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Dietary Enrichment by Almond Supplementation: Effects on Risk Factors for Cardiovascular Disease

Miss Khujesta Choudhury
Doctor of Philosophy

Aston University
December 2008

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Aston University
Dietary Enrichment with Almond Supplementation: Effects on Risk Factors for Cardiovascular Disease.
By Miss Khujesta Choudhury
For the Degree of Doctor of Philosophy

Summary

Cardiovascular disease (CVD) is the leading cause of death in Europe responsible for more than 4.3 million deaths annually. The World Health Organisation funded the Monica project (1980s-1990s) which monitored ten million subjects aged 22-65yrs, and demonstrated that coronary heart disease (CHD) mortality declined over 10 years, was due in two thirds of cases to reduced incidence of CHD (reduced risk behaviours e.g. poor diet and smoking) and one third by improved treatments.

Epidemiological evidence suggests diets rich in antioxidants decrease incidence of CVD. Regular consumption of nuts, rich in vitamin E and polyphenols reduces atherosclerosis, an important risk for heart disease. Intervention studies to date using alpha tocopherol (an active component of vitamin E) have not consistently proved beneficial.

This thesis aims to investigate the effect of almond supplementation on vascular risk factors in healthy young males (18-35yrs); mature males and females (>50yrs); and males considered at increased risk of CVD (18-35yrs) in a cohort of 67 subjects. The effects of almond intake were assessed after 25g/d for four weeks followed by 50g/d for four weeks and compared to a control group which did not consume almonds or change their diet. Cardiovascular risk was assessed by plasma lipid profiles, apolipoprotein A1, plasma nitrates/nitrates, vascular flow, BMI, blood pressure, sVCAM-1 and protein oxidation.

Systolic and diastolic blood pressures were reduced in almond supplemented volunteers but not in controls. Dietary monounsaturated fatty acids, polyunsaturated fatty acid content and total dietary fats were increased by almond supplementation. Neither sVCAM-1, venous occlusion plethysmography nor plasma nitrite levels were affected by almond intake in any independent group. No significant changes in plasma lipids, and apolipoprotein A1 were observed.

In conclusion almonds supplementation caused a reduction in blood pressure that may be due to increased sensitivity of the baroreceptors after increased monounsaturated fatty acid intake.

Keywords: Vitamin E, Cholesterol, Nuts, Blood pressure, Lipids, Monounsaturated fat.
Acknowledgements

My deepest thanks to my supervisor Professor H.R Griffiths for her guidance, support never ending patience and encouragement throughout this project. Thank you Helen.

I would like to acknowledge and give my sincere thanks to the work carried out by Mr Joe Clark in the following area of this thesis:

Data input for food diary analysis using DietPlan 6 software tool.
Carrying out nitrite assay of plasma samples.

His help is much appreciated and helped much to the completion of the project.

I would also like to acknowledge measurement of Apolipoprotein A1 were made by immunoturbimetry at Glasgow Royal Infirmary.

My sincere thanks to my friends and colleagues Dr M.M Grant, Dr S.T Russell, Kameljit McKenzie, Li Li, Christopher Dunston, Dan Gao, Rachel Willetts, Irundika Dias, and Rajitha Kolamunne, whom all have helped tremendously in this project.

Special thanks to all my volunteers whom have so graciously taken part in the study.

I would like to thank the Foods Standards Agency for funding and this project and my thanks go to the Californian Almond Board for their kind donation of almonds for the study.
بسم الله الرحمن الرحيم

"In the name of God, most compassionate, most merciful"

Dedicated to the memory of my father Altafur Rahman Choudhury,
To my mother Syeda Sangida Begum
For her complete faith and patience in all things in life.

& to my family:
Mahtab Miah Choudhury
Zahed Ahmed Choudhury
Shagufta Begum Choudhury
Muznibur Rahman Choudhury
In memory of my brother Rofiqul Hoque Choudhury
Shagufa Begum Choudhury
Firuza Begum Choudhury
Jameal Ahmed Choudhury

For my brothers who time gave no chance but gave us the opportunities,
For my sisters for their encouragement and trust in me,

& to the future generations anything is possible with faith, dedication and
determination.

Peace be upon you.
## Contents

**Title Page** 1  
**Thesis Summary** 2  
**Acknowledgments** 3  
**Dedication** 4  
**Contents** 5  
**List of Figures and Tables** 8  
**Abbreviations** 11  

### Chapter 1.0  General Introduction 15  
1.1 Dietary fatty acids and health 17  
1.2 Lipids. 21  
1.2.1 Cholesterol 21  
1.2.2 Diet as a source of cholesterol 23  
1.2.3 Low Density Lipoprotein metabolism 29  
1.2.4 High Density Lipoprotein metabolism 33  
1.2.5 ABCA1-mediated cholesterol efflux 35  
1.2.6 SR-BI-mediated cholesterol efflux 37  
1.3 Atherosclerosis 38  
1.4 The role of the endothelium in vascular function 40  
1.4.1 The role of nitric oxide in vascular function 41  
1.4.2 The role of cell adhesion molecules in vascular function 43  
1.5 Free Radicals. 47  
1.6 Scavenger Receptors 52  
1.7 Antioxidants 55  
1.7.1 Endogenous Antioxidants 56  
1.7.2 Dietary antioxidants 60  
1.8 Vitamin E 62  
1.8.1 Antioxidant effects of vitamin E 64  
1.8.2 Almonds as dietary sources of antioxidant
### Chapter 2.0 General Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Materials</td>
</tr>
<tr>
<td>2.2</td>
<td>Methods</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Study Design</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Subjects</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Protein assay by Bicinchoninic Acid Assay (BCA)</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Protein assay by RC-DC</td>
</tr>
<tr>
<td>2.3</td>
<td>Statistics</td>
</tr>
</tbody>
</table>

### Chapter 3.0 Dietary Analysis

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>3.2</td>
<td>Methods</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Food diary design</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Anthropometric measures</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Sample preparation for HPLC Vitamin E analysis</td>
</tr>
<tr>
<td>3.2.3.1</td>
<td>HPLC of Vitamin E Analysis</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
</tr>
</tbody>
</table>

### Chapter 4.0 Lipid Profiles

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>4.2</td>
<td>Methods</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Cholesterol Assay</td>
</tr>
<tr>
<td>4.2.2</td>
<td>LDL Assay</td>
</tr>
<tr>
<td>4.2.3</td>
<td>HDL Assay</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Triglycerides Assay</td>
</tr>
<tr>
<td>4.2.5</td>
<td>Apo A1 Analysis</td>
</tr>
<tr>
<td>4.2.6</td>
<td>Apo A1 Analysis in main study</td>
</tr>
<tr>
<td>4.3</td>
<td>Results</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
</tr>
<tr>
<td>Chapter</td>
<td>5.0</td>
</tr>
<tr>
<td>---------</td>
<td>-----</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>5.1.1</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>5.1.2</td>
<td>Oxidised Proteins</td>
</tr>
<tr>
<td>5.1.3</td>
<td>Soluble vascular cellular adhesion molecule-1</td>
</tr>
<tr>
<td>5.2</td>
<td>Methods</td>
</tr>
<tr>
<td>5.2.1</td>
<td>Blood Pressure</td>
</tr>
<tr>
<td>5.2.2</td>
<td>Venous Occlusion Plethysmography</td>
</tr>
<tr>
<td>5.2.3</td>
<td>Soluble vascular cell adhesion molecule-1 ELISA</td>
</tr>
<tr>
<td>5.2.4</td>
<td>Nitrite</td>
</tr>
<tr>
<td>5.2.4.1</td>
<td>Nitrite analysis using a flourometric kit.</td>
</tr>
<tr>
<td>5.2.5</td>
<td>Protein Carbonyl ELISA</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter</th>
<th>6.0</th>
<th>Proteomics</th>
<th>171</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>171</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>Methods</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>6.2.1</td>
<td>Isolation of cells from whole blood for proteomics</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>6.2.2</td>
<td>Sample Preparation for 2D Electrophoresis</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>6.2.3</td>
<td>2D Electrophoresis</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>6.2.4</td>
<td>Gel analysis</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>Results</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>6.4</td>
<td>Discussion</td>
<td>183</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter</th>
<th>7.0</th>
<th>Final Discussion</th>
<th>184</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>Future Work</td>
<td>189</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter</th>
<th>8.0</th>
<th>References</th>
<th>190</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Chapter</th>
<th>9.0</th>
<th>Appendix</th>
<th>216</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1</td>
<td>Appendix I Consent form</td>
<td>217</td>
<td></td>
</tr>
<tr>
<td>9.2</td>
<td>Appendix II Food Diary</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>9.3</td>
<td>Appendix III Pilot Study Data</td>
<td>225</td>
<td></td>
</tr>
</tbody>
</table>
List of Figures and Tables

Table A Major dietary fatty acids. 
Figure 1.1 The pathway of cholesterol biosynthesis via HMG CoA reductase. 
Figure 1.2 The formation of lipoproteins in circulation. 
Figure 1.3 The formation of HDL. 
Table B Table of risk factors. 
Figure 1.4 Nitric Oxide mediated smooth muscle cell relaxation. 
Figure 1.5 Mechanism of foam cell formation. 
Figure 1.6 Chemical structures of tocopherols and tocotrienols. 
Figure 1.7 Almond tree in fruit. 
Figure 2A A summary line of volunteer measurements taken. 
Figure 2B A BCA standard curve for calculating protein concentrations. 
Figure 2C A RC-DC standard curve for calculating protein concentration of samples. 
Figure 3.0 Alpha tocopherol standard curve from HPLC analysis of standards. 
Table 3A. Intra-batch and inter-batch coefficient of variance of quality control plasma. 
Table 3B. Percentage recovery of alpha tocopherol standards from the extraction method used for plasma alpha tocopherol. 
Figure 3.0. Estimated daily Calorie intake changes of All Group volunteers. 
Figure 3.1. Estimated daily intake of Carbohydrate of All Group Volunteers. 
Figure 3.2 Estimated daily intake of Total Sugars of All Group Volunteers. 
Figure 3.3. Estimated daily intake of Proteins of All Group Volunteers. 
Figure 3.4. Estimated daily Fat Intake of All Group Volunteers. 
Figure 3.4.1 Estimated daily Saturated Fat Intake of All Group Volunteers. 
Figure 3.4.2 Estimated daily Monounsaturated Fat Intake of All Group Volunteers. 
Figure 3.4.3. Estimated daily Polyunsaturated Fat Intake of All Group Volunteers. 
Figure 3.4.4. Estimated daily Cholesterol Intake of All Group Volunteers. 
Figure 3.5 Estimated daily Vitamin E Intake of All Group Volunteers. 
Figure 3.6 Estimated daily Fiber Intake of All Group Volunteers. 
Figure 3.7 Effect of Almond consumption on BMI of All Group Volunteers. 
Figure 3.8 Almond Supplementation On Plasma Alpha Tocopherol Levels. 
Table 4A. Intra-batch and inter-batch coefficient of variance of quality control plasma of cholesterol measurement. 
Table 4B. Intra-batch and inter-batch coefficient of variance of quality control plasma of LDL-cholesterol measurement. 
Table 4C. Intra-batch and inter-batch coefficient of variance of quality control plasma of HDL-cholesterol measurement. 
Table 4D. Intra-batch and inter-batch coefficient of variance of quality control plasma of Triglyceride measurement. 
Table 4E Intra-batch and inter-batch coefficient of variance of standard in Apolipoprotein A1 measurement. 
Figure 4.0. Effect of Almond consumption on Cholesterol intake of
Figure 4.1 Effect of Almond consumption on LDL-cholesterol levels of All Group Volunteers.

Figure 4.2 Effect of Almond consumption on HDL-cholesterol levels of All Group Volunteers.

Figure 4.3 Effect of Almond consumption on Levels of Triglycerides of All Group Volunteers.

Figure 4.4 Effect of Almond consumption on LDL/HDL ratio of all group volunteers.

Figure 4.4.1 Effect of Almond consumption on LDL/HDL in comparison to non-supplemented group.

Figure 4.5 Effect of Almond consumption on TC/LDL ratio of all group volunteers.

Figure 4.5.1 Effect of Almond consumption on TC/LDL in comparison to non-supplemented group.

Figure 4.6 Effect of Almond consumption on TC/DL ratio of all group volunteers.

Figure 4.6.1 Effect of Almond consumption on TC/HDL in comparison to non-supplemented group.

Figure 4.7 Almond supplementation effect on Plasma Apolipoprotein A1.

Figure 5.0 Blood pressure reading taken with a manual sphygmomanometer.

Table 5A Reproducibility data for VOP of a single individual on a given day and on three separate occasions.

Table 5B Intra-batch and inter-batch coefficient of variance of quality control standard of sVCAM-1 measurement.

Figure 5.1 Standard Curve of sVCAM-1

Figure 5.2 Chemical reactions involved in the measurement of NO₂ using the Griess Reagent System.

Figure 5.2.1 Standard curve of sodium nitrite using the Griess Assay

Table 5C Percentage recovery for plasma sample spiked with 10μM sodium nitrite using the Griess assay.

Table 5D Percentage recovery data for plasma sample (acid precipitation used to deplete proteins) spiked with 10μM sodium nitrite after using the griess assay.

Figure 5.2.2 Standard curve of carbonyl ELISA

Table 5E Intra-batch and inter-batch coefficient of variance of quality control plasma of carbonyl measurement.

Figure 5.3 Blood Pressure levels; effects of almond intake in Healthy Group volunteers.

Figure 5.3.1 Blood Pressure levels; effects of almond intake in Mature Group volunteers.

Figure 5.3.2 Blood Pressure levels; effects of almond intake in At Risk Group volunteers.

Figure 5.3.3 Blood Pressure levels; effects of almond intake in Control Group volunteers.

Figure 5.3.4 Percentage change in Systolic Blood Pressure from
week 0 of study to week 8 of All Groups.
Figure 5.3.5 Percentage change in Diastolic Blood Pressure from week 0 of study to week 8 of All Groups.
Figure 5.4 Vascular effects of almond supplementation on rate of blood flow in brachial artery.
Figure 5.5 Vascular effects of almond supplementation on plasma nitrite levels.
Figure 5.6 Plasma sVCAM-1 levels; effects of almond intake.
Figure 5.7 Plasma protein carbonyl levels; effects of almond intake.
Figure 6.0 Plasma Proteome master gel.
Figure 6.1 Plasma protein spot change in expression with almond supplementation. Spot ID 1301.
Figure 6.2 Plasma protein spot showing a two-fold change in expression with almond supplementation. Spot ID 4102.
Figure 6.3 Plasma protein spot showing a two-fold change in expression with almond supplementation. Spot ID 9701.
Figure 6.4 Plasma proteome profile for week 16 of At Risk group.
Abbreviations

12/15-lipoxygenase (15/12-LO)
15-lipoxygenase (15-LO)
3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA)
5-lipoxygenase (5-LO)
Acyl-coenzyme A:cholesterol acyltransferase (ACAT)
Advanced glycation end products (AGE)
Alpha Linolenic acid (LNA)
Alpha tocopherol (α-TOH)
Alpha tocopherol transfer protein (αTTP)
Amyotrophic lateral sclerosis (ALS)
Apolipoprotein (Apo)
Ascorbic acid (Vitamin C)
ATP-binding cassette A1 (ABCA1)
Bicinchoninic Acid Assay (BCA)
Body Mass Index (BMI)
Cardiovascular disease (CVD)
Catalase (CAT)
Cellular adhesion molecules (CAMs)
Cholecystokinin (CKK)
Cholesterol ester transfer protein (CETP)
Chylomicron (CM)
Chylomicron remnant (CR)
Class B1 scavenger receptor (SRB1)
Copper chaperone for SOD1 (CCS)
Coronary heart disease (CHD)
CuZnSOD (SOD1)
Dehydroascorbic acid (DHA)
Disintegrin Metalloproteinase’s (ADAMs)
Docosahexaenoic acid (DHA)
EC-SOD (SOD3)
Eicosapentaenoic acid (EPA)
Embryonic macrophages/haemocytes from drosophila (dSR C) P34
Endoplasmic reticulum (ER)
Endothelial NOS (eNOS)
Endothelin-1 (ET-1)
Endothelium-derived hyperpolarizing factor (EDHF)
Epidermal growth factor (EGF)
Esterification (Est)
Fatty acid binding proteins (FABP)
Fatty acid synthetase (FAS)
Food Frequency Questionnaires (FFQs)
Free fatty acid (FFA)
Glutamic acid-Leucine-Arginine (ELR)
Glutaredoxin (GRX)
Glutathione disulfide (GSSG)
Glutathione peroxidase (GPx)
Glutathione peroxidase (GPX)
Glycosaminoglycan (GAG)
High density lipoproteins (HDL)
Hydro (pero)-eicosatetraenoic acid (H(P)ETE)
Hydrogen peroxide (H2O2)
Hydroxyl radicals (-OH)
Hypochlorous acid (HOCl)
Immobilised pH gradient (IPG)
Inducible NOS (iNOS)
Integrin VLA-4 (α4β1)
Intercellular adhesion molecule -1 (ICAM-1)
Interleukin-8 (IL-8)
Intermediate density lipoproteins (IDL)
Lecithin:cholesterol acyltransferase (LCAT)
Lectin-like oxidised LDL receptor –1 (LOX-1)
Linoleic acid (LA)
Lipid peroxyl radical (LOO•)
Lipoprotein lipase (LPL)
Liver X receptor (LXR)
Low density lipoproteins (LDL)
LDL receptor (LDLR)
Lysophosphatidylcholine (LPC)
Macrophage receptor with a collagenous structure (MARCO)
Manganese trafficking factor (MTM1)
Membrane Tethered Matrix Metalloproteinases (MT-MMP Zn2+),
MnSOD (SOD2)
Monocyte chemoattractant protein-1 (MCP-1)
Monounsaturated fatty acids (MUFA)
Myeloperoxidase (MPO)
Neuronal NOS (nNOS)
Niemann-Pick C-1 like-1 transporter (NPC1L1)
Nitric oxide (NO)
Nitric oxide synthase (NOS)
<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inducible nitric oxide synthase</td>
<td>(iNOS)</td>
</tr>
<tr>
<td>Endothelial nitric oxide synthase</td>
<td>(eNOS)</td>
</tr>
<tr>
<td>Nuclear factor κB</td>
<td>(NF κB)</td>
</tr>
<tr>
<td>Nucleotide binding folds</td>
<td>(NBF)</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated factor receptor c</td>
<td>(PPARc)</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>(ONOO−)</td>
</tr>
<tr>
<td>Oxidation</td>
<td>(Ox)</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>(PUFA)</td>
</tr>
<tr>
<td>Pre-chylomicron transport vesicles</td>
<td>(PCTV)</td>
</tr>
<tr>
<td>Prostacyclin</td>
<td>(PGI2)</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>(PG)</td>
</tr>
<tr>
<td>Reactive nitrogen species</td>
<td>(RNS)</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>(ROS)</td>
</tr>
<tr>
<td>Recommended daily allowance</td>
<td>(RDA)</td>
</tr>
<tr>
<td>Recommended daily amounts</td>
<td>(RDAs)</td>
</tr>
<tr>
<td>Reverse cholesterol transport</td>
<td>(RCT)</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td>(SFA)</td>
</tr>
<tr>
<td>Scavenger receptor A 5</td>
<td>(SCARA-5)</td>
</tr>
<tr>
<td>Scavenger receptor class A</td>
<td>(SR A)</td>
</tr>
<tr>
<td>Scavenger receptor with C-type lectin domain</td>
<td>(SRCL-1/II)</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>(SMC)</td>
</tr>
<tr>
<td>S-nitrosylation</td>
<td>(P-S-NO)</td>
</tr>
<tr>
<td>Sodium dependent ascorbate transporters</td>
<td>(SVCT1)</td>
</tr>
<tr>
<td>Soluble VCAM-1</td>
<td>(sVCAM-1)</td>
</tr>
<tr>
<td>Sulfenic</td>
<td>(P-SOH)</td>
</tr>
<tr>
<td>Sulfinic</td>
<td>(P-SO2H)</td>
</tr>
<tr>
<td>Sulfonylic</td>
<td>(P-SO3H)</td>
</tr>
<tr>
<td>Superoxide anion radical</td>
<td>(O2−)</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>(SOD)</td>
</tr>
<tr>
<td>T memory lymphocytes CD4+</td>
<td>(CD45RO+)</td>
</tr>
<tr>
<td>The Food and Drug administration</td>
<td>(FDA)</td>
</tr>
<tr>
<td>The Food Standards Agency</td>
<td>(FSA)</td>
</tr>
<tr>
<td>The World Health Organisation</td>
<td>(WHO)</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>(TRX)</td>
</tr>
<tr>
<td>Transmembrane domains</td>
<td>(TM)</td>
</tr>
<tr>
<td>Tricarboxylic acid cycle</td>
<td>(TCA cycle)</td>
</tr>
<tr>
<td>Triglyceride transfer protein</td>
<td>(MTP)</td>
</tr>
<tr>
<td>Tumour Necrosis Factor — α- Converting Enzyme</td>
<td>(TACE;ADAM 17)</td>
</tr>
<tr>
<td>Vascular cell adhesion molecule-1</td>
<td>(VCAM-1)</td>
</tr>
<tr>
<td>Very low density lipoproteins</td>
<td>(VLDL)</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>(Vit E)</td>
</tr>
</tbody>
</table>
α tocopherol binding protein/supernatant protein factor
α-tocopheroxyl radical

(TAP/SPF)
(α-TO•)
Chapter 1.0 General Introduction

This thesis describes the effect of almond supplementation on asymptomatic volunteers to investigate the hypothesis that almonds can reduce the risk of cardiovascular disease. The investigation utilised anthropomorphic measurements, biochemical testing of lipid profiles and proteomics to establish evidence for such an occurrence. Previous epidemiological evidence supports a protective role of nut consumption on cardiovascular events (Nurse’s Health Study (Hu et al., 1998)).

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality in Westernised countries. The World Health Organisation (WHO) has reported that an estimated 17 million deaths (29.2% of deaths per year) worldwide are due to CVD. Among these deaths coronary heart disease (CHD) and cerebrovascular disease account for the majority of deaths. It is estimated that 56% of CHD and 18% of cerebrovascular disease are caused by hypercholesterolaemia. Many studies have shown the benefits of reducing cholesterol levels in preventing life threatening cardiovascular events (myocardial infarction, angina pectoris). However, it is clear that the link is not absolute and that many other genetic and environmental factors play an important role in CVD deaths.

Energy is essential to the survival of all organisms acting as the fuel in maintenance, growth and repair. Plants are able to trap energy from the sun during photosynthesis but
animals must take in chemically bound energy, which is released in the digestion of plants or other animals by way of respiration.

The modern human genome has not changed since its first appearance 100,000-50,000 years ago and remains adapted to the diet of that time (Eaton, 2006). As food sources were scarce, the body evolved to store fat for consumption at a later date to avoid starvation. Early primate nutrition consisted of foraging for food such as fruits, nuts, and other similar foods. With the appearance of the hominid the digging stick provided a greater variation in the diet allowing access to roots and tubers. About 15,000-10,000 and 30,000 years ago evidence of cereal grain use came to light with the development in agriculture (De Meester and Watson, 2007). Obtaining food proved to be a high energy requiring process with significant energy expenditure in physical activity. The consumption of saturated fats was also prevalent in early man, either from carcasses or from successful hunting of animals. However, the Neanderthals had a shorter lifespan, around 25 years, and are unlikely to have suffered with CVD, which appears in mid-life today. In addition, increased industrialization and technical advances in agriculture and food preparation have provided high energy food stuffs to a population with declining energy requirement through reduced physical activity (Bellisari, 2008).

Food shortages in famines have been a selective force in human evolution. The modern food abundance reflects possible good harvests of the past evolutionary experiences and survival dictates that genomics would influence metabolic activity to store fat for survival at times of famine (reflect modern day diets). The adaptation of the genome to
previous survival mechanisms are likely to modulate behavioural metabolic responses today, this is often referred to as the “thrifty genotype”. The term was initially coined by J.V Neel in 1962 (Neel, 1962) and later used in relation to the etiology of large babies born to diabetic mothers and also in insulin resistance, but the term is now widely adopted (Prentice, 2005).

1.1 Dietary fatty acids and health

Dietary fatty acids have mixed effects on the risk of atherosclerosis through involvement in lipid peroxidation, haemostasis, and inflammation. Dietary fats consist of a mixture of fatty acids but there will be one or two predominant fatty acids in any given food type (Table A). Fatty acids are classified by their carbon chain length, and number of double bonds they carry. Saturated fatty acids (SFA) are the major group containing no double bonds, followed by monounsaturated fatty acids (MUFA) with a single double bond, and polyunsaturated fatty acids (PUFA) with two double bonds or more. The fatty acids are categorised into families according to the position of the double bond to the closest methyl group of the carbon chain.
<table>
<thead>
<tr>
<th>Common Name</th>
<th>Formula</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauric acid</td>
<td>C12:0</td>
<td>Dairy fat, coconut oil, palm kernel oil.</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>C14:0</td>
<td>Dairy fat, coconut oil, palm kernel oil.</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>C16:0</td>
<td>Meat, palm oil.</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>C18:0</td>
<td>Meat, cocoa butter.</td>
</tr>
<tr>
<td><strong>Monounsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>C18:1 n-9</td>
<td>Olive oil, rapeseed oil, avocado, nuts (almonds).</td>
</tr>
<tr>
<td><strong>Polyunsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (LA)</td>
<td>C18:2 n-6</td>
<td>Sunflower oil, safflower oil, soybean oil.</td>
</tr>
<tr>
<td>Alpha Linolenic acid (LNA)</td>
<td>C18:3 n-3</td>
<td>Soybean oil, rapeseed oil, flaxseed.</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (EPA)</td>
<td>C20:5 n-3</td>
<td>Fish, human breast milk.</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>C22:5 n-3</td>
<td>Fish, (EPA is a precursor for DHA)</td>
</tr>
</tbody>
</table>

**Table 1** Major dietary fatty acids. Modified from (Von Eckardstein, 2005)

Fatty acids are supplied to cells largely by albumin in the circulation and are present at a total collective concentration of 0.3-2.0 mM in plasma. Fatty acids undergo activation on entry into cells and are converted to fatty acyl-CoA. This process is catalysed by acyl-CoA synthetases which will activate saturated and unsaturated fatty acids. The resultant long chain fatty acid CoA esters from both saturated an unsaturated fatty acids are effective inhibitors of adenine nucleotide translocation and therefore act as effective modulators of metabolism (Shrago et al., 1974). The fatty acids undergo β oxidation in the mitochondrial matrix to be converted to acetyl CoA which can then enter the tricarboxylic acid cycle (TCA cycle); the complete oxidation of 1 mole of palmitic acid produces 106 moles of ATP. Fatty acids are important molecules for cellular health as an energy source and also their esterification into glycerol or sterols provides important energy stores. Unsaturated fatty acids are precursors to leukotrienes, thromboxanes, and
prostaglandin hormones (Hettema et al., 2000). Cells can also utilise fatty acids in cell signalling as they are the structural skeleton of diacylglycerol and phosphoinositides.

Among the saturated fatty acids palmitic acid, lauric acid, and myristic acid cause the greatest increase in plasma cholesterol levels. Meta-analysis of studies, which used saturated fatty acids revealed an increase in total cholesterol, low density lipoprotein (LDL-cholesterol), and high density lipoprotein (HDL-cholesterol) when carbohydrates were replaced with these fatty acids in an iso-energetic replacement (Mensink et al. 2003). The effect on total cholesterol, LDL-cholesterol, and HDL-cholesterol was increased with decreasing chain length. The study also showed that lauric acid led to a greater increase in HDL-cholesterol giving a lower ratio of total cholesterol/ HDL-cholesterol reducing risk of atherosclerosis (Mensink et al. 2003).

The Mediterranean population has a low mortality rate from coronary heart disease (CHD) relative to or adjusted for gender, age, weight, and other variables (Hu, 2003). Examination of their diet shows high intakes of olive oil that contains monounsaturated fatty acids suggesting these dietary components may be conferring some beneficial effect to reduce mortality from CHD. The effect of stearic acid and oleic acid on lipoprotein levels of HDL-cholesterol show contrasting results. Studies found stearic acid to lower HDL-cholesterol (Kris-Etherton et al., 1993; Zock and Katan, 1992) while other studies showed it to lower total cholesterol, LDL-cholesterol, and HDL-cholesterol (Tholstrup et al., 1994a, 1994b). A meta-analysis by Mensink et al. (2003) reported no change in ratio of total cholesterol/ HDL-cholesterol with comparison to
carbohydrates. Oleic acid (monounsaturated fatty acid) increases levels of HDL-cholesterol and reduces levels of VLDL in comparison to stearic acid, suggesting that unsaturated fats could prove to have beneficial effects in raising HDL-cholesterol levels.

Epidemiological studies of the effects of diets with high levels of saturated fatty acids consistently show an increased prevalence of CVD (Hu et al, 1997). In contrast, dietary enrichment with monounsaturated and polyunsaturated fatty acid supplements together with folate has been shown to reduce the incidence of intermittent claudication (Carrero et al, 2005). The authors observed a reduction in circulating cholesterol and apolipoprotein (Apo) B levels and a decrease in homocysteine, which associated with benefit. These effects may be mediated through unsaturated fatty acid stimulated oxidation and degradation of Apo B in the liver (Pan et al., 2004). In contrast, dietary intake of high levels of monounsaturated fatty acids (MUFA: which are not subject to peroxidation) in comparison to PUFA has been demonstrated to reduce susceptibility of the oxidation of LDL (Berry et al. 1991). The literature is complex and the importance of different mechanisms of fatty acid oxidation and metabolism to CVD remains poorly understood.
1.2 Lipids

Lipids are a complex family of molecules which have a variety of functions in normal physiology; as energy dense molecules for metabolic activity; in membrane stability and organisation; and in intra- (e.g. ceramides) and inter-cellular signalling (e.g. steroids hormones) processes.

