The metabolism and biokinetics of vitamin E in healthy human beings and those at increased risk of cardiovascular disease

by

Yvonne Margaret Jeanes

Submitted for the degree of Doctor of Philosophy

Centre for Nutrition and Food Safety
School of Biomedical and Molecular Sciences
University of Surrey
Guildford
Surrey
GU2 7XH

© Yvonne M. Jeanes 2003
For Mark and my parents
Abstract

Vitamin E has been shown to be beneficial in the prevention of cardiovascular disease (CVD). The following research is concerned with assessing vitamin E status in healthy people and those with increased risk of CVD, who may have an altered vitamin E status.

To understand the relationship between vitamin E intake, blood concentrations and metabolism healthy subjects were given varying single doses of RRR α-tocopheryl acetate (α-TA) up to 780 mg (n=20). There was a greater uptake of α-tocopherol in plasma and erythrocytes compared with platelets and lymphocytes. No dose-response relationship was observed, however there was a positive correlation between percentage increase in α-tocopherol concentration in plasma and erythrocyte with mg urinary α-carboxyethylhydroxychroman (α-CEHC) excreted. Less than 1% of α-TA was excreted as urinary α-CEHC.

Research into the absorption of deuterium labelled (d6) α-TA showed a greater uptake into plasma and chylomicrons over 9 h when d6 α-TA was ingested with 17.5 g fat compared with 2.7 g fat (n=8). Furthermore, there was a greater uptake into chylomicrons over 9 h when d6 α-TA was ingested with toast and butter compared with cereal, cream and milk, both containing 17.5 g fat. This indicates the fat content and physical properties of a meal affect α-tocopherol absorption.

The biokinetics of newly absorbed d6 α-tocopherol into plasma, lipoproteins and blood cells was investigated in healthy subjects (n=12). d6 α-Tocopherol firstly entered the chylomicrons, followed by VLDL, then LDL and HDL. In platelets and lymphocytes there was a gradual increase in d6 α-tocopherol concentration. A third of subjects exhibited a biphasic uptake of d6 α-tocopherol in platelets, with an initial peak at 6 h.

Hypercholesterolemics are at increased risk of CVD and have altered lipoprotein metabolism therefore they may have altered vitamin E biokinetics. To investigate this, hypercholesterolemic and normolipidemic males ingested 150 mg d6 α-TA (n=16). The d6 α-tocopherol uptake into plasma was similar but there was a lower uptake of d6 α-tocopherol in low-density lipoprotein per g protein in hypercholesteroleemics compared with normolipidemics. There was also a trend towards a lower uptake of d6 α-tocopherol into erythrocytes, platelets and lymphocytes in hypercholesteroleemics. A greater
percentage of hypercholesterolemics exhibited a biphasic profile in the uptake of d6 α-tocopherol into platelets compared with normolipidemics.

Cigarette smokers are subjected to increased free radical exposure and therefore may utilise more vitamin E. Steady-state vitamin E status was measured in cigarette smokers and non-smokers (n=30). Plasma and erythrocyte α and γ-tocopherol levels were similar, however cigarette smokers had a lower concentration of α-tocopherol in platelets and α- and γ- tocopherol concentrations were lower in lymphocytes. Cigarette smokers excreted similar amounts of urinary α-CEHC, but more γ-CEHC compared with non-smokers.
Acknowledgements

I am very grateful to Dr John Lodge, my supervisor, for providing me with the opportunity and for his guidance during the course and preparation of this thesis. He was always approachable and encouraged me to attend conferences and courses. And all those ‘Wates house’ meetings, along with Wendy helped the ideas flow.

A huge thanks to Dr Wendy Hall for her invaluable contribution with recruitment, conducting studies and laboratory work. She was great to work with and has always been a source of inspiration as well as a good friend. Thank you to Anna for your encouragement over the last year and your continual optimism and kindness. And a big thanks to all my friends within the department, who have made it an enjoyable place to work.

A special mention to Mark for his tireless understanding and for being there for me when times were difficult. Also to my parents for their loving support throughout my time at University.

My thanks must also go to the people who participated in the studies and to the Medical Research Council, British Heart Foundation and Royal Society whose funding made the following research possible.
## Contents

Abstract............................................................................................................................... i  
Acknowledgements................................................................................................................ iii  
Table of Contents.................................................................................................................. iv  
List of Figures......................................................................................................................... ix  
List of Tables........................................................................................................................... xiii  
List of Abbreviations................................................................................................................ xv  
Publications resulting from research.................................................................................... xvii

### 1. INTRODUCTION........................................................................................................... 1

1.1 THE DISCOVERY OF VITAMIN E ............................................................................... 2  
1.2 CHEMISTRY OF VITAMIN E ......................................................................................... 3  
1.3 VITAMIN E INTAKE ....................................................................................................... 5  
  1.3.1 Food sources........................................................................................................... 5  
  1.3.2 Government recommendations for daily vitamin E intake .................................... 6  
  1.3.3 Vitamin E deficiency and toxicity ...................................................................... 6  
1.4 CARDIOVASCULAR DISEASE .................................................................................... 8  
1.5 CARDIOVASCULAR DISEASE: MOLECULAR FUNCTIONS OF VITAMIN E ..... 10  
  1.5.1 α-Tocopherol as an antioxidant .......................................................................... 10  
  1.5.2 Molecular functions of α-tocopherol .................................................................... 12  
    1.5.2.1 α-Tocopherol prevents LDL oxidation ............................................................... 13  
    1.5.2.2 Functional effects of α-tocopherol on monocytes and macrophages ............ 13  
    1.5.2.3 α-Tocopherol inhibits smooth muscle cell proliferation .............................. 14  
    1.5.2.4 α-Tocopherol effects on platelet adhesion and aggregation ......................... 14  
    1.5.2.5 α-Tocopherol effects on lymphocyte differentiation and proliferation .......... 14  
  1.5.3 Functional properties of γ-tocopherol ................................................................. 15  
1.6 VITAMIN E AND CARDIOVASCULAR DISEASE: OBSERVATIONAL AND  
INTERVENTION STUDIES ................................................................................................. 16  
1.7 VITAMIN E ABSORPTION AND BIOKINETICS ......................................................... 23  
  1.7.1 Absorption of vitamin E ....................................................................................... 23  
  1.7.2 Vitamin E transport within chylomicrons ............................................................ 26  
  1.7.3 Selective secretion of RRR-α-tocopherol into very low-density lipoproteins  
(VLDL); the role of α-tocopherol transfer protein (α-TTP) ........................................... 27  
  1.7.4 Post hepatic transport of α-tocopherol within lipoproteins ................................ 31  
  1.7.5 Uptake of vitamin E into blood cells and peripheral tissues ............................... 31  
  1.7.6 Intracellular distribution and regulation of α-tocopherol ....................................... 33  
1.8 VITAMIN E METABOLISM AND URINARY EXCRETION ...................................... 35  
  1.8.1 Metabolism of vitamin E to carboxyethyl hydroxychromans ............................... 35  
  1.8.2 The involvement of α-tocopherol transfer protein in vitamin E metabolism .......... 38  
1.9 ASSESSING VITAMIN E STATUS ............................................................................... 39
1.9.1 Biomarkers of α-tocopherol status .......................................................... 40
1.10 AIMS AND OBJECTIVES OF THE CURRENT RESEARCH ......................... 45
  1.10.1 Research aim ......................................................................................... 46
  1.10.2 Research objectives .............................................................................. 46

2. MATERIALS AND METHODS ....................................................................... 47

  2.1 MATERIALS .............................................................................................. 48
  2.2 METHODS .................................................................................................. 50
     2.2.1 Subjects .................................................................................................. 50
     2.2.2 Dietary intake ......................................................................................... 50
     2.2.3 Twenty-four hour urine collection ........................................................ 50
     2.2.4 Blood collection ..................................................................................... 51
     2.2.5 Plasma isolation ...................................................................................... 51
     2.2.6 Platelet isolation ..................................................................................... 51
     2.2.7 Erythrocyte isolation ............................................................................. 52
     2.2.8 Lymphocyte isolation ........................................................................... 53
     2.2.9 Isolation of lipoproteins from plasma ................................................... 54
        2.2.9.1 Isolation of chylomicrons .................................................................... 54
        2.2.9.2 Isolation of very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) by sequential ultracentrifugation .................................................. 54
        2.2.9.3 Separation of LDL subclasses by self generated gradient of iodixanol 55
     2.2.10 Determination of plasma and lipoprotein triacylglycerol ...................... 56
     2.2.11 Determination of plasma and lipoprotein cholesterol .......................... 57
     2.2.12 Determination of low-density lipoprotein (LDL) cholesterol .............. 57
     2.2.13 Determination of high-density lipoprotein (HDL) cholesterol .............. 58
     2.2.14 Determination of protein in platelets and lymphocytes ...................... 59
        2.2.14.1 Validation of urinary protein kit with the modified Lowry protein assay .................................................................................................................. 59
     2.2.15 Determination of protein in lipoproteins .............................................. 60
     2.2.16 Ascorbic acid extraction from plasma .................................................. 61
     2.2.17 Ascorbic acid quantification with HPLC with electrochemical detection 61
     2.2.18 Vitamin E extraction from blood components by solvent extraction .... 62
     2.2.19 Vitamin E extraction by saponification ................................................ 62
     2.2.20 Vitamin E quantification using HPLC with electrochemical detection 63
        2.2.20.1 Validation of δ-tocopherol as an appropriate internal standard .......... 64
     2.2.21 LC/MS quantification of vitamin E ....................................................... 65
        2.2.21.1 Development of liquid chromatography time-of-flight mass spectrometric (LC TOF MS) method to detect deuterium labelled tocopherols .................................................................................................. 66
     2.2.22 Quantification of vitamin E urinary metabolites .................................... 70
     2.2.23 Determination of urinary creatinine .................................................... 71
     2.2.24 Cotinine measurement by HPLC with UV detection ................................ 72

  2.3 STATISTICAL ANALYSIS .......................................................................... 73
3. URINARY \( \alpha \)-AND \( \gamma \)-CEHC EXCRETION AND PLASMA, ERYTHROCYTE, PLATELET AND LYMPHOCYTE \( \alpha \)-AND \( \gamma \)-TOCOPHEROL: RESPONSE TO VARYING SINGLE DOSES OF VITAMINE E

3.1 INTRODUCTION .................................................................75
3.2 AIM AND HYPOTHESIS ......................................................77
3.3 STUDY DESIGN .................................................................78
3.4 RESULTS ............................................................................78
  3.4.1 Subject characteristics ..................................................80
  3.4.2 Baseline values ............................................................80
  3.4.2.1 Baseline correlations ................................................82
  3.4.3 \( \alpha \)-and \( \gamma \)-Tocopherol concentration 24 h post ingestion of differing single doses of \( \alpha \)-tocopheryl acetate ......................................................83
  3.4.4 Urinary \( \alpha \)-and \( \gamma \)-CEHC levels after varying single-doses of \( \alpha \)-tocopheryl acetate ......................................................86
    3.4.4.1 Correlations after varying single doses of \( \alpha \)-tocopheryl acetate ..........90
  3.4.5 Plasma .................................................................90
3.5 DISCUSSION ........................................................................91
  3.5.1 Baseline \( \alpha \)-and \( \gamma \)-tocopherol concentration in plasma, erythrocytes, platelets and lymphocytes, and \( \alpha \)-and \( \gamma \)-CEHC urinary excretion ......................................................91
  3.5.1.1 Baseline correlations ................................................92
  3.5.2 Response to varying single-doses of \( \alpha \)-tocopheryl acetate in plasma, erythrocytes, platelets and lymphocytes .......93
  3.5.3 Urinary \( \alpha \)-CEHC excretion in response to varying single-doses of \( \alpha \)-tocopheryl acetate ......................................................95
  3.5.4 Conclusions ..............................................................97

4. ABSORPTION OF LABELLED VITAMIN E FOLLOWING FOUR TEST MEALS ...........................................................................99

4.1 INTRODUCTION ......................................................................100
4.2 AIM AND HYPOTHESIS ......................................................101
4.3 STUDY DESIGN .................................................................102
4.4 RESULTS ............................................................................104
  4.4.1 Subject characteristics ..................................................104
  4.4.2 Plasma and chylomicron cholesterol and triacylglycerol concentrations following four test meals ...........................................104
  4.4.3 Plasma and chylomicron uptake of \( \text{d}6 \)-RRR-\( \alpha \)-tocopherol post ingestion of \( \alpha \)-tocopheryl acetate with four test meals ...........................................106
  4.4.4 Inter-individual variation in uptake of \( \text{d}6 \)-RRR-\( \alpha \)-tocopherol ......................................................108
4.5 DISCUSSION ........................................................................110
  4.5.1 Absorption of \( \text{d}6 \) \( \alpha \)-tocopheryl acetate following four test meals ........111
    4.5.1.1 The effect of fat content on the absorption of \( \alpha \)-tocopherol ............111
    4.5.1.2 The effect of meal physical properties on the absorption of \( \alpha \)-tocopherol ......................................................113
  4.5.2 Inter-individual variation in the absorption of \( \alpha \)-tocopheryl acetate ........114
List of Figures

Figure 1.1 Chemical structure of tocopherol and tocotrienol isomers............................3
Figure 1.2 Deuterium 9 labelled RRR-α-tocopherol.......................................................4
Figure 1.3 A cross section of an artery illustrating atherosclerotic plaque progression.8
Figure 1.4 The role of α-tocopherol as a chain-breaking antioxidant adapted from
    Halliwell and Gutteridge (1999).............................................................................11
Figure 1.4 Relationship between coronary heart disease mortality rates and dietary α-
    tocopherol (mg/ habitual diet/ day)........................................................................16
Figure 1.5. Scheme of the absorption of vitamin E into the systemic circulation,
    adapted from Traber & Kayden (1989a)...............................................................27
Figure 1.6 Scheme of the transport and uptake of α-tocopherol within the systemic
    circulation adapted from Traber & Kayden (1989a)............................................29
Figure 1.7 Tocopherol metabolism to carboxyethyl hydroxychroman adapted from
    Birringer et al. (2001)...........................................................................................37

Figure 2.1 Summary of blood separation procedures for the isolation of plasma,
    erythrocytes, platelets and lymphocytes from whole blood. ..............................52
Figure 2.2 Response calibration curves for α- and γ-tocopherol in ethanol.................64
Figure 2.3 Typical total ion chromatogram and m/z scans following the injection of
    0.5 pmoles of d0, d3 or d6 α-tocopherol ................................................................67
Figure 2.4 Calibration curves for peak area versus concentration of d0, d3 and d6 α-
    tocopherol ..............................................................................................................68
Figure 2.5 Response curves used for the quantification of d0 and d6 α-tocopherol.... 69

