10.1%, p=0.060) or in the EGCG group (+0.87%, p=0.781) over the 4-week treatment period. The change from the start to the end of the treatment period was also not significantly different (p=0.083) between the two groups. The mean plasma total cholesterol level was also not significantly different between the control and the EGCG groups (p=0.089) at the end of the treatment period.

**Figure 6.5** Mean concentrations of Non-HDL-Cholesterol at the start and at the end of the treatment period. There was a significant difference in Non-HDL-Cholesterol in the control group between the pre- and post-treatment values (p=0.038). There was no significant difference in Non-HDL-cholesterol between the control and EGCG group at the end of the treatment (p>0.05). The values are Mean±SD and those, within the same group (same colour), having the same letter superscript are significantly different (p<0.05).
Table 6.2 Plasma lipids and indices of cardiovascular risk.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control group</th>
<th>EGCG group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinic 2</td>
<td>Clinic 3</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>6.56±0.80</td>
<td>7.17±0.49</td>
</tr>
<tr>
<td>Tg (mmol/l)</td>
<td>1.27±0.39</td>
<td>1.21±0.48</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.57±0.48</td>
<td>1.53±0.37</td>
</tr>
<tr>
<td>VLDL-C (mmol/l)</td>
<td>0.58±0.18</td>
<td>0.55±0.22</td>
</tr>
<tr>
<td>LDL-C / HDL-C</td>
<td>3.03±0.48</td>
<td>3.6±1.5</td>
</tr>
<tr>
<td>TC / HDL-C</td>
<td>4.46±1.17</td>
<td>5.02±1.81</td>
</tr>
<tr>
<td>Tg / HDL-C</td>
<td>0.94±0.52</td>
<td>0.92±0.71</td>
</tr>
</tbody>
</table>

Values for each clinic are Mean±SD and the p values are for differences between the two clinics in the control group and the EGCG group. **TC** – Plasma Total Cholesterol; **Tg** – Plasma Triglycerides; **HDL-C** – High Density Lipoprotein Cholesterol; **VLDL-C** – Very Low Density Lipoprotein Cholesterol; **LDL-C** – Low Density Lipoprotein Cholesterol.
Furthermore, there were no significant differences in the calculated indices of cardiovascular risk (Table 6.2) between the pre-treatment and post-treatment values for the LDL-C/HDL-C ratio in the control (p=0.237) and EGCG (p=0.204) groups, the plasma TC/HDL-C ratio in the control (p=0.353) and EGCG (p=0.196) groups and the Tg/HDL-C ratio in the control (p=0.950) and EGCG groups (p=0.292). There were also no significant differences (p>0.05) between the two groups in the change from the start to the end of the treatment period for these three indices and in the mean values at the end of the treatment period. Similarly, there were no significant differences (p>0.05) for VLDL-C (Table 6.2).

6.3.3 Dietary intakes

The participants’ dietary intakes recorded over three days before the start and before the end of the treatment period were analysed using FoodWorks 2009 Professional (Xyris Software, Qld, Australia) and are presented in Table 6.3 as daily intakes. There were no significant differences (p>0.05) between the two groups (Control vs EGCG) in the dietary intake of food (weight and energy), all the macronutrients and most of the micronutrients at the start and at the end of the treatment period. There were also no significant changes (p>0.05) within the two groups from the start to the end of the treatment period and the changes from the start to the end of the treatment period did not differ (p>0.05) between the two groups (Table 6.3).

However, the control group had a significantly lower (-20%) niacin intake at the end compared to at the start of the treatment period (p=0.022) but there was no significant change (-3.6%) in the EGCG group (p=0.703). However, the change from the start to the end of the treatment period was not significantly different (p=0.076)
### Table 6.3 Dietary intakes at the start and at the end of the treatment period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group</th>
<th>EGCG group</th>
<th>p value</th>
<th>Control group</th>
<th>EGCG group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinic 2</td>
<td>Clinic 3</td>
<td></td>
<td>Clinic 2</td>
<td>Clinic 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Pre-treatment)</td>
<td>(Post-treatment)</td>
<td></td>
<td>(Pre-treatment)</td>
<td>(Post-treatment)</td>
<td></td>
</tr>
<tr>
<td>Food Weight (g)</td>
<td>2852±736.45</td>
<td>2548.2±473.04</td>
<td>0.395</td>
<td>3502.6±1163.7</td>
<td>3242±1210</td>
<td>0.691</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>9737.8±3937.34</td>
<td>9940.6±2181.03</td>
<td>0.912</td>
<td>9521.4±2288.54</td>
<td>10088.2±4716.85</td>
<td>0.738</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>227.4±112.92</td>
<td>251.8±70.55</td>
<td>0.654</td>
<td>198.8±72.88</td>
<td>212.4±85.7</td>
<td>0.624</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>109.4±23</td>
<td>93.8±15</td>
<td>0.285</td>
<td>106.4±18.15</td>
<td>115.4±35.35</td>
<td>0.555</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>78.4±34.12</td>
<td>85.6±31.08</td>
<td>0.745</td>
<td>93.6±34.62</td>
<td>93±52.39</td>
<td>0.977</td>
</tr>
<tr>
<td>SFA (g)</td>
<td>28.80±15.69</td>
<td>31.4±6.54</td>
<td>0.726</td>
<td>35.4±13.16</td>
<td>37.8±24.12</td>
<td>0.781</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>30.2±11.21</td>
<td>33.2±14.92</td>
<td>.735</td>
<td>32.6±11.39</td>
<td>35±20.1</td>
<td>0.762</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>11.8±5.54</td>
<td>13±9.54</td>
<td>0.819</td>
<td>17.4±10.45</td>
<td>12.4±5.03</td>
<td>0.397</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>25.4±9.79</td>
<td>22.40±8.41</td>
<td>0.683</td>
<td>25.4±7.09</td>
<td>17±8.63</td>
<td>0.184</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>376.4±101.5</td>
<td>334.2±96.32</td>
<td>0.496</td>
<td>353.2±101.96</td>
<td>526.4±273.68</td>
<td>0.258</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td><strong>31.45±7.64</strong></td>
<td><strong>25.29±8.19</strong></td>
<td><strong>0.022</strong></td>
<td>27.04±5.27</td>
<td>26.08±8.32</td>
<td>0.704</td>
</tr>
</tbody>
</table>