The principal membrane lipids are phospholipids comprising of long chain fatty acids linked to phosphate containing polar head groups and cholesterol. Fatty acids can almost exclusively be synthesised in humans but there is a dietary requirement for linoleic acid (LA), alpha-linolenic acid (LNA), and for the fish oils which contain the longer-chain omega-3 fatty acids. Similarly, cholesterol can be both derived from the diet and synthesised by eukaryotic cells when the dietary supply is insufficient.

1.2.1 Cholesterol

Cholesterol is a sterol molecule synthesised by eukaryotes and is essential in mammalian life as a component in cell membranes (Maxfield et al, 2005). Cholesterol is a precursor for steroid hormones (cortisol, aldosterone, progesterone, estrogens and testosterone), vitamin D and bile acids. Cholesterol is found most abundantly in the liver, spinal cord and brain. The body derives cholesterol from two sources:

1. De novo biosynthesis of cholesterol.
2. Dietary cholesterol.
1. De novo biosynthesis of cholesterol.

De novo biosynthesis of cholesterol in the liver and intestines accounts for 10% and 15% respectively of total cholesterol produced each day. It is through the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoA reductase) pathway that endogenous cholesterol is formed. This pathway involves conversion of acetyl CoA moieties to 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) that is subsequently converted to mevalonate by HMG CoA reductase, a known target of cholesterol lowering statins. The pathway comprises of several steps as shown in Figure 1.1 and it is carefully regulated through enzyme activity and feedback mechanisms.

![Diagram of cholesterol biosynthesis pathway](image)

**Figure 1.1**  Pathway of cholesterol biosynthesis *via* HMG CoA reductase. Cholesterol biosynthesis pathway, esterification (Est), oxidation (Ox).
1.2.2 Diet as a source of cholesterol

In adults, 30-40% (60-120g/d) of the calorific intake of a western diet is provided by different fats/lipids. Triglycerides account for 90% of these calories, being the major lipid in both plants and animals; phospholipids account for 2-4g fat intake/d; cholesterol contributes 9200-600mg/d; and fat soluble vitamins that are taken in the diet account for the remainder of the dietary fat intake. Whilst fat intake is important to provide essential fatty acids and fat-soluble vitamins, the western diet is highly enriched in fatty foodstuffs that exceed the recommendation of 20% of energy from fat by the Food Standards Agency (FSA).

The dietary absorption of fats/lipids requires several digestion steps in order for it to be efficiently taken up by the intestinal cells and to eventually be released into the circulation. Digestion is the process of breaking down large food particles into smaller absorbable substances, which is a prerequisite for life (Silverthorn et al., 2001). Initial breakdown of the larger organic matter involves the formation of chyme, which is predominantly produced by mastication and mechanical mixing in the stomach (Meyer et al., 1986). This process leads to the emulsification of fats in the stomach where lipases facilitate the breakdown of triglycerides to monoglycerides and free fatty acids. In contrast, free dietary cholesterol does not undergo further digestion.

Peristalsis moves the chyme into the duodenum where the free fatty acids stimulate enterocytes to release of cholecystokinin (CKK). The hormone CKK acts on the gallbladder stimulating the release of bile and acts on the pancreatic acini that secrete
proenzymes such as lipases and co-lipases. CCK slows gastric motility and increases acid secretion, which is essential after fat ingestion. The decrease in pH in the stomach after CCK exposure causes the duodenum to release the hormone secretin, which acts on the epithelial cells that line the biliary and pancreatic ducts to release bicarbonate rich fluid and phospholipase A₂ for the hydrolysis of phospholipids. Both CCK and secretin stimulate the contraction of the pylorus and hence cause gastric emptying. Ultimately, the alkalisation of the duodenum (pH 6.5-7) leads to ionisation of fatty acids and this reduces fat droplet size.

Bile enters the duodenum helping to stabilise the coarse emulsion by acting as a detergent to form bile salt micelles from the interaction of cholesterol, triglycerides and phospholipids. Dietary cholesterol is transported by bile salt micelles to mucosal cells of the intestinal brush border for absorption (Young and Hui, 1999). Fatty acids and monotriglycerides from bile salt micelles diffuse to epithelial cells at the brush border interface. Bile salts act to deliver fats to the site of absorption and pass through the unstirred water layer (an acidic microenvironment (pH 5.3-6) surrounding the cells of the brush border). The acidic environment of the unstirred water layer protonates fatty acids, allowing dissociation from the micelles to diffuse across the lipid membrane into the enterocytes (Shiau and Levine, 1980).

Cholesterol transported across the apical membrane remains unclear. The classical view is that uptake is via diffusion across the intestinal lumen to the brush border membrane, facilitated through mixed bile salt micelles followed by passive diffusion down a
concentration gradient into enterocytes (Grundy, 1983). The opposing view is that cholesterol uptake is facilitated through protein transporters. Kinetic evidence shows second order for protein mediated cholesterol uptake by small intestine (Thurnhofer and Hauser, 1990). The class B1 scavenger receptor (SR-B1) is reported to play a role in cholesterol uptake in the intestine (Hauser et al., 1998). Other studies have shown that SR-B1 knock out mice have slightly increased intestinal absorption of cholesterol (Mardones et al., 2001) suggesting a non-essential role in cholesterol uptake. The Niemann-Pick C-1 like-1 (NPC1L1) transporter in mice has been reported to play a role in cholesterol absorption and works in cooperation with cholesterol efflux proteins ATP-binding cassette G5 (ABCG5) and ATP-binding cassette G8 (ABCG8) (Hui and Howles, 2005). NPC1L1 is expressed in human jejunum of the brush border membrane of the small intestines, and is also found in human enterocytes including the lysosomes and the mitochondria. NPC1L1 is a targeted by the drug Ezetimibe that inhibits cholesterol and phosphoesterol uptake in the intestines. Mice deficient in NPC1L1 show a reduced absorption of cholesterol and data show the approximately 70% of cholesterol absorption in mice is due to NPC1L1 (Hauser et al., 1998). Studies in mice have shown that mucin is also necessary in absorption of cholesterol (Wang et al., 2004). Uptake of radiolabelled cholesterol from the gut in hamsters, in the presence of sterol glycoside cholesterol inhibitors, caused a reduction in plasma cholesterol levels, supporting specific transporter uptake of cholesterol in the intestine (Hernandez et al., 2000). It is most likely that several mechanisms interplay in cholesterol uptake, as it is an evolutionarily conserved process in living organisms.
Once cholesterol enters the enterocytes it is esterified by acyl-coenzyme A:cholesterol acyltransferase, (ACAT), for assembly into chylomicrons (protein containing particles which facilitate transfer of lipids through the blood). The entry of fatty acids is facilitated by attachment to fatty acid binding proteins (FABP) found on enterocyte membranes from where they diffuse to the endoplasmic reticulum (ER) for esterification to form triglycerides. Cholesterol esters and newly synthesised triglycerides are transferred to Apo B48 by the action of microsomal triglyceride transfer protein (MTP). It has been suggested from in vivo studies that pre-mature chylomicrons are transported via pre-chylomicron transport vesicles (PCTV) that contain COPII proteins Sar1, Sec 24, and Sec 13/31 (Siddiqi et al., 2003) which allow fusion to the Golgi apparatus for maturation by the addition of further triglycerides. Chylomicrons are exocytosed to the lacteals from where they pass into the lymphatic system entering the venous blood supply.

Circulating chylomicrons are actively converted into chylomicron remnant particles (CR) by lipoprotein lipases found predominantly on muscle capillary endothelium and adipose tissue. The lipoprotein lipases act specifically on triglycerides within the chylomicrons to produce free fatty acids and monotriglycerides using Apo C-II as a cofactor (Kwiterovich, 2000). Both free fatty acids and monotriglycerides readily diffuse into cells leaving behind the remaining constituents that make the CR. CR are composed mainly of apolipoprotein and cholesterol which is taken up by the liver for metabolism and repackaging into lipoprotein complexes. If cholesterol is in excess then cholesterol is secreted into the bile for excretion, otherwise it is reassembled into
lipoproteins. Lipoproteins are defined as a complex of proteins conjugated with lipid components for transportation in circulation. The lipoproteins are classified by their chemical qualities and by their major function in the transport of lipids in the circulation.

In 1929 the isolation of HDL from horse serum by Michel Macheboef was the beginning of the lipoprotein classification system, as we now know it (Olson, 1998). There are five classes of lipoproteins; chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL), they are classified by their density. Characterisation of lipoproteins by ultracentrifugation led to the classification of lipoproteins based on their hydrated density while electrophoretic separation led another classification based on their electrophoretic mobility. Each of the components has a principal role to carry out in the transport of specific lipids. VLDL primarily delivers triacylglycerols and cholesterol esters from the liver to tissues throughout the body. VLDL consists of cholesterol, cholesterol esters and apolipoproteins B100, C-I, C-II, C-III, and Apo E. It is a smaller particle than the chylomicrons with a diameter between 30-75nm and with a density of <1006g/L. Nascent VLDL is initially released into circulation from the liver where Apo B100 plays a role. It then matures into VLDL once it gains Apo C-II and E from HDL. Through its circulatory pathway VLDL comes into contact with lipoprotein lipase (LPL) (located on capillary beds) that act to remove triglycerides that would be stored or used for energy purposes; as with triglyceride metabolism in chylomicrons, Apo C-II plays an essential cofactor role. Apo C-II is
transferred back to HDL with phospholipids and triglycerides in exchange for cholesterol esters, a process catalysed by cholesterol ester transfer protein (CETP) (Barter, 2000). VLDL continues to lose triglycerides as it circulates through tissues via the action of LPL, ultimately changing its lipid composition to form IDL. IDL particles have density of 1.006-1.019g/L and contain Apo E. The liver takes up IDL from plasma via the LDL receptor, interacting with the Apo E component of the IDL and then converts the particles to LDL. IDL triglycerides are hydrolysed by hepatic lipase to form LDL particles. Defects in the endogenous metabolic pathway of \( \text{VLDL} \rightarrow \text{IDL} \rightarrow \text{LDL} \) (Figure 1.2) underlie some of the dyslipoproteinaemias which are associated with premature atherosclerosis. Dyslipoproteinaemia includes alteration in a number of elements including changes in lipid levels, the distribution of lipoproteins, lipoprotein enzymes, and cell signalling via changes in receptor interactions (Jenkins et al., 2004). The change in lipoprotein distribution show decrease in HDL and elevation of small dense atherogenic LDL and IDL particles that increase the risk of atherosclerosis.
Figure 1.2  The formation of lipoproteins in circulation. The pathways for the formation of lipoproteins, very low density lipoprotein (VLDL), chylomicron (CM), chylomicron remnants (CR), free fatty acids (FFA), intermediate low density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL), low density lipoprotein receptor (LDLR).

1.2.3  Low Density Lipoprotein metabolism

LDL is a major carrier of cholesterol and triglyceride to the peripheral tissues from the liver. Each LDL particle is small enough to reach tissue fluids by passing through the vascular endothelium. LDL has an approximate size of 18-25nm with a density of 1019-1063g/L and transports 60-70% of total serum cholesterol. There is a variation in size during circulation due to changing concentrations of fatty acids during circulation.
The LDL core is composed of triglycerides and cholesterol esters and the surface outer layer consists of phospholipids and Apo B100 from VLDL/IDL (Esterbauer, 1992). The majority of phospholipids in LDL are phosphatidylcholine (PC) and sphingomyelin. Other phospholipids found in LDL include lysophosphatidylcholine; phosphatidylethanolamine; diacylglycerol; ceramide and phosphatidylinositol. Unesterified cholesterol is distributed throughout the LDL particle with the majority on the surface outer layer. Antioxidants also constitute a part of the LDL particle with each LDL particle carrying 6 molecules of α-tocopherol and lower concentrations of γ-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10 (Esterbauer, 1992). Each LDL particle contains a single Apo B100 protein of 514kD (Segrest, 2001), which plays an important role in receptor mediated clearance of cholesterol from the blood and endocytosis by tissues (Boren et al., 1998). Approximately 10% of LDL is present in the subintima at any given time facilitating cholesterol delivery to systemic tissues. LDL can deliver cholesterol to cells via two routes:

1. LDL receptor interaction.

1. The LDL receptor (LDLR) was discovered in 1974 through the study of familial hypercholesterolaemia by the Nobel Prize-winning researchers, Brown and Goldstein. The LDLR is located in clathrin-coated pits of most cells and clears up to 70% of LDL from the circulation. The LDLR is involved in maintaining cholesterol homeostasis and is able to mediate
cholesterol uptake via its interaction with Apo E and Apo B100 on lipoproteins. This receptor family contains conserved functional regions. Ligands of the receptor bind to seven complement-like cysteine-rich repeat sequences (Strickland, 2002). Other conserved regions include a cysteine-rich epidermal growth factor (EGF)-like repeat and a β-propellor domain that aids ligand uncoupling in the acidic environment of endosomes (Cybulsky et al., 2001). Another conserved region, the asparagine-proline-x-tyrosine sequence, allows adaptor proteins to link the receptor to the clathrin coated pits. Once the ligand Apo B100 on LDL binds to LDLR, the LDL particle are then internalised within clathrin coated vesicles and fuse with an acidic, late endosomal organelle. An ATP dependent proton pump reduces the pH within the vesicle (from ~7 to ~5) allowing dissociation of LDL from the receptor. The vesicle then pinches into two and the LDL receptor vesicle is recycled back to the membrane while the LDL containing vesicle fuses with lysosomes that subsequently hydrolyse cholesterol ester and triglyceride through the action of lipases. LDLR synthesis and endogenous cholesterol synthesis are regulated by a negative regulatory loop, which is dependent on levels of intracellular cholesterol. Statins act on the HMG CoA reductase to reduce biosynthesis of cholesterol. Reduction of cholesterol levels trigger sterol regulatory element binding proteins (SREBP) that translocate to the nuclei and increase expression of LDL receptor. This works to reduce LDL cholesterol further from the circulation.
2. LDL may enter cells through pinocytosis and is capable of binding to sites other than to LDLRs. This is usually a low affinity process depending on LDL levels within the circulation, occurring at a faster rate when plasma LDL concentrations are high.

The cholesterol content of the cell is regulated by a co-ordinated series of reactions. Initially, an elevation in cellular cholesterol inhibits HMG-CoA reductase catalysed de novo cholesterol synthesis by the liver and absorption from the gut. The rate of LDL-receptor synthesis is reduced and acyl-CoA-cholesterol O-acyltransferase is activated to convert cholesterol to cholesterol ester (cellular cholesterol storage form).

More than 200 independent mutations of LDLs exist each of which can contribute to hypercholesterolaemia due to poor clearance of circulating cholesterol. An individual with familial hypercholesterolaemia may inherit two defective LDLR alleles and have receptors that function poorly or not at all, predisposing them to atherosclerosis. Mutations in the Apo B100 gene cause a different form of inherited hypercholesterolaemia, as Apo B100 is the principal ligand for LDLR.

Regulation of cholesterol homeostasis is tightly regulated but where over production of reactive oxygen and nitrogen metabolites occur from inflammation, (generating oxLDL) the system becomes unbalanced and scavenger receptors such as CD36 target the modified LDL that can ultimately lead to atherosclerosis from the formation of foam cells. The cellular content of cholesterol from the uptake of oxLDL does not
regulate the receptor, which generates (peroxisome proliferator-activated receptor gamma) PPARγ ligands that targets further gene expression of itself and CD36 causing a feedback loop (Febbraio et al., 2001) that leads to a continuous uptake of oxLDL and to the formation of foam cells.

1.2.4 High Density Lipoprotein metabolism

HDL is the smallest of the lipoproteins with a diameter of 8-11nm and mediates transport of cholesterol from somatic cells to the liver for excretion into the bile. It carries 30% of blood cholesterol. This process is called reverse cholesterol transport (RCT), the most important stage being cholesterol efflux from the cells. There are at least three recognized mechanisms for cholesterol exchange between cells and plasma:

i. Aqueous diffusion

ii. ABCA1 mediated cholesterol ester transfer

iii. SR-BI mediated cholesterol efflux

HDL particles have a hydrophobic core of triglycerides and cholesterol esters and the outer layer consists of phospholipids, free cholesterol and apolipoproteins. Apo Al and Apo A-II are the major HDL protein components; Apo C-I, Apo C-II and Apo C-III are the minor protein components. Due to differences in composition, size, density, antigenicity and shape HDL is classified into subclasses (von Eckardstein, 1994). Classification of HDL was first achieved by electrophoretic separation to give two subgroups, pre-βHDL and αHDL. Further separation of pre-βHDL by polyacrylamide
gel electrophoresis shows three more HDL subclasses pre-β₁HDL, pre-β₂HDL, and pre-β₃HDL. The αHDL can also be separated in this manner to give five subclasses HDL 3c, 3b, 3a, 2b, 2a increasing in size (Barrans, 1996; Fielding, 1995). The pre-β₂HDL was discovered in 1985 and has a 67kDa mass (Kunitake, 1985) and contains two Apo A1 proteins. Apo A1 acts as an essential cofactor for esterified lecithin:cholesterol acyltransferase (LCAT) an enzyme associated with HDL that esterifies free cholesterol and converts pre-β₁-HDL to pre-β₂HDL, and pre-β₂HDL to ultimately become αHDL (Castro, 1988). Lipoproteins with Apo B100 /B48 accept a small part of the cholesterol esters from αHDL through the action of CETP leading it to be recycled back to pre-β₁-HDL (O'Connor, 1998). αHDL is also formed by the interaction of Apo A1 with SR-B1 and from the diffusion of cholesterol of cells. There is evidence from epidemiological studies of an inverse relationship between HDL₂/HDL₃ with atherosclerosis risk (Stampfer, 1991).

HDL subclass populations in the circulation are highly interchangeable and with the loss or gain of protein or lipid their density is always in flux within a range of density that encompasses them (Figure 1.3).
Figure 1.3  The formation of HDL (Adapted from von Eckardstein et al., 2001)
The blue arrows in the figure show lipid movement between the isoforms of HDL that lead to the formation of each of the forms. The red arrows indicate transfer of proteins. Cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP), hepatic lipase (HL), endothelial lipase (EL), lecithin:cholesterol acyltransferase (LCAT), and scavenger receptor B1 (SR-B1).

1.2.5  ABCA1-mediated cholesterol efflux.
Apo A1 is the major protein constituent of HDL being synthesised in the intestine and liver. It is released into the circulation in nascent HDL along with phospholipids. It is an efficient acceptor of cholesterol due to its low cholesterol content. During circulation it takes up free cholesterol from tissues through ATP-binding cassette A1 (ABCA1). Isoforms on macrophages include ABCG1/G4 that act in a similar manner. These transporters are a part of a group of ABC transporter proteins that have a pair of ATP-binding domains also called nucleotide-binding folds (NBF). There are forty-eight
ABC transporters that have been discovered and these have been divided into seven phylogenetic groups with subfamilies classified from ABCA to ABCG (Higgins, 1992; Holland, 1999). Another feature of the ABC transporters is that they have two transmembrane domains (TM) and the NBF contains Walker A and B domains that are present in ATP-binding proteins. The ABC transporters have a cassette motif upstream of the Walker B domain distinguishing them from the ATP-binding proteins (Hyde, 1990). There are twelve members of ABCA subfamily (Arnould, 2001). The ABCA1 protein was discovered during research in Tangier Disease where there is a lack of Apo A1 and HDL leading to premature atherosclerosis as a result of cholesterol deposition (Remaley, 1999). It was Dr Donald Fredrickson that identified Tangier disease and later went on to classify lipoprotein abnormalities. Mutations in the ABCA-1 receptor lead to decreased cholesterol efflux and correlates to decreased plasma HDL and increased risk of CVD (Barter, 2004). ABCA1 mediates cholesterol efflux to apolipoproteins with poor lipid content; e.g. Apo A1 and Apo E but has a poorer interaction with HDL2 and HDL3, which are the major plasma HDL particles (Wang, 2000). ABCG1 and ABCG4 mediate cholesterol efflux preferentially to HDL2 and HDL3 rather than lipid poor Apo A1 (Wang, 2000).

ABCA1 expression is regulated by the liver X receptor (LXR), a member of a superfamily of nuclear transcription factors (Willy, 1995). Oxysterols are the physiological ligands for LXR. There are two forms of LXR, LXRα and LXRβ. The LXRα is expressed on macrophages, liver, kidney and intestine while the LXRβ is expressed ubiquitously. These transcription factors regulate genes involved in RCT,
lipogenesis and cholesterol homeostasis that include ABCA1, Apo E, CETP, fatty acid synthetase (FAS), and SREBP1-c.

1.2.6 SR-BI-mediated cholesterol efflux

HDL can interact with the SR-BI receptor on steroidogenic tissues to remove the esterified cholesterol (Acton, 1996). Once the cholesterol esters have been hydrolysed by these tissues the free cholesterol can be used to form bile acids or steroid hormones. The residual HDL continues in the circulation to pick up further cholesterol or may be internalised in the liver for degradation.

Genetic variations through mutations and polymorphisms lead to increased or decreased HDL and Apo AI levels. Apo AI is initially synthesised as a precursor protein with 267 amino acids that is cleaved in the plasma to form the mature form containing 243 amino acids. The precursor form of Apo AI shows less activity in RCT than the mature form (Aldred et al., 2006). Apo AI has been sequenced and the crystal structure shows it to be in the form of a horseshoe (Roosbeek et al., 2001), which facilitates cholesterol uptake.
1.3 Atherosclerosis

The underlying cause of CVD is thought to be atherosclerosis and was first proposed in the 1950s. Atherosclerosis is a degenerative disease found widely in the animal kingdom. It causes occlusions in affected arteries or vessels that ultimately impede blood flow. Atherosclerosis has been defined by the World Health Organisation (WHO) as “a variable combination of changes of the intima of arteries consisting of focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, associated with medical changes”. It usually starts in early adolescence as found from post-mortems of young healthy men. The work carried out by Seymour Glagov showed that human arteries could enlarge to accommodate lesion occlusion (stenosis) of up to 40% of the blood vessel after which the plaques becomes obtrusive to the lumen of the vessel (Glagov et al., 1987).

Atherosclerosis is considered as a process of ageing and the prevalence of CVD will continue to increase with the world’s ageing population. The formation of atherosclerotic lesions is linked to the interactions of smooth muscle cells (SMC), endothelial cells, macrophages and lymphocytes.

Many factors contribute to the development of atherosclerosis and these can be separated into two major processes:

1. Biochemical – the accumulation and deposition of lipids, inflammatory cells, complex carbohydrates, complement, fibrous tissue and calcium deposits.
The most prevalent biochemical abnormality involved in atherosclerosis is elevated plasma LDL and the presence of arterial injury, which can be caused by toxins, viral infections, smoking, ageing, nutrient intake and also inflammatory reactions.

2. Biomechanical – hypertension is a systemic disorder and the increased pressure of blood flow through the vasculature, which is dependent on the magnitude of the force and frequency, induces shear stress endothelial activation and recruitment of inflammatory cells.

Preliminary stages of atherosclerosis consist of fatty streaks occurring from the wear and tear of blood flow through vessels. These fatty streaks are a thickening of the intima caused due to lipid laden macrophages known as foam cells, lymphocytes and smooth muscle cells. These fatty streaks can develop into lesions that have been classified from type I – VI in association with their clinical syndromes. The major atherosclerotic lesions themselves are localised to coronary arteries, carotid bifurcations, and the infra-renal abdominal aorta. Advanced lesions can lead to acute occlusions forming a blood clot (thrombus) that can cause myocardial infarction, heart attack, and stroke.

The following sections describe current knowledge on mechanisms underlying the development of CVD and which explain, at least in part, the contributions of modifiable and non-modifiable risk factors for disease development (Table B).
<table>
<thead>
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<th>Non-modifiable risk factors</th>
</tr>
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<tbody>
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<td>Age</td>
</tr>
<tr>
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<td>Male gender</td>
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<td>Hypertension</td>
<td>Heredity</td>
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<td>Obesity</td>
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<td>Physical inactivity</td>
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<td>Diet</td>
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Table B  
Table of risk factors.  
Co-morbidities such as chronic inflammatory disease and diabetes also predispose to CVD.

1.4  
The role of the endothelium in vascular function

The vascular system principally consists of arteries, veins and capillaries. Artery walls consist of an intima, media, and adventitia. The layer adjacent to blood flowing through the vessels is the intima that consists of endothelial cells. It is these endothelial cells that form the boundary between the blood and the lumen. Beneath the endothelial cells lie the sub-endothelial space and then the basement membrane that separates the intima from the vascular media. These cells function as a semi-permeable barrier regulating the transfer of small and large molecules. Under normal physiological conditions it is the endothelial cells that are under continuous stimuli, from blood pressure and blood flow. In response to such stimuli, numerous mediators are released. These messengers include vasodilators nitric oxide (NO), prostaglandins (PG), prostacyclin (PGI₂), endothelium-derived hyperpolarizing factor (EDHF), kinins, adenosine and also vasoconstrictors endothelin-1 (ET-1), reactive oxygen species (ROS), thromboxane A2 (Domenico et al., 2004). For the maintenance of healthy vessels a balance is achieved between the vasoconstrictors and vasodilators released. The changes in the blood flow
and pressure are detected though ion channels and other membrane mechanisms that are linked to the cytoskeleton of the cell. The signals are transported within the cell to the nucleus from where appropriate actions are taken. The endothelium is able to control tone and contractility of arteries and vessels, hence maintaining healthy vasculature. The interplay of this complex network of mechanisms may become disrupted leading to disease states like atherosclerosis, hypertension, pulmonary hypertension, sepsis, and inflammatory disorders (Galley and Webster, 2004).

1.4.1 The role of nitric oxide in vascular function

The enzyme nitric oxide synthase (NOS) produces nitric oxide (NO) from L-arginine. There are three isoforms of NOS; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The iNOS isoform was originally detected in macrophages and nNOS in the brain (Turko and Murad, 2002). However, these isoforms have since been reported in other tissues. NO functions by activating the enzyme guanylate cyclase which catalyses formation of cyclic GMP (cGMP) a second messenger. cGMP causes the relaxation and dilatation of vascular smooth muscle and therefore the blood vessels.
Figure 1.4  Nitric oxide mediated smooth muscle relaxation (adapted from Galley and Webster, 2004).
HDL binds to receptor SR-B1 to activate eNOS by phosphorylation of Serine 1179. HDL is also able to act through MAP to initiate the activation of eNOS. Altered phosphorylation of the enzyme by normal stimuli cause Akt to phosphorylate serine 1179 and therefore activate the enzyme (Mineo et al., 2003).

Other functions of NO include the inhibition of the adherence of platelets and leukocytes to the vessel walls, inhibition of smooth muscle cell migration and proliferation. These actions have a beneficial effect on vessels through prevention of atherosclerosis and thrombus formation (Domenico, 2004). The importance of NO in cardiovascular medicine was recognised in 1998 when Robert. F. Furchgott, Louis. J. Ignarro, and Ferid Murad received the Nobel Prize for their independent discovery that NO acts as a signalling molecule. In contrast, uncontrolled production of NO by NOS has deleterious effects. Bacterial endotoxins and cytokines can upregulate NO production through stimulation of NOS activity, leading to unwanted or excessive
vasodilatation the consequences of which maybe hypotension and septic shock (Torreilles and Guerin 1995).

1.4.2 The role of cell adhesion molecules in vascular function.

The endothelium is an important gatekeeper regulating the movement of molecules and leukocytes into the tissues from the circulation. When injury occurs to the lining of vessels a host of mechanisms come into play to aid the extravasation of leukocytes to the injured site. The up-regulation of leukocyte-specific cellular adhesion molecules (CAMs) occurs through interaction of inflammatory mediators such as TNF with endothelial cells. Monocytes, granulocytes and circulating lymphocytes are able to attach to these CAMs through their reciprocal receptors allowing leukocytes to extravasate into the tissues from the circulation. These CAMs fall into three categories; the selectins, mucins, and the integrins. Integrins are heterodimeric proteins (have large \( \alpha \) 120-170kDa (18 different types) and small \( \beta \) 90-100kDa (8 different types) subunits). They are located on leukocytes involved in cell to cell interactions and adherence to the vascular endothelium. Chemokines stimulate up-regulation of \( \alpha \)M \( \beta \)1 integrin on leucocytes that leads to binding to intercellular adhesion molecule -1 (ICAM-1). Vascular cell adhesion molecule-1 (VCAM-1) binds to integrin VLA-4 (\( \alpha 4 \beta 1 \)). VCAM-1 is located on endothelial cell surfaces and is up-regulated during inflammation. VCAM-1 can be found at sites of lesions and regions that are predisposed to form atherosclerotic lesions (Nakashima et al., 1998).
Many proteins are shed from the cell surface; these include cytokines, growth factors and adhesion molecules. The shedding of such proteins is facilitated by membrane bound or soluble proteases. Membrane Tethered Matrix Metalloproteinases (MT-MMP Zn$$^{2+}$$) and Disintegrin Metalloproteinase’s (ADAMs) are important in this process. It is Tumour Necrosis Factor – α- Converting Enzyme (TACE; ADAM 17) that has been found to be the most active enzyme in mediating the shedding of proteins. VCAM-1 is shed from cell surfaces to give soluble VCAM-1 (sVCAM-1) and this can be stimulated in vitro by phorbol esters. There are two sizes of sVCAM-1; 100kDa formed from TACE action is found in human serum and a 65kDa sVCAM-1 that is produced through neutrophil elastase cleavage of VCAM-1 on bone marrow stromal cells. Cleavage of VCAM-1 occurs near the transmembrane region (Garton et al., 2003 and 2001). High levels of sVCAM-1 have been observed in disease states such as unstable angina and atherosclerosis (Ghaias et al., 1997) and are generally measured as a marker of disease state.