Figure 3.1 Schematic figure of study protocol .............................................................78
Figure 3.2 Mean percentage changes in α-tocopherol concentrations within blood
    components between baseline and 24 h post ingestion of varying single doses of
    α-tocopheryl acetate. ..............................................................................................85
Figure 3.3 Urinary excretion of α-CEHC over 5 days when 130 mg α-tocopheryl
    acetate was ingested on day 3. ...........................................................................86
Figure 3.4 Urinary excretion of α-CEHC over 5 days when 195 mg α-tocopheryl
    acetate was ingested on day 3. ...........................................................................87
Figure 3.5 Urinary excretion of a-CEHC over 5 days when 390 mg a-tocopheryl acetate was ingested on day 3....................................................... 87

Figure 3.6 Urinary excretion of a-CEHC over 5 days when 780 mg a-tocopheryl acetate was ingested on day 3................................................................................88

Figure 3.7 Collective urinary excretion of a-CEHC above baseline from days 3, 4 and 5. ..............................................................................................................................89

Figure 4.1 Schematic figure of study protocol ...........................................................103

Figure 4.2 Chylomicron triacylglycerol concentration following ingestion of 150 mg d6 RRR-a-tocopheryl acetate with four test meals over 6 hours .........................105

Figure 4.3 Chylomicron protein following ingestion of 150 mg d6 RRR-a-tocopheryl acetate with four test meals over 6 hours...............................................................105

Figure 4.4 Plasma d6-a-tocopherol concentration, following ingestion of 150 mg d6 RRR-a-tocopheryl acetate with 4 different meals over 6 hours.............................106

Figure 4.5 Chylomicron d6-a-tocopherol per mmol triacylglycerol (TAG) following ingestion of d6 a-tocopheryl acetate with four test meals over 6 hours ..........107

Figure 4.6 Chylomicron d6-a-tocopherol per g protein following ingestion of d6 a-tocopheryl acetate with four test meals over 6 hours.................................................108

Figure 4.7 Individual d6 a-tocopherol plasma concentration profiles after ingestion of 150 mg d6-a-tocopheryl acetate with the toast and butter meal. .........................109

Figure 5.1 Schematic diagram of study protocol.......................................................120

Figure 5.2 Plasma total (d0 + d6), d0 and d6 α-tocopherol concentration profile over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate.....................................123

Figure 5.3 Chylomicron d6 α-tocopherol per g protein profile over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate .................................................................124

Figures 5.4 A. and B: VLDL, LDL and HDL d6 and d0 α-tocopherol per g protein over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate, respectively .................................................................125

Figure 5.5 Percentage d6 α-tocopherol uptake profiles for chylomicrons, VLDL, LDL and HDL over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate .......126
Figure 5.6 Erythrocyte d6 α-tocopherol corrected for packed cell volume (PCV) over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate. Plasma d6 α-tocopherol in grey shown for comparison .......................................................... 128
Figure 5.7 Total (d0 + d6), d0 and d6 α-tocopherol in platelets per g protein over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate .................................................. 129
Figure 5.8 d6 and d0 α-tocopherol profile in four subjects that had a biphasic uptake of d6 α-tocopherol and eight subjects who had a gradual d6 α-tocopherol uptake profile over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate .......... 129
Figure 5.9 Biphasic uptake of d6 α-tocopherol in platelets per g protein in 4 subjects (and chylomicron d6 α-tocopherol in grey shown for comparison) over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate ............................................. 130
Figure 5.10 Lymphocytes total (d0 + d6), d0 and d6 α-tocopherol per g protein over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate ............................................. 131
Figure 6.1 Schematic diagram of study protocol.......................................................... 144
Figure 6.2 (A) Plasma d0 and d6 α-tocopherol for NL and HC subjects (B) d0 and d6 α-tocopherol per mmol cholesterol for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .................................................. 147
Figure 6.3 (A) Chylomicron d6 α-tocopherol per g protein for NL and HC subjects (B) Chylomicron d6 α-tocopherol per mmol TAG for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .................................................. 148
Figure 6.4 VLDL d0 and d6 α-tocopherol per g protein in for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .......... 149
Figure 6.5 LDL d0 and d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .................................................. 150
Figure 6.6 HDL d0 and d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .................................................. 151
Figure 6.7 Erythrocyte d6 α-tocopherol corrected for PCV for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .......... 153
Figure 6.8 Platelets d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .............. 154
Figure 6.9 Lymphocyte d0 and d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg $RRR\alpha$-tocopheryl acetate ..........155
Figure 6.10 A d6 α-Tocopherol in LDL sub fractions at 12 hours for NL and HC per fraction, and 6.10 B d6 α-Tocopherol in LDL sub fractions at 12 hours for NL and HC per mmol cholesterol following ingestion of 150 mg $RRR\alpha$-tocopheryl acetate ..................................................................................................................156

Figure 7.1 Plasma α- and γ-tocopherol concentration per mmol cholesterol in cigarette smokers and non-smokers.............................................................................................................................................172
Figure 7.2 Erythrocyte α- and γ-tocopherol concentration corrected for packed cell volume in cigarette smokers and non-smokers........................................................................................................173
Figure 7.3 Platelet α- and γ-tocopherol concentration per g protein in cigarette smokers and non-smokers.................................................................................................................................174
Figure 7.4 Lymphocyte α- and γ-tocopherol concentration per g protein in cigarette smokers and non-smokers.................................................................................................................................175
Figure 7.5 Urinary excretion of α- and γ-CEHC excretion corrected for creatinine in cigarette smokers and non-smokers.........................................................................................................................176
List of Tables

Table 1.1 *RRR*-α-tocopherol equivalents for the various vitamin E homologues adapted from Sheppard *et al.* (1993) .................................................................................. 6

Table 1.2 Summary of the functional properties of α-tocopherol encompassing antioxidant and molecular events in the prevention of cardiovascular disease adapted from Ricciarelli *et al.* (2001) .................................................................................. 13

Table 1.3 Summary of large primary and secondary intervention trials with α-tocopherol and cardiovascular disease events adapted from Kinsky (2003)....... 18

Table 1.4 Meta-analysis of effect of high versus low vitamin E intake on cardiovascular disease mortality for observational and intervention studies from Hooper *et al.* (2001)........................................................................................................ 21

Table 1.5 Relative affinities calculated from the degree of competition for α-tocopherol transfer protein (α-TTP) adapted from Hosomi *et al.* (1997)........... 30

Table 1.6 Mechanisms of vitamin E uptake into blood cells and peripheral tissues .... 32

Table 2.1 Physiochemical data for α-, γ- and δ-tocopherol ........................................ 64

Table 2.2 Mobile phase gradient used to separate urinary metabolites and trolox ...... 71

Table 3.1 Subject characteristics for each group and total ........................................ 80

Table 3.2 Mean baseline α- and γ-tocopherol concentrations in blood components for each group and total ................................................................. 81

Table 3.3 Mean baseline urinary α- and γ- CEHC excretion for each group and total ............................................................................................................. 82

Table 3.4 Correlation matrix for baseline α-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes and urinary α-CEHC excretion .......... 82

Table 3.5 Correlation matrix for baseline γ-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes and urinary γ-CEHC excretion .......... 83

Table 3.6 α-Tocopherol concentration at baseline and 24 h post ingestion of varying single doses of α-tocopheryl acetate ..................................................... 84

Table 3.7 γ-Tocopherol concentration at baseline and 24 h post ingestion of varying single doses of α-tocopheryl acetate ..................................................... 84
Table 3.8 Urinary α-CEHC excretion up to 72 h after ingestion of varying doses of α-tocopheryl acetate ................................................................. 89
Table 3.9 Correlation matrix for α-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes 24 h following ingestion of varying single doses of α-tocopheryl acetate and incremental AUC for urinary α-CEHC excretion up to 72 h post ingestion of α-tocopheryl acetate ........................................... 90
Table 4.1 Macronutrient composition of the four test meals .................................. 102
Table 4.2 Subject characteristics (n = 8) ................................................................. 104
Table 5.1 Subject characteristics (n = 12) ............................................................... 121
Table 5.2 Area under curve (AUC), maximum concentration (C max) and time of C max for d6 α-tocopherol in plasma and lipoproteins ........................................... 122
Table 5.3 Area under curve (AUC), maximum concentration (C max) and time of C max for d6 α-tocopherol in plasma and blood cells ........................................... 127
Table 5.4 Studies investigating labelled α-tocopherol bio kinet ics ......................... 133
Table 6.1 Subject characteristics for normolipidemic and hypercholesterolemic subjects .................................................................................. 145
Table 6.2 Mean d6 α-tocopherol area under curve (AUC), in plasma and lipoproteins over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate ............... 146
Table 6.3 Mean d6 α-tocopherol area under curve (AUC) in erythrocytes, platelets and lymphocytes over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate .................................................................................. 152
Table 7.1 Subject characteristics for cigarette smokers and non-smokers ............... 170
Table 7.2 Average nutrient intake per day for cigarette smokers and non-smokers .. 171
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-TA</td>
<td>α-Tocopherol Acetate</td>
</tr>
<tr>
<td>α-TE</td>
<td>α-Tocopherol Equivalent</td>
</tr>
<tr>
<td>α-TQ</td>
<td>α-Tocopheryl Quinone</td>
</tr>
<tr>
<td>α-TTP</td>
<td>α-Tocopherol Transfer Protein</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-Binding Cassette transporter A1</td>
</tr>
<tr>
<td>all rac-α-tocopherol</td>
<td>Synthetic α-tocopherol</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variances</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under Curve</td>
</tr>
<tr>
<td>AVED</td>
<td>Ataxia with Vitamin E Deficiency</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated Hydroxytoluene</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CEHC</td>
<td>Carboxyethylhydroxychroman</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>CIU</td>
<td>Clinical Investigation Unit</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicron</td>
</tr>
<tr>
<td>Cmax</td>
<td>Concentration maximum</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>CYP 3A</td>
<td>Cytochrome 3A</td>
</tr>
<tr>
<td>CYP 4F2</td>
<td>Cytochrome 4F2</td>
</tr>
<tr>
<td>DFO</td>
<td>Deferoxamine mesylate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentacetic Acid</td>
</tr>
<tr>
<td>ECD</td>
<td>Electrochemical Detector</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
</tr>
<tr>
<td>HC</td>
<td>Hypercholesterolemic</td>
</tr>
<tr>
<td>HDL</td>
<td>High-Density Lipoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>IAUC</td>
<td>Incremental Area Under Curve</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>k Da</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LC TOF MS</td>
<td>Liquid Chromatography Time-Of-Flight Mass Spectrometry</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/Mass Spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit Of Detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit Of Quantification</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MPA</td>
<td>Metaphosphoric Acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NL</td>
<td>Normolipidemic</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidatively modified Low-Density Lipoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid Transfer Protein</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein Phosphatase</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>RRR-α-tocopherol</td>
<td>Natural form α-Tocopherol</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SR-B1</td>
<td>Scavenger Receptor class B type 1</td>
</tr>
<tr>
<td>TAP</td>
<td>Tocopherol Associated Protein</td>
</tr>
<tr>
<td>TBP</td>
<td>Tocopherol Binding Protein</td>
</tr>
<tr>
<td>t Cmax</td>
<td>Time of maximum concentration</td>
</tr>
<tr>
<td>TDC</td>
<td>Time to Digital Converter</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-Of-Flight</td>
</tr>
<tr>
<td>TOF MS</td>
<td>Time-Of-Flight Mass Spectrometry</td>
</tr>
<tr>
<td>UP</td>
<td>Urinary Protein</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEAPS</td>
<td>Vitamin E Atherosclerosis Prevention Study</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low-Density Lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
Publications resulting from research


Chapter 1

Introduction
1.1 The discovery of vitamin E

In 1922 Evans and Bishop discovered a dietary factor essential for rat fertility, which was later classified as vitamin E. Twenty-four years later Evans et al. (1936) isolated vitamin E from wheat germ oil, it was termed 'tocopherol' from the Greek meaning 'to bring forth childbirth' and later designated α-tocopherol. In the following years β-, γ- and δ-tocopherol and four tocotrienols were also isolated. Researchers have since confirmed vitamin E as the most important in vivo lipid soluble antioxidant, protecting lipoproteins and cellular membranes from oxidative damage (Burton et al., 1983; Tappel, 1962). More recently, researchers have described the involvement of α-tocopherol in cellular signalling and gene regulation (Azzi & Stocker, 2000). Vitamin E has also been proposed as beneficial in the prevention of cardiovascular disease (Devaraj & Jialal, 1998; Gey et al., 1991; Kaul et al., 2001).
1.2 Chemistry of vitamin E

Naturally occurring vitamin E consists of four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ), they all have a chromanol ring and a hydrophobic C-16 phytyl tail (Figure 1.1). Tocopherols have a saturated phytyl tail whereas tocotrienols have three double bonds situated at carbons 3', 7' and 11'. The phytyl tail anchors vitamin E within membranes, positioning the chromanol ring at the hydrocarbon interface (Halliwell & Gutteridge, 1999).

![Chemical structure of tocopherol and tocotrienol isomers](image)

**Figure 1.1 Chemical structure of tocopherol and tocotrienol isomers**
Tocopherols and tocotrienols have a chromanol ring and phytyl tail. Tocopherols have a saturated phytyl tail, whereas tocotrienols have three double bonds. Tocopherols have three chiral centres at C2', C4' and C8'. There are 4 different isomers of tocopherol and tocotrienol; α; R1 and R2 = CH3, β; R1 = CH3 R2 = H, γ; R1 = H R2 = CH3 and δ; R1 and R2 = H.

α-Tocopherol has three chiral centres on the C2' in the chromanol ring and C4' and C8' on the phytyl tail, either in the S or R configuration. Naturally occurring α-tocopherol has the R configuration at each chiral centre and hence is termed RRR-α-tocopherol (formerly d-α-tocopherol). α-Tocopherol can be synthesised by condensing trimethyl hydroquinone with isophytol. Synthetic α-tocopherol consists of
approximately equal proportions of the eight possible S and R configurations, and is termed all rac-α-tocopherol (formerly dl-α-tocopherol).

Burton and Ingold (1981) reported that the antioxidant capacity of vitamin E is primarily determined by the bond dissociation energy of the O-H bond. The chromanol ring stabilises the unpaired electron of the α-tocopheryl radical. The stereoisomers of α-tocopherol have identical antioxidant activity but differ in their biologic activity (Herrera & Barbas, 2001). RRR-α-tocopherol, the most abundant form of vitamin E in nature, has the highest biologic activity of vitamin E based on rat foetal resorption-gestation assay and relative affinity for the hepatic α-tocopherol transfer protein (Bunyan et al., 1961; Hosomi et al., 1997; Weiser et al., 1986; Weiser et al., 1996). γ-Tocopherol has only 10% of the biologic activity of α-tocopherol (Bunyan et al., 1961).

Ingold et al. (1987) synthesised deuterium labelled α-tocopherol. The deuterium can be situated on one, two or three methyl groups in the chromanol ring, these are termed d3, d6 or d9 α-tocopherol; figure 1.2 illustrates d9 α-tocopherol. The use of deuterium labelled tocopherols has enabled investigators to simultaneously measure the RRR- and all rac-α-tocopherol bioavailability and biokinetics, both in the free and ester form (Cheeseman et al., 1995; Ingold et al., 1987a; Traber et al., 1988; Traber et al., 1990a).