Values for each clinic are Mean±SD and the p values are for differences between the two clinics in the control group and the EGCG group. The bolded values and the asterix (*) denote statistical significance (p<0.05). *SFA* – Saturated Fatty Acids; *MUFA* – Monounsaturated Fatty Acids; *PUFA* – Polyunsaturated Fatty Acids.
between the two groups. The mean niacin intake was also not significantly different between the control and EGCG groups (p=0.318) at the end of the treatment.

6.3.4 Correlations

As expected, the plasma total cholesterol values in both groups and at the start and the end of the treatment period (Table 6.4) were directly and strongly correlated with LDL-C (r=0.875, p<0.001) and non-HDL-C (r=0.797, p<0.001) values and they were also related with systolic blood pressure (r=0.478, p=0.033). Also as expected, the LDL-C values were directly correlated with non-HDL-C (r=0.805, p<0.001) and the LDL-C/HDL-C ratio (r=0.523, p=0.018). The LDL-C values were also inversely correlated with SBP (r= -0.478, p=0.033).

Table 6.4 Some correlations between lipid parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol vs LDL cholesterol</td>
<td>0.875</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Cholesterol vs Non-HDL cholesterol</td>
<td>0.797</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Triglycerides vs HDL cholesterol</td>
<td>-0.603</td>
<td>0.005**</td>
</tr>
<tr>
<td>Triglycerides vs LDL cholesterol</td>
<td>-0.619</td>
<td>0.004**</td>
</tr>
<tr>
<td>Triglycerides vs Cholesterol/HDL cholesterol</td>
<td>0.456</td>
<td>0.043*</td>
</tr>
</tbody>
</table>

The r value = correlation coefficient.
*Significant at p<0.05.
**Significant at p<0.01.

The HDL-C values were inversely and strongly correlated with the values for VLDL-C (r= -0.604, p=0.005), the LDL-C/HDL-C ratio (r= -0.765, p<0.001) and the plasma TC/HDL-C ratio (r= -0.903, p<0.001). The HDL-C values were also inversely
correlated with the participants’ weight (r= -0.470, p=0.036), diastolic blood pressure (r= -0.505, p=0.023), BMI (r= -0.596, p=0.006) and WHR (r= -0.599, p=0.005).

The plasma Tg values were inversely correlated with HDL-C (r= -0.603, p=0.005), LDL-C (r= -0.619, p=0.004), the plasma TC/HDL-C ratio (r=0.456, p=0.043) and the Tg/HDL-C ratio (r=0.949, p<0.001). The plasma Tg levels were also significantly correlated with the participants’ weight (r=0.707, p<0.001), DBP (r=0.666, p<0.001), BMI (r=0.676, p=0.001) and WHR (r=0.601, p=0.005).

The plasma total cholesterol values (Table 6.5) were not significantly correlated with the dietary intake of SFA (r= -0.129, p=0.588), MUFA (r=0.013, p=0.957), PUFA (r= -0.126, p=0.595), cholesterol (r=0.160, p=0.501) and total fat (r= -0.070, p=0.769). However, the plasma TC values were significantly inversely correlated with the dietary intake of calcium (r= -0.474, p=0.035), β-carotene equivalents (r= -0.490, p=0.028) and total vitamin A equivalents (r= -0.557, p=0.011).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol vs Dietary Cholesterol</td>
<td>0.160</td>
<td>0.501</td>
</tr>
<tr>
<td>Cholesterol vs Dietary Fat</td>
<td>-0.070</td>
<td>0.769</td>
</tr>
<tr>
<td>Cholesterol vs Dietary SFA</td>
<td>-0.129</td>
<td>0.588</td>
</tr>
<tr>
<td>Cholesterol vs Total Sugars</td>
<td>-0.500</td>
<td>0.025*</td>
</tr>
<tr>
<td>Cholesterol vs Dietary Calcium</td>
<td>-0.474</td>
<td>0.011*</td>
</tr>
<tr>
<td>LDL cholesterol vs Dietary Calcium</td>
<td>-0.529</td>
<td>0.017*</td>
</tr>
<tr>
<td>HDL Cholesterol vs Dietary Calcium</td>
<td>-0.356</td>
<td>0.124</td>
</tr>
</tbody>
</table>

Table 6.5 Some correlations between plasma lipid parameters and dietary intake values.