It has been hypothesised that the initiation of atherosclerotic plaques is the result of a “response to injury” of the blood vessel wall (Ross, 1986) and it is now widely recognised that atherosclerosis is a chronic inflammatory disease of the arteries (Libby et al., 2002). The endothelium’s role as a barrier becomes compromised with selective permeability becomes disrupted allowing lipids to become modified and transported into the vessel wall (intima). Changes also occur on the surface of the endothelium allowing adhesion of leukocytes that accumulate at lesion sites. During normal physiological conditions the endothelium does not facilitate the adhesion of leukocytes;
however there are many factors that help promote this action such as an atherogenic diet. High fat diets are associated with increased levels of circulating inflammatory cytokines and C-reactive protein; these molecules stimulate endothelial adhesion molecule activation and leukocyte integrin expression (Jialal et al., 1998; Woollard et al., 2002). The up-regulation of endothelial ICAM-1 and VCAM-1 expression helps bind and recruit monocytes and T-lymphocytes in early atheroma. Studies in mice with mutant VCAM-1 (4-8% VCAM-1) expression compared to controls have reduced levels of lesion formation (Cybulsky et al., 2001). Biomechanical factors such as shear stress and disrupted blood flow at bifurcations also influence adhesion molecule production i.e an increased expression of ICAM-1 as well as up-regulation of extracellular matrix proteoglycans by arterial SMCs. Proteoglycans are heavily glycosylated glycoproteins with a protein core that is covalently attached to one or more glycosaminoglycan (GAG) chains. These GAGs are negatively charged due to sulphate and uronic acid side groups and play a role in retention of remnant particle lipids. Diets enriched with fatty acids alter glycosaminoglycan deposition and decrease retention (Rodriguez-LEE, 2007) resulting in greater circulating remnant lipid levels. Arterial SMC produce large chondroitin sulfate proteoglycans, versican and syndecan-4, as well as smaller proteoglycans decorin and biglycan that bind molecules entering the vascular cell wall such as LDL (Chait and Wight, 2000). The proteoglycans are suggested to promote lipoprotein oxidation and inflammatory response at sites of lesions in response to biomechanical stress (Lee et al., 2001). In addition, there is an upregulation of biglycan and versican mRNA and decrease in decorin mRNA in response to mechanical strain (Lee et al., 2001). Versican also has a high affinity for
LDL in vitro (Wight and Merrilees, 2004) and elevated plasma free fatty acids alter the sulphation of GAGs produce a form that has higher affinity for LDL. Studies in human atherosclerotic intima have shown that biglycans co-localise with Apo B and Apo E containing proteins and can bind LDL in vitro.

Chemoattractants secreted by endothelial cells are also responsible for the transmigration of leukocytes through the endothelial lining leading to leukocytes at sites of lesions. Monocyte chemoattractant protein-1 (MCP-1) is responsible for migration of monocytes into the intima (Austin et al., 2000), while T-cell chemoattractants exert the same action on T-lymphocytes (Mach et al., 1999). T lymphocyte accumulation at lesions have been described as showing a predisposition for T memory lymphocytes CD4+ (CD45RO+) expressing the integrin VLA-1 (Stemme et al., 1992). More recent studies have strongly impacted the absence of regulatory T cells in the aetiology of atherosclerosis and suggest that the T cell chemoattractant, CXCL10, is important in atherogenesis by modulating the local balance of regulatory versus effector T cells. Chemoattractant chemokines are cytokines secreted by cells, which are divided into four groups depending on the relative cysteine (C) residue positioning CXC, CC, C, and CX2C. The CXC chemokines are subdivided further into those containing a NH3 terminal Glutamic acid-Leucine-Arginine (ELR) and those without ELR. Early studies suggested that chemokines were specific for cell types, however, it is now understood that there is considerable cross-talk. Interleukin-8 (IL-8) belongs to the ELR containing CXC chemokines that is responsible for chemoattraction of neutrophils (Clark-Lewis et al., 1993). IL-8 works in other ways that also include
expression of adhesion molecules by neutrophils as well as acting as a chemoattractant for basophils and T lymphocytes (Larsen et al., 1989).

During inflammation, the endothelial lining increases its permeability towards plasma, leading to increased LDL entry into the interstitial fluid. Normal LDL levels in the extracellular space outside the endothelial lining are about 10% of those found in plasma. Increased permeability of the endothelium also allows circulating monocytes to enter between the cells into the sub-intimal space between the muscle and the endothelial lining where they differentiate into macrophages. The local inflammatory response is mediated through the resident tissue leukocytes and those recruited produce high levels of free radicals in response to local cytokines. Oxygen free radicals non-specifically oxidise proteins in the interstitial spaces including the apoproteins present on accumulating lipoproteins.

1.5  Free Radicals

A free radical is an atom or group of atoms that have at least one unpaired electron in their outer shell. Chemical stability is achieved by electron pairing so free radicals are unstable and highly reactive.

Moses Gomberg was the first to isolate triphenylmethyl radical and is known as the founder of free radical chemistry.
The suggested roles of free radicals in biological systems are varied and complex, and include defense against infection, mutagenesis, cell signalling and ageing. Free radicals are necessary for physiological processes and are derived from oxygen or nitrogen to form reactive oxygen species (ROS) or reactive nitrogen species (RNS). The ROS that are derived from by-product of molecular oxygen under reducing conditions are the superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (·OH). Physiological processes that produce ROS include the mitochondrial electron transport chain, NADPH oxidase and xanthine oxidoreductase. The most important biological RNS include nitric oxide (NO), peroxynitrite (ONOO⁻) and nitrosothiols are produced following nitric oxide synthase activity. Although H₂O₂ is not a free radical it is involved in the production of oxygen free radicals through its breakdown into ·OH in the presence of transition metal ions according to the Haber-Weiss reaction (Cheeseman and Slater, 1993). ·OH is highly reactive, damaging and can initiate lipid peroxidation (Tien and Aust, 1982), protein oxidation and DNA oxidation.

The damaging effects of free radicals have been implicated in many disease states including Parkinson’s disease, Alzheimer’s disease, rheumatoid arthritis, diabetes mellitus and atherosclerosis. Free radicals are also produced by environmental agents e.g. in cigarette smoke and tar; these agents are suggested to promote development of CVD by increasing free radical formation and decreasing antioxidant stores.

OH⁻ radicals frequently attack biological molecules (including lipids) by abstracting hydrogen. O₂⁻ reacts with nitric oxide (NO⁻), a free radical produced by several cell
types (especially phagocytes and vascular endothelial cells) to give peroxynitrite, which is also a potent lipid oxidising and nitrating agent. Lipid peroxidation is a highly significant consequence of oxidative stress in injured human arterial walls, contributing to the development of atherosclerotic lesions. It was in 1979 that Chisolm and colleagues stated that oxidized LDL was damaging to arterial wall and that it may be an important factor in atherogenesis (Hessler et al., 1979). Entry of LDL into the extracellular space consequently leads to oxidative modifications that produce lipid hydroperoxides, lysophospholipids, and carbonyls. The modifications in the lipids play a key role in up-regulating expression of adhesion molecules, chemoattractants, and proinflammatory cytokines. Oxidative modification of LDL is thus a critical factor in the progression of atherogenesis.

Many mechanisms have been suggested to catalyse LDL oxidation such as 15/12-lipoxygenase, metal ions, ceruloplasmin, myeloperoxidase (MPO) by formation of hypochlorous acid (HOCl), and NADPH oxidase.

The lipoxygenases are non-heme iron dioxygenases that can react with PUFA to insert oxygen forming hydro (pero)-cicosatetraenoic acid (H(P)ETE) (Funk et al., 2000). Fogelman and colleagues reported that malondialdehyde produced by the lipoxygenase pathway could lead to Schiff-bases with the epsilon amino groups of Apo B100 lysine residues of LDL (Fogelman et al., 1980). Oxidation of lysine residues of LDL by formation of aldehydes can be measured through their carbonyl content. The lipoxygenase that have been investigated in atherosclerosis include the reticulocyte-
type 15-lipoxygenase (15-LO) in humans and also a closely linked lipoxygenase in mice 12/15-lipoxygenase (15/12-LO), and 5-lipoxygenase (5-LO). The expression of 15-LO has been found at lesion sites of foam cells linking it to atherosclerosis (Ylä-Herttuala et al., 1991). The expression of 5-LO is observed in monocytes/macrophages, mast cells, neutrophils, and dendritic cells and is up-regulated in atherosclerosis.

MPO is a peroxidase enzyme found in neutrophils that catalyse the reaction of H₂O₂ and chloride anions during the respiratory burst producing HOCl; during this reaction heme is required as a cofactor. The H₂O₂ can act as a halogenating or a peroxidising agent and is able to oxidise tyrosine to a tyrosyl radical; both H₂O₂ and tyrosyl radicals are cytotoxic and normally help eliminate pathogens through the respiratory burst. HOCl has been implicated in LDL oxidative modification and atherogenesis (Heinecke et al., 1997). Evidence to support the association has shown HOCl-mediated protein modification, dityrosine and 3-chlorotyrosine at human atherosclerotic lesions (Leeuwenburgh et al., 1997). HOCl can react with epsilon amino groups of Apo B100 lysine residues leading to formation of N-chloramines. These N-chloramines derivatives N(alpha)-acetyl-lysine chloramine and taurine chloramines are capable of oxidising Apo B100 cysteine residues (Carr et al., 2000).

Cellular mechanisms have been proposed to play a role in the direct oxidation of Apo B100 through copper mediated oxidation of tryptophan residues. The oxidation of tryptophan is suggested to be an initiation step in the oxidation of LDL. Alteration to apolipoproteins at the arterial wall leads to an antigenic response (Stemme et al., 1995)
and presence of circulating autoantibodies to oxidised LDL (oxLDL) may be either pathogenic or protective. Apo B100 oxidation alters the conformation of LDL so it is no longer recognisable by LDLRs on cell surfaces (Stocker, 1999). Instead, oxLDL is taken up by scavenger receptors and macrophages transform into foam cells by this uncontrolled uptake of oxLDL; a major factor of atherosclerosis (Fuhrman et al., 1997).

These oxLDL stimulate endothelial cells to release MCP-1 (Young and McEneny, 2001), which is responsible for further monocyte accumulation at the arterial wall through upregulation of endothelial VCAM-1 (Libby et al., 2001). Monocyte accumulation at the atherosclerotic site is also promoted by lysophosphatidylcholine (LPC) that constitutes 40% of oxLDL and is formed during oxidation of LDL. LPC promotes atherosclerosis by inhibiting eNOS, increasing expression of the scavenger receptor LOX-1, by causing release of vascular adhesion molecule and leading to accumulation of leukocytes in the vicinity. oxLDL also functions to up-regulate angiotensin II receptors, these receptors are implicated in endothelial cell dysfunction as well inducing LOX-1 mRNA and ultimately protein expression (Li et al., 1999).
1.6 Scavenger Receptors

Scavenger receptors are found on many different cell types and their presence on macrophages was first discovered by Goldstein and Brown when investigating the accumulation of LDL in lesions of patients with familial hypercholesterolaemia (Goldstein et al., 1979). Scavenger receptors were named for their ability to bind modified LDL and not the native form of LDL. Scavenger receptors have expanded to also include receptors capable of binding of certain polyanionic ligands. Krieger and co-workers have classified these scavenger receptors into eight different groups (A, B, C, D, E, F (SREC I/II), G (SR-PSOX) and H (fascinil, epidermal growth factor (EGF)-like FEEL-1, FEEL-2)) according to their structural moieties (Mardones et al., 2001).
Scavenger receptor class A (SR A) bind a broad range of ligands from modified LDL; endotoxins and lipoteichoic acid of bacteria; glycated proteins (advanced glycation end products, AGE); and β-amyloid fibrils (El Khoury et al., 1996). SR A are highly conserved and have three isoforms derived from alternative splicing of a single gene SR A1, SR AII, and SR AIII (Freeman et al., 1990). The SR AIII form is non-functional and remains in the endoplasmic reticulum (Gough et al., 1998). SR A on macrophages are able to uptake modified LDL and accumulate cholesterol within cells without any feedback inhibition as these receptors are not subject to regulation by cellular cholesterol levels. The class A receptors also include scavenger receptor A 5 (SCARA-5) (Jiang et al., 2006), this receptor is unable to endocytose modified LDL and is restricted to the mucosal epithelium. Other class A receptors include scavenger receptor with C-type lectin domain (SRCL-I/II) that are expressed in vascular endothelial cells; and macrophage receptor with a collagenous structure (MARCO) (Elomaa et al., 1995). MARCO expression can be induced in response to infection by bacteria leading to the formation of long dendritic processes that may enhance phagocytosis.

Class B scavenger receptors include CD36 and SR-BI are capable of forming functional dimers and multimers. CD36 and SR-BI both recognise hypochlorite modified LDL which is a product of oxidative burst by phagocytic cells found at lesions (Marsche et al., 2003). CD36 is an 88 kDa glycosylated glycoprotein that is expressed on many cell types including macrophages, endothelial cells, and monocytes (Malad et al., 2002). The structure of these molecules is essential to the functional role that they play. The
CD36 cytoplasmic C terminus aids internalisation and degradation of oxLDL (Malaud et al., 2002). Other studies have also shown that the C terminus cytoplasmic domain mediates oxLDL nuclear factor κB (NF κB) signalling (Lipsky et al., 1997). SR-BI is expressed on macrophages, monocytes, hepatocytes and dendritic cells (Terpstra et al., 2000). CD36 receptor activation encourages activation of peroxisome proliferator-activated factor receptor c (PPARc) leading to further lipid accumulation in macrophages (Daviet et al., 1997). Scavenger receptor CD36 also mediates adhesion of macrophages to activated platelets through platelet glycoprotein thrombospondin that may enhance recruitment of macrophages to the endothelial injury site (Bodart et al., 2002).

The class C scavenger receptor was found in embryonic macrophages/haemocytes from drosophila (dSR C) (Abrams et al., 1992) and can bind bacteria and acetylated LDL (acLDL) (Ramet et al., 2001). Whether a mammalian counterpart exists, remains unknown.

Class D scavenger receptors bind liposomes that are rich in phosphatidylserine and also oxLDL and include CD68 that is expressed in lysosomes and late endosomes of macrophages. Other members of the group include lysosomal membrane glycoproteins Lamp-1, Lamp-2, and Lamp-3. Both Lamp-1 and Lamp-2 are widely distributed while Lamp-3 expression increases with the maturation of dendritic cells and is stimulated by LPS and TNFα (de Saint-Vis et al., 1998). However, modified LDL does not bind to Lamp receptors.
The class E scavenger receptor family has only one member; the lectin-like oxidised LDL receptor–1 (LOX-1). LOX-1 is expressed on endothelial cells (Sawamura et al., 1997) and is capable of binding AGE modified proteins (Jono et al., 2002). LOX-1 also recognises apoptotic cells (Oka et al., 1998) possibly through the exposed phosphatidylserine (PS) of aged/apoptotic cells. Exposure of PS acts as a procoagulant and at sites of atherosclerosis. Inhibition of apoptosis by ox-LDL may prevent the clearance of apoptotic cells in the region. LOX-1 also binds to fibronectin (Shimaoka et al., 2001) that suggests a role in adhesion to the matrix and to platelets through interaction with exposed phospholipid epitopes. LOX-1 also enhances endothelin-1 release from endothelial cells triggering vasoconstriction and vessel occlusion (Kakutani et al., 2000).

1.7 Antioxidants

Antioxidant mechanisms are in place to deal with ROS produced during normal metabolism. Antioxidants are defined as molecules that inhibit oxidation or reactions promoted by oxygen and peroxides. In biological systems, the definition has been further refined to by Halliwell to highlight that antioxidants are usually present in small concentrations compared to the biomolecules they protect and can prevent or reduce the extent of oxidative damage to biomolecules (Halliwell, 1990).

Antioxidant mechanisms have evolved to protect and repair enzymes to remove free radical damage to lipids, proteins, and DNA. Antioxidants are synthesised
endogenously and are also derived from the diet. Antioxidants help to maintain a balance between free radicals, biological function and free radical damage to biomolecules. Biological systems have enzymatic antioxidants that include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Dietary antioxidants include vitamins and minerals that can be either natural or synthetic. Dietary antioxidant molecules such as vitamin E, vitamin C, carotenoids, and polyphenolic compounds those are present in both intracellular and extracellular compartments to deal with reactive oxygen species and oxidants.

1.7.1 Endogenous Antioxidants

Protein thiol groups are susceptible to oxidative modifications through formation of disulfide bonds between thiol groups of different proteins; within the protein itself, and through thiols and glutathione. Oxidative modifications result in sulfenic (P-SOH), sulfenic (P- SO₂H), sulfonic (P-SO₃H), and also S-nitrosylation (P-S-NO) of protein thiols. The cellular thiol redox state is controlled by the glutaredoxin (GRX) and thioredoxin (TRX) systems (Holmgren, 1985). The GRX is a family of proteins that utilise GSH to donate hydrogen for ribonucleotide reductase and was first discovered in Escherichia coli of TRX negative mutants (Holmgren, 1976). GSH is a tripeptide molecule made of glycine, cysteine, and glutamate that are ubiquitous with all cell types capable of producing it. Intracellular levels of GSH range from 1-10mM (Franco et al., 2007). The GSH distribution within the cell is mainly localised to the cytoplasm (85-90%) with the rest being distributed in mitochondria, nuclear matrix and peroxisomes (Lu, 2000). Plasma levels of GSH are relatively low 0.01mM due to high
rates of oxidation and catabolism. Free radicals, ROS, and reactive nitrogen species (RNS) readily oxidise GSH to glutathione disulfide (GSSG). The ratio of GSH:GSSG is an indicator of cellular redox state and under normal physiological conditions is >10 (Woollard et al., 2002). The ratio is also a measure of cellular antioxidant capacity, however this ratio is also affected by the NADPH/NADP⁺ as well as TRX_red/oxidised levels. Once GSH is oxidised to GSSG it can again be reduced to GSH by NADPH-dependent glutathione reductase. Glutathione peroxidase (GPx) utilises GSH to remove hydrogen peroxide within cells by condensation reaction by forming a disulfide bridge with the –SH groups of cysteine.

\[ 2 \text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O} \]

GSSG can be reduced back to GSH by the action of glutathione reductase. There are two types if GPx; those containing selenium and those without any selenium. The GPx without selenium is not capable of reacting with hydrogen peroxide but is capable of reacting with artificial hydroperoxides. Selenium is required in animal and human diets as an essential cofactor for GPx. GSH plays many roles that include acting as a store for cysteine. GSH can conjugate with NO to form a S-nitrosogluthathione adduct that can then be cleaved by TRX system to release GSH and NO (Fang et al., 2002). Levels of GSH need to be maintained as GSH is necessary for cell proliferation (Aw, 2003). If the ratio of GSH/GSSG slides towards a cellular oxidative state along with changes in cellular redox state, this triggers signalling pathways that lead the cell towards apoptosis (Mukuddem-Petersen et al., 2005).
In 1969 Irwin Fridovich and Joe McCord isolated the enzyme superoxide dismutase (McCord and Fridovich, 1969) previously known as the metalloprotein, erythrocuprein. SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide, suggested to be a more stable ROS than superoxide anion.

\[ 2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \]

Many SODs have been identified and three distinct types have been classified in mammalian cells in accordance to localisation of the enzyme. The cytosolic SOD is a CuZnSOD (SOD1) that is usually a homodimer; the mitochondria contain MnSOD (SOD2) that functions as a tetramer (Weisiger and Fridovich, 1973); and extracellular EC-SOD (SOD3) is also a tetramer that has a C terminal heparin-binding domain (Marklund, 1982; Szasz et al., 2007). Cytosolic SOD1 is constitutively expressed and is present in mitochondria and the nuclear intermembrane space (Bannister et al., 1987). SOD1 enters the mitochondria as an Apo-enzyme and once inside, requires a copper chaperone for SOD1 (CCS) to convert it to the functional form (Wong et al., 2000). The CCS within the mitochondria loads copper to the SOD1 enzyme (Maxfield and Tabas, 2005). SOD2 interacts with manganese trafficking factor M1 (MTM1) so that it can be converted into its active form. After inactivation of MTM1 in yeast, high doses of manganese supplementation were required to restore SOD2 function (Luk et al., 2003). Mutations in SOD1 can lead to Amyotrophic lateral sclerosis (ALS) or “Lou Gehrig’s Disease” which is a neuro-degenerative disorder of motor neurons of the brain and spinal cord. SOD1 has also been linked to Down’s syndrome when over-
expressed (Groner et al., 1994). Deficiency in SOD2 activity have been associated with eye diseases including optic neuropathy (Qi, 2003), development of age-related macular degeneration (Justilien et al., 2007) which may be ameliorated by SOD2 gene delivery. SOD3 binds to the extracellular matrix and cell surfaces via a heparin binding domain and carriers a genetic variant of SOD3 leading to high circulating SOD3 levels have increased vascular disease (Chu et al., 2006). The mutation identified mutation prevents endothelial uptake of SOD3 thus plasma levels rise and the risk of ROS-dependent CVD increases (Sentman et al., 2001).

Catalase (CAT) is another important endogenous antioxidant enzyme, which acts on hydrogen peroxide that is generated by SOD, and other enzymes to decompose it to water and oxygen. CAT is found in peroxisomes where it also acts on a number of other substrates like methanol, ethanol, phenol, and nitrates (Oshino et al., 1973). CAT was found in 1811 when Thenard, who discovered hydrogen peroxide, found that its degradation of was catalysed by an unknown substance. Later in 1863 Schönbein identified it as a certain “ferment”; it was eventually Leow in 1901 that named this enzyme catalase (Zamocky and Koller, 1999). Later in 1927, Warburg illustrated that the active centre of CAT contained iron. CAT is a tetramer with four porphyrin heme groups at the catalytic centre that allow the CAT to react with hydrogen peroxide. A reduction in CAT activity is indirectly related to the development of CVD by causing an increase in blood pressure (Singh, 1996).
1.7.2 Dietary antioxidants

The two vitamins that are considered to have major antioxidant roles, at least in vitro, are vitamin C and vitamin E. As essential vitamins, recommended daily amounts (RDAs) originally set by the Department of Health in 1979 to define the quantity required of a certain nutrient was needed by different groups of the population. This has more recently been replaced by dietary reference values. Ascorbic acid (vitamin C) is a water soluble antioxidant in plasma, which acts as a multifunctional antioxidant in biological systems and an intake of 40mg/day is considered sufficient by the UK Food Standards Agency. Ascorbic acid was identified as a vitamin, vital for health, for its role in the prevention of scurvy as a cofactor for collagen biosynthesis; in 1747, James Lind demonstrated that citrus fruit could be used treatment and prevention of scurvy. Ascorbic acid is known chemically as hexuronic acid and was established as being the same as vitamin C in 1932 by Svirbely and Szent-Györgyi. Humans cannot synthesise ascorbic acid as they lack the enzyme L-gluonolactone oxidase to convert D-glucose to L-ascorbic acid, shown by Brown in 1959, and hence deficiencies in dietary intake of ascorbic acid lead to scurvy. Ascorbic acid is a reducing agent and is capable of scavenging singlet oxygen, superoxide, hydrogen peroxide, hydroxyl radicals, hydroperoxyl radicals, hypochlorous acid to give semidehydroascorbate. The structure of ascorbic acid is related to glucose and has an enediol group that allows electron lability making it capable of accepting and donating electrons. When ROS oxidise ascorbic acid, it forms ascorbyl radical initially and disproportionate to dehydroascorbic acid (DHA) and ascorbic acid. The DHA can then be used as an electron donor in cell metabolism. Transportation of ascorbic acid is carried out by sodium dependent
ascorbate transporters SVCT1 and SVCT2. Ascorbic acid levels are maintained with the help of erythrocytes that carry ~30% of the whole blood total vitamin C and therefore help preserve antioxidant defence in whole blood (Meyer et al., 1986). DHA is enters cells through the GLUT family of transporters GLUT1, GLUT3, and GLUT4, which, cannot transport ascorbic acid. The red blood cells have GLUT1 that can preferentially uptakes DHA compared to glucose (Montel-Hagen et al., 2008). The levels of ascorbate in the brain are ten times higher, with the expression of GLUT1 in the blood brain endothelial barrier (Agus et al., 1997). DHA is formed during conditions of oxidative stress outside cells (Wang et al, 1997). ROS are able to activate glucose transporters in cells, which coincide with the reduction of ascorbic acid (Maellaro et al, 1994). The increased glucose transport in oxidative stress enhances reducing agents like NADH/NADPH via glycolysis, which are required to recycle extracellular ascorbic acid. Ascorbic acid defends biomolecules against ROS and oxidative damage; it also is suggested to work in association with vitamin E to prevent damage by free radicals to cell membranes. At cell membranes tocopherol and ubiquinol play the primary role of defense against free radical damage but one of the functions of ascorbic acid lie in the regeneration of vitamin E after it has been oxidised in membranes and plasma lipoproteins.

The LDL particle itself carries lipophilic antioxidants including \(\alpha\)-tocopherol, \(\gamma\)-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10 (Hevonoja et al, 2000). Each particle of LDL has between 6-12 \(\alpha\)-tocopherols. Other complexes that act as
antioxidants are GPx, and SOD. GPx works by decomposing hydrogen peroxide and phospholipid hydroperoxides in plasma and intracellularly.

Epidemiological studies in Finland and the UK have shown that ascorbic acid deficiency in humans is a risk factor for myocardial infarction (Nyyssonen et al., 1997; Khaw, 2001). However, no association between low plasma ascorbate (<28uM) and increased risk if CVD was observed in an independent US cohort (Loria et al., 2000).

1.8 Vitamin E

Vitamin E is a fat soluble molecule consisting of 4 tocopherols and 4 tocotrienols. The most active form of vitamin E is \( \alpha \)-tocopherol (\( \alpha \)-TOH). The recommended daily allowance (RDA) for vitamin E in the US is 10-20mg/d for an adult; infants less than 2kg require 4-6mg/d of \( \alpha \)-tocopherol, however, the UK FSA states that 4mg/day vitamin E as part of a balanced diet for men and 3mg/day for women. Evans and Bishop (1922) discovered that deficiency in vitamin E caused loss of fertility in rats through reabsorption of the foetus. Levels of \( \alpha \)-tocopherol in humans range from 5-35\( \mu \)M in plasma, with the highest levels only achievable by dietary supplementation. The tocopherols consist of an aromatic ring and a long saturated phytol chain. The different number and position of the methyl groups on the aromatic ring give the four differing types of tocopherols: \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \). The tocotrienols have the same structure as their counterparts and therefore labelled \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) correspondingly, however, the phytol side chain has double bonds at positions 3', 7', and 11'.
Figure 1.6  Chemical structures of tocopherols and tocotrienols (adapted from Wang and Quinn, 1999).

Alpha tocopherol is the predominant fraction of vitamin E found in membranes at approximately 1 αTOH molecule to 1000 lipid molecules. This is due to alpha tocopherol transfer protein (αTTP) that preferentially takes up the αTOH form (Sato et al., 1993). Mutations in αTTP lead to low levels of α-TOH; deficiency in vitamin E can cause ataxia; an autosomal disease that impairs the coordination of voluntary
movements (Marzouki et al., 2005). Another protein that interacts with αTOH is α
tocopherol binding protein/ supernatant protein factor (TAP/SPF). TAP binds other
hydrophilic ligands and has a CRAL-TRIO domain that is a conserved element within
the protein and accommodates hydrophobic ligands. This vicinity forms a pocket into
which the hydrophobic ligand can sit. This domain is also found in αTTP whose
crystal structure reveals such a cleft for the αTOH to bind (Stocker et al, 1999). High
concentrations of TAP are found in the liver, brain, and prostate organs. α-TOH is
involved in signal transduction pathways and is reported to activate protein phosphatase
A which goes on to inactivate protein kinase C downstream; these alterations to the
signalling cascade may ultimately induce changes in activity or expression of proteins
within the cell (Azzi., 2004). αTOH is an effective antioxidant in vitro and it has been
assumed that it also fulfils this role in vivo.

1.8.1 Antioxidant effects of α tocopherol

The phytol tail of αTOH allows the molecule to position itself within the membrane
bilayer and the aromatic ring is located at the surface. It is highly reactive with ROS
and once it is oxidised, it is able to regenerate itself through its interaction with
vitamin C and glutathione. During excessive ROS attack, the levels of αTOH can be
depleted leaving membrane and LDL lipids open to attack. αTOH is a one electron
antioxidant and therefore cannot protect against two electron attack from oxidants
such as hypochlorous acid (Upston et al., 2003). αTOH is not a chain breaking
antioxidant and can show both pro and anti-oxidant activity. The former is dependent
on the formation of α-tocopheroxyl radical (α-TO•). This is produced from the
reaction with lipid peroxyl radical (LOO•) and is less reactive; nevertheless, alpha-tocopheroyl radical has been shown to initiate lipid peroxidation in human LDL (Witting et al., 1997). The α-TO• produced exhibits increased stability compared to the lipid peroxide as the electron is delocalised into the aromatic ring structure.