![Figure 1.2 Deuterium 9 labelled RRR-α-tocopherol](image)
Chapter 1

1.3 Vitamin E intake

1.3.1 Food sources

Plant and products made from them are the predominant dietary source of vitamin E. Nuts, seeds, vegetable oils and some grains are rich sources of vitamin E, differing in their proportions of the different vitamin E homologues (Bauernfeind, 1980). Wheat germ, safflower oil and sunflower oil are all rich sources of α-tocopherol. The amount of vitamin E within foods at the point of consumption is difficult to assess as it depends upon the effects of processing, storage and preparation (Expert Group on Vitamins and Minerals, 2003). γ-Tocopherol is the most abundant form of vitamin E within the American diet, due to their high consumption of corn and soybean oil (Jiang et al., 2001; McLaughlin & Weihrauch, 1979; Swanson et al., 1999). However, a lower proportion of γ-tocopherol rich oils are consumed within the UK (NDNS, 2003). Vitamin E supplements are widely available, both as RRR and all rac-α-tocopherol. These supplements are usually esterified with acetate or succinate at the reactive hydroxyl group within the chromanol ring, thereby increasing the molecule’s stability and extending the products shelf life. RRR-α-tocopherol naturally occurs unesterified in food.

Previously, international units (IU) have been used to report vitamin E activity in foods. IU were assigned to vitamin E homologues stating the specific activity of 1 mg all rac-α-tocopheryl acetate, therefore, 1 IU is equivalent to 0.67 mg RRR-α-tocopherol (Expert Group on Vitamins and Minerals, 2003). IU have since been superseded by RRR-α-tocopherol equivalents (α-TE) and RRR-α-tocopherol has been assigned an activity of 1 mg α-TE per mg tocopherol. The relative activities of the tocopherols and tocotrienols are listed in table 1.1.
Table 1.1 RRR-α-tocopherol equivalents for the various vitamin E homologues adapted from Sheppard et al. (1993)

<table>
<thead>
<tr>
<th>Vitamin E homologue</th>
<th>Activity as α-TE (mg/mg compound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRR-α-tocopherol</td>
<td>1.0</td>
</tr>
<tr>
<td>RRR-β-tocopherol</td>
<td>0.5</td>
</tr>
<tr>
<td>RRR-γ-tocopherol</td>
<td>0.1</td>
</tr>
<tr>
<td>RRR-δ-tocopherol</td>
<td>0.03</td>
</tr>
<tr>
<td>RRR-α-tocotrienol</td>
<td>0.3</td>
</tr>
<tr>
<td>RRR-β-tocotrienol</td>
<td>0.05</td>
</tr>
<tr>
<td>All rac-α-tocopheryl acetate</td>
<td>0.74</td>
</tr>
</tbody>
</table>

The activities of γ and δ-tocotrienol are unknown.

1.3.2 Government recommendations for daily vitamin E intake

The Department of Health report on dietary reference values for the United Kingdom, published in 1991, concluded that daily intakes of 4 mg and 3 mg of α-TE can be adequate for men and women respectively (Committee in Medical Aspects of Food Policy, 1991). The Panel also reported that vitamin E requirements depend on dietary polyunsaturated fatty acid (PUFA) intake. PUFAs have a greater number of carbon double bonds in comparison to saturated and monounsaturated fatty acids and are thus more susceptible to oxidation. Vitamin E is utilised in the process of preventing lipid peroxidation in vivo (Tappel, 1962). Muggli (1994) reported the mean dietary ratio of mg α-TE : g PUFA in Britain to be over 0.6. This was reported as adequate, although there is no general agreement on what ratio to recommend (Committee in Medical Aspects of Food Policy, 1991). In 2002 Brigelius-Flohe et al. reported that the United States and German governments had increased their dietary recommendations for vitamin E intake to 15 mg vitamin E/ day.

1.3.3 Vitamin E deficiency and toxicity

Plasma concentrations are often used to assess vitamin E status, these are influenced by lipid levels and hence are often expressed as a tocopherol:cholesterol ratio. Plasma tocopherol concentrations less than 11.6 μmol per litre or < 2.25 μmol per mmol cholesterol indicate vitamin E deficiency (Thurnham et al., 1986). A standard test for vitamin E deficiency is ex vivo oxidative susceptibility of erythrocytes to dilute hydrogen peroxide (Farrell et al., 1977). Clinical manifestations of vitamin E deficiency rarely develop from dietary inadequacy. The most common
cause of vitamin E deficiency is chronic malabsorption from an underlying gastrointestinal, pancreatic or hepatic origin. Conditions that result in vitamin E deficiency include; abetalipoproteinemia, chronic cholestatic hepatobiliary disorders and cystic fibrosis. A genetic defect in the α-tocopherol transfer protein (α-TTP) results in vitamin E deficiency; this disorder is termed ataxia with isolated vitamin E deficiency (AVED). Severe vitamin E deficiency causes dysfunction in neuromuscular, vascular and reproductive systems and is characterised by spinocerebelar ataxia and myopathies (Sokol, 1993).

Vitamin E is considered to have low toxicity. A recent publication by the Food Standards Agency (2003) reported that 540 mg α-TE per day as a safe upper level of consumption over a lifetime (Expert Group on Vitamins and Minerals, 2003). There have been several large-scale supplementation studies from which data on the adverse effects of vitamin E have been collated. The majority of supplementation studies have not reported any adverse outcomes from vitamin E supplementation. The Alpha-Tocopherol, Beta-Carotene (ATBC) cancer prevention study investigated the effects of 50 mg all rac-α-tocopherol in male cigarette smokers and an increase in mortality from haemorrhagic stroke in hypertensive smokers was observed (Leppälä et al., 2000). However, although biologically plausible, the significance of this finding is uncertain. A meta-analysis conducted by Vivekananthan et al. (2003), which included four studies, revealed no significant difference in incidence of all-cause strokes for subjects treated with vitamin E or controls.
1.4 Cardiovascular disease

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the Western world. CVD is a chronic, multi-factorial disease and the aetiology can be attributed to the accumulation of risk factors. CVD encompasses coronary heart disease (CHD), stroke and peripheral vascular disease. In the UK it is the most common cause of premature death and 110,000 people die of CHD each year (Department of Health, 2000).

Atherosclerosis interlinked with thrombosis is fundamental in the pathogenesis of CHD (Zaman et al., 2000). Considerable evidence suggests that oxidative stress and inflammation are central to atherosclerosis (Berliner et al., 1995; Ross, 1999). The development of an atherosclerotic lesion is shown in figure 1.3. Possible causes of endothelial dysfunction leading to atherosclerosis include elevated and modified LDL; free radicals caused by cigarette smoking; hypertension, diabetes mellitus and genetic alterations (Ashwell, 1998; Griendling & Alexander, 1997; Steinberg, 1997; Talmud & Humphries, 2001).

![Figure 1.3 A cross section of an artery illustrating atherosclerotic plaque progression](image)

*Figure 1.3 A cross section of an artery illustrating atherosclerotic plaque progression.*

Fatty streaks initially consist of lipid-laden monocytes and macrophages (foam cells) together with T lymphocytes. Later they are joined by various numbers of smooth muscle cells. The steps involved in this process include smooth-muscle migration, T-cell activation, foam cell formation, platelet adherence and aggregation and the adherence and migration of leucocytes. Illustration from Ross et al. (1999)
Raised plasma low-density lipoproteins (LDL) is a well recognised risk factor for CVD (Keys A, 1970; Martin & et al, 1986; Rose & Shipley, 1986; Steinberg et al., 1989). LDL, which may be oxidatively modified, can induce endothelial dysfunction (Griendling & Alexander, 1997; Stokes et al., 2002b). Furthermore, LDL can become trapped within the arterial wall, here they undergo further oxidation and are internalised into macrophages leading to the formation of foam cells (Steinberg et al., 1989). LDL are heterogeneous, and have been classified into sub populations; large buoyant, intermediate or small, dense LDL (Griffin, 1997). A predominance of small dense LDL is considered more atherogenic than large, buoyant LDL (Berneis & Krauss, 2002; Griffin, 1999).

Epidemiological studies have associated cigarette smoking with CVD (Doll et al., 1994; Doyle et al., 1964). Several mechanisms for the increased risk have been proposed, these include; increased oxidative stress (Cross et al., 1998; Morrow et al., 1995), disturbed lipoprotein metabolism by increasing insulin resistance and lipid intolerance (Eliasson et al., 1997). The products of cigarette smoking damage vascular endothelium, leading to increased secretion of adhesion molecules which enhance the binding of platelets and monocytes (Blann et al., 1998) and increased platelet aggregation (Lehr et al., 1997; Rival et al., 1987).
1.5 Cardiovascular disease: molecular functions of vitamin E

Vitamin E has traditionally been known as a lipophilic antioxidant, however a number of non-antioxidant roles have been discovered, broadening the role of α-tocopherol in disease prevention. α-Tocopherol has been shown to inhibit cells crucial in the progression of atherosclerosis both directly and via the inhibition of low-density lipoprotein (LDL) oxidation, suggesting a purpose for its selective retention within the body (Azzi et al., 2003).

1.5.1 α-Tocopherol as an antioxidant

Vitamin E is the principle chain-breaking antioxidant in cellular and intracellular membranes and lipoproteins halting lipid peroxidation (Burton et al., 1983; Tappel, 1962). Polyunsaturated fatty acids within phospholipids are most susceptible to lipid peroxidation by free radical attack. The antioxidant function of vitamin E maintains the structural integrity of cells.

The ability of α-tocopherol to inhibit the oxidation of LDL in vitro and ex vivo was the original rational behind the protective role of vitamin E in CVD (Devaraj et al., 1997; Esterbauer et al., 1997). The oxidative modification of LDL is considered a key step in the progression of atherosclerosis (Ross, 1993; Ross, 1999; Steinberg et al., 1989).

α-Tocopherol inhibits lipid peroxidation by scavenging lipid peroxyl (LOO•) radicals faster than they can react with adjacent lipid moieties within membranes (Halliwell & Gutteridge, 1999), thus preventing propagation of a chain reaction. The hydroxyl group on α-tocopherol is responsible for lipid peroxyl scavenging activity as demonstrated in figure 1.4. During radical scavenging, α-tocopherol oxidises to an intermediate tocopheroxyl radical, which can be recycled or undergo further oxidation. The non-radical products of tocopherol oxidation vary including α-substituted tocopherones and epoxy (hydroperoxy) tocopherone depending upon what the tocopheryl radical reacts with. These products readily hydrolyse to tocopherylquinone and epoxyquinone respectively. α-Tocopherylquinone is the primary product of tocopherol oxidation and has been detected in human tissues and plasma (Murphy & Kehrer, 1987; Vatassery, 1994). Some of these tocopherylquinone products can be regenerated to α-tocopherol in vivo (Moore & Ingold, 1997).
Figure 1.4 The role of α-tocopherol as a chain-breaking antioxidant adapted from Halliwell and Gutteridge (1999).

α-Tocopherol can scavenge lipid peroxyl radicals (LIPID-OO*), react with O₂⁺ (superoxide radical) and quench singlet oxygen. The tocopheryl radical might also react with a further peroxyl radical to give non-radical products. The pathway of regeneration of α-tocopherol by ascorbate is also shown.

Within LDL membranes there is approximately ten molecules of α-tocopherol for every 400 polyunsaturated, oxidisable lipid groups (Esterbauer et al., 1992). α-Tocopherol levels would be rapidly depleted unless physiological mechanisms existed to reduce the tocopheryl radical back to tocopherol. Moore and Ingold (1997) measured the conversion of tocopherylquinone to tocopherol in humans by giving a male volunteer deuterium labelled tocopherylquinone and detecting deuterium labelled tocopherol. A number of in vitro and indirect in vivo studies have demonstrated the ability of ascorbate to convert the tocopheryl radical back to tocopherol (Kagan et al., 1992; May et al., 1998). However, direct evidence to prove that this happens in vivo is
limited (Hamilton et al., 2000). Ubiquinol can also recycle the tocopheryl radical and there is limited evidence that glutathione is also able to in some membranes, although, the ascorbate-dependent recycling system is likely to be the most important in vivo (Halliwell & Gutteridge, 1999).

Theoretical models and some in vitro studies have indicated that α-tocopheryl radicals may propagate lipid peroxidation within lipoproteins (Bowry et al., 1992; Bowry & Stocker, 1993; Neuzil et al., 2001). However, many researchers believe the importance of pro-oxidation reactions of α-tocopherol in vivo is questionable. In vivo α-tocopherol predominately acts as an antioxidant due to the continual presence of co-antioxidants, such as ascorbate, to convert the tocopheryl radical back to tocopherol (Azzi & Stocker, 2000; Frei et al., 1988).

1.5.2 Molecular functions of α-tocopherol

Vitamin E, specifically α-tocopherol, has the ability to influence cellular events by mechanisms not related to its antioxidant function (Table 1.2). The specificity of α-tocopherol in these processes explains to a large extent why it is selectively retained within the body compared to other vitamin E homologues.

Atherosclerosis involves a combination of oxidative and inflammatory processes with α-tocopherol playing an important role in inhibiting its progression (Kaul et al., 2001). α-Tocopherol has been shown to reduce lipid peroxidation (Esterbauer et al., 1997) and to inhibit platelet adhesion and aggregation (Jandak et al., 1989; Steiner, 1983; Steiner, 1991; Steiner & Anastai, 1976) and smooth muscle cell proliferation (Azzi et al., 1998; Chatelain et al., 1993; Clement et al., 1997). It also exerts anti-inflammatory effects on monocytes (Cachia et al., 1998) and improves endothelial function (Faruqi et al., 1994) in vitro and ex vivo.