The r value = correlation coefficient.
*Significant at p<0.05.
The LDL-C values were also significantly and inversely correlated with the dietary intake of calcium (r= -0.529, p=0.017) and also of retinol (r= -0.450, p=0.046). The HDL-C values were also significantly and inversely correlated with the dietary intake of vitamin A equivalents (r= -0.456, p=0.043) but not with the dietary intake of β-carotene equivalents (r= -0.379, p=0.100) and calcium (r= -0.356, p=0.124). There were also significant and direct correlations between the plasma triglyceride values and the dietary intake of retinol (r=0.542, p=0.014) and calcium (r=0.574, p=0.008).

6.3.5 Capsule consumption compliance and adverse effects

As estimated by counting the number of capsules left in the containers brought in by the participants at the end of the study, compliance with the instructions for taking the capsules was relatively high in both groups. It was estimated that the control group consumed on average 3.9 capsules/day, which equated to a 98.3% compliance rate, while the EGCG group consumed on average 3.7 capsules/day, which equated to a 92.9% compliance rate but there was no significant difference (p=0.245) between the two groups in compliance. At the end of the study, when the participants were asked to guess which group they were assigned to, only one from each group guessed correctly, three guessed wrongly and five said that they did not know.

At the end of the treatment period, five participants (four in the placebo group and 1 in the EGCG group) reported on some adverse effects during the treatment period, which they believed may have been related to the capsules they were taking. One different symptom was reported by the four participants in the control group who reported adverse effects: one participant reported a headache, which was not an unusual occurrence, one reported some dry belching during the first week of the study, one reported one bout of diarrhoea and one reported more frequent visits to the bathroom to
defecate during the course of the study. However, these reported symptoms did not pose much concern to the participants and all four voluntarily continued with the consumption of their capsules and completed the 4-week treatment period.

One participant from the EGCG group reported some hay fever symptoms including sneezing, itchy throat and excess mucus from day 7 of the 4-week treatment period until the 6th day after the end of the study period (post-treatment). This participant also voluntarily continued with the consumption of the capsules and finished the study because the symptoms were not of major concern.
6.4 Discussion

In this Chapter, it was hypothesised that ‘pure EGCG would lower blood cholesterol in humans with moderately elevated cholesterol levels, if given at a higher dose than 300mg/day, as suggested by Hill et al.[428]. Furthermore, based on the results of the EGCG absorption study in Chapter 5, the main aim of the study was to determine if pure EGCG given for four weeks in the form of capsules taken without food on an empty stomach would lower blood cholesterol in humans with moderately elevated cholesterol.

A pilot study in 10 participants, 6 males and 4 females, with moderate hypercholesterolaemia between 5.5-7.5mmol/l, was conducted to test the hypothesis. The study was a 4-week, double blind, randomised, placebo-controlled, parallel design intervention trial with 1g EGCG/day given in two 500mg doses, one in the morning and the other in the evening. The participants were provided with placebo (Control group) or 250mg EGCG (EGCG group) capsules and instructed to consume two capsules at least 30min prior to their morning meal and two capsules at least 30min prior to their evening meal, and to do this daily for 28 consecutive days (4 weeks).

The aim was achieved in that all 10 participants completed the study. Fasting blood samples were collected at baseline and at the end of the study and plasma total cholesterol, LDL cholesterol, HDL cholesterol and plasma triglycerides and the ratios of LDL-C/HDL-C, HDL-C/TC and Tg/HDL-C, were determined.

The hypothesis that ‘pure EGCG would lower blood cholesterol in humans with moderately elevated cholesterol levels, if given at a higher dose than 300mg/day, was not directly supported. At the end of the treatment period, the total plasma cholesterol was not significantly different (p=0.089) in the EGCG group compared to the control group (Table 6.2).
Importantly however, the hypothesis was partially supported because, at the end of the treatment period, the LDL cholesterol was 25% lower in the EGCG group (p=0.026) compared to the placebo group (Figure 6.4). This difference was due to a 15.9% increase in plasma LDL cholesterol (p=0.016), which occurred in the placebo group during the 4-week treatment period, while there was no significant change (+6.8%, p=0.365) in the EGCG group.

Therefore, it appeared that there may have been a general increase in the LDL cholesterol during the 4-week treatment period and that the EGCG was able to prevent the increase in the treatment group. This was also reflected in the non-HDL cholesterol (Figure 6.5); it also increased 13.4% in the control group (p=0.038) while it remained unchanged (+2.1%) in the EGCG group (p=0.559) over the 4-week treatment period. Although there were no significant changes, a similar trend was observed for the plasma total cholesterol concentrations (Table 6.2); the values were +10.1%, (p=0.060) for the control group and +0.87% for the EGCG group (p=0.781) over the 4-week treatment period.

The findings of this pilot study in humans are only partially consistent with previous research in animal models for hypercholesterolaemia [242, 401, 402, 408, 413-416, 432, 433, 469, 516, 517], and with the findings in the animal model experiment presented in Chapters 3 and 4. In our rabbit study, pure EGCG reduced cholesterol by 85% (p=0.021) in the treatment group while it remained unchanged in the control group at the end of the treatment. These results were entirely consistent with a study by Bursill et al. [242], which reported that administration of a crude catechin extract reduced blood cholesterol levels by 60% by up-regulating the hepatic LDL-receptor and reducing cholesterol synthesis.
The findings of this pilot study are consistent with some of the previous studies in humans and suggest that effects may be more readily seen on LDL cholesterol rather than on plasma total cholesterol. Furthermore, a 2-week stabilisation period before the treatment period, and a 4-week treatment period may not be sufficient for reaching new metabolic steady states in cholesterol metabolism [600].