Despite the epidemiological evidence in support of a protective role for vitamin E against cardiovascular disease, intervention studies have been consistent. The MRC/Heart Protection Study supplemented with 600 mg vitamin E, 250 mg vitamin C, and 20 mg beta-carotene daily for five years in more than 20,000 subjects with existing cardiovascular disease did not show any benefit. A meta-analysis of antioxidant efficacy in primary and secondary prevention did not show any beneficial effect but rather an increase in mortality (Bjelakovic et al., 2007). The elimination of free radicals with excessive intakes of antioxidants may be disrupting essential mechanisms such as apoptosis, detoxification and phagocytosis that may ultimately have detrimental effects. Possible reasons for the lack of benefit may be due to different dosages of αTOH used in the different studies. A study carried out by Roberts et al, (2007) investigated the dose response of $RRR$-α-tocopherol intake on plasma F2-isoprostane concentrations and found that to gain significant affects of supplementation doses of 1600 and 3200 IU/day are required (Robert et al., 2007). Most studies have used lower levels of dosage and this may be indicative of little or no effect of supplementation.
1.8.2 Almonds as dietary sources of antioxidant nutrients

Maintaining levels of antioxidants through the diet is postulated to protect against CVD through the scavenging of ROS and free radicals. Almonds are one of the richest dietary sources of the antioxidant vitamin E. In addition, they contain betulinic acid, oleanolic acid, and ursolic acid that have been reported to have anti-inflammatory anti-cancer activities (Takeoka and Doa, 2003). Polyphenols are known to scavenge free radicals and to inhibit the oxidation of LDL are also found in almonds and include tannins, rhamnetin, quercetin and kaempherol aglycones (Pinelo et al., 2004). Almonds also contain high levels of arginine, a precursor for NO production and all the essential amino acids except for methionine required by the body. The combination of bioactive constituents in almonds may increase benefit from additive effects of individual components over a single supplement. Almond supplementation studies have shown significant decrease in total cholesterol and LDL cholesterol (Spiller et al., 1998) (Lovejoy et al., 2002), however, the mechanism for this remains unknown.

The UK Food Standards Agency has recommended that the UK diet should include at least five portions of fruit and vegetables per day (www.eatwell.gov.uk/healthydiet/nutritionessentials/fruitandveg/) as epidemiological evidence suggests that “people who eat lots of fruit and vegetable are less likely to develope chronic diseases such as coronary heart disease and some cancers”.

In addition, many epidemiological studies have provided evidence for an association between increased nut consumption with a lower risk of heart disease and diabetes. An
examination of a cohort of women from The Nurses Health Study found that frequent nut consumption reduced the risks of fatal coronary heart disease and non-fatal myocardial infarction (Hu et al., 1998). In another analysis of the US Physicians' Health Study it was also suggested that nut consumption caused a reduction in sudden cardiac death (Albert et al., 2002). Other metabolic studies carried out show a cholesterol-lowering effect of taking nuts is greater than expected (Kris-Etherton et al., 1999) and again a protective role against heart disease and diabetes. The Food and drug administration (FDA) has released a statement approving the health claim that eating 42.8g/d nuts may lead to decreasing risks of CHD (Brown, 2003).

The almond (*Prunus dulcis* (Mill) D.A. Webb) belongs to the family Rosaceae, and is a fruit of this small deciduous tree. It belongs to the same classification as the peach in the genus *Prunus*. Almonds lack the fleshy outer layer of plums and cherry’s that also belongs to *Prunus* and instead has a hull that contains the edible kernel otherwise known as the nut. The almond tree is native to southwest Asia and its flowers are white or pale pink. Domestication and production of almonds has reached approximately 1.5 million tones. The major producers of this nut include the Mediterranean region Greece, Spain, Morocco, Portugal, Syria, Turkey but the largest producer is The United States.
Figure 1.6  **Almond tree in fruit** (adapted from Sañchez-Pérez et al., 2008)

There are two forms of almond trees the ones with white flowers producing the sweet almonds and the ones with pink flowers producing the bitter almonds. Bitter almonds contain a fixed oil and emulsion and were in the past used in medicine. It is still used today in aromatherapy. Bitter almonds contain amygdalin that produces breakdown products of cyanide and benzaldehyde. Eating bitter almonds is dangerous and a dose of twenty bitter almonds is lethal to adults (www.botanical-online.com/alcaloidesametllerangles).

There are some concerns that increased almond intake may increase weight gain as nuts besides chestnuts contain high levels of fat. Investigations into almond consumption and Body Mass Index (BMI) have found no relationship between increased dietary nuts and weight gain. Nut consumption rather leads to increased stool fat, and some enhancement of satiety (Sabate, 2003). The lipids comprise 50% of the total weight and
are found in intracellular oil vesicles. The cell wall acts as a barrier against enzyme action and metabolism and therefore fewer calories may be absorbed.

A reduction in cholesterol levels can reduce risk of CVD. There are several components in almonds that can act to achieve this goal. Almonds contain 70% MUFA, which can aid in decreasing cholesterol levels by replacing SFA. Subjects that have been fed diets rich in MUFA and PUFA have shown to have lower total and LDL cholesterol (Spiller et al., 1992). It is however diets containing higher MUFA than PUFA that show a greater resistance to LDL oxidation (Bonanome et al., 1992). Despite this evidence, a study by Hyson et al. (2002) did not confirm a reduction in oxidisability of LDL following 66g/d dietary almonds in healthy men and women over six weeks. However, these authors observed an increase in HDL cholesterol, a reduction in LDL cholesterol and triglycerides after almond consumption. In a recent study by Jaceldo-Siegl et al. (2004), individuals were supplemented with almonds and the levels of MUFA, PUFA, fibre, vegetable protein, α tocopherol, copper and magnesium increased to the recommended daily allowance (RDA). A decrease was also observed in dietary trans fatty acids, Na, cholesterol and sugars therefore intake of almonds led to positive nutrient adjustment aiding disease prevention (Jaceldo-Siegl et al., 2004).

Almond research is leading to the identification of many other bioactive compounds that may help to explain the mechanistic actions of these nuts. Phytochemicals in almonds may have certain beneficial effects. Certain polyphenols have been identified in almond skins. Polyphenols scavenge free radicals and have been reported to protect
DNA and LDL against oxidation. Rhamnetin, which constitutes 4.5% of total almond hull weight, was found along with quercetin and kaempherol aglycones (Pinelo et al., 2004). Another investigation into antioxidant capacity of almond skin revealed quercetin, isorhamnetin, quercitin, kaempferol 3-O-rutinoside, isorhamnetin 3-O-glucoside, and morin as the key flavonoids. Almond skin extracts containing these flavonoids successfully inhibited LDL oxidation (Wijeratne et al., 2006). Hulls of almonds are also a rich source of triterpenoids (1%); betulinic acid that is reported to have anti-inflammatory properties and to be an anti-HIV agent; oleanolic acid reported to have anti-cancer activities and ursolic acid (Takeoka et al., 2003). Other compounds include 3-prenyl-4-O-β-glucopyranosyloxy-4-hydroxybenzoic acid, catechin, and protocatechuic acid. Protocatechuic acid is a benzoic acid derivative found in fruits and vegetables that has a ten-fold higher antioxidant activity than αTOH. Investigations into this compound show chemoprotective effects on the colon and in oral carcinogenesis of rats (Sang et al., 2002). As well as these antioxidants a sphingolipid 1-O-beta-D-glucopyranosyl-(2S,3R,4E,8Z)-2-[(2R)-2-hydroxyhexadecanoylamino]-4,8-octadecadiene-1,3-diol constituent has been identified (Sang et al., 2002). Studies carried in animals showed that increased intake of sphingolipids had beneficial effects in reducing LDL cholesterol while increasing high density HDL cholesterol.

Almonds are one of the richest sources of dietary antioxidants. Antioxidants can work as free radical quenchers and prevent damage to biological molecules in disease states. Almonds also contain high levels of vitamin E that reduces the risk of LDL oxidation known to lead to atherosclerosis the underlying cause of CVD (Jambazian et al., 2005).
Almond supplementation can improve these levels in the diet and hence reduce the possible risks of CVD (Jambazian et al., 2005). One ounce (~28g) almonds contain ~7.4mg αTOH and smaller quantities of the other tocopherol isoforms. Incorporation of almonds into the diet raises αTOH levels while reducing the other isoforms of vitamin E (Jambazian et al., 2005).

Whilst intervention studies with vitamin E have not consistently shown beneficial effects on cardiovascular disease risk, a previous study reported that supplementation with 200IU/d of vitamin E for two weeks can cause an increase in the levels of Apo A1, an important factor for reversed cholesterol transport (Aldred et al., 2006). There is evidence for primary prevention of CVD where dietary changes such as consumption of fruits, nuts, fish and whole grain foods have led to beneficial lipid lowering effects. This ultimately leads to a reduced risk of developing these CVD (Hu and Willett, 2002). This thesis will investigate whether intake of dietary vitamin E in foodstuffs through increasing dietary almond intake, can modulate risk factors for CVD in non-symptomatic adults.
The aim of this research is to undertake a vitamin E enrichment diet using almonds in three distinct populations and to examine effects on risk factors for cardiovascular disease. This work will specifically examine almond-diet induced changes in the following CVD risk factors; after 4 and 8 weeks of dietary almond consumption of 24g/d (week 0 – week 4) and 50g/d (week 4 – week 8).

- cholesterol level and distribution
- plasma nitrites and protein oxidation
- flow-mediated dilatation
- blood pressure
- sVCAM-1 levels

Proteomics will be employed to determine the effects of foodstuffs on the plasma protein expression profile.

This study will investigate the outcome measures in individuals > 50yrs to determine whether the effects of almond intake differ from those observed in the subjects aged 18-35yrs. This will identify whether dietary antioxidant enrichment through almond consumption offers age-dependent benefit or risk.

The project is designed to allow data collection over all seasons eliminating any bias in dietary intake with time of year.
Chapter 2.0  General Methods

2.1 Materials

All reagents were obtained from Sigma-Aldridge (Poole, Dorset, UK) and solvents were from Fisher (Loughborough, UK) unless otherwise stated. Mouse anti-human Apolipoprotein A1 was from Serotec (Oxford, UK) mouse antidinitrophenol, peroxidase-conjugate was from Sigma. EconoPac 10 DG columns for desalting, Biolytes 3/10, immobilised pH gradient strips (3-10), precast Tris-glycine gradient gels (10-20%) and tributylphosphine were from BioRad, UK. Disposable plastic ware was from Greiner Bio-one and Nunc maxisorb plates for ELISA were from Thermo Fisher Scientific (Roskilde, Denmark). Assays for cholesterol determination were from Randox (Country Antrim, UK). The nitrite/nitrate assay was purchased from Cayman Chemicals (Ann Arbor, USA). Lymphprep: A ready to use solution for the isolation of pure lymphocyte suspensions was obatined from AXIS-SHIELD, Norway. It contains Sodium Diatrizoate 9.1% (w/v), Polysaccharide 5.7% (w/v). All water used was from a MILLIPORE water purification system. (18ohms)
2.2 Methods

2.2.1 Study Design
This thesis presents the work from two intervention studies. The first small pilot study was undertaken to establish the validity of methods, to identify any potential problems with the study design and to confirm that the proposed consumption of almonds was tolerable by the participants.

Pilot Study Design
Six volunteers willing to consume almonds were recruited for the pilot study; three females and three males. Volunteers that were recruited were non-smokers; were not taking any multivitamins; and had a body mass index below 30. Subjects were asked to complete a food diary (Appendix II). At the initial meeting they were also advised to continue with their normal diet throughout the study period and recommended not to take any other nut food product other than those that were supplied. The volunteers were also advised not to heat/cook the almonds in any way or soak them and were advised to continue with their normal daily lifestyle. Blood samples were collected before and after the consumption of 50g/d of almonds over a four week period for the pilot study. Patient demographic and anthropometric measurements were taken included weight, height and blood pressure. Volunteer compliance was calculated from the number of bags of almonds returned throughout the study.

Main study design
The effect of almond consumption (25 g/d for 4 weeks followed by 50 g/d for 4 weeks) was evaluated in this study. The study recruited a total of n = 67 subjects into a treatment controlled washout study. Patient anthropomorphic measurements of blood pressure; flow-mediated dilation; height; weight; and a 20ml fasting blood samples were collected at time intervals described below. Volunteers were asked to complete a food diary. At the initial meeting they were also advised to continue with their normal diet and lifestyle throughout the study period and recommended not to take any other nut food product other than those that were supplied. The volunteers were also advised not to heat/cook the almonds in any way or soak them.
Initial measurements were taken at week 0 and then they were given 25g/d almonds to consume over 4 weeks. This was followed by another set of measurements at week 4 after which they were given 50g/d of almonds to consume over another 4 week period. A third set of measurements was taken at week 8 (figure 2A). Compliance was monitored by almond bag return.

<table>
<thead>
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<th>8</th>
<th>16</th>
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<tr>
<td>Measurements taken:</td>
<td>50g/d Almonds</td>
<td>50g/d Almonds</td>
<td>No Almonds</td>
<td></td>
</tr>
<tr>
<td>KEY</td>
<td>Blood sample (10mls)</td>
<td>Height</td>
<td>Weight</td>
<td>Blood Pressure</td>
</tr>
</tbody>
</table>

**Figure 2A.** A summary line of volunteer measurements taken.

2.2.2 Subjects

Volunteers were recruited by a variety of methods; email or from personal contact with subjects known to members of the research laboratory; follow-up from a presentation at Think-Tank in Birmingham; patients attending the eye clinic at Aston University or by local advertisements in the media. Prior to admission to the study, volunteers were interviewed to determine whether they met the study inclusion criteria.

Potential volunteers were excluded if they fulfilled any of the following exclusion criteria:

- Current smokers
- Taking multivitamins
- Taking regular medication, or on medication for CVD, hypertension and lipids
- Diabetics
- Women of child bearing age (16-49yrs)
All eligible volunteers signed a consent form, which was approved by the University ethics committee (Appendix I). The study followed the principles laid out in the Helsinki declaration, ethical approval for this study was granted by Aston University Human Science Ethical Committee (REG/00/174).

A power calculation was made using G*Power 3.0.10 to determine the number of subjects required per group to observe an effect of almond intake on vascular risk factors. The effect size was calculated from the previous study of Aldred et al. (2006), which described an increase in Apo A1 concentration to 248+153 mg/dL after 400IU vitamin E supplementation per day for 4 weeks from a baseline level of 95.8+126mg/dL (mean + SD, n=11 per group). This indicated an effect size of 1.076 for change in Apo A1 on supplementation. The sample size required using a paired two-tailed analysis and giving a significance of p<0.05 was 12 per group with a 90% power to observe the effect.

Following recruitment, blood samples were taken after a 12hour overnight fast where water was allowed to be consumed is required. Blood samples used from volunteers to define which subject group they would be allocated to. Volunteers were divided into four groups according:

- Males (age range of 18-35yrs) n=16 and referred to as “healthy male” of which n=15 completed the study.
- Males having a BMI >25, cholesterol levels > 5.17 mmol, blood pressure > 140/90 (age range of 18-35yrs) referred to as “at risk group” n=16 of which n=16 completed the study.
- Male and post-menopausal women (age range >50yrs) n=20 referred to as “Mature” of which n=18 completed the study.
- Treatment control (all ages) n=15 and referred to as “control” of which n=14 completed the study.

Subjects were randomized to either active intervention or control groups.
2.2.3 Protein assay by Bicinchoninic Acid Assay (BCA)

Bicinchoninic acid (BCA) reagent solution: containing bicinchonine acid, sodium carbonate, sodium tartrate and sodium bicarbonate in sodium hydroxide (0.1M, PH 11.25) (Sigma).

Copper (II) sulphate pentahydrate solution: 4% (w/v) solution (Sigma).

Protein standard: BSA (1.0mg/ml in sodium chloride (0.15M) and sodium azide (0.05%) (Sigma).

A flat bottomed 96 well costar plate was used for protein determination by the BCA assay. A standard curve was constructed using 1mg/ml BSA (bovine serum albumin). Triplicate samples of BSA at 0, 0.2, 0.4, 0.6, 0.8, and 1.0mg/ml were plated (10μl/well) diluent distilled water used. Samples for the protein carbonyl ELISA (10μl) were tested in triplicate. BCA (bicinchoninic acid solution) (4% v/v copper sulphate in a 50:1; 200μl/well) was added to all wells. The plate was incubated for 30mins at 37°C then allowed to cool for 10mins before the absorbance was read at 490nm using a Dynex MRX microplate reader and unknowns calculated using the standard curve.

\[
R^2 = 0.9993
\]

**Figure 2B.** A BCA standard curve for calculating protein concentrations.
2.2.4 Protein assay by RC-DC

The protein concentration of pooled plasma samples after albumin and IgG depletion was determined using RC-DC. Samples were pre-diluted to 1:2, 1:5 with PBS as the diluent. BSA standards prepared 1.5mg/ml, 1mg/ml, 0.75mg/ml, 0.5 mg/ml, 0.25mg/ml, and 0.125mg/ml and placed on ice along with plasma samples. Plasma and standards (5μl) were dispensed in triplicate into labelled eppendorf tubes. Reagent 1 (Biorad, 25μl/tube) was added and the tube was vortexed. Reagent 2 (Biorad 25μl/tube) was added and the tube was vortexed. The samples and standards were centrifuged for 13,000 rpm for 3 minutes. The tubes were allowed to drain on tissue paper for ~ 10 minutes until no fluid remained in the tubes. Reagent S (5μl) was added to Reagent A (250μl) and this was inverted to mix prior to addition to samples and standards (25μl/tube) and allowed to stand until pellet dissolved. Reagent B (200μl/tube) was added to all tubes and incubated for 15 minutes at room temperature. Samples and standards were dispensed into a 96 well plate (200μl/well). The absorbance read at 630nm and protein concentration determined from a standard curve.

![Graph](image)

**Figure 2C.** An RC-DC standard curve for calculating protein concentration of samples.
2.3 *Statistics*

Statistical analysis of data was carried out using Graphpad prism (Copyright © 1994-1999 by GraphPad Software All rights reserved.) using the following methods according to the data under investigation. Initially, data was assessed for Gaussian distribution using the Kolmogorov-Smirnov (KS) test. All datasets were found to be normally distributed therefore parametric statistics were employed. The datasets, which were analysed in the pilot and the main study, were for those subjects who completed the intervention only owing to the requirement for paired statistical tests. Compliance analysis, measured as bag return, was included for use as a covariate in subsequent analysis. Previous intervention studies have indicated high levels of inter-individual variability in response to nutrients, necessitating the use of paired statistics. Intention to treat analysis was not possible as paired comparison analyses were employed. Two tailed, paired Student’s t-test was used to compare differences within groups at start (week 0) of the study to the end point of the study (week 8). Comparison of column means for more than two time points or between more than two groups the one-way analysis of variance was used in conjunction the repeated measures analysis, and where required a Tukeys Multiple Comparison test was used. P values found to be < 0.05 were significant. The power calculation was undertaken to reduce the risk of a type II error, the failure to detect a real effect due to small sample size. This indicates that a sample size of 12 was required to observe a significant effect; however, this calculation was based only on the earlier dataset of vitamin E effect on Apo A1 where doses of vitamin E used were higher than those taken by almond-consuming subjects.
Chapter 3.0 Dietary Analysis

3.1 Introduction

The diet and lifestyle of populations has changed a great deal over the last century with regards to exercise and food availability. The abundance of refined foods along with a less active lifestyle has contributed to a rise in obesity and particular in Western countries (Flegal et al., 2002). In the last few decades much evidence has been gathered indicating that dietary patterns can influence cardiovascular health.

As the purpose of this thesis is to understand the effects of almond intake on cardiovascular disease risk, it is important that the following possible confounders are addressed before conclusions can be drawn;

1. What is the influence of including almonds in the diet on nutrient intake?
2. Is there an effect of study participation alone on nutrient intake?
3. Can dietary almond compliance be determined quantitatively?
4. Are there any adverse effects of calorie-dense almond intake on body mass?

Dietary assessment methods are used to analyse nutrient intakes. Several methods are available and method choice depends on study purpose. While Food Frequency Questionnaires (FFQs) are used for large population samples and measure qualitative changes (Pietinen et al., 1988), they tend to show over estimation of food and energy intake (Kabagambe et al., 2001; Schroder et al., 2001). For small sample sizes, a 24-hour recall assessment is also used after the consumption of food and is therefore not likely to influence dietary behaviour but this requires a knowledgeable interviewer to gather all the necessary information. The most appropriate measure
for short term effects of intervention studies is the use of food diaries to assess behavioural and dietary intake changes. The optimal length of diet diary reporting is a subject of debate, but the majority of researchers favour using a three day recorded food diary which does not require weighing of portion size (Khaw et al., 2001; Yancey et al., 2000; Crawford et al., 1994). However, food recording will have an element of misreporting and this will be more apparent in certain food types where foods considered bad for health (those high in fats and sugars) would be underestimated (Lafay et al., 2000). The assessment of food intake in a control group was also undertaken in order to address the effect of study participation on food diary recording.

As food diary assessments are likely to be misreported and there is a need to assess intake of the test food (almonds), the validation of food consumption as assessment through biomarkers from blood, urine or hair has been proposed (Isaksson, 1980; Khaw et al., 2001). Studies using 24 hour urine nitrogen excretion are in agreement (80-100%) with dietary intakes as estimated in diet records (van-Staveren et al., 1985; Kipnis et al., 2001; Subar et al., 2003). Similarly, fruits, nuts and some vegetables are the principal supply of dietary vitamin C, carotenoids and vitamin E and measurement of plasma micronutrients has been used as an indicator of fruit and vegetable consumption. (Khaw et al., 2001; Stampfer et al., 1991; Talegawkar et al., 2007; Jacques et al., 1993). However, levels of correlation between reported intake and plasma levels decreased when supplemented individuals are omitted from the results. Nevertheless, in this study an attempt to quantitatively assess almond intake by change in plasma alpha tocopherol level was undertaken as almonds are one of the richest natural dietary sources of vitamin E.
Cardiovascular disease risk increases with body mass index and the almonds consumed are energy dense (200kcal/25g). Therefore the effect of study participation on body mass index was evaluated in those taking almonds and in the control group to remove any confounding from weight gain on cardiovascular risk.

In summary, this chapter estimates the compliance of subjects with respect to almond consumption, the effect of almond intake on total nutrient intakes, and any impact of almond intake on anthropometric measures that are associated with cardiovascular risk.
3.2 Methods

3.2.1 Food diary design

In a pilot study, several food diaries were examined for their reported reliability in informing on dietary nutritional intakes. It was important to develop a questionnaire which would provide accurate information on lipid intake as this is one of the study outcome measures. There was also concern that consumption of almonds would provide a significant calorific load and that subjects may alter their diets in order to compensate for almond intake.

Another consideration that was taken into account in designing the food diary was the number of days to be reported. It has been reported that greater accuracy is recorded if fewer study days are reported with the optimal number of days being 3-4 days to include a weekend day.

Based on the evidence described in the introduction to this chapter and the findings of the pilot study, the food diary was based on three days and included information about portion sizes. The food diary is shown in Figure 2.y. Three-day diet records were analyzed by using the DietPlan6 software programme (Forestfield Software, Horsham).
3.2.2 Anthropometric measures

Anthropometric measurements are used in nutritional assessments and for adults usually include height, weight, body mass index (BMI). The BMI is an estimate of body composition that correlates an individual's weight and height to lean body mass. Therefore, BMI is an index of weight adjusted for height. Body mass index is calculated by dividing weight in kilograms by height in meters squared and multiplying by 100. Height was measured using a telescopic measuring rod after volunteers had removed their shoes. Weight was recorded as the average of two independent measurements on weighing scales. Subjects were considered at risk for cardiovascular disease if their BMI was greater than 25kg/m².

3.2.3 Sample preparation for HPLC Vitamin E analysis

Vitamin E is the major lipid soluble antioxidant in vivo which has variably been reported to affect cardiovascular risk. Almonds are enriched in vitamin E and this may contribute to the potentially beneficial effects of almonds on cardiovascular risk parameters. To determine whether consumption of almonds modified plasma vitamin E levels, analysis of plasmas was undertaken pre- and post-supplementation.

Plasma aliquots (300µl) were thawed from -80°C and transferred to a fresh 2ml centrifuge tube to which 300µl of ethanol (containing 80µM tocopherol acetate) was added. The samples were vortexed for 10sec. Then 1.5ml of hexane (containing 12.5mg/L BHT (2, 6-Di-tert-butyl-4-methylphenol)) was added to all samples. These were vortexed for 60sec and then centrifuged for 5000 x g for 2min. The organic phase was collected and placed into a fresh centrifuge tube; the remaining
pellet was further extracted again with 1.5ml hexane (containing 12.5mg/L BHT). The sample was again vortexed for 60sec and centrifuged for 5000 x g for 2min. The organic phase was collected and placed into another appropriately labelled fresh centrifuge tube. The vial containing organic phase was evaporated to dryness under reduced pressure. After the evaporation, methanol (200µl) was added to each vial to dissolve the remaining pellet. Alpha tocopherol standards 80, 40, 20, 10, and 5µM were prepared using this method, but after the first extraction there was no separation of the organic phase and the standard were then evaporated under vacuum (Eppendorf concentrator 5301 Hamburg). The pellet was reconstituted with 300µl methanol (Rizzo et al, 2000)

3.2.3.1 HPLC of Vitamin E Analysis

Reverse phase chromatography was employed to analyse alpha tocopherol levels in plasma. The HPLC system constituted the Dionex AS50 autosampler, GP50 gradient pump, and the UVD 170U detector. The mobile phase consisted of methanol HPLC (100%) with a flow rate of 1ml/min and the alpha tocopherol peak was detected at 292nm (UVD 170U detector). Samples were prepared as described in the sample preparation method. Standards and plasma samples were run in duplicate with 50µl injection onto a Phenomenex Gemini 5 micron C18 110A column. Alpha tocopherol was eluted by methanol at 7.56 minutes and tocopherol acetate was eluted at 9.62 minutes. An “unknown plasma x” was also used as an internal standard to monitor fluctuations between readings. The unknowns were calculated using the standard curve.
A typical standard curve is shown in Figure 3A with an $r^2 = 0.9972$ calculated using Spearman's rank regression analysis.

![Graph showing a linear relationship between concentration and peak area. The line is labeled $r^2 = 0.9972$.]

**Figure 3.0** Alpha tocopherol standard curve from HPLC analysis of standards.

To assess tocopherol recovery from plasma samples, plasma was spiked with 40μM tocopherol acetate and the level of tocopherol was reported pre- and post-spiking. The difference in tocopherol concentration detected between these two measurements was then expressed as a percentage of the amount of tocopherol added to calculate tocopherol recovery.
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</tr>
</tbody>
</table>

**Table 3A.** Intra-batch and inter-batch coefficient of variance of quality control plasma.

<table>
<thead>
<tr>
<th>Alpha Tocopherol Standards (mM)</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>93</td>
</tr>
<tr>
<td>40</td>
<td>89</td>
</tr>
<tr>
<td>80</td>
<td>90</td>
</tr>
</tbody>
</table>

**Table 3B.** Percentage recovery of alpha tocopherol standards from the extraction method used for plasma alpha tocopherol.

Tocopherol was corrected for the internal standard, tocopherol acetate. To evaluate the reproducibility of the assay, quality assurance plasma was analysed four times on one day and on ten separate days. The results are presented in Table 3A.
3.3 Results

Food intake assessment by a three day estimated food diary record found little change in the estimated macronutrient intake of the diet in the healthy group (18-35yr male) after almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks. Food diary assessment with DietPlan6 estimated significant increases in micronutrient vitamin E levels.

A three day estimated food intake was assessed with DietPlan6 software. Participants who completed the food diary before the study started and who completed the diary after completion of the study were used in the dietary assessment. Of the 14 volunteers in the healthy group only 6 (43%) completed the required food records to enable assessment. The following nutrients; carbohydrates, total sugars, protein, fat, saturated fat, cholesterol, sodium, and fibre showed no significant change after almond supplementation in the diet of the participants. There was a significant increase \((P<0.0026)\) in the estimated level of vitamin E intake (Fig.3.5). No changes in the calorific intake were observed during the study period (Fig. 3.0)

A three day estimated food record analysis with DietPlan6 for the mature group (>50yrs male and female), showed no significant effects of almond supplementation (24g/d for four weeks followed by 50g/d for a further four weeks) on estimated intakes of carbohydrates, total sugars, protein, saturated fat, cholesterol, sodium, fibre, and vitamin C. Estimated increases in the level of fat intake, monounsaturated fat, polyunsaturated fat and vitamin E levels were significant.
Completion of food diary records by participants in the group was 82%. Only completed food diaries were assessed with the DietPlan6 software and used in analysis. The analyses for the following macronutrient estimated intakes; carbohydrates, total sugars, protein, saturated fat, cholesterol, sodium, fibre, and vitamin C, showed no significant changes in their levels over the study period (25g/d for four weeks followed by 50g/d for a further four weeks). Levels of fat intake significantly increased ($P < 0.0027$; Fig. 3.4), accounted for by increases in certain types of fats these included monounsaturated fat ($P < 0.0001$; Fig 3.4.2) and polyunsaturated fat ($P < 0.0012$; Fig 3.4.3). Estimated levels of vitamin E intake ($P < 0.0001$; Fig. 3.5) in this group were also significantly increased. There was a trend of increasing estimated calorie intake over the study period for mature group volunteers (Fig. 3.0).