A large proportion of cellular events affected by α-tocopherol can be attributed to the inhibitory effect of α-tocopherol on protein kinase C (PKC) activity. PKC is an enzyme with a central role in the regulation of cellular signalling. α-Tocopherol inhibits PKC at a cellular level by activating protein phosphatase (PP2A) which in turn causes dephosphorylation of PKC (Ricciarelli et al., 1998).
Table 1.2 Summary of the functional properties of α-tocopherol encompassing antioxidant and molecular events in the prevention of cardiovascular disease adapted from Ricciarelli et al. (2001)

<table>
<thead>
<tr>
<th>Functions of α-tocopherol</th>
<th>Proposed mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Protects LDL from oxidative damage</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>2. Reduces the respiratory burst in monocytes and neutrophils</td>
<td>Decrease in PKC activity</td>
</tr>
<tr>
<td>3. Reduces the release of cytokines (IL-1β) from monocytes</td>
<td>5-lipoxygenase pathway</td>
</tr>
<tr>
<td>4. Decreases monocytes-endothelial adhesion</td>
<td>NF-κB inhibition</td>
</tr>
<tr>
<td>5. Inhibits platelet adhesion and aggregation</td>
<td>Decrease in PKC activity</td>
</tr>
<tr>
<td>6. Inhibits smooth muscle cell proliferation</td>
<td>Decrease in PKC activity</td>
</tr>
<tr>
<td>7. Down regulates ox LDL scavenger receptors in macrophages</td>
<td>Gene regulation</td>
</tr>
<tr>
<td>8. Enhances lymphocyte differentiation and proliferation</td>
<td>Possibly antioxidant</td>
</tr>
</tbody>
</table>

1. (Devaraj et al., 1997; Esterbauer et al., 1997) 2. (Cachia et al., 1998; Devaraj et al., 1996) 3. (Devaraj & Jialal, 1999; Wu et al., 1999) 4. (Faruqi et al., 1994; Islam et al., 1998) 5. (Jandak et al., 1989; Steiner, 1991; Steiner & Anastai, 1976) 6. (Chatelain et al., 1993) 7. (Devaraj et al., 2001; Teupser et al., 1999) 8. (Lee & Wan, 2002). LDL: low-density lipoprotein, oxLDL: oxidatively modified LDL: PKC: Protein kinase C, IL-1β: interleukin 1β

1.5.2.1 α-Tocopherol prevents LDL oxidation

Oxidatively modified LDL (oxLDL) are central in the aetiopathology of atherosclerosis. OxLDL increase adherence of monocytes to the endothelium and are taken up by macrophages within the arterial sub-endothelium forming foam cells (Steinberg, 1997). The ability of vitamin E to inhibit LDL oxidation ex vivo has been extensively studied (Devaraj et al., 1997; Esterbauer et al., 1997).

1.5.2.2 Functional effects of α-tocopherol on monocytes and macrophages

Monocytes adhere to the endothelium and penetrate into the sub-endothelium differentiating into macrophages. α-Tocopherol appears to influence monocytes and macrophages in a number of ways. α-Tocopherol enrichment of monocytes in vitro decreases monocytes-endothelial cell adhesion (Devaraj et al., 1996; Devaraj & Jialal, 2000; Martin et al., 1997). The mechanism for decreased monocytes-endothelial cell adhesion is due to a decrease in the expression of cellular adhesion molecules on the monocytes by inhibiting NF-κB activation (Cominacini et al., 1997; Islam et al., 1998). Cell culture experiments have shown α-tocopherol inhibits superoxide anion production by impairing the assembly of the NADPH-oxidase by a PKC dependant mechanism (Cachia et al., 1998; Devaraj et al., 1996). The release of superoxide anions from monocytes and neutrophils can induce LDL oxidation (Li & Cathcart,
Monocytes and macrophages also secrete several cytokines including Interleukin-1β (IL-1β); these contribute towards the pathogenesis of atherosclerosis (Bevilacqua et al., 1984; Maziere et al., 1996; Wu et al., 1999). α-Tocopherol inhibits the release of IL-1β via the inhibition of the 5-lipoxygenase pathway (Devaraj et al., 1996; Devaraj & Jialal, 1999).

OxLDL are taken up by scavenger receptors on macrophages which ultimately forming foam cells. α-Tocopherol has been shown to down regulate the expression of the cholesterol scavenger receptors SR-A and CD36 in monocytes and macrophages ex vivo (Devaraj et al., 2001; Teupser et al., 1999). The possibility of reduced oxLDL uptake into macrophages may reduce foam cell formation in vivo.

### 1.5.2.3 α-Tocopherol inhibits smooth muscle cell proliferation

Smooth muscle cell proliferation is central in the progression of atherosclerosis. α-Tocopherol inhibits vascular smooth muscle cell proliferation in cell culture by PKC inhibition (Boscoboinik et al., 1991; Chatelain et al., 1993; Tasinato et al., 1995).

### 1.5.2.4 α-Tocopherol effects on platelet adhesion and aggregation

Platelet adhesion and aggregation is a key factor in thrombosis and CVD pathogenesis (Handin, 1996). α-Tocopherol modulates platelet adhesion and aggregation as demonstrated in supplementation studies and ex vivo experiments (Calzada et al., 1997; Steiner, 1991; Steiner, 1999). Several studies have shown that inhibition of platelet aggregation occurs by a PKC dependent mechanism (Freedman et al., 1996; Freedman & Keaney, 2001; Mabile et al., 1999). Additionally the expression of glycoprotein IIb, a specific receptor for platelet aggregation, is down-regulated by α-tocopherol (Chang et al., 2000). α-Tocopherylquinone also inhibits platelet aggregation in vitro (Freedman & Keaney, 2001).

### 1.5.2.5 α-Tocopherol effects on lymphocyte differentiation and proliferation

Peripheral lymphocytes, predominately T cells, localise to atherosclerotic lesions during the pathogenesis of atherosclerosis (Hansson, 1997; Song et al., 2001). Within the arterial intima, lymphocytes secrete pro-inflammatory cytokines which
activate macrophages (Bach et al., 1997). α-Tocopherol enhances lymphocyte differentiation and proliferation in vitro and ex vivo, possibly via interleukin-2 production, which induces proliferation and differentiation (Beharka et al., 1997; Meydani et al., 1990; Meydani et al., 1986; Moriguchi & Muraga, 2000). α-Tocopherol protects lymphocytes from oxidative damage (Brennan et al., 2000; Metzger et al., 1980; Schneider et al., 2001). Lee & Wan (2002) hypothesised that this was the mechanism behind its effects on lymphocytes.

1.5.3 Functional properties of γ-tocopherol

Researchers have predominately focused on α-tocopherol due to its selective retention within the body and its greater antioxidant capacity (Burton & Ingold, 1981; Traber & Kayden, 1989b). However, γ-tocopherol represents the major dietary source of vitamin E in the American diet (Jiang et al., 2001) and it has only recently been considered potentially important (Galli et al., 2003; Kontush et al., 1999; Ruiz-Rejon et al., 2002).

Although α-tocopherol has a greater antioxidant capacity, γ-tocopherol has a greater ability to neutralise the toxic nitrogen dioxide (Cooney et al., 1993). γ-Tocopherol is able to form a stable nitro adduct, due to its unsubstituted carbon in the chromanol ring (Figure 1.1), compared with the relatively reactive nitrite ester formed from α-tocopherol reaction with nitrogen dioxide (Christen et al., 1997; Hoglen et al., 1997). γ-Tocopherol also inhibits platelet aggregation and delays thrombus formation in rats and ex vivo (Saldeen et al., 1999). It has been hypothesised that this occurs by a mechanism modulating nitric oxide rather than PKC (Liu et al., 2003). Both γ-tocopherol and the γ-tocopherol metabolite, 2,7,8-trimethyl 1-2-(β-carboxyethyl)-6-hydrochroman (γ-CEHC), possess anti-inflammatory properties due to the inhibition of cyclooxygenase activity in macrophages and epithelial cells (Jiang et al., 2000).
1.6 Vitamin E and cardiovascular disease: observational and intervention studies

Antioxidant vitamins including vitamin E have been implicated in the prevention of cardiovascular disease (CVD) for a number of years (Ross, 1993; Steinberg et al., 1989). The more recent findings of the involvement of vitamin E in cellular events (detailed in section 1.5) are further reason to believe vitamin E has a central role in CVD prevention.

Observational studies have predominately shown that diets habitually high in vitamin E are inversely correlated with CVD mortality (Knekt et al., 1994; Kushi et al., 1996; Losonczy et al., 1996; Rimm et al., 1993; Sahyoun et al., 1996; Stampfer et al., 1993). This relationship is clearly illustrated in figure 1.4 from a study reported by Bellizzi et al. (1994). However, there was no relationship found between dietary vitamin E and CVD mortality in the 25 year follow up of the Seven Countries Study (Kromhout et al., 1996).

![Figure 1.4 Relationship between coronary heart disease mortality rates and dietary α-tocopherol (mg/habitual diet/day).](image)

Figure 1.4 Relationship between coronary heart disease mortality rates and dietary α-tocopherol (mg/habitual diet/day).

●: European countries (Au; Austria, Be; Belgium, De; Denmark, Fi; Finland, Fr; France, Ge; Germany (West), Gr; Greece, Ir; Ireland, Is; Israel, It; Italy, NL; Netherlands, No; Norway, Po; Portugal, Sp; Spain, Sw; Sweden, Sz; Switzerland, UK; United Kingdom) ▲: Non-European countries (Al; Australia, Ca; Canada, IC; Iceland, Ja; Japan, Ma; Malta, NZ; New Zealand, US; United States). Correlation for all counties; r = -0.6, European countries; r = -0.8. (Bellizzi et al., 1994)
Chapter 1

The relationship between plasma levels of vitamin E and CVD is less clear. The WHO/MONICA cross-cultural study reported an inverse correlation between plasma α-tocopherol levels and CVD mortality rates (Gey et al., 1991) and lower plasma vitamin E levels were observed in patients with coronary artery disease compared with controls (Singh et al., 1995). However, there are several studies that did not find an association between blood levels of vitamin E and CVD (Hense et al., 1993; Kok et al., 1987; Salonen et al., 1985). There is relatively small variation in plasma vitamin E levels within populations, thus correlations are less likely, compared with the greater variation observed in international studies (Gey et al., 1993). Interestingly, γ-tocopherol levels in plasma have recently been shown to correlate inversely with CVD (Kontush et al., 1999; Ohrvall et al., 1996).

Observational studies have associated α-tocopherol with a reduction in CVD events, but they cannot provide a causal relationship. Table 1.3 shows a summary of large vitamin E intervention trials that have been carried out during the last decade. These test the hypothesis that vitamin E can prevent LDL oxidation and cellular progression of atherosclerosis and therefore have a positive effect on either primary or secondary prevention of CVD. The studies differ in their selection of subjects, the population and hence habitual diet and lifestyle, the type and dose of vitamin E and whether combined with other antioxidant supplements, the study duration and the selected endpoints measured. The combination of these differences make it difficult to compare and interpret the collective outcomes. Collectively the study outcomes are inconsistent between a positive effect and no effect after vitamin E supplementation (Table 1.3).
Table 1.3 Summary of large primary and secondary intervention trials with α-tocopherol and cardiovascular disease events adapted from Kinsky (2003)

<table>
<thead>
<tr>
<th>Study (country)</th>
<th>No. (CVD events)</th>
<th>Study design</th>
<th>Dose α-T (duration)</th>
<th>CVD events reported</th>
<th>Primary endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linxian (China)</td>
<td>27,056 (523)</td>
<td>1°</td>
<td>30 mg* 5-8 years</td>
<td>Death from stroke</td>
<td>+</td>
</tr>
<tr>
<td>ATBC (Finland)</td>
<td>29,133 (1723)</td>
<td>1°</td>
<td>50 mg 5-8 years</td>
<td>Death from all CVD</td>
<td>↔/−</td>
</tr>
<tr>
<td>ASAP (Finland)</td>
<td>520 (6)</td>
<td>1°</td>
<td>270 IU* 6 years</td>
<td>Death from all CVD</td>
<td>+</td>
</tr>
<tr>
<td>PPP (Italy)</td>
<td>4495 (109)</td>
<td>1°</td>
<td>300 mg 3.6 years</td>
<td>Death from all CVD</td>
<td>↔</td>
</tr>
<tr>
<td>VEAPS (USA)</td>
<td>353</td>
<td>1°</td>
<td>400 IU 3 years</td>
<td>Non-fatal MI and stroke</td>
<td>↔</td>
</tr>
<tr>
<td>CHAOS (England)</td>
<td></td>
<td>2°</td>
<td>400-800 IU 1.4 years</td>
<td>Decrease in non-fatal MI</td>
<td>+</td>
</tr>
<tr>
<td>GISSI (Italy)</td>
<td>11,324</td>
<td>2°</td>
<td>250 IU 3-5 years</td>
<td>No effect on fatal and non-fatal CVD</td>
<td>↔</td>
</tr>
<tr>
<td>HOPE (USA/Canada)</td>
<td>9541</td>
<td>2°</td>
<td>400 IU 4.5 years</td>
<td>No effect on fatal and non-fatal CVD</td>
<td>↔</td>
</tr>
<tr>
<td>SPACE (Israel)</td>
<td>196</td>
<td>2°</td>
<td>800 IU 1.4 years</td>
<td>Decrease in CVD</td>
<td>+</td>
</tr>
<tr>
<td>HPS (England)</td>
<td>&gt; 20,536</td>
<td>1°/2°</td>
<td>660 IU* 5 years</td>
<td>No effect on fatal and non-fatal CVD</td>
<td>↔</td>
</tr>
</tbody>
</table>

Linxian China trial (Blot et al., 1993; Mark et al., 1998). ATBC; Alpha Tocopherol Beta Carotene (Virtamo et al., 1998). ASAP; Antioxidant Supplementation in Atherosclerosis Prevention (Salonen et al., 2000). PPP; Primary Prevention Project (Collaborative group of the primary prevention project, 2001). VEAPS; The Vitamin E Atherosclerosis Prevention Trial (Hodis et al., 2002). CHAOS; Cambridge Heart and Antioxidant Study (Stephens et al., 1996). GISSI; Gruppo Italiano per lo Studio Della Sopravivenza nell’Infarto Miocardico (Gruppo Italiano per lo Studio della Sopravivenza nell’Infarto miocardico, 1999). HOPE; Heart Outcomes Prevention Evaluation (The Heart Outcomes Prevention Evaluation Study Investigators, 2000). SPACE; Secondary Prevention Antioxidants of Cardiovascular disease in End stage renal disease (Boaz et al., 2000). HPS; Heart Protection Study (Heart Protection Study Collaborative Group, 2002)

CVD: cardiovascular disease, MI: myocardial infarction, 1°/2°: primary or secondary prevention study design, αT: α-tocopherol, IU: international unit, * Combined supplementation with other antioxidants, + positive effect, − negative effect, ↔ no effect

CVD is a chronic multifactorial disease that starts in childhood and slowly progresses through life (Ross, 1999). The intervention studies tabled were carried out predominantly in middle-aged people and it is very likely that the stage of the disease was reasonably advanced in the study populations and is known to be so in the secondary prevention trials. The antioxidant and molecular functions of vitamin E,
detailed in section 1.5, implicate its involvement in the early stages of the disease, protecting the endothelium and LDL from oxidative damage (Simon et al., 2001). Therefore, as stated by Brigelius-Flohe et al. (2002), subjects with pre-existing CVD are not the ideal target group for vitamin E supplementation. Supplementation from an early age is also not feasible as the number of CVD events would be very small in the early stages of the disease to effectively measure outcome. The end points measured were predominately CVD events rather than atherosclerosis progression itself. Although, the VEAPS and ASAP trials both reported the progression of atherosclerosis by measuring change in intima media thickness, the VEAPS study reported no effect, whereas the ASAP study reported a retarded progression with vitamin C and E supplementation (Hodis et al., 2002; Salonen et al., 2000).