For example, the present LDL cholesterol findings reflect those in a study in which the LDL cholesterol increased significantly by 6.3% over a 3-week treatment period in a control group of women but remained unchanged in the women treated with a catechin extract [407]. However, a randomised parallel study by Inami et al.[601] in 40 human volunteers reported that there was no significant decrease in plasma LDL cholesterol after four weeks of treatment despite evidence of systemic effects on the oxidation of LDL cholesterol, which was significantly decreased by over 11% with the administration of a catechin extract.

In a 6-week study, Erba et al.[438], observed an 11% lowering of LDL cholesterol with a catechin extract. However, similar to the present results, there were no significant effects on plasma total cholesterol. In a longer controlled human intervention study over a period of 12 weeks Nagao et al. [436] also reported a significant lowering of LDL cholesterol by a catechin extract but again no effect on plasma total cholesterol. However, there may also be a difference between men and women in their cholesterol response to catechins; in the 3-week study by Nantz et al. [407], there was a significant lowering of both LDL cholesterol and plasma total cholesterol with a catechin extract in men but not in women. In the present study, there was no difference between the groups in gender makeup and the numbers were too small to look for gender differences.
From the 3-week study by Nantz et al. [407], it also appears that the baseline level of LDL cholesterol is a factor which affects whether the catechins can lower the LDL cholesterol; there was only a significant lowering of LDL cholesterol (-7%) when the subjects had baseline LDL cholesterol above 2.5mmol/l. There was no change in LDL cholesterol in the subjects with baseline LDL cholesterol below 2.5mmol/l. Therefore, there may have been significant effects on LDL cholesterol in the present study because the participants had baseline LDL cholesterols well above 2.5mmol/l (Figure 6.4).

Interestingly, in the studies where there were significant effects, especially on LDL cholesterol, only moderate amounts of catechins were used [407, 436, 438]. Therefore, the suggestion by Hill et al.[428], that the main reason for the limited changes in the health parameters in some studies is likely to be the relatively low amounts of EGCG administered, usually no more than 300mg/day, may not necessarily be correct. Other factors may be involved, including the consumption of the catechins with food [587], which, as illustrated in Chapter 5, can dramatically lower the systemic absorption of EGCG.

In the present study, the possibility that background dietary factors were responsible for the differences observed in the LDL cholesterol, was also investigated (Table 6.5). The dietary factors most likely to have been involved such as saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, total fat and dietary cholesterol were all not significantly related to plasma cholesterol levels (p>0.05).

However, the intake of dietary calcium showed a significant inverse relationship with plasma (r= -0.474, p=0.011) and LDL cholesterol (r= -0.529, p=0.017). These findings are in contrast with many previous studies which have related a higher dietary intake of calcium with a higher plasma cholesterol level and an increased incidence of
coronary heart disease [602-606]. This relationship is largely due to the food group that dietary calcium mostly comes from, dairy products high in saturated fats which can increase blood cholesterol. Therefore, the type and quantity of dietary fat in this food group may possibly override any cholesterol lowering effect of calcium [607].

In the present study, dietary calcium (Table 6.5) was positively and highly correlated with saturated fat intake (r=0.624, p=0.003) but not with total fat (r=0.545, p=0.13), a finding consistent with the dietary calcium coming primarily from the dairy food group. Some studies have indicated that milk and dairy products have the capacity to lower plasma lipids [608, 609]. However, this effect may be due to other bioactive substances in milk and milk products [610, 611]. Interestingly, a study in obese Zucker rats [612] identified that calcium could dose-dependently increase the intestinal absorption of cholesterol and increase cholesterol synthesis while paradoxically lowering plasma cholesterol. This finding was explained by an increase in the faecal excretion of bile acids causing an increase in the conversion of cholesterol to bile acids [265], a pathway which can account for nearly 50% of the daily elimination of cholesterol from the body [613].

The results of the present pilot study indicated that daily consumption of 1g EGCG per day in two separate doses of 500mg (morning and evening) was well tolerated and did not cause any major side effects. Four of the five participants in the treatment group did not report significant disturbances but one participant reported hay fever-like symptoms after the first week, which may have been related to the trial. However, the symptoms were not unbearable and the subject voluntarily completed the study. Furthermore, more of the participants on the placebo gelatine capsules (4 out of the 5 subjects) than on the EGCG capsules (1 out of 5 subjects) reported other minor adverse effects.
This finding is consistent with other studies in humans using EGCG as a functional component in extracts [419, 588, 589]. These studies have shown very good tolerance to these catechin extracts with only mild side effects reported such as gastrointestinal complaints (abdominal bloating, flatulence and nausea) and central nervous system stimulation (agitation and restlessness), all of which appear to be primarily due to the high caffeine content (7% w/w) of the preparations [589]. Additionally, doses of pure EGCG up to 1.6g/day have been shown to be well tolerated without any adverse cardiovascular, clinical chemistry or physical findings [420].

The main limitation of this study was the low number of participants and the results cannot be extrapolated to the general population at large with moderate hypercholesterolaemia. As discussed above, the acclimatisation and treatment periods may also have been too short. However, as a pilot study, it did show promise in that 1g/day EGCG in 2 doses of 500mg was well tolerated and that significant positive differences were observed for LDL cholesterol to warrant further trials with more significant subject numbers and with longer stabilisation periods to prevent the LDL cholesterol increases seen in the present study. The mechanisms of action will also need to be looked at, especially those worked out in animal models of hypercholesterolaemia, including in Chapters 3 and 4: an increase in the LDL-receptor, a reduction in cholesterol synthesis, and less likely, an increase in the faecal excretion of cholesterol.