Risk group volunteers (18-35yrs male) who completed the three day estimated food diaries reported no significant changes carbohydrates, total sugars, protein, fat, saturated fat, cholesterol, sodium, and fibre intake over the study period (25g/d for four weeks followed by 50g/d for a further four weeks). Estimated vitamin E intakes were significantly increased compared to the start of the study, and increased intakes in monounsaturated fat and vitamin C were reported. Risk group participant diaries which were completed both before and after almond enrichment (71%) were used in the food diary analysis. Food record assessment was carried out with DietPlan6 software and no significant increases in the macronutrients (carbohydrates, total sugars, protein, fat, saturated fat, cholesterol, sodium, and fibre) of the at risk participants were reported during the study time.
frame (25g/d for four weeks followed by 50g/d for a further four weeks). Estimated increases in the intakes of vitamin E were found to be significant ($P<0.0006$; Fig. 3.5). There was a general trend in the diet of the participants for reduced saturated fat intake and cholesterol compared to the start of the study. Despite the observation of reduced fat intake there was a significant increase in estimated monounsaturated fat ($P<0.0195$; Fig 3.4.2) intake in the group.

*For control group participants (18-35yrs male), food intake assessment showed no significant changes in estimated nutrient intake of carbohydrates, total sugars, protein, fat, saturated fat, monounsaturated fat, cholesterol, sodium, vitamin E, and vitamin C and fibre over eight weeks. However, the analysis did show significant decreases in the estimated polyunsaturated fat intake.*

The volunteers of the control group did not undergo almond supplementation during the study period but were asked to complete estimated food records at the same time points given to the other three groups. The diaries from volunteers of the group who completed both before and after study food diaries (83%) were used in this analysis. Although no dietary intervention was introduced for these participants, the food diaries revealed that intakes of polyunsaturated fats were significantly decreased ($P<0.0352$; Fig 3.4.3).
Plasma Vitamin E levels of the almond supplemented groups showed no significant increase. The BMI analysis of participants of the study showed an increase in the healthy group volunteers.

Volunteers of the healthy group consisted of male between the ages of 18-35yrs and were shown to increase BMI during the almond supplementation (25g/d for four weeks followed by 50g/d for a further four weeks) period (Fig. 3.7). The plasma alpha tocopherol measured using HPLC as a marker for almond intake during the study showed no change in participants with only a small increasing trend of the healthy group (Fig. 3.8). Dietary and plasma vitamin E correlation of mature group data showed no correlation with almond supplementation (Fig. 3.9).
Figure 3.0. Estimated daily Calorie intake changes of All Group volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=11) Data represent changes in daily calorie intake over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.1. Estimated daily intake of Carbohydrate of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=11) Data represent changes in daily carbohydrate intake over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.2 Estimated daily intake of Total Sugars of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=11). Data represent changes in daily sugar intake over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.3. Estimated daily intake of Proteins of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=11). Data represent changes in protein over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.4. Estimated daily Fat Intake of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14) with significant increases in fat intake (paired t test P< 0.0027); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=11). Data represent changes in fat over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.4.1  Estimated daily Saturated Fat Intake of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=10). Data represent changes in saturated fat intake over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.4.2 Estimated daily Monounsaturated Fat Intake of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs, paired t-test (P<0.0079); (B) Mature male and female volunteers <50yrs (n=14) paired t-test (P<0.0001); (C) At risk group, male volunteers between the ages of 18-35yrs (n=10) paired t-test (P<0.0195); (D) Control group not supplemented with almonds (n=11). Data represent changes in monounsaturated fat over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.4.3. Estimated daily Polyunsaturated Fat Intake of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14) paired t test ($P<0.0012$); (C) At risk group, male volunteers between the ages of 18-35yrs (n=10); (D) Control group not supplemented with almonds (n=11) paired t-test ($P<0.0352$). Data represent changes in polyunsaturated fat over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.4.4. Estimated daily Cholesterol Intake of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=10). Data represent changes in cholesterol intake over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.5  Estimated daily Vitamin E Intake of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs, paired t-test ($P<0.0026$); (B) Mature male and female volunteers <50yrs (n=14) paired t-test ($P<0.0001$); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9), paired t-test ($P<0.0006$); (D) Control group not supplemented with almonds (n=10). Data represent changes in vitamin E intake over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.6  Estimated daily Fiber Intake of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A) Healthy male volunteers (n=6) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=14); (C) At risk group, male volunteers between the ages of 18-35yrs (n=9); (D) Control group not supplemented with almonds (n=10). Data represent changes in fiber intake over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.7  Effect of Almond consumption on BMI of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=14) between the ages of 18-35yrs, paired t-test ($P<0.0367$); (B) Mature male and female volunteers <50yrs (n=17); (C) At risk group, male volunteers between the ages of 18-35yrs (n=16); (D) Control group not supplemented with almonds (n=12) paired t-test ($P<0.0297$). Data represent changes in BMI over 3 days up to week 0 (before) and up to week 8 (after).
Figure 3.8  Almond Supplementation On Plasma Alpha Tocopherol Levels.
Volunteers were supplemented with 25g/d almonds for 4 weeks followed by 50g/d almonds for another four weeks. (A) Healthy group male volunteers between 18-35yrs (n = 14); (B) Mature group volunteers male and female >50yrs supplemented with almonds (n = 15); (C) At Risk male volunteers between 18-35yrs supplemented with almonds (n = 14); (D) Control group volunteers not supplemented with almonds (n = 12).
3.4 Discussion

Nutritional epidemiology is carried out to analyse the relationship between food intake and disease states like cardiovascular disease and cancer. In this study where almond supplementation was used to examine possible beneficial effects in healthy and at risk, young and mature male populations the estimated food records were used to assess nutrient intake of the participants. These food records were used to assess if any changes in dietary behaviour would take place when individuals were participating in the study and to establish if nutrient intake maybe affected by consumption of almonds additionally to their normal diet.

Consumption of 50g almonds contributed an additional 300kcal to the dietary energy intake, although no significant intake in calories was reported in the food diaries. In the older group, there was a trend towards increase in calories intake with a mean change from 2050kcal/d to 2268kcal/d. The subjects were advised to maintain their normal diets, although the diet diaries suggests that subjects may have accommodated the additional calorie load by reducing consumption of other foods as the calories intakes of supplemented individuals was not increased by 300k uniformly.

In fact, 19 subjects reported a reduced calorie intake. This may represent under-reporting as evaluation of BMI for the healthy group subjects comparing week 0 and week 8 showed a significant increase. Under-reporting in diet diaries has been established for a long time and in 1990 Livingstone et al. used a sub-sample of the Northern Ireland Diet and Health to describe this in males and females. The study
used a 7 day food diary, 14 day food diary, and double labeled water technique to assess energy intake and energy expenditure. The findings reported were a bias towards under-reporting of 219% in men and 218% in women for energy intake. Further analysis found that under-reporting was not uniform in male and females but subjects of the middle and lower thirds were more likely to under-report while those of the top third showed agreement with energy intake and expenditure. The findings demonstrated the different bias towards food reporting within populations (Livingstone et al., 1990). Subjects in other studies have reported to omitting foods due to inconvenience; self-consciousness; or feelings of shame in a 7 day weighed food diary analysis (Macdiarmid et al., 1998).

An independent study reported by Jaceldo-Siegal showed an effect of adding almonds (52g) to a self-selected habitual diet resulting in improved quality of fats with increased MUFA, and PUFA levels (Jaceldo-Siegal et al., 2004); the data reported in this chapter confirm the findings of this study in the mature individuals. The study by Jaceldo-Siegal also showed improvement in magnesium, alpha tocopherol, fibre, as well as reduction in trans-fatty acids, cholesterol and sugar intake which met with recommendations for CVD prevention along with other nutrient changes reported with the almond supplemented diet.

In the subject groups where the food diary return was less than 50%, no significant effect of dietary nuts was observed but this may be due to a lack of statistical power in the analysis against a background of variable reporting. The food diary analysis for this study shows there was minimal impact on the participants’ dietary habits in respect to the protein and carbohydrate intake. The level of fat was found to be
increased in the mature group along with monounsaturated fats and polyunsaturated fats; this may be due to increased intake of almonds as observed with an increased trend in calories. The trend for increased monounsaturated fats is also observed in the healthy and at risk group, supporting the suggestion that it may be due to almond intake. Almonds are known to contain high levels of monounsaturated fats and polyunsaturated fats that could account for the increases seen. Fat intakes at baseline were reported to be similar in all groups ranging from 74-85g/d but on almond supplementation these levels changed in healthy and mature subjects to 98g/d and 96g/d respectively. The at risk group however showed little change in fat intake 80g/d to 79g/d and the control group reported initial intake of 79g/d which declined to 73g/d at the end of the study duration and may be evidence of under-reporting in the at risk group.

There was a shift towards increased vitamin E and monounsaturated fat levels as would be expected with the intake of the almonds during the study duration. Trends in the nutrient intakes would however suggest that the dietary habits were not altered during the study duration as no significant changes were found in the other nutrients investigated from the food diary. The changes in vitamin E intake significantly increased in all supplemented groups (healthy, mature, and at risk groups). There was no change observed in the control group who did not take almonds and therefore suggests that the increase was most likely due to the intake of almonds in the supplemented groups.

Within the at risk group, caloric intake was predominantly stable with those with reporting high caloric intake at baseline reducing their levels over the study duration
possibly as an impact of participation in the study. The mean value of the levels of fat intake, saturated fat and cholesterol intake was reported to decrease within the group. The changes in dietary intake of the fat food stuffs indicate a change in dietary habits. People are known to report “good eating” habits rather than “bad eating” habits in studies such as this.

Analysis of the control group diaries showed no significant changes in nutrient intake levels within the group other than for polyunsaturated fatty acid intake. However, the control group showed a trend towards reduced intakes of cholesterol and vitamin E levels, and this may also indicate that reporting of foods containing these nutrients were either eliminated from the diet during the study or not reported in the food records. These findings are in line with other studies reporting underreporting of food intake and possibly of certain food groups.

Once the volunteers had completed participation of the study they were asked how the food records were kept and if they were a real representation of their dietary habits. Some volunteers reported choosing days that were convenient to write in the food diaries while others claimed they did not eat certain foods to avoid the complication of recording it in the food diary. This clearly indicated that the simple aspect of keeping a food record alters people’s eating patterns and that this may not have been affected by simply just participating in the study without food diary logs.

The purpose of collecting food diaries was to establish that diets were not significantly changed during the study period other than for the intervention. This allows any effects observed to be more confidently attributed to almond
intervention. To establish a baseline, diaries were collected at week -1 and then diaries were re-collected during the final week of 50g almond intervention. Of the 64 subjects who completed the 8 week study, 60 completed the diary at week -1, however, completed food diaries were returned after week 7 by only 40 subjects. This represents a 62.5% dietary survey completion rate, which is not a true representation of the population. There was a better return by older adults and those with higher BMI, however; again this may lead to biased evidence, as obese individuals are known to underreport food intakes. A study of obese men demonstrated under-recording of foods especially that of fat intake that leads to bias in studies including obese individuals (Goris et al., 2000) For this reason, vitamin E intake determined by food diary and plasma levels of vitamin E were only correlated in the mature group where compliance to food diaries >77%.

Analysis of food diaries was using DietPlan 6, showed a wide variability in nutrient intakes with up to a five-fold difference in reported nutrient intake for carbohydrates, total sugars, and vitamin E between individuals. The use of a three day food record for diet aimed to minimize week to week variability for individual subjects and this has been examined in the control group who were asked to complete food diaries on two separate occasions in the absence of an intervention. There was a greater than 10% increase in the total sugars, cholesterol, and fibre with a greater than 10% decline in saturated fats, monounsaturated fats, and polyunsaturated fats reported by the control group. The reported dietary analysis in the nutrients for the control group was not significant except for polyunsaturated fats. Food diary records will always have an element of misreporting and therefore is essential to have measurements of markers to verify the dietary intake reported.
It has been suggested that plasma and urinary biomarkers of vitamin C could provide useful indices of the dietary intake, however, in this study the importance of vitamin E was under investigation and levels were measured using HPLC. Plasma levels of vitamin E in the form of alpha tocopherol were measured in conjunction with almond supplementation, as almonds are a rich source of vitamin E. There was no significant change in plasma vitamin E with the consumption of almonds. The levels were already above the recommended levels of 15mg/d at baseline, which may have saturated the plasma levels of the vitamin; therefore the vitamin E provided by almond intake of 15mg/d in 50g almonds was not sufficient to alter the alpha tocopherol levels measured in the plasma. Those with low levels of vitamin E at baseline did show increase in their levels but this was not significant. There was no effect of almond supplementation on plasma vitamin E levels when all almond supplemented groups were analysed together.

There are many nutritional software programmes available for use since the advent of nutritional epidemiology in the early 1950s (Hoover, 1987). With the onset of so many programmes to choose from, the nutrient databank used in these programmes is essential in choosing the best analysis tool. The Dietplan 6 food analysis software used in the study contains the UK food tables from McCance & Widdowson's The Composition of Foods. The data within the McCance & Widdowson’s food tables are based from UK nutrient databank that contains the nutritional information of foods that are frequently consumed in the UK. This makes it suitable software to use in our study instead of using foreign programmes and databases. The accuracy of computer analysis has been raised, however, various studies have reported
accuracy of computer programmes to 7% or 15% for energy intake with ~15% for particular nutrients (Lee et al., 1995; Nieman et al., 1992). Comparison studies carried out on several programmes found them to be accurate within to 5% of nutrients (McCullough et al., 1999).

During analysis of the three day diaries completed by study subjects, The Dietplan 6 software frequently was not able to provide accurate values for many vitamins and minerals in some of the foodstuffs e.g. the vitamin C content for an orange drink of unknown brand could not be determined but instead was given a + value which indicates that the nutrient was there in a significant amount but in an unknown concentration. In other cases, the value d was used to denote that a nutrient value derived or deduced and the e: value to indicate an estimated numerical value for that nutrient.

Flavonoids have been suggested to offer some cardio-protective effect through free radical scavenging and increased bioavailability of NO. Although almonds contain flavonoids during the time that this study was undertaken there was no data available from the Widdowson database. Future measurements of almonds may provide insight into whether dietary intake of almonds can alter the levels and have any beneficial affects.

In summary, significant change in fat and vitamin E intakes were most likely due to high monounsaturated fats in almonds but as no changes were observed in other nutrients. The diets of those subjects consuming almonds were probably not modified in any other way.
Chapter 4.0 Lipid Profiles

4.1 Introduction

Cholesterol has had a long standing association with atherosclerosis and coronary heart disease (CHD). Dietary clinical interventions studies in the early 1960’s led to the understanding that CHD could be reduced by dietary changes. The connection had been speculated as early as 1916 by C.D. DeLangen when he found that natives of Indonesia had lower blood cholesterol compared to Dutch colonists (DeLangen, 1916) and that it maybe attributed to the differences in diet. In 1961 The American Heart Association (AHA) made the recommendation that individuals at high risk of developing CHD should modify their diets to avert heart attacks. The AHA recommended that the total calorific intake should be reduced and fats should consist of no more than ~25-30% of the total calories (1965).

E.H Ahrens Jr carried out research demonstrating that dietary changes can change blood cholesterol levels (Ahrens, 1979). A large scale cholesterol lowering trial in 1984 by the National Institute of Health (NIH) of a randomised, double blind study demonstrated significant reductions in CVD end points due to reduced cholesterol levels. The cholesterol levels were lowered by using bile acid binding resin, cholestyramine (now statins) in the 1980’s and 1990’s are used in hypercholesterolemic patients.

Methods for determining total cholesterol levels are essential in diagnosing patients that are at high risk. Several methods have been developed over time utilizing enzymatic methods to convert cholesterol esters into free cholesterol for measurement. Cholesterol oxidation was initially described by Turfitt in 1944 by microbial enzymes whilst
investigating soil sterols in acidic conditions (Turfitt, 1944). Preparations of cholesterol oxidase from *Nocardia sp.* were first used by Richmond to assay cholesterol (Richmond, 1973). The step of the cholesterol assay would involve the use of cholesterol esterase and in 1974 Tarbuttona and Allain (Allain et al., 1974) had developed a method for assaying total cholesterol using both these enzymes. The reaction of these enzymes produces hydrogen peroxide and phenol that is used to quantify the cholesterol. The phenol reacts with 4-amoantipyrine or Amplex Red in the presence of peroxide to produce a colorimetric change or a fluorescent end product.

Gofman was the first to successfully characterise the lipoproteins and then went on to carry out a collaborative clinical study to establish if lipoproteins measurements would be a better predictor of cardiovascular events than total cholesterol. The findings reported in 1956 found lipoprotein measurements were just as good as total cholesterol measurements. Later studies showed the importance of lipoprotein fractions in the development of atherosclerosis.

Statins have been successfully shown to reduce LDL levels by ~25% that can lead to a drop in CHD mortality by 30-40% during a period of 5-6yr intervention period. Treatments starting earlier in life could possibly be expected to have greater reductions. The Heart Protection Study found treatment with Simvastatin (40mg daily over a five year period) of a wide range of high-risk patients (20 536 UK adults between the ages of 40-80yrs) lowered LDL-cholesterol on average by 1.0mmol/L reducing CVD end points such as myocardial infarction, stroke, revascularisation, by approximately a quarter (2002). Statins lower LDL-cholesterol and non-HDL (IDL) cholesterol to a similar extent, the ATP III has set the target therapy to 30mg/dL due to the association of the atherogenic potential of remnant lipoproteins in hypertriglyceridaemic patients.
A class of drug that elevates HDL-cholesterol levels are the fibrates several studies where patients present with high triglycerides and low HDL-cholesterol (diabetic, metabolic syndrome) it has been shown to reduce risks of CHD events (Robins et al., 2003). Nicotinic acid also raises HDL-cholesterol and helps reduce CHD risk (Canner et al., 1986). The FDA have approved the use of combinational therapy of statins and nicotinic acid after studies showed a decrease in LDL-cholesterol accompanied with an increase in HDL cholesterol (Bays et al., 2003). Other combinational therapies approved have included statins with ezetimibe marketed as vytorin. Recent trials with this combinational therapy have given rise to caution with some preliminary trials showing an increased risk of cancer (Struthers, 2008).

The method for separating lipoproteins was initially carried out by preparative ultracentrifugation (Havel et al., 1955). This method is expensive and requires both time and highly trained individuals. Further methods have been developed that do not require this ultracentrifugation step. A precipitation method using Sodium Dodecyl Sulfate and heparin with $\text{Mn}^{2+}$ was developed (Wilson and Spiger, 1973). It was found that the heparin preparations themselves were inconsistent causing varied degrees of precipitation of VLDL and LDL with the different preparations. Another method by Burstein and Samaillé (Burstein et al., 1970) used phosphotungstic acid and $\text{Mg}^{2+}$ to precipitate VLDL and LDL. These methods have been compared and the later was found to be a reliable and an inexpensive technique for determining HDL cholesterol.

Diet is a major source for triglycerides in the circulation. Measurement of triglycerides in conjunction with other lipid analysis has been used in the diagnosis of certain diseases e.g. hyperlipoproteinaemia, diabetes mellitus, nephrosis, and extrahepatic
biliary obstruction. Determination of triglycerides is commonly carried out by enzymatic hydrolysis resulting in the release of glycerol and free fatty acids. Glycerol is then quantified by a colorimetric or fluorimetric method.
4.2 Methods

4.2.1 Cholesterol Assay

A Randox kit (CHOD-PAP reagent CH201) was used to analyse the plasma samples that were taken at the time intervals described. Plasma samples were thawed from -80°C to room temperature prior to being analysed. Plasma (10μl), standard (10μl) each was placed into separate vials and 1000μl of reagent mixture was added to each vial from the kit. The mixtures were inverted several times to ensure components were mixed and then incubated at 37°C for 5 minutes until the samples were analysed at 500nm. Cholesterol levels were calculated by the method provided in the kit notes:

\[
\text{Cholesterol concentration} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{Concentration of standard}
\]

The assay works on the principle of enzymatic hydrolysis and oxidation of cholesterol. The production of hydrogen peroxide reacts with phenol and 4-aminoantipyrine in the presence of peroxidase to form quinoneminine that is measured as an indirect measure of cholesterol.

\[
\text{Cholesterol ester + H}_2\text{O} \xrightarrow{\text{Esterase}} \text{Cholesterol + Fatty acids}
\]

\[
\text{Cholesterol + O}_2 \xrightarrow{\text{Oxidase}} \text{Cholestene-3-one + H}_2\text{O}_2
\]

\[
2\text{ H}_2\text{O}_2 + \text{ Phenol + 4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine + 4 H}_2\text{O}
\]
Table 4A  Intra-batch and inter-batch coefficient of variance of quality control plasma of cholesterol measurement.

4.2.2  LDL Assay

LDL levels were measured using a Randox kit (CH3151 LDL cholesterol standard; ST1018 HDL cholesterol standard; HE2613 multisera control level 3). Plasma (50µl), or standard (50µl) each in separate vials was added to precipitation reagent (500µl). Samples were mixed by inversion and then allowed to stand at room temperature for 10mins. Samples were centrifuged at 4000 xg for 15mins. Supernatant was removed and 50µl was incubated with 1000µl of reagent mixture, for analysis using the cholesterol kit (CHOD-PAP reagent CH201) as described in the method above.

Table 4B  Intra-batch and inter-batch coefficient of variance of quality control plasma of LDL-cholesterol measurement.

4.2.3  HDL Assay

A Randox kit (CH203-HDL cholesterol precipitation; ST1018 HDL cholesterol standard) was employed to analyse HDL levels in plasma. Plasma (100µl) or standard (100µl) were each added to precipitation buffer (200µl). Samples were inverted to mix
and then allowed to stand for 10 mins at room temperature. Samples were then centrifuged at 4000 \( X_g \) for 10 mins. The supernatant was recovered and 100\( \mu \)l was used in the analysis using the cholesterol kit (CHOD-PAP reagent CH201) as described in the method above.

<table>
<thead>
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<th>Sample Description</th>
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<th>Intra-batch C.V ((n=3))</th>
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<tr>
<td>Quality control Plasma X</td>
<td>2.53</td>
<td>0.29</td>
</tr>
</tbody>
</table>

**Table 4C** Intra-batch and inter-batch coefficient of variance of quality control plasma of HDL-cholesterol measurement.

### 4.2.4 Triglycerides Assay

A Randox kit (TR1697) was used to analyse plasma triglyceride levels. Reagent enzyme (R1b, 1.7ml) added to reagent buffer (R1a, 100ml). Plasma/standard (5\( \mu \)l) aliquotted in triplicate and Reagent mix (500\( \mu \)l) added. Samples were inverted to mix and then allowed to sit for 10 minutes at room temperature (20 – 25 ℃). Reagent mix used as a blank and samples were dispensed into a 96 well plate (200\( \mu \)l/well) and read at 500nm.

\[
\text{Triglyceride concentration (mg/dL)} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 200
\]

\[\text{Triglyceride} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acids}\]

\[\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}\]

\[\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2\]

\[2H_2O_2 + 4\text{-aminoantipyrine} + 4\text{ chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + HCl + 4H_2O\]
<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Inter-batch C.V ($n=3$)</th>
<th>Intra-batch C.V ($n=3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quality control Plasma X</td>
<td>3.46</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 4D  Intra-batch and inter-batch coefficient of variance of quality control plasma of Triglyceride measurement.

4.2.5 *Apo A1 Analysis*

Apolipoprotein A1 is the major lipoprotein in HDL and it has been shown to be an independent protective factor against cardiovascular disease. In a previous study of vitamin E supplementation in healthy adults, Aldred et al (2006) demonstrated that apolipoprotein A1 levels were increased after consumption of 200IU vitamin E for 2 weeks. In order to investigate whether almond consumption could cause an increase in apo A1, a competition ELISA was employed to determine the plasma apo A1 levels before and after dietary enrichment with almonds in the pilot study. Apo A1 (2μg/ml) 50μl/well was applied to a 96 well nunc plate. The plate was incubated for 1 hour at 37°C. The plate was washed three times in washing buffer (PBS/Tween 20 (0.05% in 1L PBS)). The plate was blocked with milk (marvel, 4% w/v) 200μl/well and left overnight at +4°C. The plate was washed three times in washing buffer.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Inter-batch C.V ($n=3$)</th>
<th>Intra-batch C.V ($n=3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>5.06</td>
<td>5.28</td>
</tr>
</tbody>
</table>

Table 4E  Intra-batch and inter-batch coefficient of variance of standard in Apolipoprotein A1 measurement.
<table>
<thead>
<tr>
<th>A</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<th>10</th>
<th>11</th>
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<tbody>
<tr>
<td>B</td>
<td>40</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>1.25</td>
<td>0.625</td>
<td>0.3125</td>
<td>0.15625</td>
<td>Blank</td>
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<td></td>
<td>μg/ml</td>
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<td>2.5</td>
<td>1.25</td>
<td>0.625</td>
<td>0.3125</td>
<td>0.15625</td>
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<tr>
<td>D</td>
<td>40</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>1.25</td>
<td>0.625</td>
<td>0.3125</td>
<td>0.15625</td>
<td>Blank</td>
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</table>

Standard Apo AI (40μg/ml; 50μl/well) added to wells in triplicate. Doubling dilutions were carried to prepare a standard curve. Wells B3 –B11; C3-C11; and D3-D11 were plated with milk (marvel, 4% w/v, 25μl/well). Standard (40μg/ml) 25μl removed and placed into the next well along the row and aspirated and 25μl removed again and continued along the row of standards. From wells B10, C10, and D10 25μl was discarded. For the blank milk (25μl) was plated in triplicate as indicated in the diagram of plate. All plasma samples were prediluted 1:200 with milk as the diluent and plated in triplicates (25μl/well). The primary antibody anti-Apo AI (Goat polyclonal to human apolipoprotein A-I, Abcam) was added to all wells (final dilution 1:10000, 25μl/well). The plate was incubated at 37°C for 2 hours. The plate was washed three times in washing buffer. The secondary antibody (anti-goat IgG horse radish peroxidase conjugate, Serotec) added to all wells at a dilution of 1:2000 using blocking buffer as a diluent (Tween-20 1% v/v in PBS, 200μl/well), incubated for 1 hour at 37°C. The plate was washed and substrate (10ml citrate-phosphate buffer (0.15M, pH 5), 8μl hydrogen peroxide, and 1 o-phenyldiamine tablet) was added (50μl/well). The plate was
incubated in the absence of light for 2mins to allow colour development. The colour reaction was stopped with the addition of 2M sulphuric acid (50μl/well). The absorbance was read at 490nm using a Dynex MRX microplate reader and unknowns calculated using the standard curve.

Experimental modifications were introduced to improve results these included using HDL (40μg/ml) as the competitor rather than Apo AI, and plasma sample prepared to a 1:100 dilution. Varying concentrations of HDL and Apo AI were used as competitor and coating agent see below.

<table>
<thead>
<tr>
<th>Coating agent</th>
<th>Competitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL 0.5μg/ml</td>
<td>HDL (40μg/ml) / Apo AI (40μg/ml)</td>
</tr>
<tr>
<td>HDL 1μg/ml</td>
<td>HDL (40μg/ml) / Apo AI (40μg/ml)</td>
</tr>
<tr>
<td>HDL 2μg/ml</td>
<td>HDL (40μg/ml) / Apo AI (40μg/ml)</td>
</tr>
<tr>
<td>Apo AI 0.5μg/ml</td>
<td>HDL (40μg/ml) / Apo AI (40μg/ml)</td>
</tr>
<tr>
<td>Apo AI 1μg/ml</td>
<td>HDL (40μg/ml) / Apo AI (40μg/ml)</td>
</tr>
<tr>
<td>Apo AI 2μg/ml</td>
<td>HDL (40μg/ml) / Apo AI (40μg/ml)</td>
</tr>
</tbody>
</table>

4.2.6 Apo Al Analysis in main study

Volunteer plasma samples were sent to the Division of Cardiovascular and Medical Sciences, University of Glasgow for Apo Al analysis, which was undertaken using a turbidimetric assay.
4.3 Results

Almonds intake of 25g/d over four weeks followed with 50g/d almonds for four weeks had no effects on plasma cholesterol levels.

Cholesterol levels of participants in all groups were measured to determine if almond consumption had any affect on total plasma cholesterol levels. Results of the cholesterol analysis show no significant changes in cholesterol levels in the Healthy Group, Mature Group, At Risk Group or the Control Group (Fig. 4.0). The total plasma cholesterol levels were not significantly higher in the at risk, healthy and mature groups compared to control groups at baseline.

Almonds intake of 25g/d over four weeks followed with 50g/d almonds for four weeks reduced LDL-cholesterol levels in the Healthy Group but had no significant effects on LDL-cholesterol in other groups.

LDL-cholesterol levels were measured in the all participants of the study. LDL-cholesterol showed a significant reduction in the healthy group ($P < 0.001$) after almond consumption of 25g/d for four weeks followed by 50g/d for four weeks (Fig. 4.1). Analysis of the remaining groups shows no significant changes in their LDL-cholesterol due to almond consumption during the study.

Almonds intake of 25g/d over four weeks followed with 50g/d almonds for four weeks had no effects on plasma HDL-cholesterol levels.

HDL-cholesterol levels were analysed in all participants and showed no significant changes with almond consumption of 25g/d for four weeks followed by 50g/d for four weeks in all groups. There was also no change in HDL-cholesterol levels in the control group whom were not consuming any almonds (Fig. 4.2).
Almonds intake of 25g/d over four weeks followed with 50g/d almonds for four weeks had no effects on plasma triglyceride levels.