The studies summarised in table 1.3 were predominately carried out in populations with adequate vitamin E status as measured by baseline plasma vitamin E. It has been reported that individuals with plasma vitamin E concentration < 27.5 µM may be at greater risk of CVD (Gey et al., 1993). The studies do not distinguish between those with low or high baseline plasma vitamin E. This may have masked any effect of supplementation in those with sub optimum levels before supplementation. The genetic background of the subjects was also not generally considered. There are known polymorphisms that respond differently to diets and could therefore have had quite a large influence on sub groups within the studies (Paoloni-Giacobino et al., 2003).

The α-tocopherol dose varied between all rac and RRR- isoforms and the amount taken varied considerably from 30 mg to 800 IU between studies (Table 1.3). all rac and RRR α-tocopherol are equally absorbed, however, all rac α-tocopherol is metabolised to a greater extent (Traber et al., 1998a), thus producing a larger concentration of metabolites within the body compared with supplementation with RRR α-tocopherol. Within the studies the amount absorbed between subjects would have also varied due to the well-documented variable amount of vitamin E absorbed and the large inter-individual variation in absorption. Vitamin E needs to be consumed with fat for optimal absorption (Cohn et al., 1992b; Cohn, 1997); this was not always considered in the trials. As a consequence the amount of vitamin E entering the systemic circulation would not have been homogenous within the studies.
A number of the studies gave vitamin E in combination with other antioxidants such as vitamin C, β-carotene or selenium. The ability of vitamin C to regenerate the tocopheryl radical to tocopherol *in vivo* is generally accepted and believed to be influential in the ability of α-tocopherol to act optimally (Hamilton *et al.*, 2000). The ASAP study used combined vitamin E and C supplementation and they reported retarded progression of carotid atherosclerosis (Salonen *et al.*, 2000).

Several systematic reviews and meta-analyses have reported on the collective outcome of the intervention trials. They conclude that the trials have failed to unequivocally demonstrate that vitamin E supplementation has a positive role in slowing the progression of CVD (Asplund, 2002; Hooper *et al.*, 2001; Pryor, 2000; Vivekananthan *et al.*, 2003). This is in contrast with the positive findings of observation studies and summarised in table 1.4 by a meta-analysis of both observation and intervention studies.
### Table 1.4 Meta-analysis of effect of high versus low vitamin E intake on cardiovascular disease mortality for observational and intervention studies from Hooper et al. (2001)

<table>
<thead>
<tr>
<th>Study</th>
<th>High vitamin E (n/total)</th>
<th>Low vitamin E (n/total)</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observational studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knekt (&gt;3 IU)</td>
<td>58/1709</td>
<td>98/1709</td>
<td>0.58 [0.41-0.83]</td>
</tr>
<tr>
<td>Kushi (&gt;30 IU)</td>
<td>50/6897</td>
<td>52/6897</td>
<td>0.66 [0.65-1.42]</td>
</tr>
<tr>
<td>Sahyoun (&gt;30 IU)</td>
<td>22/145</td>
<td>26/145</td>
<td>0.82 [0.44-1.52]</td>
</tr>
<tr>
<td>Stampfer (≤200 IU)</td>
<td>17/17449</td>
<td>25/17449</td>
<td>0.68 [0.37-1.26]</td>
</tr>
<tr>
<td>Rimm (≤400 IU)</td>
<td>16/7982</td>
<td>26/7982</td>
<td>0.61 [0.33-1.15]</td>
</tr>
<tr>
<td>Losonczy (?)</td>
<td>19/359</td>
<td>1082/10819</td>
<td>0.50 [0.32-0.80]</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>182/34541</td>
<td>1309/45001</td>
<td>0.67 [0.46-0.83]</td>
</tr>
<tr>
<td><strong>Intervention studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATBC (50 IU)</td>
<td>853/14564</td>
<td>870/14569</td>
<td>0.98 [0.89-1.08]</td>
</tr>
<tr>
<td>GISSI (300 IU)</td>
<td>310/5660</td>
<td>329/5664</td>
<td>0.94 [0.80-1.10]</td>
</tr>
<tr>
<td>PPP (300 IU)</td>
<td>22/2231</td>
<td>26/2264</td>
<td>0.86 [0.48-1.52]</td>
</tr>
<tr>
<td>HOPE (400 IU)</td>
<td>342/4761</td>
<td>328/4780</td>
<td>1.05 [0.90-1.23]</td>
</tr>
<tr>
<td>CHAOS (≥400 IU)</td>
<td>53/1035</td>
<td>44/967</td>
<td>1.13 [0.75-1.71]</td>
</tr>
<tr>
<td>SPACE (800 IU)</td>
<td>9/97</td>
<td>15/99</td>
<td>0.57 [0.24-1.38]</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>1589/28348</td>
<td>1612/28343</td>
<td>0.98 [0.89-1.06]</td>
</tr>
</tbody>
</table>

Test for heterogeneity p=0.3
Test for overall effect p=0.002

Knekt (Knekt et al., 1994), Kushi (Kushi et al., 1996), Sahyoun (Sahyoun et al., 1996), Stampfer (Stampfer et al., 1993), Rimm (Rimm et al., 1993), Losonczy (Losonczy et al., 1996). ATBC; Alpha Tocopherol Beta Carotene (Virtamo et al., 1998). GISSI; Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto Miocardico (Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto miocardico, 1999). PPP; Primary Prevention Project (Collaborative group of the primary prevention project, 2001). HOPE; Heart Outcomes Prevention Evaluation (The Heart Outcomes Prevention Evaluation Study Investigators, 2000). CHAOS; Cambridge Heart and Antioxidant Study (Stephens et al., 1996). SPACE; Secondary Prevention Antioxidants of Cardiovascular disease in End stage renal disease (Boaz et al., 2000). IU: international unit, CI: confidence interval

The observational studies may represent the beneficial effect of lifelong high habitual intake of vitamin E compared with comparatively short-term high dose supplementation, however, observational studies are inherently confounded by other lifestyle and dietary factors. Although the intervention trials may have reduced the confounding factors, there are discrepancies between their study design and the theory behind vitamin E’s role in CVD prevention.

The lack of a conclusive positive effect of vitamin E on CVD prevention has lead some researchers to question the underlying hypothesis of LDL oxidation in the pathology of atherosclerosis (Jialal & Devaraj, 2003). However, CVD is multifactorial
and the expectation that one micronutrient can slow the progression of atherosclerosis may have been unrealistic.

Knowledge of the regulation and biokinetics of vitamin E within the body and the molecular effects of vitamin E on various cell types involved in atherosclerosis has been rapidly increasing, however, it is still not fully elucidated. It is important to understand these aspects of vitamin E in order to interpret the past intervention studies and possibly for the design of future studies. It may not be possible to conduct the type of study required to conclusively determine the role of vitamin E in CVD prevention, as mentioned by Pryor (2000). As yet, there is no agreed biomarker to accurately determine vitamin E status (Morrissette & Sheehy, 1999) and until this is resolved it will prove difficult to assess the effect of vitamin E status in disease states. Likewise, until an optimal vitamin E status is determined it is unknown who may benefit from an increased intake of vitamin E. Plasma vitamin E concentration is not an ideal biomarker and therefore the cut off, >27.5 μM for adequate vitamin E status, as suggested by Gey et al. (1993) may be too simplistic (discussed in detail in section 1.9.1).
1.7 Vitamin E absorption and biokinetics

Vitamin E, a lipophilic compound, is associated with lipoproteins during absorption and transport within the hydrophobic environment of the systemic circulation. Vitamin E status is closely regulated. The relative proportions of vitamin E isoforms within the diet are not reflected in plasma or tissue concentrations (Jiang et al., 2001; Traber & Kayden, 1989b). Plasma vitamin E concentration is a saturable process and large supranutritional doses of α-tocopherol only result in a 2-3 fold increase from baseline concentration (Dimitrov et al., 1991; Jialal et al., 1995).

1.7.1 Absorption of vitamin E

Vitamin E absorption is reliant on the same intraluminal, membrane and intracellular events that are required for lipid absorption. Lipids must be emulsified and hydrolysed before they can penetrate across the brush border membrane of the enterocytes, this is dependent upon pancreatic function, biliary secretion and micellar formation (Carey et al., 1983). No specific transport proteins have been identified to facilitate vitamin E absorption into enterocytes.

The stomach has an important role in fat digestion, it mechanically breaks up large lipids into smaller particles. There is no appreciable metabolism of α-tocopherol within the stomach and gastric emptying of vitamin E follows lipids (Borel et al., 2001). Partially digested food leaves the stomach and enters the small intestine triggering the secretion of bile into the lumen. Mixed micelles form and are composed of monoglycerides, fatty acids (from lipid hydrolysis) and biliary secretions. Vitamin E is solubilised within these micelles (Carey & Small, 1970; Kayden & Traber, 1993). Patients with cholestatic liver disease or biliary obstruction are unable to secrete bile and thus cannot effectively absorb lipids, or vitamin E, due to the inability to form mixed micelles (Jeffrey et al., 1987; Sokol et al., 1983).

Pancreatic secretions include pancreatic lipase and esterase, these hydrolyse lipids and cleave tocopheryl esters respectively, enabling tocopherol absorption (Carey et al., 1983; Muller et al., 1976). The importance of pancreatic secretions is demonstrated in patients with cystic fibrosis, who are unable to produce sufficient pancreatic secretions and consequently vitamin E passage through the brush border is impaired (Harries & Muller, 1971).
Vitamin E is believed to enter the enterocytes with lipids by passive diffusion. This process is non-saturable, non-carrier mediated and unaffected by metabolic inhibitors (Hollander et al., 1975; Traber et al., 1990b). Following investigations into patients with coeliac disease it has been proposed that the main site for vitamin E absorption is in the proximal small intestine (Muller et al., 1974).

Vitamin E absorption is affected by the amount and composition of lipid with which it is ingested. Bile micelles with lipids have a greater capacity to solubilise vitamin E than pure bile salt micelles in vitro (Cohn et al., 1992b). However, the amount of fat required for maximal vitamin E absorption is currently undetermined (Melia et al., 1996; Parks & Traber, 2000). Long chain polyunsaturated fatty acids (PUFA) ingested with vitamin E reduce its absorption compared to ingestion with saturated fatty acids and medium chain triacylglycerol (Gallo-Torres, 1970; Meydani et al., 1987; Tijburg et al., 1997; Weber et al., 1964). It was hypothesised that vitamin E is utilised within the intestine to protect PUFAs from oxidative damage and subsequently there is less vitamin E available for absorption.

Once within the enterocytes, vitamin E is incorporated into chylomicrons with the newly absorbed lipid (Bisgaier & Glickman, 1983). Although the mechanism of chylomicron assembly has been thoroughly investigated, it remains unknown how vitamin E is incorporated into the chylomicrons (Brigelius-Flohe et al., 2002). Chylomicrons are secreted into the mesenteric lymph and then into the systemic circulation via the thoracic duct. Patients with abetalipoproteinemia are unable to assemble chylomicrons, and as a consequence vitamin E accumulates within the enterocytes. The ingested vitamin E cannot be transported without chylomicron production, hence plasma vitamin E concentrations are extremely low (Kayden, 1972; Muller et al., 1974).

There are only a few human studies which have attempted to measure the fractional dose of vitamin E absorbed (Kelleher & Losowsky, 1970; MacMahon, 1970) (Blomstrand & Forsgren, 1968), the investigators used faecal recovery of radioactive α-tocopherol or collected lymph via a thoracic duct cannula. Investigators agree that vitamin E absorption is incomplete, however the amount absorbed is very variable. Using the faecal recovery of radioactive α-tocopherol, vitamin E absorption has been estimated to be between 50 – 86% (Kelleher & Losowsky, 1970; MacMahon, 1970). These values are likely to be greater than the actual amount as any losses of
radioactivity are included in the calculated amount absorbed (Kayden & Traber, 1993). Measurement of vitamin E absorption by thoracic duct cannulation in two cancer patients showed that 21% of free tocopherol was absorbed over a 24 hour period (Blomstrand & Forsgren, 1968). It is currently unknown why vitamin E is only partially absorbed, considering lipid absorption is almost complete (Carey et al., 1983).

The effect of the dose of dietary vitamin E on the absorption efficacy is also not known and the studies performed so far are few and inconclusive. Traber et al. (1986b) reported that increasing amounts of \( \alpha \)-tocopherol supplementation decreased the percentage of dose absorbed in animals. However, a dose-response increase in newly absorbed \( \alpha \)-tocopherol into plasma was observed up to 150 mg.

Several studies have investigated the plasma appearance of various forms of vitamin E, they have unanimously concluded that \( \text{RRR-} \alpha \text{-, all rac-} \alpha \text{- and } \gamma \)-tocopherol are absorbed without discrimination (Traber et al., 1990a; Traber & Kayden, 1989b). Cheeseman et al. (1995) demonstrated that the absorption of \( \text{RRR-} \alpha \)-tocopherol was not different when orally administered as the free phenol, acetate or succinate ester.

Individuals vary greatly in their rate and magnitude of lipid and vitamin E absorption (McNamara et al., 1987; Roxborough et al., 2000). This inter-individual variation is a result of the gastrointestinal handling of lipids and tocopherols. This encompasses variability in the secretion of bile and pancreatic lipases and processes involved in the packaging of chylomicrons and their release into the systemic circulation. Genetic variation has been proposed as the major determinate of variation in lipid metabolism (Ye & Kwiterovich, 2000). Within the enterocytes, polymorphism in apo B or the chylomicron assembly protein, microsomal transfer protein, may influence the rate of chylomicron secretion and therefore appearance of newly absorbed \( \alpha \)-tocopherol in the systemic circulation (Bergeron & Havel, 1997; Hussain, 2000; Peacock et al., 1995). Fatty acid composition impacts on the postprandial handling of dietary triglycerides (Tinker et al., 1999) and would therefore presumably impact upon vitamin E transport (Parks & Traber, 2000).
1.7.2 Vitamin E transport within chylomicrons

Vitamin E incorporated within chylomicrons enters the systemic circulation via the lymphatic pathway, illustrated in figure 1.5. Chylomicrons are substrates for endothelial bound lipoprotein lipase, which catalyses triglyceride hydrolysis, facilitating the transfer of lipid products and vitamin E to peripheral tissue (Nelsson-Ehle et al., 1980; Traber et al., 1985). The importance of this pathway is demonstrated in patients with lipoprotein lipase deficiency who have low vitamin E levels within adipose tissue but have higher than normal levels in plasma (Kayden, 1983). The loss of triglycerides from chylomicrons and the acquisition of apolipoprotein E from high-density lipoproteins (HDL) lead to the formation of chylomicron remnants. During this process excess surface area is created from the chylomicrons and the resultant phospholipids, apolipoproteins and vitamin E are transferred to HDL (Traber & Kayden, 1989a). Finally the liver takes up the chylomicrons remnants, which are thought to still contain a substantial proportion of the newly absorbed vitamin E (Traber & Sies, 1996).
Figure 1.5. Scheme of the absorption of vitamin E into the systemic circulation, adapted from Traber & Kayden (1989a)

VE: vitamin E, CM: chylomicron, HDL: high-density lipoprotein, Apo E: apolipoprotein E

The enterocytes within the intestine secrete vitamin E within chylomicrons into the lymphatic circulation; they enter the blood via the thoracic duct. A large proportion of the ingested vitamin E is excreted in the faeces without being absorbed. The chylomicrons are catabolised within the circulation by lipoprotein lipase, which transfers fatty acids and vitamin E to peripheral tissues. The resultant chylomicron remnants have excess surface area, which is transferred to HDL along with vitamin E. The chylomicron remnants acquire apolipoproteins E from HDL and are subsequently taken up into the liver.