Finally, the bioavailability of EGCG was not measured in this study because it would have been somewhat of a repetition of the study already described in Chapter 5. However, it is acknowledged that the bioavailability of EGCG may have been different because the participants were allowed to eat 30 min after taking the supplement instead of 4 h as described in Chapter 5. However, in the clinical setting, ‘taking prescription drugs 30 minutes before a meal’ is a very common instruction.
In conclusion, the results of this pilot study have shown that pure EGCG suppressed the rise in LDL and non-HDL cholesterol compared to a placebo. This finding could not be ascribed to differences in the dietary intake values for macronutrients, including saturated fat for example, and most of the micronutrients, although the dietary intake of calcium may have had an impact.
Chapter 7. General Discussion and Conclusions

Cardiovascular disease still remains the single leading cause of death and disability in the world and it is unlikely that a significant favourable change will be achieved in the near future [4]. The negative effect of high blood cholesterol levels, especially high levels of LDL cholesterol, on the development of atherosclerosis has been well established in numerous large population based studies [37-43]. Therefore, cholesterol lowering therapies, including the use of potential hypocholesterolaemic compounds found in a variety of different foods, including green tea (GT), are becoming increasingly popular.

Although the good health promoting properties have been related with GT consumption for several centuries in the traditional Chinese and Japanese cultures [352, 356] it is only relatively recently that the health effects of GT have been investigated in the western scientific literature [352]. Although there are a number of compounds found in GT, the beneficial effects have primarily been ascribed to its most predominant compounds, the polyphenolic compounds called the catechins and, of the main four catechins, the most prevalent catechin, EGCG.

Based mostly on reports of cholesterol lowering effects of GT catechin extracts, the main aim of the present study was to determine the effect of pure EGCG on blood cholesterol 1) in the cholesterol-fed, hypercholesterolaemic rabbit model and 2) in humans with moderately elevated blood cholesterol levels. The working hypothesis for the study was that ‘pure EGCG would lower cholesterol in hypercholesterolaemic rabbits and humans’.

The main outcomes of the study were the findings that: 1) EGCG lowered blood cholesterol levels in the cholesterol-fed hypercholesterolaemic rabbit model by possibly up-regulating the hepatic LDL-receptor and decreasing cholesterol synthesis by
inhibiting it at the level of the enzyme squalene epoxidase (Chapter 3) but not by increasing the faecal excretion of cholesterol and other sterols (Chapter 4); 2) EGCG was absorbed best when administered in capsule form without food (Chapter 5) and 3) EGCG kept LDL cholesterol lower in humans with moderately elevated cholesterol levels, compared to a placebo over a 4-week parallel intervention trial (Chapter 6). The present chapter provides a summary and discussion of the most significant findings in this thesis.

7.1 Mechanisms by which pure EGCG lowered blood cholesterol in the cholesterol-fed hypercholesterolaemic rabbit model

The cholesterol lowering effects of GT catechins in animal studies [401, 402, 408, 413-416, 432, 433, 469, 516, 517] have primarily been ascribed to EGCG, the most abundant catechin found in GT. Furthermore, it has been postulated that the hypocholesterolaemic effect of the GT catechins occurs through three mechanisms of action; up-regulation of the LDL receptor [242, 434], reduction of cholesterol synthesis [242] and inhibition of cholesterol absorption leading to an increased faecal excretion of sterols [402, 408, 413-416]. Despite un-dismissible evidence that GT catechins have these effects, *in vivo* cholesterol lowering studies with pure EGCG are relatively scarce.

Therefore, the main aim of the study presented in Chapters 3 and 4 was to determine the effect of pure EGCG on blood cholesterol in the cholesterol-fed hypercholesterolaemic rabbit model. It was hypothesised that ‘pure EGCG would lower blood cholesterol levels by up-regulating the LDL-receptor (Chapter 3), reducing cholesterol synthesis (Chapter 3) and increasing the faecal excretion of neutral and/or acidic sterols (Chapter 4)’.
The most significant outcome from this animal study was that serum cholesterol was effectively lowered by 85% in the group of six rabbits receiving 2% (w/w) EGCG incorporated into their rabbit chow along with 0.25% (w/w) cholesterol. Although this finding is consistent with other similar animal studies done with GT extracts [242, 401, 424, 434, 468, 469], this is the first study to demonstrate the effect with pure EGCG in the cholesterol-fed hypercholesterolaemic rabbit model.

Furthermore, the results presented in Chapter 3 were consistent with the cholesterol lowering having been achieved at least in part by an increase in the LDL-receptor (+59%), which is a primary pathway for the removal of cholesterol from the circulation (Figure 7.1). The findings were also consistent with previous in vitro studies, which had shown an increase in the LDL-receptor of human liver HepG2 cells treated with a GT extract [410] or pure EGCG [411, 412], and with animal intervention trials in rabbits and rats [242, 434], which had indicated that GT extracts with a high content of EGCG increased their hepatic LDL-receptor.

Therefore, the present results, taken together with the previous cell and animal findings, strongly suggest that EGCG is the primary active component of GT and GT extracts and that up-regulation of the LDL-receptor plays a pivotal role in the lowering of blood cholesterol by EGCG. Furthermore, the increase in the hepatic LDL-receptor implies that EGCG needs to be absorbed from the intestines and travel to the liver - for it to have such a systemic effect (Figure 7.1).