Plasma triglyceride levels were analysed in volunteers of all groups at each time point. Data analysis showed no significant changes in triglyceride levels of the healthy group and mature group, or the At Risk group who were consuming almonds 25g/d for four weeks followed by 50g/d for four weeks. The Control group showed no changes in plasma triglyceride levels (Fig. 4.3).

Almond intake of 25g/d over four weeks followed by 50g/d for a further four weeks had no effect on LDL/HDL gave time point. LDL/HDL was significantly different between supplemented and non-supplemented control group. Ratio of TC/LDL and TC/HDL showed no significant changes within groups of supplemented and un-supplemented groups. TC/LDL comparison of all almond supplemented groups with non-supplemented control group showed significant differences between them.

The ratio of LDL/HDL, TC/LDL, and TC/HDL were calculated in participants of all groups. Data showed significant difference in LDL/HDL ratios between the almond supplemented group and the non-supplemented group (Fig. 4.4.1) and also in TC/LDL ratios (Fig. 4.5.1).

Almonds intake of 25g/d over four weeks followed with 50g/d almonds for four weeks did not change levels of apolipoprotein A1 in the healthy group, mature group, increased risk group, and control group.

The investigation of plasma Apo A1 level demonstrated no alteration due to almond supplementation in any of the groups. No trends were observed either observed in the groups for the Apo A1 (Fig. 4.7).
Figure 4.0. Effect of Almond consumption on Cholesterol intake of All Group Volunteers.

Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=14) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=18); (C) At risk group, male volunteers between the ages of 18-35yrs (n=14); (D) Control group not supplemented with almonds (n=13). Data represent cholesterol levels before 0 weeks and at week 4 and 8.
Figure 4.1  Effect of Almond consumption on LDL-cholesterol levels of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A) Healthy male volunteers (n=13) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=16); (C) At risk group, male volunteers between the ages of 18-35yrs (n=14); (D) Control group not supplemented with almonds (n=12). Data represent LDL-cholesterol levels at 0 weeks and at week 4 and 8.
Figure 4.2  Effect of Almond consumption on HDL-cholesterol levels of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A)Healthy male volunteers (n=14) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=18); (C) At risk group, male volunteers between the ages of 18-35yrs (n=14); (D) Control group not supplemented with almonds (n=13). Data represent HDL-cholesterol levels at 0 weeks and at week 4 and 8.
Figure 4.3  Effect of Almond consumption on Levels of Triglycerides of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50d/d for a further four weeks in (A)Healthy male volunteers (n=14) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=18); (C) At risk group, male volunteers between the ages of 18-35yrs (n=14); (D) Control group not supplemented with almonds (n=13). Data represent levels of Triglycerides at 0 weeks and during study duration at 4 and 8 weeks.
Figure 4.4  Effect of Almond consumption on LDL/HDL ratio of All Group Volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=13) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=17); (C) At risk group, male volunteers between the ages of 18-35yrs (n=14); (D) Control group not supplemented with almonds (n=11). Data represents ratio of LDL/HDL at weeks 0, 4 and 8.
Figure 4.4.1  Effect of Almond consumption on LDL/HDL in comparison to non-supplemented group.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks (n=44) comparison to the control non-supplemented group (n=11) at time points of 0, 4 and 8 weeks. Difference in LDL/HDL at weeks 4 of almond supplemented and non-supplemented group (*) P<0.04.
Figure 4.5  Effect of Almond consumption on TC/LDL ratio of all group volunteers.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=13) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=17); (C) At risk group, male volunteers between the ages of 18-35yrs (n=14); (D) Control group not supplemented with almonds (n=11). Data represents the ratio of TC/LDL at weeks 0, 4 and 8.
Figure 4.5.1  Effect of Almond consumption on TC/LDL in comparison to non-supplemented group.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks (n=44) comparison to the control non-supplemented group (n=11) at time points of 0, 4 and 8 weeks. Difference in TC: LDL at 0 weeks of almond supplemented and non-supplanted group (*) P<0.01 and at 4 weeks (**) P<0.008.
Figure 4.6  Effect of Almond consumption on TC/HDL ratio of all group volunteers. Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks in (A) Healthy male volunteers (n=14) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n=17); (C) At risk group, male volunteers between the ages of 18-35yrs (n=16); (D) Control group not supplemented with almonds (n=12). Data represents ratio of TC/HDL at weeks 0, 4 and 8.
Figure 4.6.1 Effect of Almond consumption on TC/HDL in comparison to non-supplemented group.
Almond supplementation of 25g/d for four weeks followed by 50g/d for a further four weeks (n=50) data compared to control non-supplemented group (n=14) at time points of 0, 4 and 8 weeks; (non-significant (NS)).
Figure 4.7  Almond supplementation effect on Plasma Apolipoprotein A1.
Volunteers were supplemented with 25g/d almonds for 4 weeks (week 0-4) followed by 50g/d for another four weeks (week 4-8). (A) Healthy group male volunteers between 18-35yrs (n = 14); (B) Mature group volunteers male and female >50yrs supplemented with almonds (n = 17); (C) At Risk male volunteers between 18-35yrs supplemented with almonds (n = 14); (D) Control group volunteers not supplemented with almonds (n = 12). Data represent plasma Apo A1 levels at week 0, 4, and 8.
4.4 Discussion

In this almond supplementation study of 25g/d for four weeks followed by 50g/d for a further four weeks no significant differences on lipid profiles were observed on the young healthy male group (18-35yrs); mature male and female group (<50yrs); young males with increased risk (18-35yrs); and non supplemented control group (18-35yrs) apart from an overall change in the mean LDL-cholesterol level in the young healthy male group. No changes in levels of Apo A1 were observed.

One possible contributing factor to the lack of change is the variation between subjects seen for each analyte. This variation appears bigger than the variation that would be expected from a normal population. Internal standard plasma was included into each assay batch to determine inter-assay sample variation and to identify whether poor assay reproducibility may be contributing to the range of data observed to rule out the possibility of technical error and validate the data.

Epidemiological studies of nut consumption and CHD mortality have suggested that nuts are cardioprotective, beneficial effects (Hu et al., 1998; Kris-Etherton et al., 2001). The fatty acid composition (high levels of MUFA and PUFA) of nuts is a contributing factor in lowering LDL-cholesterol and hence CHD risk (Grundy, 1987). Frequent nut consumption of 1-4 times a week has been reported to reduce risk of CHD mortality by ~25% (Dreher et al., 1996). An almond intervention study carried out by Spiller et al showed a reduction in total cholesterol and LDL-cholesterol while levels of HDL-cholesterol were maintained (Spiller et al., 1998). The study carried by Spiller et al. used 100g/d almonds over a 4 week period and was carried out in conjunction with an olive oil based diet and a dairy based diet in hyperlipidaemic patients. Where levels of
l lipids are already elevated as in the above study a more potent effect of almonds is observed which has not been the case in this study looking at normal lipidemic individuals. However an almond study of increasing almond intake of 0%, 10%, and 20% of total energy carried out by Sabate et al. in normal healthy individuals did show a decrease in total cholesterol, LDL-cholesterol with an increase in HDL-cholesterol with the consumption of a 20% (68g/d) almond intake over 4 weeks (Sabate et al., 2003). Similar studies with individuals already at risk have shown beneficial effects of almond supplementation.

Almond studies that provided <50g/d were not able to demonstrate beneficial changes in LDL-cholesterol levels (Jenkins et al., 2002; Sabate et al., 2003). There were trends observed in total cholesterol of the increased risk group showing a slight decline with 25g/d and at 50g/d, this trend is repeated in levels of LDL-cholesterol for this group and the healthy group. However, these earlier studies did show that HDL-cholesterol levels were increasing in a healthy group. The results described in this chapter are based on using 25g/d almonds for four weeks followed by 50g/d almonds for a further four weeks may not have reached a threshold level for normal healthy individuals to see an affect on their total cholesterol, LDL-cholesterol, and HDL-cholesterol levels.

Almond studies have not been shown to have significant impact on triglyceride levels in comparison to the control diets of these studies (Jenkins et al., 2002; Sabate et al., 2003; Spiller et al., 1998). This study also showed no changes in the triglyceride levels in conjunction with almond consumption.

The incidence of CVD has been positively linked to elevated total cholesterol, LDL-cholesterol as well as Apo B. There is also a negative correlation between CVD and
levels of HDL-cholesterol and Apo A1 levels. Florvall et al. carried out a study to establish whether Apos B and A1 would be useful markers for cardiovascular mortality and morbidity; results showed Apo A1 to be the better risk marker against LDL-cholesterol, HDL-cholesterol and also ratios of ApoB/ApoA1 (Florvall et al., 2006). Nut studies have illustrated rises in levels of apo A1 with reductions observed in apo B with pecan intake (Rajaram et al., 2001). Measurement of Apo A1 in this study showed no significant changes due to almond consumption. Studies carried out by Aldred et al. with alpha tocopherol (268 mg/d over 28 days) showed elevated levels of Apo A1 demonstrating rises in alpha tocopherol can lead to increased Apo A1 levels. Almonds are a rich source of vitamin E and this study provided the recommended nutrient intake of 15mg/d when participants were consuming 50g/d almonds. These levels of vitamin E (15mg/d) were not sufficient to increase levels of Apo A1 and larger quantities would be required to see a similar effect.

The use of lipoprotein ratios as predictive tools for cardiovascular events has been widely under investigation. The ratio of total cholesterol (TC) to HDL (TC/HDL) was evaluated for predictive power in the Framingham Heart Study where it had a greater power than TC alone or LDL-cholesterol in identifying future CHD events in individuals (Kinosian et al., 1994). Other ratios investigated are the LDL/HDL, a multivariate analysis showed no effect on CVD outcomes (Hadaegh et al., 2006).

The findings of this study show no real change in the ratio analysis of lipids. There are significant differences between almond supplemented individuals and those not supplemented in their ratio. Notable the baseline levels are different when comparing groups that consumed almonds to those that did not (control group). In the case of TC/LDL ratio the baseline mean value is lower and this declines further at week four.
and eight, with the opposing effect on LDL/HDL ratio. The TC/HDL ratio shows little difference between the almond supplemented groups and those not supplemented. These results would suggest that participation of volunteers did affect dietary and possible lifestyle habits and small changes are observed in the control group. The almond supplemented groups had an intake of additional fats from the almonds into their diet that would increase their calorific intake compared to non-supplemented groups. The results here do not show this increase but neither a decrease.

A greater number of participants of the study would have enabled a larger population evaluation and possibly given more insight into greater effects of almond intake on lipid levels, as the study already showed some trends emerging in declining cholesterol and LDL-cholesterol levels in some of the groups investigated. In summary this study demonstrated that almond intake of 25g/d for four weeks followed by 50g/d for four weeks does not significantly alter levels of cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, and Apo A1 levels.
Chapter 5.0 Vascular Health

5.1 Introduction

Vascular function is an important component of many disease states. The endothelium in particular is a key regulator of cardiovascular system homeostasis. The key elements involved in homeostatic regulation by the endothelium include the maintenance of low levels of oxidative stress along with a relaxed vascular tone. The endothelium maintains this role with mediators that include nitric oxide, prostacyclin (PGI₂), angiotensin II, and endothelin 1 (ET-1) (Vane et al., 1990). The endothelium is also involved in regulating the permeability of the vasculature to plasma components that includes adhesion of platelets and leukocytes. Endothelial function can become impaired by a number of factors leading to “endothelial dysfunction” which is characterised by a malfunction of the homeostatic regulation. Endothelial dysfunction is associated with a number of features prevalent in patients with coronary atherosclerosis, and more recently in the progression of atherosclerosis itself and cardiovascular events (Suwaidi et al., 2000). Onset of endothelial dysfunction can be demonstrated by an increase in adhesion molecules, increased oxidative stress, and increased proinflammatory and prothrombotic factors, along with abnormal vascular tone. These factors contribute to the malfunction of the vasculature that includes endothelium dependent vasodilation (Drexler, 1997) and is an underlying factor in the development of atherosclerosis. Reports of patients with cardiovascular risk factors such as hypertension (Perticone et al., 2001) have demonstrated endothelial dysfunction as an independent predictor of cardiovascular events. Endothelial dysfunction is caused due to multiple factors.
There a body of evidence that reactive oxygen species are the major contributing factor.

To investigate the effect of almond intake on vascular health the following measurements were undertaken; measurement of blood pressure, venous occlusion plethysmography (VOP), plasma nitrite, soluble vascular cell adhesion molecule -1 (sVCAM-1), and plasma protein carbonyl levels.

5.1.1 Nitric Oxide

Measurement of NO• is via its measurement of its oxidative metabolism products nitrite (NO₂⁻) and nitrate (NO₃⁻). NO• has only a short life of less than 2 milliseconds (Liu et al., 1998) and is rapidly scavenged. It interacts rapidly with available substrate and heam prosthetic group of soluble guanylate cyclase producing an increase in cyclic guanosine 3'5'-monophosphate (cGMP). NO• can also react with other blood components to produce N-nitrosamines (RNNOs), iron nitrosyls, S-nitrosothiols (RSNOs), nitrated lipids, and nitrated proteins (Nagababu and Rifkind, 2007). cGMP is involved in various signalling pathways and is involved in regulating vascular tone and platelet function of the cardiovascular system. Downstream effects of NO• signalling through cGMP include cGMP-dependent protein kinase, cAMP-dependent kinase, cyclic nucleotide-gated channels and regulation of phosphodiesterase (Lucas et al., 2000) that are involved in the modulate blood pressure regulation by smooth muscle relaxation (Warner et al., 1994); platelet aggregation (Buechler et al., 1994); and other effects. NO• can also directly affect transition metal containing enzymes and transcriptional factors such as cyclooxygenase 2 (COX-2) (Goodwin et al., 1998). Other direct interactions
of NO• are the formation of methaemoglobin (methHb) and NO₂⁻ from its reaction with oxyhemoglobin. Oxyhemoglobin acts as a buffer and carrier for NO• with the NO₂⁻ being a reliable source of measurement of NO• catabolism (Gow et al., 1999).

The levels of NO• vary in different biological situations as it is produced in response to different stimuli. NO• undergoes several reactions with biologically available molecules these include the following:

\[
\begin{align*}
NO + O_2^- & \rightarrow ONO_2^- + H^+ \rightarrow NO_3^- + H^+ \\
2NO + O_2^- & \rightarrow N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- \\
NO + NO_2 & \rightarrow N_2O_3 + H_2O \rightarrow 2NO_2^- 
\end{align*}
\]

In human plasma nitrite has a short half life of minutes, where as nitrate has a longer half-life of approximately eight hours (Kleinbongard et al., 2006). Levels of plasma nitrite measurement have been reported to be incalculable to levels of up to 20µmol/L (Pelletier et al., 2006; Tsikas, 2005). NO₃⁻ is the major metabolic product of NO• and in red blood cells is biologically inactive as opposed to NO₂⁻. Studies in vivo have shown that levels of NO₂⁻ rather than NO₃⁻ are more indicative of eNOS release of NO• (Kleinbongard et al., 2003; Lauer et al., 2001; Misko et al., 1993). NO₂⁻ can act to release NO• with subsequent reaction with deoxyhaemoglobin (Giustarini et al., 2008) therefore also acting as a store for NO•.
5.1.2 Oxidised Proteins

Oxidative injury to proteins, lipids and DNA can have serious damaging effects. Damage to proteins can have immediate impact on a cell as many function as enzymes. Protein oxidation have been found in many disease states including diabetes and neurodegenerative diseases as well as in the aging process (Chevion et al., 2000). A measure of oxidative damage inflicted by free radicals is an essential way to assess levels of oxidative stress. The damaging effects are caused by many different agents including reactive oxygen species (superoxide (\(\text{O}_2^\cdot\)), hydroxyl (\(\text{OH}^\cdot\)), peroxyl (\(\text{RO}_2^\cdot\)), alkoxy (\(\text{RO}^\cdot\)), hydroperoxy (\(\text{HO}_2^\cdot\)), (Berlett and Stadtman, 1997), HOCI (Hazan and Heinecke, 1997; Kettle, 1996), and ionising radiation (Zbikowska et al., 2006). Methods available for protein oxidation rely on the detection of changes in the proteins after free radical attack; these include the production of protein carbonyls, loss of thiol groups, or detection of protein antigenic changes due to oxidation (Jackson, 1999).

Plasma LDL can be oxidatively modified leading to formation of carbonyl groups (Yan et al., 1997). Oxidative modification of LDL is one of the pathological conditions of atherosclerosis and hence a damaged vascular system. Oxidation of certain amino acyl moieties occurs within Lys, Pro, Thr and Arg (Amici et al., 1989; Stadtman, 1990) leading to formation of aldehydes and ketones on side chains that can be detected by a carbonyl assay. Oxidation of these amino acids produces stable moieties that make it possible to detect and store (Shacter, 2000). Assays for detecting carbonyls frequently involve the derivatisation of carbonyl groups carried out by dinitrophenylhydrazine (DNPH) that leads to the formation 2,4-dinitrophenylhydrazone (DNP) (Levine et al., 1990). The 2,4-dinitrophenyl (DNP)
hydrazone product can then be detected and measured by various means. The enzyme-linked immunosorbent assay (ELISA) is one method that utilises antibodies to DNP to quantify the levels of protein carbonyl (Buss et al., 1997). The method was developed by Buss et al. (Buss et al., 1997) and Carty et al. (2001) producing a sensitive method that is reproducible and requiring only small quantities of protein (µg). The ELISA method has been used to evaluate oxidative stress for patients undergoing coronary surgery (Pantke et al., 1999) and it was found that the method was suitable for the evaluation of serum oxidative stress of the patients. The technique is widely used to measure protein oxidation.

5.1.3 Soluble vascular cellular adhesion molecule-1

Inflammatory processes have long been associated with progression of atherogenesis and atherosclerosis. During the process of atherosclerosis adhesion molecules play a key role in the progression of the disease. At sites of atherosclerotic lesion elevated levels of cellular adhesion molecules ICAM and VCAM have been found from increased levels of corresponding mRNA. VCAM-1 has been implicated in the early development of atherosclerosis (Cybulsky et al., 2001; O'Brien et al., 1993). The role of soluble adhesion molecules in pathological stages of coronary artery disease is not entirely known. At sites of endothelial damage, endothelial cells release soluble forms of the cellular adhesion molecules. Soluble forms of the cellular adhesion molecules can be identified and measured in plasma and serum, and have been demonstrated to increase in certain diseased states (Gearing and Newman, 1993). Levels of soluble adhesion molecules have been proposed as a measure of risk in cardiovascular health (Mulvihill et al., 2002). Further studies in diet have illustrated that diets that induced
hyperhomocysteinaemia corresponded to rises in sVCAM-1 expression at arterial walls (Hofmann et al., 2001). Studies in mice deficient in ICAM-1 or VCAM-1 demonstrate a lesser degree of progression to atherosclerosis when subjected to atherosclerotic stimuli (e.g. deletion of apolipoprotein E). In mice the deficiency of VCAM-1 demonstrated a greater role in the development of atherosclerosis when compared to ICAM-1 (Cybulsky et al., 2001). Recent studies into acute cerebral ischemia have shown elevated levels of sVCAM-1 and endothelin-1 indicating an inflammatory state or possible endothelial dysfunction in this disease state (Brondani et al., 2007). As endothelial dysfunction is one of the stages of the inflammatory pathway towards CVD it is under much investigation. A study into flavanol rich coco diet of hypercholesterolaemic postmenopausal women has shown improvement of vascular health as assessed with sVCAM-1 that declined over the study period (Wang-Polagruto et al., 2006). In patients with acute coronary syndromes it was also found to be a strong predictor of further cardiovascular events (Postadzhiyan et al., 2008).

Increasing interest in levels of adhesion molecules has supported the development of ELISA methods for their measurement and these are commercially available. These ELISA kits are capable of measuring nanogram concentrations of the adhesion molecules. The measurement can be limited as circulating levels of adhesion molecules can bind to receptors in circulation (Sfikakis and Tsokos, 1997). Also levels measured would not reflect whether if there is amplified synthesis or declining clearance of the adhesion molecules (Gearing and Newman, 1993) and if the forms that are detected are active (Meager et al., 1996).
Almond supplementation effect on these biomarkers would give an understanding into the mechanistic changes occurring. These markers have previously been shown to elucidate risk of CVD and if they can be reduced by almond intake could lead to benefit.
5.2 Methods

5.2.1 Blood Pressure

For blood pressure measurements, volunteers were asked to sit down and rest their arm on a table, so that the brachial artery was level with the heart. A sphygmomanometer cuff was then wrapped around the subjects’ upper arm just above the brachial artery.

Pressure of the cuff is increased to 180mmHg until the blood flow of the brachial artery compressed and exceeds systolic pressure causing the artery to collapse. The cuff is then slowly deflated to allow the artery to open causing turbulence as blood flow starts to pass through the artery causing Korotkoff sounds that normally occurs at 120mmHg giving the systolic blood pressure reading. As the cuff is continues to deflate the pressure drops below the diastolic pressure where blood flow returns to normal at approximately 80mmHg.
Blood pressure measurement was taken in triplicate to ensure standardisation of results.

5.2.2 *Venous Occlusion Plethysmography*

Blood flow in the human forearm was monitored non-invasively by venous occlusion plethysmography. Subjects in the study were asked to participate in blood flow measurement using venous plethysmography. The forearm was inserted into a cuff and then the venous flow was stopped at about 40mmHg using a sphygmomanometer for 10sec whilst still allowing arterial flow. The cuff measures the rise in arm volume corresponding to the stop of venous flow. When the pressure on the venous flow was released the rate at which the blood flow returned to normal was calculated as the rate of blood flow. The rate at which the fluid volume leaves the forearm is indicative of the efficiency of venous return; a slow return shows a low venous flow, and is indicative of underlying vascular problems.

To evaluate the reproducibility of the assay, one volunteer was asked to present for venous occlusion plethysmography on three successive days. The results are reported in Table 5.0 below;

<table>
<thead>
<tr>
<th>Venous plethysmography</th>
<th>Within batch CV</th>
<th>Between batch CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((n = 6))</td>
<td>((n = 3))</td>
</tr>
<tr>
<td>Repeated analysis on one volunteer</td>
<td>9.56</td>
<td>7.91</td>
</tr>
</tbody>
</table>

**Table 5A** Reproducibility data for VOP of a single individual on a given day and on three separate occasions.
5.2.3 Soluble vascular cell adhesion molecule-1 ELISA

Vascular cell adhesion molecule 1 (VCAM-1) is an endothelial molecule which is critical for the recruitment of leukocytes to inflammatory sites. Levels of VCAM-1 expression on endothelial cells are increased by inflammation and there is increased shedding of this receptor as a soluble molecule into the plasma. Clinical studies have shown that elevated serum concentrations of cell adhesion molecules such as inter-cellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin (ESEL) and P-selectin (PSEL) may be independent risk factors for atherosclerosis and cardiovascular disease (CVD). Woollard et al (2006) have shown that intake of vitamin E reduces sVCAM-1 in healthy volunteers.

In this study, plasma sVCAM-1 levels were assessed using an ELISA kit from IDS Ltd (DC 850-580-096). Plasma was pre-diluted 1:50 using standard buffer diluent. Plasma (100μl) was applied to wells in duplicate. Standard (50ng/mL) was added to wells in duplicate. The following 5 pairs of wells directly below the standard (50ng/mL) was used to make a standard curve with a 100μl/well standard buffer diluent in each of the wells. Then 100μl was removed from the top standard (50ng/mL) and added to the well directly beneath. This was mixed using aspiration and then 100μl removed and added to the well beneath until all five wells containing doubling dilutions of standards. From the last well, 100μl was discarded. For the blank, standard buffer diluent (100μl) was plated in duplicate. A positive control (100μl/well) was included in the wells indicated below. The detection antibody (anti- sVCAM-1) was pre-diluted with biotinylated antibody diluent as indicated in the kit protocol.
Anti-VCAM-1 was added to all wells (50µl/well) and the plate incubated at 37°C for one hour. The plate was washed three times with the wash buffer provided. Streptavidin-HRP (100µl/well) was applied to all wells. The plate was incubated at room temperature for 30mins. The plate was washed three times and TMB (100µl/well) was added. The plate was incubated in the absence of light for 12-15min for colour development. The reaction was stopped with sulphuric acid (100µl/well). The absorbance was read immediately at 450nm using a Dynex MRX microplate reader. Levels of sVCAM-1 were calculated from the standard curve.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Inter-batch C.V ((n=3))</th>
<th>Intra-batch C.V ((n=3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>10</td>
<td>1.66</td>
</tr>
</tbody>
</table>

**Table 5B** Intra-batch and inter-batch coefficient of variance of quality control standard of sVCAM-1 measurement.
5.2.4 Nitrite

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular. A means of investigating nitric oxide formation is to measure nitrite (NO$_2^-$), which is one of two primary, stable and non-volatile breakdown products of NO. This assay relies on a diazotization reaction that was originally described by Griess in 1879. Through the years, many modifications to the original reaction have been described.

The Griess Reagent System is based on the chemical reaction shown in Figure 1, which uses sulfanilamide and N-1-napthylethlenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO$_2^-$ in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium. The nitrite sensitivity is dependent on the matrix (Figure 2). The limit of detection is 2.5µM (125pmol) nitrite (in ultrapure, deionized distilled water).
Figure 5.2 Chemical reactions involved in the measurement of NO$_2$. using the Griess Reagent System.

The Griess assay was used to examine the nitrite content of the plasma samples. Sodium nitrite (1mM) was used to prepared standard curve 500µM; 25µM; 5µM; and 2.5µM. An aliquot of 50µl of each standard was pipetted into a 96 well float bottom (costar) this was done in triplicate. Plasma samples was diluted 1:5 (distilled water) prior to addition of Griess reagent 50µl. The plate was allowed to incubate for 15mins in the absence of light. Absorbance was measured at 570nm using a Dynex MRX microplate reader and unknowns calculated using the standard curve.

To assess nitrite recovery from plasma samples, plasma was spiked with 10µM nitrite and the level of nitrite was reported pre- and post-spiking. The difference in nitrite concentration detected between these two measurements was then expressed as a percentage of the amount of nitrite added to calculate nitrite recovery. The recovery results are shown in Table 5.2.
In order to minimise the interference from plasma proteins, a number of depletion techniques were attempted and their results are shown in Table 5.3

![Graph showing absorbance (570nm) vs. concentration (mM) with a correlation coefficient of R² = 0.9993.]

**Figure 5.2.1** Standard curve of sodium nitrite using the Griess Assay.

<table>
<thead>
<tr>
<th>Percentage recovery after 10μM spiking (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma sample</td>
</tr>
</tbody>
</table>

**Table 5C** Percentage recovery for plasma sample spiked with 10μM sodium nitrite using the Griess assay.

<table>
<thead>
<tr>
<th>Percentage recovery after 10μM spiking (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma sample</td>
</tr>
</tbody>
</table>

**Table 5D** Percentage recovery data for plasma sample (acid precipitation used to deplete proteins) spiked with 10μM sodium nitrite after using the Griess assay.
5.2.4.1 Nitrite analysis using a flooumetric kit.

Plasma sample nitrates were measured by Mr Joe Clark using a flooumetric assay kit according to the manufactures instructions (Cayman Chemical Company Cat. No. 780051).

5.2.5 Protein Carbonyl ELISA

The protein content of the plasma samples was determined using the BCA method. Carbonyl standards were prepared to a concentration of 20μg/ml. Standards and samples (50μl/well) was plated onto a 96 well Nunc plate in triplicate and incubated for an hour at 37°C. The plate was washed three times with PBS/Tween 20 (0.05% in 1L PBS). Dinitrophenyl hydrazine was added to wells and the plate was incubated at room temperature for an hour. The plate was again washed and then blocked with blocking buffer 200μl/well (PBS/Tween 20 0.05%) and left over night at +4°C. The following day the plate was washed three times and the primary antibody 50μl of anti-DNP (mouse IgE, Sigma, 1:1000) was added to all wells, followed by three washes. The peroxidase labelled secondary antibody (rat anti-mouse IgE, Serotec (1:5000)) was added (50μl/well) to all wells and incubated for an hour at 37°C. The plate was washed and substrate (10ml citrate-phosphate buffer (0.15M, pH 5), 8μl hydrogen peroxide, and 1 o-phenyldiamine tablet) was added (50μl/well). The plate was incubated in the absence of light for 15-30mins to allow colour development. The colour reaction was stopped with the addition of 2M sulphuric acid (50μl/well). The absorbance was read at 490nm using a Dynex MRX microplate reader and unknowns calculated using the standard curve.
**Figure 5.2.2** Standard curve of carbonyl ELISA

![Graph showing absorbance vs. concentration](image)

**Table 5E**  Intra-batch and inter-batch coefficient of variance of quality control plasma of carbonyl measurement.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Inter-batch C.V ($n=3$)</th>
<th>Intra-batch C.V ($n=3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quality control Plasma X</td>
<td>1.7</td>
<td>1</td>
</tr>
</tbody>
</table>

The graph illustrates the relationship between absorbance at 490nm and concentration in ng/ml, with a coefficient of determination ($R^2$) of 0.9804.
5.3 Results

Blood pressure changes were monitored before the start and at the end of the study and demonstrated that intake of 25g/d almonds for four weeks followed by 50g/d almonds for a further four weeks elicited significant change in the healthy, mature, and at risk groups.