1.7.3 Selective secretion of RRR-α-tocopherol into very low-density lipoproteins (VLDL); the role of α-tocopherol transfer protein (α-TTP)

The liver has a central role in maintaining plasma vitamin E concentrations and the selective retention of RRR-α-tocopherol within the body as illustrated in figure 1.6 (Traber et al., 1990c; Traber et al., 1990a; Traber & Kayden, 1989b). α- and γ-Tocopherols are equally absorbed into chylomicrons, however, VLDL only contain α-tocopherol (Traber & Kayden, 1989b). Using deuterium labelled RRR-α- and all rac-α-tocopherol investigators have established that RRR-α-tocopherol compared with other stereoisomers is selectively secreted in VLDL (Traber et al., 1988; Traber et al., 1990a).
Vitamin E isomers other than \textit{RRR} \( \alpha \)-tocopherol are presumed to be excreted in bile or by routes as yet undetermined (Brigelius-Flohe \textit{et al.}, 2002). Bjørneboe \textit{et al.} (1987) gave an intravenous injection of chylomicrons labelled with \[^3\text{H}]\alpha\)-tocopherol into rats, 14-20\% of the label was excreted in the bile within the first 24 hours. Increases in bile tocopherol concentrations have also been shown in humans following 300 mg \( \alpha \)- and \( \gamma \)-tocopherol supplementation (Traber & Kayden, 1989b). The mechanism by which vitamin E is secreted into bile is not known. Mustacich \textit{et al.} (1996) demonstrated that \( \alpha \)-tocopherol secretion into bile requires microtubules and suggested a relationship between the phospholipid phosphatidylcholine and biliary output of \( \alpha \)-tocopherol. Interestingly, it has been demonstrated that \( \alpha \)-tocopherol secreted into the bile of rats was reabsorbed into the enterohepatic circulation along with phosphatidylcholine (Mustacich \textit{et al.}, 1998) and enterally infused phosphatidylcholine inhibited \( \alpha \)-tocopherol absorption (Koo & Noh, 2001). The exact meaning of these findings is not clear and further studies are needed to elucidate vitamin E excretion in bile and its reabsorption.
Chapter 1

Figure 1.6 Scheme of the transport and uptake of α-tocopherol within the systemic circulation adapted from Traber & Kayden (1989a)

α-T: α-tocopherol, VE: vitamin E, α-TTP: α-tocopherol transfer protein, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein.

Following chylomicron remnant uptake, the liver secretes VLDL. α-Tocopherol transfer protein (α-TTP) selectively transfers RRR-α-tocopherol into VLDL during its assembly. The other homologues of vitamin E are excreted, presumably into bile. Whilst in the circulation VLDL is catabolised to LDL by lipoprotein and hepatic lipase. Approximately half of VLDL is converted to LDL the remainder is taken up by the liver. During catabolism of VLDL, excess surface area and α-tocopherol is transferred to HDL. Peripheral tissues take up α-tocopherol within LDL via the LDL receptor.

The discrimination between the different homologues and stereoisomers is dependant upon the hepatic α-tocopherol transfer protein (α-TTP), a protein that has been purified and characterised in human liver (Kuhlenkamp et al., 1993). α-TTP belongs to a family of cytosolic lipid-binding and transfer proteins known as the Sec 14 protein family (Kaempf-Rtzoll et al., 2003b). α-TTP preferentially binds RRR-α-tocopherol, the fully methylated chromanol ring, the phytyl side chain and R configuration at the C2 position being essential in the binding of tocopherol (Burton et al., 1998; Hosomi et al., 1997; Ingold et al., 1987b). The relative affinities of the different vitamin E homologue and stereoisomers for α-TTP have been determined by Hosomi et al. (1997) as shown in table 1.5.
Table 1.5 Relative affinities calculated from the degree of competition for α-tocopherol transfer protein (α-TTP) adapted from Hosomi *et al.* (1997)

<table>
<thead>
<tr>
<th>Vitamin E</th>
<th>Relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRR-α-Tocopherol</td>
<td>100%</td>
</tr>
<tr>
<td>SRR-α-Tocopherol</td>
<td>11%</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>38%</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>9%</td>
</tr>
<tr>
<td>δ-Tocopherol</td>
<td>2%</td>
</tr>
<tr>
<td>α-Tocotrienol</td>
<td>12%</td>
</tr>
<tr>
<td>Trolox*</td>
<td>9%</td>
</tr>
</tbody>
</table>

* Trolox is a tocopherol analogue without a phytol side chain

α-TTP transfers α-tocopherol between intrahepatic compartments. It has been demonstrated that it transfers α-tocopherol between liposomes and mitochondria *in vitro* (Mowri *et al.*, 1981). The mechanism by which α-TTP might add RRR-α-tocopherol to VLDL during its assembly remains unknown. Using brefeldin to disrupt the Golgi apparatus, Arita *et al.* (1997) showed that inhibition of VLDL assembly did not affect α-tocopherol secretion. They speculated that α-tocopherol is not assembled into VLDL in the liver cell but rather becomes associated with VLDL after it is secreted. They also reported inhibition of α-tocopherol secretion by 25-hydroxycholesterol, a potent modulator of cholesterol metabolism, linking α-tocopherol secretion to cellular cholesterol metabolism and/or transport. Oram *et al.*, (2001) suggested that this pathway may involve the ATP-binding cassette transporter A1 (ABCA1), a transport protein for cholesterol and phospholipids.

There are a number of identified genetic defects in α-TTP within the human population. Ataxia with vitamin E deficiency (AVED) is a syndrome characterised by neurological abnormalities characteristic of those associated with vitamin E deficiency (Sokol, 1993). Patients with AVED have extremely low plasma vitamin E levels, even though they are able to absorb vitamin E and have normal transport within chylomicrons. However, they have an impaired ability to incorporate vitamin E into VLDL (Traber *et al.*, 1990c). Transfer of vitamin E between lipoproteins is important in α-TTP deficient patients who depend upon this transfer process to incorporate vitamin E from chylomicrons into their VLDL, LDL and HDL (Schuelke *et al.*, 1999; Traber, 1994).

Plasma levels of vitamin E are tightly regulated, large intakes of vitamin E do not increase plasma concentration greater than ~80 μM (Dimitrov *et al.*, 1991; Jialal *et
This saturable process is believed to be due to the saturation of α-TTP within the liver and hence the inability to incorporate additional α-tocopherol into VLDL (Traber, 1994).

1.7.4 Post hepatic transport of α-tocopherol within lipoproteins

α-Tocopherol associated with VLDL is secreted from the liver into the systemic circulation during the fasted state. VLDL is catabolised by lipoprotein lipase and hepatic lipase, illustrated in figure 1.6, approximately half of secreted VLDL are converted to LDL whereas the remainder are returned to the liver (Parhofer et al., 1991). During VLDL catabolism some α-tocopherol is transferred to HDL and peripheral tissues, however the majority of α-tocopherol stays within the delipidated VLDL and either returns to the liver or remains in the LDL.

α-Tocopherol associated with LDL is taken up by peripheral tissues via the LDL receptor (Cohn & Kuhn, 1989). Fibroblasts expressing the LDL receptor have been shown to take up α-tocopherol in vitro (Traber & Kayden, 1984). Wantanabe rabbits with defective LDL receptors do not have reduced tissue concentration of vitamin E (Cohn et al., 1992a). Therefore, although considered an important pathway, LDL receptor uptake is not crucial in maintaining tissue α-tocopherol concentration.

α-Tocopherol is constantly exchanged between lipoproteins via the action of phospholipid transfer protein (PLTP) (Kostner et al., 1995). α-Tocopherol readily exchanges from HDL to other lipoproteins (Traber et al., 1992b). This is less rapid with chylomicrons and VLDL, but during delipidation excess surface is created allowing the exchange of tocopherol between lipoproteins. Furthermore, HDL has been shown to be a more effective donor of α-tocopherol to erythrocytes compared with other lipoproteins (Kayden & Bjornson, 1972; Kostner et al., 1995).

1.7.5 Uptake of vitamin E into blood cells and peripheral tissues

The mechanisms that regulate peripheral cellular uptake and tissue concentration of vitamin E are not fully elucidated. The rate at which newly absorbed vitamin E is taken up by tissues varies. For example, plasma and erythrocytes have relatively quick turnover, whereas this process is slower in muscles and the brain (Burton & Traber, 1990; Traber et al., 1994). Vitamin E uptake from lipoproteins...
involves a number of mechanisms, listed in table 1.6, and it is a combination of these that regulate vitamin E status.

Table 1.6 Mechanisms of vitamin E uptake into blood cells and peripheral tissues

<table>
<thead>
<tr>
<th>Vitamin E uptake mechanism</th>
<th>Cell/ Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lipoprotein lipase</td>
<td>Peripheral</td>
</tr>
<tr>
<td>2. Chylomicron remnant uptake</td>
<td>Liver</td>
</tr>
<tr>
<td>3. Tocopherol binding protein</td>
<td>Erythrocytes</td>
</tr>
<tr>
<td>4. LDL receptor</td>
<td>Lymphocytes and Peripheral</td>
</tr>
<tr>
<td>5. Scavenger receptor class B type 1 receptor</td>
<td>Peripheral</td>
</tr>
<tr>
<td>6. Phospholipid transfer protein</td>
<td>Endothelium</td>
</tr>
<tr>
<td>7. Passive diffusion</td>
<td>Lipoproteins and cells</td>
</tr>
</tbody>
</table>

1. (Sattler et al., 1996; Traber et al., 1985) 2. (Traber & Sies, 1996) 3. (Wimalasena et al., 1982) 4. (Cohn & Kuhn, 1989; Traber & Kayden, 1984) 5. (Mardones et al., 2002) 6. (Kostner et al., 1995) 7. (Oram et al., 2001; Traber et al., 1992b)

Vitamin E distribution in blood cells has only been documented for erythrocytes. Monocytes, neutrophils, lymphocytes and platelets are all functionally affected by vitamin E but little is known about uptake mechanisms. Uptake by these blood cells is presumed to occur during cell formation and/ or passive diffusion from circulating lipoproteins. Lymphocytes have LDL receptors, therefore presumably obtain vitamin E from internalising LDL (Norman et al., 1999; Wilund et al., 2002). Erythrocytes do not have LDL receptors and it has been suggested neither do platelets, and therefore obtain their vitamin E from a different pathway (Kaempf et al., 1994).

Vitamin E binding sites have been identified and characterised for erythrocytes and are thought to facilitate the transfer of vitamin E from plasma to the erythrocytes (Kitabchi & Wimalasena, 1982b; Wimalasena et al., 1982). Vitamin E binding sites have been reported for other peripheral cells such as liver, cultured aortic endothelial cells and rat adrenal cells (Dutta-Roy, 1999; Kitabchi et al., 1980; Kunisaki et al., 1993). However, (Kitabchi & Wimalasena, 1982a) reported from preliminary studies that neither platelets nor polymorphonuclear cells have tocopherol binding.

The scavenger receptor class B type 1 receptor (SR-B1), a membrane bound protein, selectively transports cholesteryl esters from HDL into cells and is capable of transferring vitamin E (Goti et al., 2001; Mardones et al., 2002). This has been shown by uptake from HDL in various cultured cells and SR-B1 knockout mice have reduced concentration of α-tocopherol in bile and some tissues but not others (Mardones et al.,
Vitamin E status has been shown to up and down regulate SR-B1 protein levels within liver tissue (Witt et al., 2000).

The ATP-binding cassette transporter A1 (ABCA1), an ATP-binding cassette protein, transports cellular cholesterol and phospholipids to lipid-poor HDL apolipoproteins and facilitates cellular α-tocopherol secretion (Oram et al., 2001). It is possible that ABCA1 co-transport α-tocopherol, cholesterol and phospholipids to the cell surface where the lipid complex is solubilised and removed by apolipoproteins (Oram & Vaughan, 2000). Oram et al. (2001) suggested that SR-B1 and ABCA1 operate together to transport α-tocopherol between tissues by the HDL reverse cholesterol transport pathway. HDL in vitro is capable of removing α-tocopherol from cells by passive diffusion as well as by the aforementioned active processes.

Genetic polymorphisms in certain genes can influence lipoprotein metabolism and therefore vitamin E tissue uptake and biokinetics (Ye & Kwiterovich, 2000). These encompass apolipoproteins, lipid processing enzymes and lipoprotein receptor genes. Together these would ultimately affect α-tocopherol concentration in tissue and may account for the inter individual variation in response to vitamin E supplementation (Roxborough et al., 2000).

1.7.6 Intracellular distribution and regulation of α-tocopherol

Intracellular α-tocopherol transport, resulting in the accumulation of α-tocopherol in certain cellular and intracellular membranes, is only partially understood. α-Tocopherol distribution is influenced by its lipid solubility and is hence associated with membrane and tissue lipid (Blatt et al., 2001). The lipophilic nature of α-tocopherol means that it requires specific transfer proteins for transport through the hydrophobic cell compartments. Dutta-Roy et al. (1994) reported that α-tocopherol accumulates within the cells where oxygen radical production is greatest and thus where it is required most as an antioxidant namely in the mitochondria membrane and the endoplasmic reticulum. There are a number of tocopherol binding proteins (TBP) that have been identified, it is unclear how many exist and which mechanisms regulate tocopherol transfer between peripheral cells (Dutta-Roy, 1999).

α-TTP is involved in the selective retention of RRR α-tocopherol within the body (Burton et al., 1998; Hosomi et al., 1997; Ingold et al., 1987b). α-TTP, although
predominately found within hepatocytes it is also expressed in the human brain and placenta, the rat brain, spleen, lung and kidney and the mouse uterus (Copp et al., 1999; Hosomi A et al., 1998; Kaempf-Rtzoll et al., 2002; Kaempf-Rtzoll et al., 2003a). An intracellular 15 kDa TBP that specifically binds α-tocopherol is present in all major tissues and it seems likely that it is a general intracellular carrier of α-tocopherol (Dutta-Roy et al., 1993; Dutta-Roy, 1999). The mechanism of how this TBP transports intracellular α-tocopherol is still unknown.