Although less strong, the results presented in Chapter 3 were also consistent with the cholesterol lowering having been achieved at least in part by inhibition of cholesterol synthesis at the level of squalene epoxidase (SE) [239, 241, 245, 247, 452, 473], a key rate limiting enzyme in the cholesterol biosynthetic pathway (Section 1.2.1). This was observed using the serum lathosterol to squalene ratio, which is a way of
looking at the eventual conversion of squalene, the substrate for SE, down the cholesterol synthesis pathway to lathosterol, a close precursor for cholesterol [479]. Therefore, the present results identified the effect of EGCG on the SE enzyme and its possible impact on serum cholesterol as an avenue for further research. Furthermore, the apparent inhibition of the SE enzyme also implies that EGCG needs to be absorbed from the intestines - for it to have such a systemic effect (Figure 7.1).

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

The results presented in Chapter 3 also identified that the EGCG treatment also increased the liver CD36 protein in the rabbits. Although CD36 is referred to as a scavenger receptor, which can interact with modified LDL and some native lipoproteins[336, 474], the effect of EGCG on this protein is entirely novel and it is not known whether up-regulated CD36 could play a role in lowering blood cholesterol.
Therefore, the present results identified the effect of EGCG on CD36 as an avenue for further research. However, as for the systemic effects of EGCG on the LDL receptor, its effect on the hepatic CD36 protein also implies that the catechin needs to be absorbed from the intestines and travel to the liver - for it to have such a systemic effect (Figure 7.1).

In contrast to previous findings in the mice, rats and hamsters [401, 402, 408, 413-416, 432, 433, 469, 516, 517], the results presented in Chapter 4, showed that the faecal excretion of neutral and acidic sterols was not significantly increased by EGCG in the rabbits. Therefore, the present results indicated that EGCG did not have the expected intra-luminal effect in the intestines of the rabbit, most likely due to its longer and different small intestine compared to mice, rats and hamsters [526]. Furthermore, the systemic effects of EGCG reported in Chapter 3, the increase in the LDL-receptor and possibly a decrease in cholesterol synthesis, rather than intra-luminal effects on intestinal cholesterol and bile acid absorption, are likely to be the main mechanisms of action by which EGCG lowered the rabbits’ serum cholesterol in this study (Figure 7.1).

### 7.2 Maximising the systemic absorption of EGCG in humans

Based on the results from the study in Chapters 3 and 4, it appeared that EGCG needed to be absorbed from the intestine in order to have effects on systemic mechanisms of action which led to a lowering in serum cholesterol in the cholesterol-fed hypercholesterolaemic rabbit model. It was also reasonable to conclude that EGCG would also need to be absorbed from the intestines in order to have cholesterol lowering effects in humans.

Therefore, the aim of the study presented in Chapter 5 was to investigate the best of three methods used to administer EGCG, in order to maximise its systemic
absorption in humans and thereby maximise its potential cholesterol lowering effect. It was hypothesised that ‘pure EGCG would be absorbed better when given as a capsule without food and give the highest plasma EGCG concentrations when given in this format compared to when given as a capsule with a breakfast’. However, it was also hypothesised that ‘incorporating EGCG in a food product like a strawberry sorbet would improve the absorption of EGCG compared to taking the catechin in capsule form with a breakfast’.

Consistent with previous human trials that looked at the absorption of EGCG taken with and without food [380, 420, 440, 528, 572], the results presented in Chapter 5 indicated that pure EGCG was best absorbed when taken in capsule form without any food after an overnight fast compared to when the EGCG was taken in capsule form with a breakfast of cereal and milk or whether the catechin was incorporated in a strawberry sorbet.

There are several plausible explanations for the finding that food dramatically lowers the absorption of EGCG, including a change to more alkaline pH values in the upper portion of the small intestine due to the food intake [560], an increase in transit time for EGCG through the alkaline environment of the small intestine in the presence of food that is not favourable for EGCG stability [534, 541] and the binding of EGCG by food components like protein [562], which could make the EGCG unavailable for absorption. In the study presented in Chapter 5, the protein component of the cereal and milk breakfast and the strawberry sorbet was inversely related to the absorption of EGCG expressed as AUC over the period of 8 hours. Therefore, the results were consistent with the protein content of food having a negative impact on the absorption of EGCG from the intestine.
Based on the findings of the study in Chapter 5, ingesting EGCG in capsules with water on an empty stomach was taken to be the most appropriate method for the oral delivery of EGCG in the pilot study investigating the cholesterol lowering potential of EGCG in humans with moderately elevated blood cholesterol, as presented in Chapter 6.

7.3 Pilot study with EGCG in humans with moderately elevated cholesterol

Based on the study in Chapters 3 and 4, it appeared that EGCG needed to be absorbed from the intestine in order to have effects on systemic mechanisms of action which led to a lowering in serum cholesterol (Figure 7.1). Furthermore, based on the findings of the study in Chapter 5, ingesting EGCG in capsules with water on an empty stomach was the most appropriate method for the oral delivery of EGCG. Therefore, the aim of the pilot study presented in Chapter 6 was to determine if pure EGCG, given for four weeks, in capsule form before meals, could lower blood cholesterol in humans. It was hypothesised that ‘pure EGCG would lower blood cholesterol in humans with moderately elevated cholesterol levels between 5.5-7.5 mmol/l’.