The diastolic pressures of the healthy group significantly decreased over the eight week intervention (paired t test $P < 0.0205$). However, there was no significant decrease in systolic pressure, although a trend towards a decrease is evident. The mature group blood pressures demonstrated a decreasing trend over the study period but only systolic blood pressure was significantly decreased (paired t test $P < 0.0015$). The At Risk group also demonstrated a significant reduction in systolic blood pressure (paired t test $P < 0.0054$) with a trend towards decreasing diastolic pressure. Control group subjects who were not supplemented with almonds but whom had their blood pressure also taken at the same intervals as the volunteers that were consuming almonds showed no significant changes or trends in declining blood pressure.

*Plasma sVCAM-1 levels measured at time points of 0, 4, and 8 weeks during almond supplementation of 25g/d almonds for four weeks followed by 50g/d almonds for a further four weeks did not significantly alter in any of the groups during the study.*

Levels of plasma sVCAM-1 in the healthy group of volunteers show a trend of decreasing from week 4 to week 8 as seen by the percentage differences compared to week 0. For the mature group and the at risk group, sVCAM-1 levels remained
stable with a greater variation in week 8 measurement seen in both groups towards increasing levels of sVCAM-1. The control group that did not undergo almond intervention showed a trend towards increasing levels of sVCAM-1 observed with a greater variation towards increased levels as opposed to those seen in the at risk group where variation of the results lie both increasing and decreasing levels.

Protein carbonyl levels did not alter during almond supplementation (25g/d almonds for four weeks followed by 50g/d almonds for a further four weeks) of the healthy, mature, and at risk group volunteers. No changes were observed in the non-supplemented group.

Levels of plasma protein carbonyls measured in the healthy group (male volunteers between the ages of 18-35yrs; n = 13) showed an increasing variation at week 8 (Fig.5.3.9). The results of statistical analysis of data show no significant changes in the levels and also no trends from the data can be ascertained. The mature (male and female participants <50yrs) and at risk group (male volunteers between the ages of 18-35yrs) did not show any change in protein carbonyl level over eight weeks of almond supplementation.

Plasma nitrite levels in almond supplemented groups (healthy, mature, and at risk, 25g/d almonds for four weeks followed by 50g/d almonds for a further four weeks) were not significantly changed. The control group that was not supplemented with almonds also showed no change in plasma nitrite levels.

The nitrite analysis was carried out on filtered plasmas from the healthy group, mature group, and increased at risk groups but showed no changes from almond intake. Plasma nitrite observed were within the range of those previously reported
Figure 5.3  Blood Pressure levels; effects of almond intake in Healthy Group volunteers.
Healthy male volunteers (n=14) between the ages of 18-35 yrs supplemented with 25g/d almonds from week 0 to 4, and then 50g/d almonds from week 4 to 8. Data represent systolic and diastolic before study at week 0 followed by data after four weeks of almond intake (25g/d) followed by further almond intake (50g/d for four weeks) at week 8 at end of study duration. Diastolic pressure changes significant (paired t test $P < 0.0205$).

Figure 5.3.1  Blood Pressure levels; effects of almond intake in Mature Group volunteers.
Mature volunteers (>50yrs male and female; n=17) supplemented with 25g/d almonds from week 0 to 4, and then 50g/d almonds from week 4 to 8. Data represent systolic and diastolic before study at week 0 followed by data at end of study (four weeks of almond intake (25g/d) followed by further almond intake (50g/d for four weeks)) at week 8. Systolic pressure changes significant (paired t test $P < 0.0015$).
Figure 5.3.2  Blood Pressure levels; effects of almond intake in At Risk Group volunteers.

At risk male volunteers (n=14) between the ages of 18-35yrs supplemented with 25g/d almonds from week 0 to 4, and then 50g/d almonds from week 4 to 8. Data represent systolic and diastolic before study at week 0 followed by data at end of study (four weeks of almond intake (25g/d) followed by further almond intake (50g/d for four weeks)) at week 8. Systolic pressure changed significantly (paired t test $P<0.0054$).

Figure 5.3.3  Blood Pressure levels; effects of almond intake in Cotrol Group volunteers.

Male volunteers (n=11) between the ages of 18-35yrs not supplemented with any almonds. Data represent changes of systolic and diastolic pressure from at week 0 (start of study) and at end of study week 8; corresponding to other group’s intake of almonds (25g/d almonds from week 0 to 4, and then 50g/d almonds from week 4 to 8).
Figure 5.3.4  Percentage change in Systolic Blood Pressure from week 0 of study to week 8 of All Groups.
Healthy Group (n=14) between the ages of 18-35yrs; Mature Group (n=17) over the age of 50yrs; At Risk Group (n=14) between the ages of 18-35yrs; and Control Group (n=11) between the ages of 18-35yrs. All groups except control group supplemented with 25g/d almonds from week 0 to 4, and then 50g/d almonds from week 4 to 8. Data represent percentage changes of systolic blood pressure compared to week 0 (start of study) to week 8 (end of study). Central line represents median value with top and bottom lines represent range of data within groups.

Figure 5.3.5  Percentage change in Diastolic Blood Pressure from week 0 of study to week 8 of All Groups.
Healthy Group (n=14) between the ages of 18-35yrs; Mature Group (n=17) over the age of 50yrs; At Risk Group (n=14) between the ages of 18-35yrs; and Control Group (n=11) between the ages of 18-35yrs. All groups except control group supplemented with 25g/d almonds from week 0 to 4, and then 50g/d almonds from week 4 to 8. Data represent percentage changes of diastolic blood pressure compared to week 0 (start of study) to week 8 (end of study). Central line represents median value with top and bottom lines represent range of data within groups.
Figure 5.4  Vascular effects of almond supplementation on rate of blood flow in brachial artery. Volunteers were supplemented with 25g/d almonds for 4 weeks followed by 50g/d for another four weeks. (A) Healthy group male volunteers between 18-35yrs (n = 13); (B) Mature group volunteers male and female >50yrs supplemented with almonds (n = 16); (C) At Risk male volunteers between 18-35yrs supplemented with almonds (n = 13); (D) Control group volunteers not supplemented with almonds (n = 11).
Figure 5.5  Vascular effects of almond supplementation on plasma nitrite levels.
Volunteers were supplemented with 25g/d almonds for 4 weeks followed by 50g/d for another four weeks. (A) Healthy group male volunteers between 18-35yrs (n = 11); (B) Mature group volunteers male and female >50yrs supplemented with almonds (n = 12); (C) At Risk male volunteers between 18-35yrs supplemented with almonds (n = 13); (D) Control group volunteers not supplemented with almonds (n =5).
Figure 5.6 Plasma sVCAM-1 levels; effects of almond intake.
Data represent plasma sVCAM-1 at week 0 followed by data from four weeks of almond intake (25g/d) at week 4, followed plasma sVCAM-1 changes at week 8 after almond intake (50g/d for four weeks) in (A) Healthy male volunteers (n = 14) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n = 17); (C) At risk male volunteers between the ages of 18-35yrs (n = 14); (D) Control group not supplemented with almonds (n = 11).
Figure 5.7  Plasma protein carbonyl levels; effects of almond intake.
Data represent plasma protein carbonyl at week 0 followed by data from four weeks of almond intake (25g/d) at week 4, followed plasma protein carbonyl changes at week 8 after almond intake (50g/d for four weeks) in (A) Healthy male volunteers (n = 13) between the ages of 18-35yrs; (B) Mature male and female volunteers <50yrs (n = 17); (C) At risk male volunteers between the ages of 18-35yrs (n = 14); (D) Control group not supplemented with almonds (n = 12).
5.4 Discussion

To investigate the effects of almond supplementation on vascular function and measures of oxidative stress in participants, the blood pressure, sVCAM-1, nitrite, FMD, and protein carbonyl levels were analysed before and after dietary modification with almonds. The principal finding of the analysis of these markers of vascular health showed a significant reduction in blood pressure of all almond-supplemented groups (healthy, mature, and at risk). The other measures however showed no significant changes.

Blood pressure measurements were taken with an automated blood pressure monitor. This method does not give absolute values, which can be achieved by the auscultatory method using a stethoscope and sphygmomanometer. However triplicate readings were taken to ensure accurate and consistent results.

The systemic blood pressure is controlled by a number of different mechanisms but the baroreceptor reflex carries out the primary role. The baroreceptor reflex consists of specialised neuronal cells of the aortic arch, carotid sinuses, and other regions of the cardiovascular system all relaying blood pressure changes back to the medulla in the brain. The sympathetic nervous system and the rennin-angiotensin-aldosterone system both feed into this reflex arc. The baroreceptors are stretch-sensitive nerve endings that fire action potentials to activate/deactivate the central nervous system (CNS) in accordance to changes in blood pressure. Increases in blood pressure results in increased stretch of the receptors leading to increased firing of action potentials. These afferent impulses are integrated by the CNS leading to reflex
activation of the sympathetic or parasympathetic pathways of the autonomic nervous system (ANS). In the case of increased systemic blood pressure the parasympathetic pathways are more elevated leading cardiac chronotropy. The sympathetic pathways are also affected in an inhibition of the pathways, causing a reduction of vascular tone and other factors. The decrease in blood pressure leads to the opposing effects (Monahan, 2007) and therefore acts in a reflex arc.

The sensitivity of the baroreceptor reflex is associated with CVD risk, and reduced or impaired sensitivity has been shown to increase mortality of patients following myocardial infarction (La Rovere et al., 1988). Atherosclerosis has been ascribed to reduced baroreceptor activity in animal (Angell-James, 1974) models and humans (Vlachakis et al., 1976). Further studies in the field have also demonstrated that baroreceptor reflex dysfunction can promote the development of atherosclerosis in rats and inflammation may play a possible role in this (Cai et al., 2005). Lipid abnormalities in obese hypertensives have been associated to impairment of the baroreceptor reflex leading to increased blood pressure and cardiovascular events (Gadegbeku et al., 2002). The cellular mechanism of baroreceptor response to sympathetic nervous system is believed to be dependent on angiotensin II. A recent intervention study with losartan showed reduced risk from cardiovascular events in older adults. To investigate whether this was due to antagonism of angiotensin II signalling, a further investigation was carried out in normotensive subjects (Yee and Struthers 1998).

Previous studies have described the modulation of baroreceptor reflex sensitivity in metabolic syndrome following cashew or walnut intake over eight weeks. Subjects
maintained on a normal diet did not show any change in baroreceptor reflex sensitivity. A walnut diet (20% of energy) showed a reduction in the baroreceptor reflex sensitivity, while the cashew nut diet showed elevated baroreceptor reflex sensitivity after eight weeks of supplementation. However, no significant effect on blood pressure was noted in these subjects. The principal difference in composition between cashews and walnuts was the unsaturated fatty acid content, with MUFA contributing 15% of total energy from the cashew intervention compared with 11% of energy in control and walnut diets. In contrast, in the walnut diet, PUFA contributed 21% of energy whereas in the control or cashew supplemented diets; PUFAs contributed 8.5% of energy. As previously described in Chapter 3 (food diary), the almonds provided by the Californian Almond board contained 68% of fats as monounsaturated fats. Intake of these almonds for 8 weeks significantly reduced the systolic blood pressure in the mature and at risk subjects but diastolic pressure remains unaffected. In healthy subjects, the diastolic but not systolic blood pressure is significantly reduced with almond supplementation. Reduction in diastolic blood pressure has been considered as a primary clinical endpoint for reducing risk of cardiovascular event, however, Izzo et al. have reported that systolic blood pressure reduction was a more important target for reduction in hypertension (Izzo et al., 2000).

The reasons underlying the effects of almond intake on diastolic BP in healthy and systolic BP in mature and at risk younger subjects is unclear; it may relate to an increase in the set point of the baroreceptor reflex during hypertension as mature subjects (132.3+5.2mmHg) and those at risk (128.7 + 2.8mmHg) had elevated systolic BP compared to healthy subjects (118.6 + 2.5mmHg) or controls
(120+3.1mmHg) at week 0. It is also reported that ageing of the vasculature reduces sensitivity of the baroreceptor reflux as seen during postural hypotension. There are no independent reports of the effects of nut intake on blood pressure as the principal outcome measure. Most reports have shown changes in lipid profiles in conjunction with nut consumption.

Local vasoconstrictors include nitric oxide produced by eNOS can reduce basal blood pressure by causing vasodilation. Organic nitrates have been used for treatment of CHD, and the first reported use of nitroglycerin in relieving angina was in 1879 (Murrell, 1879). The organic nitrates used are pro-drugs and subsequently undergo de-nitration to liberate NO leading and causing vasodilation. In conditions of hypertension and hypercholesterolaemia levels of NO are decreased. This has been suggested to be due to increased free radical production; the superoxide anion interacts with NO at a diffusion controlled rate and decreases bioavailability of NO. The production of NO by endothelial cells is controlled by the constitutively expressed eNOS and activated by calcium influx. It requires the presence of cofactors such as tetrahydrobiopterin and Zn for co-ordination of BH4 in addition to the substrate arginine. A study delivering eNOS gene into hypertensive rats demonstrated a reduction in systemic blood pressure (Lin et al., 1997). Also, arginine was also administered at 7.5 g/L for 11 weeks after eNOS gene delivery to give a greater reduction in blood pressure (Lin et al., 1997). Almonds are enriched in arginine and are a substrate for eNOS for the production of NO and its actions on blood pressure. Others have reported that intracellular arginine levels are not rate limiting, and to examine whether levels of NO were increased following almond intake, nitrite levels were measured.
Studies using oral supplementation of L-arginine (3g) in women with preclampsia have shown reduction in blood pressure, which was associated with an increased endothelial bioavailability or production of NO (Rytlewski et al., 2005). Biochemically, arginine levels within cells are more than sufficient for NO synthesis by NOS (Bredt and Snyder., 1990). However, co-localisation of arginine transporters (cationic amino acid transporter-1) may supply NOS directly with L-arginine (McDonald et al., 1997). Arginine is also able to stimulate the release of vasodilator hormones such as insulin that may play a role in blood pressure changes.

For the measurement of NO, the proteins were removed using a 10kDa filter to reduce interference for the measurement of nitrite levels. There was no change in nitrite levels in almond supplemented groups. The levels of nitrite and nitrate are not the only biological derivatives and NO can react with thiol groups of proteins in S-nitrosylation, to form S-nitrosothiols (RSNOs). Almonds may not have increased production of NO or it may have already been dissipated into other systems so would not be measurable in the nitrite form.

Protein modification by free radicals is known to lead to the oxidation of LDL-cholesterol and development of atherosclerosis. Flavonoids and vitamin E possess antioxidant capacities and have an inverse relationship with cardiovascular disease. A study on flavonoids from almond skin have been demonstrated to work with vitamin C and E in reducing LDL oxidation in hamsters (Chen et al., 2005). The levels of protein oxidation in this study were measured using a carbonyl ELISA of the plasma samples. The results showed no change in levels with almond supplementation of the three groups. Previous studies carried out by our lab to
investigate vitamin C effects also showed no change in carbonyls levels in healthy subjects, but a reduction was only seen in subjects with low vitamin C status at baseline (Carty et al., 2001).

Another method of assessing endothelial function is to measure FMD, which has the added advantage of being a non-invasive method. It has been shown that in CVD risk factors such as hypertension, diabetes, dyslipidaemia, and smoking is negatively associated with FMD (Celermajer et al., 1993). FMD may also be used as predictive tool for future CVD events (Gokce et al., 2003). From the findings of the study there were no significant changes in FMD in individual groups, however this may reflect a lack of power relative to the error of the measurement. The changes in FMD were shown to increase in healthy and mature group participants of the study but not to a significant extent. With an ultrasound Doppler flow method of the brachial artery would give more accurate data and any subtle changes in vasodilation maybe possible to be recorded. However the FMD measurements were taken multiple times to ensure consistent data. The measurements were also taken during the early morning to prevent diurnal variation in the data. Changes in FMD are influenced by sympathetic activity (Hijmering et al., 2002) and is difficult to estimate the importance of eNOS derived NO in blood pressure regulation (Gamboa et al., 2007).

Plasma levels of sVCAM-1 did not change significantly after almond intervention. Generally sVCAM-1 levels are increased in plasma during atherosclerosis, inflammatory disorders, myocardial infarction and ischemic stroke (Bevilacqua et al., 1993; Gearing and Newman, 1993). VCAM-1 is present on activated endothelial
cells, tissue macrophages, dendritic cells, bone marrow fibroblasts, myoblasts and myotubes (Rigott and Power, 1993). The study reported in this chapter study is looking at a healthy population and a non-symptomatic at risk population therefore the changes in sVCAM-1 levels may be to small to be significant as individuals are not in a disease state. It has been reported that hypertensive older male populations have increased cell adhesion molecules (DeSouza et al., 1997) and although a mature population was studies here, no differences in the sVCAM-1 levels were detected. Previous studies carried out by our lab which supplemented individuals with vitamin E (400IU/d) showed a decrease in the levels of sVCAM-1(Woollard et al., 2006). The levels of vitamin E supplied by the almonds (50g/d) only provided 15mg, which were not enough to increase plasma levels and therefore were unlikely to affect the plasma sVCAM-1 levels as observed.

In summary the results of the effects of almond supplementation on vascular health demonstrated improvements in blood pressure in the almond supplemented groups that may be related to the beneficial affects of MUFA. Future work in this area of research should include methods to investigate this affect that could include measurement of plasma levels of MUFA, and amino acid analysis for measurement of arginine levels.
Chapter 6.0 Proteomics

6.1 Introduction

The sequencing of the human genome in 2002 has revealed 30,000-40,000 genes. However, the proteomes arising from these genes are a complex mixture and number more than was initially thought of when the number human genes were revealed. The greater number of proteins are due to slicing and post-translational modifications that allow the generation of a more than a million structures. All modifications of the proteome from the gene encoding it can lead to varied functionality of that protein. The proteome is defined as the:

"the protein complement expressed by a genome" (Schweigert, 2007).

The proteome reflects the interactions of the cell/organism to external influences that affect the intracellular aspects of the proteome, which can differ from cell to cell. A feature of proteomics is the understanding of the phenotype in conjunction with particular protein function and modifications that are required for that phenotype. Post-translational modifications include phosphorylation, ubiquitination, and proteins can also undergo methylation, acetylation, glycosylation, oxidation and nitrosylation. Proteomics techniques can lead to greater understanding of structure, functionality, and protein-protein interactions in disease states and therefore can be used as a diagnostic tool. Clinical proteomics focuses on biomarkers for diagnosis and for target of drug
therapy. With particular emphasis on the development of serological biomarkers for
detection of cancer and cardiovascular disease, proteomics is leading the way forward.

Proteomics can examine the presence or absence of proteins, and can also detect
changes in levels and modifications to those proteins after synthesis. To assess the
proteome of biological samples (cells, tissues, plasma, and urine) requires specialised
technology and skill. Methods used most commonly in proteomics combine 2-D
polyacrylamide gel electrophoresis (2-DE) and Mass Spectrometry (MS). 2-DE consists
of iso-electric focussing in the first dimension that allows separation of proteins based
on charge and polyacrylamide gel electrophoresis (Person et al., 2003) and the second
dimension of the electrophoresis separates the proteins further according to size/mass.
The combination of 2-DE and MS allows proteins to be resolved as spots on a gel
however their resolution and reproducibility by 2-DE is an ongoing area of research.
Plasma protein levels can range by a factor of 100,000 with the key proteins (albumin,
immunoglobulins, transferring, fibrinogen, and hetoglobulin) making up ~90% of these.
These high abundance proteins overshadow the low abundance proteins that could be of
importance. Therefore plasma albumin is depleted plasma of before 2-DE is carried out
on samples (Aldred et al., 2004). In this study proteomics is employed to analyse the
impact of almond consumption on in vivo plasma proteome in a non hypothesis driven
manner and the elimination of high abundance protein albumin allowed investigation
into small changes.
6.2 Methods

6.2.1 Isolation of cells from whole blood for proteomics

A 10ml fresh blood samples was taken from the volunteers into EDTA glass tubes vacutainer tubes (Becton, Dickinson and Company, Warwickshire UK). Blood was diluted 1:1 in PBS/0.1%BSA. Lymphprep (15ml) was aliquotted into each 50ml tube and 20ml diluted blood was very gently and slowly layered on top of the lymphprep. The gradient was spun down at 160 x g for 15 minutes at 20°C. Plasma (15ml) was aspirated from the top of the tube and discarded to remove platelets. The remaining gradient was spun down at 350 x g for 20 minutes at 20°C. The red blood cells sediment to the bottom of the tube. A thin layer of cotton-like cells appeared at the interface between the lymphprep and plasma. The plasma was removed from above the cells until 0.5cm remained on top of the lymphprep. About 3-4mls of cells was harvested from the interface using a squeezing plastic pipette. This was washed once with PBS prior to storage as a pellet at -80°C until required for proteomics.

6.2.2 Sample Preparation for 2D Electrophoresis

An Aurum Serum Protein Mini Kit (Biorad #732-6701) was used to deplete albumin and IgG from the plasma samples prior to 2D electrophoresis. The procedure was followed as instructed by the kit. Plasma (10µl) from each subject in the “at risk” group was pooled together into four independent aliquots for each of the different time points (0, 4, 8, and 16 weeks). Pooled plasma (60µl) was placed in a clean vial with serum protein binding buffer (180µl).
A serum protein column was placed into a test tube provided with kit and the left to stand for 10 minutes allowing the resin to settle. Serum binding buffer (1ml) added to wash column. The column was placed in 2ml collection tube and centrifuged 20 seconds at 10,000 x g. The collection tube was discarded and a yellow cap added to the bottom of the column and was then placed into fresh collection tube. Plasma samples (200μl) which had been previously prepared were added to the column. The column was gently vortexed and allowed it to sit for 5 minutes before being vortexed again and the column was allowed to sit for a final 5 minutes. The yellow cap was removed from the column and the column was returned to its collection tube. This was then centrifuged at 10,000 x g for 20 seconds and the eluate was labelled “unbound”. A further 200μl of serum protein binding buffer was added to the column which was again centrifuged with the “unbound” collection tube for 10,000 x g for 20 seconds. This “unbound” sample contained the albumin- and IgG-depleted pooled sample used for 2D Electrophoresis. This separation was carried out for pooled plasmas from all time points 0, 4, 8, and 16 weeks.

6.2.3 2D Electrophoresis

Following protein determination, pooled plasma samples (50μg) depleted of albumin and immunoglobulins were analysed in triplicate by 2D electrophoresis. The samples were evaporated under vacuum in siliconized centrifuge tubes (Eppendorf concentrator 5301 Hamburg) for 1hour. Rehydration buffer (urea (8 M), thiourea (2 M), CHAPS 2%, \(N\)-decyl-\(N\), \(N\)-dimethyl-3-ammonio-1-propane sulphonate (SB3-10) (2%), Destreak (Amersham Pharmacia Biotech, 12μl/ml), Tris HCL (40mM) and biolytes 3/10 (0.5%))
was added to the reduced samples (180μl/sample). Samples were rehydrated into IPG strips (pH 3-10 non-linear, Biorad, overnight, 20°C). Proteins were then focussed using the electrophoresis tank (LKB 2117 multiphor II electrophoresis unit) and 13cm v 1.5cm filter paper wicks added to both ends of the strips. Mineral oil was added to cover the IPG strips. Proteins were focussed into the gels under the following conditions: 0V-500V for 500Vh, then 500V-3500V for 3500Vh after which the strips were held at 3500V for 96kVh (Amersham Pharmacia Biotech, EPS 350 XL, with a Grant LT D6G cooling system). The strips were stored at -80°C until required for the second dimension separation.

The IPG strips were removed from the -80°C freezer. Equilibration buffer (urea (8M), glycerol (20%), SDS (2%), tributyl phosphine (TBP; 2mM), Tris (0.375M) pH 8.8) was added. Strips were rotated to equilibrate in 7mls buffer (Dynal sample mixer) for 20mins. The IPG gels were removed from the equilibration buffer and positioned above a polyacrylamide gel (4-20% Criterion gel, Biorad). They were set in place with agarose gel (10%). The gel was electrophoresed for 80mins at 150V in a Bio-Rad criterion tank. Gels were removed and fixed (ethanol 40%, acetic acid 10%) over night and stained with silver/flamingo stain (Biorad) for eight hours. Gel analysis of spots was carried out using PD Quest (Bio-Rad).
6.2.4 Gel analysis

Gels containing proteins of interest were scanned by calibrated imaging densitometer (BioRad GS-710 Pharos FX) and assessed using PD-Quest (BioRad). A master gel was created for each time point of the study (0, 4, 8, and 16 weeks) to allow statistical analysis of the effects of almond supplementation at these time points. Protein spots were identified as (a) proteins that were consistently present in all time points of the almond supplementation (b) proteins with altered increased decreased expression or disappeared on almond supplementation, and (c) proteins that only present upon almond supplementation. Statistical analyses of these changed protein profiles with almond supplementation were carried within the software, allowing changes in fold expression to be identified. Protein spots showing a 2 fold change, where the set included proteins spots whose quantity in supplemented groups was at least 2 times that of the corresponding spot in non-supplemented groups, were selected for future study (Aldred et al., 2006).
6.3 Results

Analysis of the spots by PD-Quest revealed three protein spots that were shown to change their expression two fold with almond supplementation of the increased risk group plasma proteome.

Almond supplementation of the increased CVD risk group (males between the ages of 18-35yrs) showed changes in the plasma proteome profile after almond supplementation when gels were analysed by PD-Quest software. On average gels showed 260 number of spots and three spots were shown to change expression by a two fold difference. Three protein spots from the gel analysis showed consistent change during the supplementation period between weeks 4 and 8 compared with week 0 and 16 time points.
Figure 6.0  Plasma Proteome master gel.
Pooled plasma sample depleted of albumin and immunoglobulin of increased risk group at different time points of almond supplementation (0 weeks no almond supplementation, 4 weeks 25g/d almond supplementation, 8 weeks 50g/d almond supplementation, 16 weeks no almond supplementation.)
Figure 6.1  Plasma protein spot change in expression with almond supplementation. Spot ID 1301.
Pooled plasma sample depleted of albumin and immunoglobulin of at risk group at different time points of almond supplementation (A) 0 weeks no almond supplementation, (B) week four of 25g/d almond supplementation, (C) week eight of 50g/d almond supplementation, (D) weeks sixteen of washout four weeks after almond supplementation. Gels are representative of three independent experiments.
Figure 6.2  Plasma protein spot showing a two-fold change in expression with almond supplementation. Spot ID 4102.
Pooled plasma sample depleted of albumin and immunoglobulin of at risk group at different time points of almond supplementation (A) 0 weeks no almond supplementation, (B) week four of 25g/d almond supplementation, (C) week eight of 50g/d almond supplementation, (D) weeks sixteen of washout four weeks after almond supplementation. Gels are representative of three independent experiments.
Figure 6.3  Plasma protein spot showing a two-fold change in expression with almond supplementation. Spot ID 9701.

Pooled plasma sample depleted of albumin and immunoglobulin of at risk group at different time points of almond supplementation (A) 0 weeks no almond supplementation, (B) week four of 25g/d almond supplementation, (C) week eight of 50g/d almond supplementation, (D) weeks sixteen of washout four weeks after almond supplementation. Gels are representative of three independent experiments.
Figure 6.4  Plasma proteome profile for week 16 of At Risk group.
Pooled plasma sample depleted of albumin and immunoglobulin of at risk group (male between the ages of 18-35yrs; n = 11) supplemented with almonds (0 weeks no almonds, 0-4 weeks 25g/d almonds, 4-8 weeks 50g/d almonds, 8-16 weeks no almonds consumed). Image representative of three independent experiments stained with flamingo.
6.4 Discussion

Epidemiological studies have shown that plasma vitamin E levels have a negative correlation with CVD risk but have not, in the most part, been proven in large cohort studies such as the GISSI. Other effects of vitamin E (alpha tocopherol the most active component) have suggested a role in cellular signalling. Micronutrient antioxidant function is thought to partially occur through changes in gene expression as reported for vitamin C and E (Jackson et al. 2002). Investigations of alpha tocopherol as a cell signalling molecule have led to findings demonstrating its ability to inhibit protein kinase C (PKC) and 5-lipoxygenase in smooth muscle cells (Azzi et al., 2000). A previous alpha tocopherol supplementation study has utilised proteomics to investigate the effect of alpha tocopherol on plasma proteins (Aldred et al., 2006) and identified an increase in plasma isoforms of apo A1. The scavenger receptor CD36, collagenase and alpha tocopherol transfer protein gene regulation are likely to occur by alpha tocopherol (Teupser et al., 1999). Therefore, the proteome of an organism can be affected by dietary nutrients. For a more detailed review, see Griffiths and Grant (2006).

Preliminary findings from proteomic analysis of the plasmas from the increased CVD risk group have identified three protein spots that increase in expression over time with almond supplementation. It is interesting to speculate whether these are related to changes in vascular function observed after almond intake. Further analysis of these spots by MS will identify these proteins and may provide greater understanding of the effect of almond supplementation on plasma proteome in conjunction with other affects of almond intake discussed in this thesis.
Dietary habit has long been linked to risk of cardiovascular disease and with a world-wide ageing population; particular emphasis is focused on food stuffs that have favourable effects on health span. The food industry is seeking to label products with healthy effects for reducing risk of cardiovascular disease. Nut intakes have been linked to beneficial effects and reduced CHD events (Fraser et al., 1992) as they are a good source of MUFAs and PUFAs. The FDA has allowed the labelling of nut packages to state that “42g daily as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease” in response to evidence for the beneficial effects of consuming nuts.