A 46 kDa TBP, the tocopherol associated protein (TAP), has recently been described (Zimmer et al., 2000). TAP is reportedly expressed in all tissues although it is most abundant in the brain, liver and prostate (Brigelius-Flohe et al., 2002; Zimmer et al., 2000). In the presence of α-tocopherol, TAP translocates to the nucleus and activates reporter gene transcription (Yamauchi et al., 2001). Porter (2003) reported that TAP is identical to supernatant protein factor, which stimulates the synthesis of cholesterol. Further studies are needed to clarify the role of TAP (Manor, 2003).
1.8 Vitamin E metabolism and urinary excretion

Vitamin E is metabolised in the liver and specific metabolites have been detected in plasma and urine (Galli et al., 2002; Schultz et al., 1995). It is hypothesised that they are excreted into bile and reabsorbed via the intestine into the systemic circulation before urinary excretion (Hattori et al., 2000; Lodge et al., 2001).

The sole vitamin E metabolite products initially identified had an oxidised chromanol ring, these were derived from vitamin E acting as an antioxidant (Burton & Ingold, 1993; Simon et al., 1956). The primary oxidation product is tocopherylquinone, reduced to tocopherylhydroquinone (Siegel et al., 1997), this and other tocopherol oxidation products are conjugated to yield the glucuronate (Drevon, 1991). These products are excreted into bile or further degraded in the kidneys to tocopheronic acid and excreted in the urine (Burton & Ingold, 1993; Simon et al., 1956). Due to the great interest in the antioxidant function of vitamin E, early studies investigating vitamin E metabolism concentrated on these oxidised products, urinary tocopheronolactones, the so called Simon metabolites (Brigelius-Flohe & Traber, 1999; Burton & Ingold, 1993; Simon et al., 1956). However, Schultz et al. (1995) reported tocopheronolactone was actually a product of oxidation during sample preparation within the laboratory and not an in vivo oxidation product. There is still some disagreement on the ex vivo oxidation of carboxyethyl hydroxycromans (CEHC) to tocopheronolactone (Pope et al., 2000).

1.8.1 Metabolism of vitamin E to carboxyethyl hydroxycromans

Schultz et al. (1995) described a urinary metabolite of vitamin E with an intact chromanol ring, which was identified as 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxycroman (α-CEHC), shown in figure 1.8. Eleven years earlier δ-CEHC was the first urinary metabolite of vitamin E to be discovered with an intact chromanol ring (Chiku et al., 1984). γ-CEHC was first described as α-LLU, a novel natriuretic factor that inhibits the 70 pS ATP-sensitive K⁺ channel in the thick ascending limb cells of the kidney (Wechter et al., 1996). α- and γ-Tocotrienols are excreted as α- and γ-CEHC (Lodge et al., 2001). α- and γ-CEHC have been identified within both the urine and plasma of humans (Galli et al., 2002; Schultz et al., 1995). Previously, α-CEHC was only detected following supplementation (Schultz et al., 1995). The development of more sensitive detection assays revealed that α-tocopherol is
metabolised to α-CEHC without supplementation (Galli et al., 2002; Radosavac et al., 2002; Stahl et al., 1999; Swanson et al., 1999).

The HepG2 cells, a human hepatoblastoma cell line, are capable of synthesising the CEHC metabolites and have been instrumental in characterising the metabolic pathway of vitamin E (Birringer et al., 2001; Parker & Swanson, 2000). Birringer et al. (2001) used HepG2 cells to demonstrate that tocopherols are metabolised by side chain ω-oxidation and consecutive β-oxidation of the phytol tail followed by stepwise removal of two- or three-moieties, ultimately yielding the 3'-carboxychromanol metabolite. Figure 1.8 shows the intermediate products that have been identified. Songtag and Parker (2002) reported that the initial oxidation of the terminal methyl group is catalysed by cytochrome P450 isoform 4F2. They demonstrated that the previously proposed CYP 3A does not possess tocopherol ω-hydroxylase activity, whereas CYP 4F2 exhibited clear NADPH-dependant ω-oxidation of α- and γ-tocopherol. CYP 4F2 catabolism demonstrated in vivo and in vitro discrimination between α- and γ-tocopherol, with greater rate of oxidation for γ-tocopherol (Songtag & Parker, 2002). The enzyme systems that degrade the side chain have yet to be identified.
Figure 1.7 Tocopherol metabolism to carboxyethyl hydroxychroman adapted from Birringer et al. (2001)
Side chain degradation starts with the ω-hydroxylation catalysed by a cytochrome P450 enzyme in microsomes. The hydroxyl group is further oxidised to a carboxylic residue in the cytosol. Subsequent β-oxidation is thought to take place in peroxisomes. Metabolites identified so far are α-CMHHC, α-γ- and δ-CMBHC (Pope et al., 2001) and α-γ- and δ-CEHC. CYP: cytochrome
1.8.2 The involvement of α-tocopherol transfer protein in vitamin E metabolism

Since RRR-α-tocopherol is selectively retained within the body it is degraded to α-CEHC to a lesser extent than the other isoforms of vitamin E (Lodge et al., 2001; Swanson et al., 1999; Traber et al., 1998a). It is generally believed that α-TTP mediates this through the removal of RRR-α-tocopherol from the metabolism pathway (Schuelke et al., 2000). Swanson et al. (1999) reported that a substantial amount of dietary γ-tocopherol is excreted as γ-CEHC, whereas most α-tocopherol consumed is retained within the body.

Schuelke et al. (2000) detected high concentrations of urinary α-CEHC in patients with defective α-TTP despite the fact that they had extremely low plasma vitamin E concentrations. This study supports the case that vitamin E metabolism does not require α-TTP as there was no difference in α-CEHC excretion in α-TTP deficient patients following all-rac-α-tocopherol or RRR-α-tocopherol supplementation.
1.9 Assessing vitamin E status

Vitamin E status can be assessed by measuring dietary intake, clinical examination or biochemical or physiological biomarkers. Dietary measurement is limited by inadequate food databases, the large variability of vitamin E within foods and the inherent problems of people reporting dietary intake. Clinical examination lacks sensitivity, vitamin E deficiency syndromes develop over long periods of time and symptoms of overt toxicity remains unknown (Expert Group on Vitamins and Minerals, 2003). Reliable biomarkers of vitamin E status need to ensure sufficient sensitivity to detect greater \( \alpha \)-tocopherol usage and increased requirements within selected tissues. It is not possible in many cases to obtain this information from humans, therefore indirect biomarkers of tissue \( \alpha \)-tocopherol status, that can be obtained non-invasively, are used (for example blood, urine and saliva). Assumptions are made when using these indirect biomarkers to assess \( \alpha \)-tocopherol status in individuals and how this may relate to disease risk.

Vitamin E is predominately located within biological membranes and adipose tissue. Vitamin E prevents phospholipid peroxidation thereby protecting membrane integrity. \( \alpha \)-Tocopherol has important non-antioxidant functions, these processes would be compromised by vitamin E inadequacy which may increase disease risk. Hence it is important to have reliable indices of vitamin E status. Vitamin E status encompasses vitamin E adequacy in all cellular and intracellular membranes of all tissues. There is no storage organ that releases vitamin E on demand. The bulk of vitamin E within the body is localised in adipose tissue, but is not readily mobilised (Schaefer et al., 1983; Traber & Kayden, 1987). There is limited information on the vitamin E content of human tissues and the rate of turnover. Burton et al. (1998) reported a fast turnover of vitamin E in plasma and liver with the slowest being in adipose tissue and nerves, as determined by uptake of deuterium labelled \( \alpha \)-tocopherol in terminally ill patients.

Certain tissues are exposed to greater oxidative stress and therefore may require greater amounts of vitamin E, for example, skeletal muscle after strenuous exercise, the lungs of cigarette smokers and atherosclerotic arteries with chronic inflammation. It is not clear whether sites of increased vitamin E usage become deficient in vitamin E or if there is increased mobilisation of vitamin E to the site.
Evidence so far is contradictory, there is more \( \alpha \)-tocopherol in atherosclerotic plaque compared with healthy arterial tissue (Iuliano et al., 2003). However, cigarette smokers have lower vitamin E concentration in alveolar fluid compared with non-smokers (Pacht et al., 1986). Understanding how different tissues respond to oxidative stress \textit{in vivo}, their ability to retain or mobilise vitamin E (Elsayed, 2001) and how vitamin E is regulated between tissues is fundamental in the interpretation of biomarkers of vitamin E status.

\( \alpha \)-Tocopherol status is affected by dietary intake (total vitamin E and ratio of isoforms ingested), \( \alpha \)-tocopherol usage (either oxidised or metabolised) and the availability and efficiency of other compounds to recycle oxidised vitamin E (such as ascorbic acid and glutathione). \( \alpha \)-Tocopherol is selectively retained within the body and therefore its status should be considered separately to other isoforms of vitamin E. Researchers are still in the early stages of determining the importance of \( \gamma \)-tocopherols' role within the body (at present government recommendations do not include \( \gamma \)-tocopherol), in the future, attention may be focused at determining \( \gamma \)-tocopherol status and requirements (Committee in Medical Aspects of Food Policy, 1991; Galli et al., 2003).

\textit{1.9.1 Biomarkers of \( \alpha \)-tocopherol status}

Plasma concentrations of \( \alpha \)-tocopherol are commonly used to assess vitamin E status. However, it is generally recognised that steady-state plasma \( \alpha \)-tocopherol is not an ideal indicator of vitamin E status as plasma vitamin E is closely regulated. The relative proportions of vitamin E isoforms within the diet are not reflected in plasma or tissue concentrations (Jiang et al., 2001; Traber & Kayden, 1989b). Plasma vitamin E concentration is a saturable process (Dimitrov et al., 1991; Jialal et al., 1995), influenced by plasma lipids (Horwitt et al., 1972; Thurnham et al., 1986) and there is limited evidence to show that it is not sensitive to altered vitamin E concentrations within cells or tissues (Mezzetti et al., 1995; Simon et al., 1997). Plasma vitamin E is often corrected for cholesterol to remove the confounding effect of plasma lipids. The consequence of higher circulating \( \alpha \)-tocopherol, due to raised plasma lipids, on tissue uptake is not fully understood.
Steady-state plasma α-tocopherol concentration is the combined total of α-tocopherol within lipoproteins. The primary function of lipoproteins is to transport lipids to and from peripheral tissues. α-Tocopherol is there to protect lipids from peroxidation and α-tocopherol distribution is primarily due to its lipid solubility (Blatt et al., 2001). α-Tocopherol content of LDL may be a useful biomarker of vitamin E status, although, interestingly in unsupplemented individuals it does not correlate with plasma α-tocopherol (Ziouzenkova et al., 1996).

Tissue α-tocopherol uptake and retention is a complex process and not directly related to plasma levels (detailed in section 1.7.5). Simon et al. (1997) reported similar plasma α-tocopherol corrected for cholesterol in normocholesterolemic and hypercholesterolemic men, however, there was significantly lower α-tocopherol concentration in the erythrocytes of hypercholesterolemic subjects. Steady state levels of plasma α-tocopherol corrected for cholesterol have been reported to be similar in cigarette smokers and non-smokers (Dietrich et al., 2003; Duthie et al., 1993; Marangon et al., 1998). Mezzetti et al. (1995) reported that plasma α-tocopherol was not related to α-tocopherol levels within arterial tissue and Pacht et al. (1986) reported lower α-tocopherol within the alveolar fluid of cigarette smokers despite similar plasma levels. Two studies investigating the biokinetics of α-tocopherol in cigarette smokers compared with non-smokers have demonstrated that cigarette smokers utilise α-tocopherol differently to non-smokers and this difference cannot be detected in steady state plasma α-tocopherol (Munro et al., 1997; Traber et al., 2001).

Researchers have been interested in the vitamin E content of erythrocytes and their susceptibility to oxidation as an indicator of vitamin E deficiency (Miyake et al., 1991). α-Tocopherol is located within the membranes of erythrocytes and may therefore better represent α-tocopherol status in cellular membranes. It has been proposed that erythrocyte α-tocopherol is a better indicator of vitamin E status in hypercholesterolemic and obese subjects compared with plasma α-tocopherol concentration (Nishida et al., 1982; Simon et al., 1997). However, α-tocopherol concentration within erythrocytes is not specific enough in cigarette smokers. The majority of studies have reported erythrocyte α-tocopherol in cigarette smokers and non-smokers to be similar, whereas biokinetic studies have shown them to differ in
their handling of α-tocopherol (Brown et al., 1997; Brown et al., 1998; Duthie et al., 1991; Munro et al., 1997; Traber et al., 2001). Further research is required to determine whether erythrocyte α-tocopherol is related to other tissue α-tocopherol utilisation and concentration.

α-Tocopherol concentration in platelets has been proposed as a useful biomarker of vitamin E status, by Lehmann (1981) and Vatassery et al. (1983), as they are not influenced by plasma lipid levels. α-Tocopherol concentration within platelets may reflect a direct functional biomarker of vitamin E as platelet adhesion and aggregation are influenced by α-tocopherol (Steiner, 1983). It is unknown if increased usage of vitamin E within the body results in less α-tocopherol within platelets and is currently undetermined how vitamin E concentration within platelets is reflected in other tissue levels.

Kaempf et al. (1994) proposed that cells with LDL receptors, such as lymphocytes, might be more useful biomarkers of vitamin E status than erythrocytes or platelets. Lehmann et al. (1988), Meydani et al. (1990) and Lenton et al. (2000) are the only investigators who have measured the α-tocopherol content of lymphocytes. α-Tocopherol concentration within lymphocytes may represent a functional biomarker of vitamin E status in relation to chronic inflammation (Lehr, 2000; Ross, 1999). It is not known if α-tocopherol content in lymphocytes reflects that in other tissue levels. α-Tocopherol concentrations within other mononuclear and polynuclear leucocytes have also received very little attention. Due to the low circulating volume of leucocytes, compared with erythrocytes, large blood samples are required to measure vitamin E concentration, which is not always practical.

α-Tocopherol concentration within adipose tissue is a long-term indicator of vitamin E intake (Handelman 1994). Adipose tissue α-tocopherol is not readily mobilised and therefore does not represent a sensitive indicator of vitamin E status. Tissues could be vitamin E deficient but the α-tocopherol but levels within adipose tissue may remain unchanged for a number of months or years.

Vitamin E metabolites (tocopherylquinone or α-CEHC) may prove to be useful biomarkers of vitamin E status. Tocopherylquinone (TQ) is the primary product of tocopherol oxidation in vivo. TQ levels could be an indicator of greater oxidative stress, and thus an increased requirement for vitamin E. The measurement of TQ needs
great care (especially when measuring $\alpha$-T:$\alpha$-TQ ratio) as any oxidation of tocopherol during sample preparation would produce inaccurate data. Tocopherolactone, a urinary metabolite of TQ, has been suggested as a biomarker for vitamin E oxidation in vivo (Pope et al., 2000). However, it remains unclear whether or not it is an artefact of CEHC extraction. Schultz et al. (1995) proposed urinary $\alpha$-CEHC as an indicator of adequate vitamin E status on the premise that $\alpha$-tocopherol is only metabolised when plasma levels exceed 7-9 $\mu$mol/g lipid. Since this suggestion was made, $\alpha$-CEHC has been shown to be produced without supplementation and in AVED patients who have a defective $\alpha$-TTP and extremely low plasma vitamin E levels (Schuelke et al., 2000).