The results of the pilot study (Chapter 6) in 10 individuals (6 males and 4 females) showed that pure EGCG suppressed a rise in LDL cholesterol seen with the placebo. While there was no difference between the two groups in LDL cholesterol at the start of the 4-week intervention period, the LDL cholesterol was 25% lower in the EGCG group (3 males and 2 females) compared to the placebo group (3 males and 2 females). This difference was due to a 15.9% increase in plasma LDL cholesterol in the placebo group while there was no significant change in the EGCG group.

The results of the pilot study also indicated that the daily consumption of 1g EGCG, in two separate doses of 500mg in the morning and in the evening without food,
everyday for 28 days was well tolerated and did not cause any major side effects. Furthermore, the differences in LDL cholesterol could not be ascribed to differences or changes in the background dietary intake values for macronutrients, including saturated fat, and most of the micronutrients, although the dietary intake of calcium may have had an inverse impact.

The LDL cholesterol findings in the present study were consistent with other studies which have 1) found that LDL cholesterol increased in a control group and not in a group given a GT catechin extract [407] and 2) found a significant lowering of LDL cholesterol with a GT catechin extract compared to control with no significant effects on plasma total cholesterol [438].

Therefore, it appears that the effects of EGCG may be seen when LDL cholesterol is determined but its effects may be missed when only total blood cholesterol is measured. Nonetheless, it is well established [37-43] that high levels of LDL cholesterol are the “bad cholesterol”, as referred in the popular press, because it is the cholesterol associated with this lipoprotein, which is a major risk factor for the development of atherosclerosis (Section 1.3.1.5).

7.4 Study limitations

In both the rabbit and human studies described in this thesis, the fundamental limitation was a low number of rabbits or humans. However, in the rabbit study, the amount of EGCG used at 2% (w/w) in the diet would be supra-pharmacological in humans, and the likelihood of seeing changes were high based on a previous study in the same number of rabbits using a GT catechin extract [242]. Therefore, as hypothesised in Chapter 3, six rabbits in each group was sufficient to observe
significant differences for the systemic effects of EGCG on blood and liver cholesterol and on the LDL-receptor and the lathosteryl/squalene ratio.

However, in Chapter 4, where no significant effects were seen on the faecal excretion of neutral and acidic sterols, it may have been that there was more variation in this body function between the rabbits and that having a higher number of rabbits may have shown significant differences. However, the fact that GT catechin extracts and EGCG have been observed to increase the faecal sterol excretions when similar numbers of mice, rats and hamsters were used [401, 402, 432, 433, 469, 516, 517], mitigates this possibility.

In both human studies, the EGCG absorption study (Chapter 5) and the pilot EGCG intervention study (Chapter 6), a limited number of participants were recruited, four and ten, respectively. However, despite the relatively small number of participants in the EGCG absorption study (four), the results were dramatically different for EGCG taken with or without food. The study was also a crossover study, where each participant was given the EGCG in the three different ways on three separate occasions, in order to minimise variation in the results. The results unequivocally revealed that the EGCG was best absorbed when it was consumed in capsule form without any food after an overnight fast, a finding which supported the results of a previous study by Chow et al.[440], in which a GT catechin extract was given to ten subjects.

As all pilot studies are, the EGCG intervention trial with five subjects in each group (Chapter 6) was somewhat underpowered. However, despite the low numbers, the LDL cholesterol was found to be significantly lower (−25%) in the EGCG group compared to the placebo group at the end of the 4-week treatment period, a finding which also supported previous studies [407, 438], which found significant reductions in LDL cholesterol with GT catechin extracts compared to placebo but with no significant
effects on plasma total cholesterol. This finding can also be ascribed to the increase in LDL-cholesterol in the placebo group.

7.5 Future directions

The work in this thesis has provided interesting and novel findings on the cholesterol lowering effects of EGCG in rabbits and humans and the rabbit study has provided evidence for the mechanisms of action for these effects. However, as discussed in Section 7.4, the studies have their limitations, especially the EGCG intervention pilot study.

The findings of the rabbit study described in Chapters 3 and 4 have certainly identified that EGCG can lower serum cholesterol in the cholesterol-fed hypercholesterolaemic rabbit model and that this can be primarily ascribed to systemic effects: an increase in the hepatic LDL-receptor and possibly a decrease in cholesterol synthesis as defined by the lathosterol to squalene ratio, but not to the intra-luminal effect of increasing the faecal excretion of sterols (Figure 7.1).

Although the results for the up-regulation of the LDL-receptor were clear-cut and entirely consistent with 1) the results from previous studies using a GT catechin extract in the same rabbit model [242] and in a rat model [434] of hypercholesterolaemia and 2) the results from studies with the human liver HepG2 cell line [410, 411], the results for the down-regulation of cholesterol synthesis were not as consistent with the previous rabbit and rat studies [242, 434].

In the previous rabbit study with a GT catechin extract, a 60% decrease in the whole body cholesterol synthesis index (the lathosterol/cholesterol ratio) was observed, but it was not observed in the present study or in the previous rat study [434]. However,
in the present study, another index of cholesterol synthesis, the lathosterol/squalene ratio was significantly lower in the EGCG treated group compared to control.