Almonds have been investigated for beneficial effects and have been reported to reduce plasma levels of cholesterol and LDL-cholesterol. The cholesterol lowering effects of almonds were reported by Spiller et al. whom examined consumption of 100g daily of almonds on hypercholesterolaemic patients. The findings by Spiller et al. demonstrated a decrease in total cholesterol (9%) and of LDL-cholesterol of (12%) while no change in HDL-cholesterol was observed (Spiller et al., 1992). A study in healthy subjects was also shown to reduce total cholesterol (4%) and LDL-cholesterol (6%) when fat intake was replaced with almond oil (35g) and whole almonds (66g) (Hyson et al., 2002). Further research in the field yielded a dose response relationship on the lipid lowering effects of almond intake, 28g and 56g of almond intake was found to reduce LDL-cholesterol by 4.7% and 9.9% respectively (Jenkins et al., 2002). A 7g/day almond intake was calculated to reduce LDL-cholesterol by 1% from the dose response relationship of almonds and reduced lipids from the study carried out by Jenkins el al. However, this effect was not reproduced in the study reported in this thesis and considering differences
in study design, it appears that both duration and amount of almond consumption are important for lipid-lowering effects.

From the previous evidence of the lipid lowering capacity of almonds, this study aimed to investigate such effects in three populations against a control diet group; healthy young males (18-35 yrs), mature males and females (>50 yrs), and young males with increased risk of developing CVD. The study examined almond supplementation at two different intakes of 25g/d for four weeks followed by 50g/d for a further four weeks in order to establish if smaller quantities of almond consumption in these populations could confer some beneficial effects. There were no differential sensitivities to or selective benefits from almond intake in any of the groups studied. Further from the dietary analysis carried out there were changes in MUFA, SFA, vitamin E as expected with almond intake, but this was not reflected in the biochemical analysis of the lipids. This brings into question the compliance of subjects to almond consumption, even though this was assessed with bag return. A free-living study cannot guarantee the intake of the given food at the correct dosage but it is however more comparable to what would normally happen in the general population. This therefore does provide a real result in relation to the population.

Almonds are a rich source of αTOH providing 27.75mg αTOH /100g almonds as well as containing phenolic and polyphenolic compounds that can potentially have antioxidant effects in disease states. Some αTOH supplementation studies have previously reported some beneficial effects of supplementation in reduced risk of CVD; however other studies have reported no benefit. The Health Professionals Study reported an association between α TOH (≥100IU) intake with 60% incidence of coronary heart disease in men compared to other individuals of the study (Rimm et al., 1993). The Cambridge Heart
Antioxidant Study (CHAOS) investigated the stratified effects of alpha tocopherol (800IU, 400IU) supplementation in patients with coronary atherosclerosis and reported a reduction in non-fatal myocardial infarction with treatment over a year (Stephens et al., 1996). However, neither the MRC/BHF study nor a combined meta-analysis revealed any reduction in risk of cardiovascular events with αTOH consumption. While the original epidemiological evidence of an inverse relationship between plasma αTOH and CVD remains true, it is more likely that αTOH is a biomarker of a healthy diet and that the beneficial effects of the diet are either unrelated to αTOH or require that αTOH is consumed with a series of other micronutrients present in foods like nuts, if there is to be any beneficial effect. Whether alpha-tocopherol exerts an antioxidant effect in vivo or whether it regulates gene expression has been widely discussed by Azzi. To partly address these questions in the almond intervention study, plasma protein carbonyl levels were measured to evaluate any antioxidant effect and sVCAM-1 was measured as a possible marker of alterations in VCAM-1 expression. Neither was changed by almond intake, nor were plasma levels of α TOH in any of the subjects. As α TOH levels were already high in subjects at baseline, and then it is possible that adequate control of gene expression or antioxidant activity is already present in the subjects under study.

A 28g serving of almonds provides 164kcal provided mainly from its fat content which is 49.4% of the weight, and 67% of which is attributed to MUFA. There have also been reports of reduced susceptibility of LDL-cholesterol to oxidising damage with increased intake of MUFAs. In addition, increased MUFAs are reported to alter baroreceptor sensitivity and reduce blood pressure. This thesis has identified that almond intake is effective in reducing blood pressure in all subjects groups independent of CVD risk or age.
Almonds are also rich in the amino acid arginine, which is a precursor for nitric oxide production in a reaction catalysed by NOS. It has been suggested that the cellular level of arginine can determine the activity of NOS and if this hypothesis was true in the current study, then the intake of dietary almonds would be expected to increase nitric oxide production and increase FMD responses. While there was an increase of FMD between weeks 4 and 8 for all almond supplemented subjects, there was no selective effect based on age or CVD risk profile. No change in plasma nitrite levels were recorded, however, recent studies have suggested the more important biologically active forms of NO may be conjugated to protein thiol groups for release during stress and will not have been measured by the Griess assay used here.

The absorption and bioavailability of αTOH from foods has an important role in the overall effect of almond intake. A study investigating αTOH supplementation with the consumption of fat-free milk was shown to increase levels of plasma α-TOH levels (Leonard et al., 2004). The bioavailability of α TOH is also influenced by prandial status and that, for optimum uptake fat is also additionally required.

The main findings of this study demonstrated a reduction in blood pressure from almond supplementation. Further investigation into the washout following 50g/d almonds would provide evidence of a return to the initial baseline levels at of biochemical markers. The returning levels of biomarkers to baseline would show a real affect on Almond intake. The washout period also would allow observations of the time scale to which the biomarkers change and how lasting this effect is on almond withdrawal.
The assessment of the at risk group engaged volunteers with different risk categories that included a BMI >25, cholesterol levels >5.17mmol, and blood pressure >140/90 due to low numbers of volunteers. This does not allow interpretation of the data to be based on one specific risk category and further investigation into each specific risk individually would bring greater understanding of almond supplementary effect on them.
7.1 Future Work

The immediate finding of this study warrants greater investigation of the vascular effects of almond intake. The positive improvement in blood pressure and FMD indicate that almonds can affect vascular health in healthy populations and in the case of blood pressure it is demonstrated in both mature and younger populations. These effects are most likely to be due to the MUFA content of the almonds, and therefore determination of plasma levels of MUFA could provide more insight into this.

Investigating vascular health by means of amino acids analysis of plasma samples would give an insight into possible changes of arginine levels with almond intake and possible increases of NO in this manner. Receptors of L-glutamate and serotonin are implicated in sympathetic nerve activity that may be modulated by NO to elicit changes in blood pressure as well. NO can lead to nitration of proteins so protein nitrosothiols could also be investigated.

Proteomic analysis was incomplete in this study and further analysis of the spots that changed in intensity by mass spectrometry would identify these proteins and allow greater understanding protein regulation with almonds intake. Proteomic analysis of the remaining groups to confirm or deny the regulation of the same proteins as identified with the at risk group would establish what proteins almond intake could affect.


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Kwiterovich, P. O., Jr. (2000). The metabolic pathways of high-density lipoprotein, low-density lipoprotein, and triglycerides: a current review. Am J Cardiol 86, 5L-10L.


Libby, P. (2001b). What have we learned about the biology of atherosclerosis? The role of inflammation. Am J Cardiol 88, 3J-6J.


HDL cholesterol: the Veterans Affairs HDL Intervention Trial (VA-HIT). Diabetes Care 26, 1513-1517.


adhesion or C-reactive protein levels but reduces soluble vascular adhesion molecule-1 in the plasma of healthy subjects. Redox Rep 11, 214-222.


9.0 Appendix

9.1 Appendix 1

Consent form
ASTON UNIVERSITY

HUMAN SCIENCE ETHICAL COMMITTEE

CONSENT FORM FOR VOLUNTEERS

This pro-forma has been produced by the Human Science Ethical Committee for the guidance of investigators. You should insert the necessary information under each heading and the form as you intend to use it should be submitted to the Committee before the start of your project.

PROJECT TITLE

Diet enrichment with vitamin E containing food: effect on lipid profile and vascular function

RESEARCH WORKERS, SCHOOL AND SUBJECT AREA RESPONSIBLE

Dr Helen Griffiths
Khujesta Choudhury

EXPLANATION OF ANY POSSIBLE HAZARDS AND THE PROCEDURES TO BE USED

The purpose of this study is to look at the effect of diet on cardiovascular health. This research may help us to better understand the relationship between the food we eat and heart disease, helping doctors and scientists to offer better dietary advice.

Participants will be asked to eat a small packet of commercially available almonds (vitamin E containing food) each day, at their convenience, for up to 8 weeks. We would like to take a small blood sample from the volunteer’s arm before any change in diet, then after 4 and 8 weeks. This will be taken by a qualified member of staff using sterile techniques. We would also like to take a measurement of the blood flow in the volunteer’s arm with a special instrument. This is safe and painless and only takes a few seconds to do.

If you agree to take part, we will need you to fill in a short questionnaire about your diet and general health. This will be sent out to you before you have give any blood samples.
There are no particular risks involved in this project. Although this project may have no direct benefit to you, it may provide important information about benefits of vitamin E rich food in the diet that will help reduce cardiovascular disease in the future.

CONFIDENTIALITY OF INFORMATION

The confidentiality of personal information and the anonymity of all volunteers involved in this investigation will be preserved in the following way:

Aston University

Illustration removed for copyright restrictions

VOLUNTEER'S STATEMENT

I have read and understand the above explanation. I have had the opportunity to discuss it with the investigators and to ask any questions. I agree to take part in the above project and I have been informed that I am free to withdraw at any time.

Signed: .................................................................

Dated: .................................................................

JGW/HSEC
26 5 00
9.2 Appendix II  

Food Diary
Initial Food Diary
Of Vitamin E Study
Personal Number_____

Please could you take the time to fill in this three-day food diary so that we may ascertain the amount of Vitamin E that you already consume in your daily diet. Two days should be chosen from Monday to Friday and one day from the weekend.

The information you provide will be strictly confidential and no identification will be used in reporting the data. We would be happy to report the findings of our investigation to you if you wish to know.

Thank you.
FOOD DIARY

Remember to mention the way in which the food was prepared and especially for meat if it was roasted fried etc. Use portions sizes like litre, tablespoon, teaspoon, or just take a look at the packaging. If eating take-out please mention that it was take out and the case of pizza what size pizza was ordered (number of slices) and tell us how many slices were consumed. For alcohol use: pints and glasses of wine or shot to specify quantities. Also for hot drinks specify cups or mugs of tea or coffee.

Thank You.

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9.3  Appendix III  Pilot Study
Pilot Study: Investigation of Almond Supplementation (50/d for four weeks) on a small cohort of Free-living individuals

1.0 Introduction
Cardiovascular disease (CVD) is a growing concern in the UK where the annual cost of CVD was just below £1,750 million in 1999; 53% of which was the cost of drugs alone. An additional £5,300 million was spent on care associated with coronary heart disease (CHD). During 2002, CVD caused 39% of deaths in the UK (238,000 people; http://www.heartstats.org). The rate of morbidity and mortality, and the increasing costs of medical intervention have lead to greater research into CVD prevention.

1.1 Atherosclerosis
The underlying cause of CVD is thought to be atherosclerosis, which was proposed in the 1950's. Atherosclerosis defined by the World Health Organisation (WHO) is "a variable combination of changes of the intima of arteries consisting of focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, associated with medical changes". Atherosclerosis is a degenerative disease found widely in the animal kingdom. It causes occlusions in affected arteries or vessels that ultimately impedes blood flow.

1.2 Risk Factors for CVD
Several risk factors contribute to atherosclerosis some of which are controllable and others that are not

**Controllable risk factors**
- Cigarette smoking,
- Obesity,
- Hypertension,
- Elevated cholesterol,
- Elevated triglycerides.
- low plasma tocopherol
- low plasma ascorbate

**Uncontrollable risk factors**
- gender,
- age,
- family history,
- heredity.
1.3 Almonds as rich dietary sources of antioxidant nutrients

Maintaining levels of antioxidants through the diet is postulated to protect against CVD through the scavenging of ROS and free radicals. Almonds are one of the richest dietary sources of the antioxidant, vitamin E. In addition, they contain betulinic acid; oleanolic acid; ursolic acid that have been reported to have anti-inflammatory anti-cancer activities (Takeoka and Doa 2003). Polyphenols which are known to scavenge free radicals and to inhibit the oxidation of LDL are also found in almonds and include tannins, rhamnetin, quercetin and kaempherol aglycones (Pinelo et al, 2004) Almonds also contain high levels of arginine, a precursor for NO production and all the essential amino acids except for methionine required by the body. The combination of bioactive constituents in almonds may have increased benefit over single supplements from the additive effects of the individual components. Indeed, almond supplementation studies have shown significant decrease in total cholesterol and LDL-cholesterol (Spiller et al, 1998; Lovejoy et al, 2002), however, the mechanism for these remains unknown.

Foods rich in antioxidant nutrients may afford a greater benefit than a single antioxidant supplement and help reduce the risk of CVD. Dietary nutrients of foods can influence protein expression that maybe related to antioxidants.

This pilot study aims to investigate whether vitamin E enrichment with almond supplementation can induce changes in plasma cholesterol distribution and vascular function.
2.0 Materials and Methods

2.1 Study Design and Volunteers

All volunteers recruited for the study signed consent forms approved by the University ethics committee (Appendix I).

Six volunteers were recruited for the pilot study, three females and three males. Volunteers that were recruited were non-smokers; were not taking any multivitamins; and had a body mass index below 30. Subjects were asked to complete a food diary and a food questionnaire (Appendix II). At the initial meeting they were also advised to continue with their normal diet throughout the study period and recommended not to take any other nut food product other than those that were supplied. The volunteers were also advised not to heat/cook the almonds in any way or soak them. Blood samples were collected before and after the consumption of 50g/d of almonds over a four week period for the pilot study. Patient demographic measurements were taken included weight, height and blood pressure. Volunteer compliance was calculated from the number of bags of almonds returned throughout the study.

Figure A Time line for pilot study measurements undertaken.

All material used in the experiments were from Sigma Aldrich unless otherwise stated.
2.1.1 Sample preparation and Storage
Blood samples were centrifuged at 498 Xg (Heraeus instruments labofuge 400R) for 10mins within 2 hours of collection. The plasma was separated and aliquoted into centrifuge tubes with appropriate labeling. These plasma aliquots were stored at -80°C until required for analysis. Aliquots were not freeze-thawed and each aliquot was disposed of after testing.

2.2 Cholesterol Assay
See Chapter 4.0 Lipid Profiles section 4.2.1 of thesis for full details.

2.2.1 LDL Assay
See Chapter 4.0 Lipid Profiles section 4.2.2 of thesis for full details.

2.2.2 HDL Assay
See Chapter 4.0 Lipid Profiles section 4.2.3 of thesis for full details.

2.3 Plasma Nitrite
The griess assay was used to examine the nitrite content of the plasma samples. Sodium nitrite (1mM) was used to prepared standard curve 50μM; 25μM; 5μM; and 2.5μM. An aliquot of 50μl of each standard was pipetted into a 96 well flat bottom (Costar) in triplicate. Plasma samples were diluted 1:5 (distilled water) prior to addition of Griess reagent (50μl). The plate was allowed to incubate for 15mins in the absence of light. Absorbance was measured at 570nm using a Dynex MRX microplate reader and unknowns calculated using the standard curve.

2.4 sVCAM-1 ELISA
See Chapter 5.0 Vascular Health section 5.2.3 of thesis for full details.

2.5 Protein Carbonyl ELISA
See Chapter 5.0 Vascular Health section 5.2.4 of thesis for full details.

2.7 Bicinchoninic Acid Assay (BCA)
See Chapter 2.0 General Methods section 2.2.3 of thesis for full details.
2.8 Sample preparation for HPLC Vitamin E analysis

Plasma aliquots of 200μl were thawed from -80°C and transferred to a fresh 2ml centrifuge tube and 200μl of ethanol (containing 40μM tocopherol acetate) was added. The samples were vortexed for 10sec. Then 1.5ml of hexane (containing 12.5mg/L BHT (2, 6-Di-tert-butyl-4-methylphenol)) was added to all samples. These were vortexed for 60sec and then centrifuged for 5000 Xg for 2min. The organic phase was collected and placed into a fresh centrifuge tube; the remaining pellet was further extracted again with 1.5ml hexane (containing 12.5mg/L BHT). The sample was again vortexed for 60sec and centrifuged for 5000 Xg for 2min. The organic phase was collected and placed into another appropriately labelled fresh centrifuge tube. The vial containing organic phase was evaporated to dryness under reduced pressure. After the evaporation, methanol (200μl) was added to each vial to dissolve the remaining pellet. Alpha tocopherol standards 80, 40, 20, 10, and 5μM were prepared using this method, but after the first extraction there was no separation of the organic phase and the standard were then evaporated under vacuum (Eppendorf concentrator 5301 Hamburg). The pellet was reconstituted with 200μl methanol (Rizzo et al, 2000)

2.8.1 HPLC Vitamin E Analysis

Reverse phase chromatography was employed to analyse alpha tocopherol levels in plasma. The HPLC system constituted the Gilson 811C dynamic mixer; Gilson 307 pump; Gilson 306 pump; Gilson 819 injection module; and Gilson 215 liquid handler. The mobile phase consisted of methanol HPLC (100%) with a flow rate of 1ml/min and the alpha tocopherol peak was detected at 292nm (Gilson 170 diode array detector). Samples were prepared as described in the sample preparation method. Standards and plasma samples were run in duplicate with 50μl injection onto a Phenomenex Gemini 5micron C18 110A column. Alpha tocopherol was eluted by methanol at 6.60min and tocopherol acetate was eluted at 8.35mins. An “unknown plasma x” was also used as an internal standard to monitor fluctuations between readings. The unknowns were calculated using the standard curve.
2.9 Apo Al Analysis

A competition ELISA was employed to determine the plasma apo A1 levels before and after dietary enrichment with almonds in the pilot study. Apo Al (2µg/ml) 50µl/well was applied to a 96 well nunc plate. The plate incubated for 1 hour at 37°C. The plate was washed three times in washing buffer (PBS/Tween 20 (0.05% in 1L PBS)). The plate was blocked with milk (marvel, 4% w/v) 200µl/well and left overnight at +4°C. The plate was washed three times in washing buffer.

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Standard apo Al (40µg/ml; 50µl/well) added to wells in triplicate. Doubling dilutions were carried to prepare a standard curve. Wells B3 –B11; C3-C11; and D3-D11 were plated with milk (marvel, 4% w/v, 25µl/well). Standard (40µg/ml) 25µl removed and placed into the next well along the row and aspirated and 25µl removed again and continued along the row of standards. From wells B10, C10, and D10 25µl was discarded. For the blank milk (25µl) was plated in triplicate as indicated in the diagram above. All plasma samples were pre-diluted 1:200 with milk as the diluent and plated in triplicates (25µl/well). The primary antibody anti-apo Al (Goat polyclonal to human apolipoprotein A-I, (Abcam Plc UK) was added to all wells (final dilution 1:10000, 25µl/well). The plate was incubated at 37°C for 2 hours. The plate was washed three times in washing buffer. The secondary antibody (anti-goat IgG horse radish peroxidase conjugate, Serotec) added to all wells at a dilution of 1:2000 using blocking buffer as diluent (Tween-20 1% w/v in PBS, 200µl/well), incubated for 1 hour at 37°C. The plate was washed and substrate (10ml citrate-phosphate buffer (0.15M, pH 5), 8µl hydrogen peroxide, and 1 o-phenyldiamine tablet) was added (50µl/well). The plate was incubated in the absence
of light for 2mins to allow colour development. The colour reaction was stopped with the addition of 2M sulphuric acid (50μl/well). The absorbance was read at 490nm using a Dynex MRX microplate reader and unknowns calculated using the standard curve.

Experimental modifications were introduced to improve results these included using HDL (40μg/ml) as the competitor rather than apo Al, and plasma sample prepared to 1:100 dilution. Varying concentrations of HDL and Apo Al were used as competitor and coating agent see table below.

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<td>- 2μg/ml</td>
<td>HDL (40μg/ml) / Apo Al (40μg/ml)</td>
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Statistical Analysis
Data was assessed for statistical significance using the students t-test (paired) where P<0.05 was considered significant. Analysis was conducted using PRISM software. Also one-way analysis of variance ANOVA was employed with the Tukeys post analysis test.
3.0 Results

3.1 Apo AI

From the primary method of Apo AI detection the colour development on the plate was too rapid and therefore the standards were not readable. Hence 50μl/well of the solution was removed and the plate re-read. The results were still not dependable. Following the modification of using HDL as the competitor, the ELISA colour development was again rapid and did not produce successful results and therefore further modifications were introduced. Standards achieved with Apo AI (0.5μg/ml) as coating agent was and using HDL as the competitor were reasonable. The ELISA was re-run with these new conditions with plasma samples. Results show a general trend in declining Apo AI levels after consumption of almonds (50g/d) for 4 weeks (Fig. 1). Data produced was not consistent or reliable and therefore raises questions concerning the antibodies used. Pilot plasma sent to (Glasgow Royal Infirmary, Glasgow, UK) for Apo AI analysis at present.

3.2 Pilot Study

For the initial pilot study 6 subjects were recruited half of whom were female and male. Of the 6 subjects, 2 dropped out within the first week of the study. The percentage compliance of almond consumption was 98.57%. The volunteer BMI calculated before and after the study showed no significant changes over this period (Fig 2a). Blood pressure readings show a significant decline in diastolic pressure (P<0.005, using a Students paired t-test; Fig. 2c) with no significant change in the systolic pressure (Fig 2b). The pilot study showed that the supplementary intake of almonds in the diet (50g/d for four weeks) had no significant effect on total plasma cholesterol levels (Fig 3). The pilot study period did not cause a significant reduction in HDL and LDL cholesterol (Fig. 4), although there was a trend for lower cholesterol levels in LDL and HDL. Dietary almond (50g/d for 4 weeks) enrichment had no effect on plasma nitrite levels (Fig. 5), however significantly reductions of plasma sVCAM-1 levels (P < 0.05 using Students t-test paired; Fig. 6). A non-significant decrease in the rate of blood flow was observed after almond intake (Fig. 7). Increased formation of plasma protein carbonyl content occurred during the pilot study period (consuming 50g/d for 4 weeks) but this was not significant (Fig. 8). The concentration of alpha tocopherol increased from the baseline levels after the consumption of almonds (50g/d) for 4 weeks (Fig. 9).
Figure 1, Apolipoprotein A1 levels with almond supplementation
Apo A1 levels before (week 0) and after (week 4) consumption of almonds (50g/d) for 4 weeks. Results are expressed mean before ± SEM and mean after ± SEM.

Figure 2, Effect of almond supplementation on BMI and Blood Pressure
BMI of pilot volunteers before and after the consumption of 50g/d of almonds over 4 weeks (A). Systole pressure before and after the consumption of 50g/d of almonds over 4 weeks (B). Diastole pressure before and after the consumption of almonds (50g/d) over 4 weeks (P<0.005, using a Students paired t-test) (C). Results are expressed as box and whisker plots where the central line represents the median; the bars indicate the range; and the box indicates upper and lower quartiles (n=5).
Figure 3, Effect of almond supplementation of cholesterol levels
Cholesterol levels before and after the consumption of 50g/d of almonds over 4 weeks. Results are expressed mean before ± SEM and mean after ± SEM (n=5) dietary enrichment.

Figure 4, Effect of almond supplementation on HDL-cholesterol and LDL-cholesterol
HDL and LDL levels before and after almond intake, (A) HDL levels before and after almond intake (50g/d for four weeks). (B) LDL levels before and after almond intake (50g/d for four weeks). Results are expressed as mean ± SEM before and mean ± SEM (n=5) after supplementation. Results are expressed as box and whisker plots where the central line represents the median; the bars indicate the range; and the box indicates upper and lower quartiles (n=4).
Figure 5. Almond supplementation effect on plasma nitrite levels

Measurement of nitrite levels before and after dietary almond (50g/d for 4 weeks) consumption. Results are expressed mean ± SEM before and mean ± SEM (n=5) after dietary enrichment.

Figure 6. Almonds supplementation effect on plasma sVCAM-1

Change in plasma sVCAM-1 levels before and after the consumption of almonds (50g/d for 4 weeks, * = P < 0.05). Results are expressed as mean ± SEM before and mean ± SEM (n=5) after dietary enrichment.
Figure 7. Effect of almond supplementation of rate of flow of the brachial artery.

Blood flow before and after almond consumption (50g/d for 4 weeks). Results are expressed mean ± SEM before and mean ± SEM (n=5) after dietary enrichment.

Figure 8. Almond supplementation effect on plasma protein carbonyls

Carbonyl levels before and after the consumption of almonds (50g/d) for 4 weeks. Results are expressed as mean ± SEM before and mean ± SEM (n=5) after dietary enrichment.
Figure 9. **Almond supplementation effects on plasma alpha tocopherol levels**

Plasma alpha tocopherol concentrations before and after the consumption of almonds (50g/d) for 4 weeks. Results are expressed as mean ± SEM before and mean ± SEM (n=5) after dietary enrichment.
4.0 Discussion

The pilot study has demonstrated the feasibility of examining the role of almonds in the diet on some markers of CHD. There were no side effects on the volunteers consuming 50g/d almonds for 4 weeks and overall compliance was 98.57% based on bags of almonds returned. Analysis of weight and blood pressure revealed no significant effects. Systolic pressure showed no dramatic changes, however, a statistically significant (p<0.005) decrease in diastolic pressure was observed. Almond based diets have been shown to reduce total cholesterol and LDL-cholesterol. (Spiller et al, 1998) Positive lipid lowering effects may be due to a number of bioactive constituents of almonds, w-9 fatty acids, phytochemicals, and amino acid ratio of arginine: lysine have all been implicated in improving lipid profiles. The BMI did not change significantly for the pilot group or the main study group. Previous studies on nut consumption have shown a general trend in reducing the BMI of those individuals that consumed nuts (Sabate 2003), the reduction in BMI being attributed to increased fat in the stools and elevated satiety levels as a cofactor.

The intake of almonds in the pilot study led to increased levels of plasma alpha tocopherol as previously shown in other studies (Jambazian et al, 2005.) Almonds (28g) contain 7.4mg of alpha tocopherol and the recommended daily allowance for adults in the UK is 15mg/d. The levels in this study have yet to be adjusted for recovery using the tocopherol acetate standard curve and are higher compared to alpha tocopherol levels found in other studies. This may reflect sample concentration during preparation. Importantly, the study is incomplete and there is an insufficient number of volunteers to power the analysis for detecting any differences less than 50% change.

Plasma cholesterol concentrations fell within the pilot study, with reductions in both LDL and HDL cholesterol levels. Almonds contain high quantities of monounsaturated fatty acids (MUFA), and studies previously shown a lipid lowering affect of taking diets high in MUFA and polyunsaturated fatty acids (PUFA) (Fraser 1999) (Kris-Etherton Et al, 1999). The levels of HDL and Apo Al are negatively correlated with cardiovascular disease. The pilot study shows a declining trend in HDL cholesterol. There have been no significant effects on HDL reported from other nut studies. HDL carries out reverse cholesterol transport in which apolipoprotein Al (Apo Al) plays a major role. Any variation within Apo Al would inversely affect HDL levels. The Apo Al protein is initially synthesized as a precursor and previous work by our lab have shown that this precursor increases with increased intake of alpha tocopherol (Aldred et al., 2006).
However this precursor form of Apo A1 may not be transforming into its mature form leading to inefficient HDL formation which may contribute to the results seen here. Nevertheless this requires further analysis with increased subject numbers. Measurement of Apo A1 was undertaken in order to identify possible effects of diet on Apo A1 expression by developing an ELISA to measure Apo A1 levels in plasma. The results were inconsistent and unreliable, so at present plasma samples have been dispatched to Glasgow Royal Infirmary Glasgow, UK for analysis.

This study has measured vascular reactivity through examining levels of nitric oxide effects as flow-mediated dilation. Almonds are a rich source of the antioxidant micronutrient alpha tocopherol, and contain high quantities of arginine (9-11%), a precursor of nitric oxide. Nitric oxide that is a vasodilator released from normal endothelial cells. Beneficial effects such as reducing platelet aggregation, inhibiting smooth muscle proliferation, and preventing monocyte adherence to endothelium are attributed to nitric oxide. These mechanisms all contribute to reducing atherosclerosis (Fraser 1999). Other studies that have examined supplementation with L-arginine have shown reduced plaque formation and reduced vascular oxidative stress in male rhesus monkeys (Dhawan et al, 2005). In the pilot study, nitrite levels showed a non-significant increase indicative of increased peroxynitrite formation and possible loss of bioavailable nitric oxide. The results are consistent with the results of the flow-mediated dilatation data, which show a decline in blood flow rate following venous occlusion.

Another marker of vascular dysfunction is an increase of plasma sVCAM-1. sVCAM-1 was examined in subjects pre- and post- dietary enrichment. VCAM-1 is involved in recruiting leukocytes to sites of inflammation and overall plays a role in the formation of atherosclerotic plaques. A significant decline in sVCAM-1 was observed following almond supplementation. Reduced sVCAM-1 may suggest a decrease in expression of endothelial surface expression of sVCAM-1 or a reduced rate of cleavage by surface metalloproteinases (Garton et al, 2003). These changes of sVCAM-1 may be modulated through the micronutrients found in almonds.
5.0 Conclusion

In summary, the preliminary data so far demonstrates a real possibility in reducing total plasma cholesterol levels in normal healthy subjects following dietary almond enrichment by reducing LDL-cholesterol. However, the decline in HDL levels observed in the pilot data needs further investigation; further measurements taken in the main study, together with the increased power of larger sample size will allow more confident conclusions to be drawn.
6.0 References

See Chapter 8.0 References of thesis.