Therefore, further studies are needed to determine whether the effects of EGCG around the ‘squalene part’ of the cholesterol biosynthetic pathway (Figure 1.8) can lead to substantial lowering in plasma or LDL cholesterol [239, 241, 245, 247]. Furthermore, although a study by Abe et al. [247] has reported that EGCG can non-competitively inhibit the SE enzyme in rats, there is potential for EGCG inhibiting the other two important enzymes, which are also important components in this part of the cholesterol biosynthetic pathway (Figure 1.8).

The results in Chapter 3, also found that EGCG caused a significant increase of CD36 protein in the liver of the rabbits. Although what is known about specific aspects of CD36 signalling involves CD36 having the capacity to bind and deliver biologically active lipids to cells [489, 490], the mechanisms by which this facilitation occurs remains unclear. Furthermore, although CD36 is referred to as a scavenger receptor, which can interact with modified LDL and some native lipoproteins [336, 474], the effect of EGCG on this protein is entirely novel and it is not known whether up-regulated CD36 could play a role in lowering blood cholesterol.

Clearly, this is an interesting avenue for further research. For example, whether EGCG affects CD36 in animals not fed cholesterol and whether the up-regulation of CD36 described in this thesis was a direct effect of EGCG on the expression of the liver protein or due to a decrease in oxidised forms of cholesterol or fatty acids related to the strong antioxidant capacity of EGCG, are questions which could be investigated.

The findings of the EGCG absorption study (Chapter 5) identified that pure EGCG was best absorbed when it was taken without food on an empty stomach after an overnight fast. Furthermore, it was seen that incorporating it in a strawberry sorbet did
not improve its absorption over other foods. Clearly further studies are needed to
determine if any other sort of packaging could improve its intestinal absorption.
Therefore, studies could be done to determine whether the use of currently
available encapsulation techniques could help preserve the EGCG in the alkaline
environment of the small intestine and increase its absorption.

In alkaline environments, such as found in the small intestines, EGCG is well
known to undergo epimerisation and oxidation, which could undoubtedly cause its poor
bioavailability. In support of this, an in vitro study by Dube et al. [614] identified that
the use of some very potent reducing agents such as tris(2-carboxyethyl)phosphine
(TCEP) provided the best protection to a variety of different catechins including EGCG
under very alkaline conditions. However, TCEP is not approved as a food component
and cannot be used in humans. Nonetheless, the encapsulation of EGCG in combination
with other very potent reducing agents used as common food preservatives, such as
ascorbate (vitamin C) and butylated hydroxytoluene (BHT), could lead to the
development of EGCG formulations, which are more stable in the alkaline environment
of the small intestine.

A recent study by Ru et al. [569] also identified that EGCG could be successfully
encapsulated in oil-in-water emulsions and stabilised with the use of proteins
(carragenan and β-lactoglobulin). However, although the use of proteins for the
encapsulation of nutraceuticals is very common, the findings from the EGCG
absorption study in Chapter 5, suggest that the use of proteins for encapsulation may
have a negative effect on the systemic absorption of EGCG encapsulated in this way.
Therefore, the use of encapsulation materials that are not protein based, such as
liposomes, need to be investigated to determine whether they can improve the systemic
absorption of EGCG and thus increase its potential cholesterol lowering properties.
On the other hand, some studies suggest that the delivery of EGCG down to the lower small intestines, the ileum, where the absorption of EGCG is likely to occur, may not be the main issue. For example, a study in human ileostomy subjects found that 51% of EGCG imbibed as green tea was able to survive intact all the way down the small intestine to the ileum [615]. This paper suggested that the low absorption of EGCG and other catechins was more likely to be due to a block in intestinal cells which caused the catechins to remain in the intestinal cells long enough to get sulphated or glucoronidated and then shifted back out into the lumen rather than through the cells into the portal circulation (Figure 7.2).

Figure 7.2 Regulation of ECG uptake and efflux by specialised cellular transporters in intestinal cells. ECG – Epicatechin-3-gallate; MCT – Monocarboxylate transporter; MRP2 – Multi-drug associated protein 2; P-gp – P-glycoprotein; Adapted from [616]

In support of this, there is evidence that the uptake and efflux of EGCG by intestinal cells is not a passive phenomenon but is regulated by specialised cellular transporters [616, 617]. Although the study was mostly done with ECG, the paper by
Vaidyathan and Walle [616], indicated that these cellular transporters could be responsible for actively taking the catechins into the intestinal cells and then actively returning most of them back into the lumen rather than into the portal circulation (Figure 7.2). Therefore, understanding which cellular transporters are involved in the trafficking of EGCG through the intestinal cells and how they are regulated is clearly an important area for further research. An increased understanding of this process could possibly ultimately lead to being able to increase the systemic absorption of EGCG.

The results of the human pilot study in Chapter 6 revealed that EGCG suppressed the rise in LDL cholesterol in moderately hypercholesterolaemic humans compared to a placebo. Therefore, the results were sufficient to warrant further studies be done with higher numbers of moderately hypercholesterolaemic subjects. Apart from higher numbers, these studies should ensure that the LDL cholesterol is better stabilised during the lead in period prior to the intervention and consideration should be given to also running the studies over at least 12 weeks to ensure enough time is given to reach any new metabolic steady state for cholesterol metabolism during the intervention period. A cross-over design should also be used to increase the statistical power to find significant effects.

7.6. Conclusions

In conclusion, 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 compared to control. The human pilot study also showed that it was safe to give this amount of EGCG over this 4-week period, as no serious adverse effects were noted.
8. References

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132. !!! INVALID CITATION !!!