In a healthy population it is unknown whether people deficient in vitamin E still produce $\alpha$-CEHC. The regulation of $\alpha$-CEHC production is not fully understood, however it can be hypothesised that extent of $\alpha$-CEHC metabolism, in people with functioning $\alpha$-tocopherol transfer protein ($\alpha$-TTP), may represent the amount of vitamin E excess to requirements within the body (Schuelke et al., 2000; Schultz et al., 1995). $\alpha$-CEHC probably indicates $\alpha$-TTP function and dietary intake but may not be reflective of tissue vitamin E status hence more research is required into $\alpha$-CEHC regulation before it can be considered a useful biomarker.

The requirement for vitamin E and uptake by different tissues will vary between individuals due to the diverse functionality of vitamin E. There is large inter-individual variation in the absorption of vitamin E and uptake into erythrocytes as described by Roxborough et al. (2000). The variations observed include differing activity of enzymes and proteins involved in chylomicron packaging, cellular and tissue uptake, metabolic rate, blood volume, lipid concentration in blood and ratio of lipoproteins (Brigelius-Flohe et al., 2002; Ye & Kwiterovich, 2000). These factors could indicate a large variation in requirements for $\alpha$-tocopherol for optimal supply of vitamin E to all tissues.

In conclusion, no single biomarker for $\alpha$-tocopherol status is known to be representative of $\alpha$-tocopherol status and the measurement of a number of biomarkers are needed to observe whether vitamin E biokinetics and tissue levels are altered in different sub populations and disease states. It is important to gain an understanding of vitamin E status to further elucidate the role of $\alpha$-tocopherol in preventing disease progression. It is also important to understand the extent of inter-individual variation
in the healthy population as this may mask small differences in vitamin E biomarkers. It is difficult to estimate vitamin E requirements on a physiological basis until we have a clear understanding of how different tissues respond to vitamin E intakes, their ability to retain vitamin E and how the mobilisation of vitamin E is regulated.
1.10 Aims and objectives of the current research

It has been well documented that vitamin E is essential for optimal health. Vitamin E protects biological membranes from free radical damage and lipid peroxidation, maintaining membrane integrity. There is also mounting evidence for the role of vitamin E in preventing the pathogenesis of cardiovascular disease (CVD), both as an antioxidant and at a molecular level.

Optimal vitamin E status has yet to be defined, this is primarily due to the difficulties in assessing vitamin E status within humans. The majority of studies have used steady-state plasma vitamin E levels to assess vitamin E status, however, plasma vitamin E concentration is hepatically regulated, saturable, influenced by plasma lipids and limited evidence shows that it is not sensitive to altered concentrations in cells or tissues. A number of steady-state and functional biomarkers have been suggested, although these have not been properly standardised and are not universally accepted. Urinary metabolites (α- and γ- CEHC) have been suggested as potential biomarkers of adequate vitamin E status, however, there is limited information on the exact metabolic pathways and the amount of α-tocopherol converted to α-CEHC. At present there is no adequate biomarker to reliably and accurately measure vitamin E status. Throughout the work presented in this thesis a combination of biomarkers will be used to assess vitamin E status.

It is important to understand and be able to determine vitamin E status when investigating possible factors relating to disease risk. Scientific publications covering vitamin E status in people with increased risk of CVD are limited. Some risk factors for CVD such as dislipidemia and cigarette smoking may predispose these people to an altered vitamin E status. To examine vitamin E within these sub populations further investigation into vitamin E status in healthy individuals is first required.
1.10.1 Research aim

The aim of this research was to use a more holistic approach in assessing vitamin E status, the purpose being to gain a greater understanding of vitamin E status in the healthy population and in those with an increased risk of CVD with a possible altered vitamin E status.

1.10.2 Research objectives

1. To investigate the response of vitamin E concentration within blood components and urinary excretion of vitamin E metabolites to supplemental α-tocopherol in healthy volunteers.

2. To compare the biokinetics of supplemental α-tocopherol between hypercholesterolemic and normolipidemic volunteers

3. To determine steady-state levels of vitamin E status in cigarette smokers compared with non-smokers.
Chapter 2

Materials and Methods
2.1 Materials

The reagents used for sample isolation and assays in the studies reported in Chapters 3, 4, 5, 6 and 7, are listed below;

L-ascorbic acid (Sigma-Aldrich Chemical Co., Poole, UK)
Acetonitrile HPLC grade (Fisher Scientific Ltd, Loughborough, UK)
Bio-Rad Protein assay dye reagent concentrate (Bio-rad laboratories Gmbh, Muchen)
Butylated hydroxytoluene (Sigma-Aldrich Chemical Co., Poole, UK)
Citric acid (Fisher Scientific Ltd, Loughborough, UK)
Cholesterol reagent (ACE™, Alfa Wasserman B.V., 3440 AL Woerden, The Netherlands, supplied by Randox laboratories ltd, Antrim, UK)
Copper sulphate (CuSO₄) (Sigma-Aldrich Chemical Co., Poole, UK)
Cotinine (Sigma-Aldrich Chemical Co., Poole, UK)
Creatinine kit (Randox laboratories ltd, Antrim, UK)
Deferoxamine mesylate (DFO) (Sigma-Aldrich Chemical Co., Poole, UK)
Diethylenetriaminepentacetic acid (DTPA) (Sigma-Aldrich Chemical Co., Poole, UK)
Diethylether (J.T. Baker, Scientific Chemical Suppliers ltd)
Diammonia hydrogen orthophosphate (BDH, Poole, UK)
1-Dodecane sulfonic acid sodium salt for ECD (Sigma-Aldrich Chemical Co., Poole, UK)
Ethanol (absolute) (Hayman Ltd, Witham, Essex, UK)
Folin Ciocalteu reagent (BDH, Poole, UK)
High-density lipoprotein kit (Randox laboratories ltd, Antrim, UK)
Histopaque-1077 (Sigma-Aldrich Chemical Co., Poole, UK)
Lithium perchlorate 99.9 % (Sigma-Aldrich Chemical Co., Poole, UK)
Low-density lipoprotein kit (Randox laboratories ltd, Antrim, UK)
Optiprep™ (XIS-SHIELD Poc AS, Oslo, Norway)
Methanol (LC-MS Chromasolv) (Riedel-deHøn from Sigma-Aldrich Chemical Co., Poole, UK)
Methanol HPLC grade (Fisher Scientific Ltd, Loughborough, UK)
Metaphosphoric acid (MPA) (Sigma-Aldrich Chemical Co., Poole, UK)
Perfluorodecalin (Fluorochem, Derbyshire, UK)
Phosphate-buffered saline (PBS) tablets (Oxoid Ltd, Basingstoke, Berkshire, UK)
Phosphate-buffered saline (PBS) tablets (Oxoid Ltd, Basingstoke, Berkshire, UK)
Potassium bromide (KBr) (Fisher Scientific Ltd, Loughborough, UK)
Potassium hydroxide (KOH) (BDH, Poole, UK)
Sodium acetate (Sigma-Aldrich Chemical Co., Poole, UK)
Sodium azide 99.5 % (Fisher Scientific Ltd, Loughborough, UK)
Sodium bromide (NaBr) (Fisher Scientific Ltd, Loughborough, UK)
Sodium carbonate (NaCO₃) (Sigma-Aldrich Chemical Co., Poole, UK)
Sodium dodecyl sulphate (SDS) (BDH, Poole, UK)
Sodium potassium tartrate (Sigma-Aldrich Chemical Co., Poole, UK)
Tris[hydroxymethyl]aminomethane 99% (Sigma-Aldrich Chemical Co., Poole, UK)
Triglycerides reagent (ACE™, Alfa Wasserman B.V. 3440 AL Woerden, The Netherlands, supplied by Randox laboratories ltd. Antrim, UK)
Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) 97% (Sigma-Aldrich Chemical Co., Poole, UK)
Urinary protein kit (Randox laboratories ltd., Antrim, UK)
Vitamin E capsules (Holland and Barrett, Nuneaton, Warwickshire) Ingredients listed were RRR-α-tocopheryl acetate, gelatine and glycerine shell and soya bean oil.
α-CEHC (>99 %) BSAF, Germany
α-Tocopherol (>99 %) (Fluka Biochemika, Switzerland)
β-glucuronidase (Sigma-Aldrich Chemical Co. Poole, UK)
γ-CEHC (>99 %) (Cayman chemicals, USA)
γ-Tocopherol (>99 %) (Fluka Biochemika, Switzerland)
δ-Tocopherol (>90 %) (Sigma-Aldrich Chemical Co. Poole, UK)
RRR-α-5, 7-(CD₃)₂-tocopheryl acetate (d6) a gift from Cognis Nutrition and Health
all rac-α-5 (CD₃)-tocopheryl acetate (d3) a gift from Cognis Nutrition and Health
2.2 Methods

2.2.1 Subjects

Subjects were recruited by poster advertisements placed around the University of Surrey and Guildford town centre, emails were sent to university staff and advertisements were placed in a number of local newspapers. Subjects were required to complete a personal information sheet (Appendix I), those reporting gastrointestinal problems or any other problems that could possibly affect the studies were not used. Subjects were excluded if they took dietary supplements within 6 months before the study period. Subjects recruited were aged between 20 and 60 years.

The University of Surrey Advisory Committee on Ethics approved the studies documented within this thesis. Subjects supplied written, informed consent before starting a study, they were reimbursed to cover time and inconvenience incurred by participating in a study. The studies took place in the Clinical Investigation Unit at the University of Surrey.

2.2.2 Dietary intake

Habitual dietary intake was recorded using previously validated EPIC 7-day food diaries (Bingham et al., 1997). The diaries were analysed using Diet 5 for Windows (USDA release 12, 1998, Robert Gordon University), this software did not distinguish between the different forms of vitamin E within the diet.

2.2.3 Twenty-four hour urine collection

Volunteers were provided with a 2.5 litre container containing 0.5 g sodium azide as preservative. They were instructed to start collecting once their first morning urination had been discharged and continue collecting until after the first urination the following morning. Volunteers were requested to keep the sample out of direct sunlight and the container was collected from the subject before mid-day. The volume was recorded and two 50 ml aliquots were taken and kept at -20°C until analysis, the samples were analysed within six months.
2.2.4 Blood collection

A suitably trained person drew the blood samples from an antecubital vein in the forearm of subjects, in the studies described in Chapters 3, 5, 6 and 7. For the study detailed in Chapter 4, the blood samples were taken from an intravenous cannula inserted into an antecubital vein in the forearm under local anesthetic by a trained doctor. The blood samples were aliquoted into 10 ml tubes containing ethylenediaminetetraacetic acid (EDTA). Subjects were requested to fast (no food or drink except water) for 12 h before the start of each study.

2.2.5 Plasma isolation

For the isolation of plasma from whole blood the EDTA tubes containing the blood samples were centrifuged at 1550 g for 10 minutes at 4 °C. The plasma was carefully removed for lipoprotein isolation, as detailed in section 2.2.9.

During the studies detailed in Chapters 3, 5, 6 and 7 plasma was isolated after the removal of platelets and erythrocytes (as illustrated in figure 2.1), to minimize the amount of blood required from the volunteers.

Plasma samples were aliquoted into cryo tubes, containing 10 μl butylated hydroxytoluene (BHT) (1 mg/ ml ethanol), snap frozen in liquid nitrogen and stored at −80 °C until analysis; the samples were analysed within six months.

2.2.6 Platelet isolation

Platelets were isolated from whole blood using a method described by Lehmann et al (1988) (Lehmann et al., 1988). Ten ml whole blood was centrifuged at 280 g for 14 minutes at a pre-cooled temperature of 2 °C. The platelet rich plasma was transferred into a flat-bottomed glass tube and subsequently spun at 1120 g for 15 minutes at 2 °C, the bottom layer was used to isolate erythrocytes (section 2.2.7). The platelet poor plasma was transferred into a cryo tube (and stored at −80 °C until analysis), leaving a visible opaque disc of platelets at the base of the tube. A 2 ml Tris buffer (134 mmol/L NaCl, 5 mmol/L KCl and adjusted to pH 7.4 with HCl) was added and gently aspirated with a Pasteur pipette to agitate the platelets. The platelets were spun at 1120 g for 15 minutes at 2 °C. The supernatant was removed leaving the washed platelets at the base of the tube, 1 ml Tris buffer was aliquoted into the tube
with the platelets and gently aspirated with a Pasteur pipette. The platelets were aliquoted equally, in duplicate, into cryo tubes (containing 10μl BHT (1 mg/ ml ethanol)), snap frozen in liquid nitrogen and stored at −80 °C until analysis. Samples were analysed within six months.

Figure 2.1 Summary of blood separation procedures for the isolation of plasma, erythrocytes, platelets and lymphocytes from whole blood.

2.2.7 Erythrocyte isolation

Erythrocytes were isolated from the infranatant after the platelet rich plasma was removed (illustrated in figure 2.1). The sample was centrifuged at 1550 g for 10 minutes at 4 °C. The plasma was removed and the leucocytes ‘buffy coat’ discarded. The erythrocytes were washed by addition of 4 ml saline, then mixed by inversion. The erythrocytes and saline solution was centrifuged at 700 g for 10 minutes at 4 °C, the supernatant was then discarded. This washing process was repeated twice. The washed packed cells (erythrocytes) were measured for haematocrit. Through capillary action the erythrocytes travelled up the capillary tube, these capillary tubes were
placed in a haemo centrifuge (Heraeus Biofuge, Kendro laboratory products GmbH, Germany) and centrifuged at 13,000 g for 10 minutes. The percentage haematocrit was determined by placing the capillary tube against a chart supplied by the manufacturers and reading the percentage packed cells. The remaining erythrocytes were equally aliquoted into cryo tubes (containing 10 μl BHT (1 mg/ ml ethanol) and 10 μl deferoxamine mesylate (DFO) (1 mg/ ml ethanol)), snap frozen in liquid nitrogen and stored at -80 °C until analysis. Samples were analysed within six months.

2.2.8 Lymphocyte isolation

Lymphocytes were isolated from whole blood using Histopaque-1077, a solution of polysucrose and sodium diatrizoate, adjusted to the density of 1077 g/ ml as instructed by the manufacturers. During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sediment, whereas, lymphocytes and other mononuclear cells remain at the plasma-Histopaque-1077 interface. The manufacturers state a 68 ± 13% recovery of lymphocytes from whole blood.

Ten ml Histopaque-1077 was aliquoted into a 50 ml centrifuge tube and allowed to reach room temperature before 10 ml whole blood was carefully layered on top, so as not to disturb the Histopaque-1077. The tube was centrifuged at 400 g for 30 minutes at room temperature. The plasma layer was removed to within 0.5 ml of the opaque interface, the 0.5 ml near the opaque interface was discarded. The opaque interface, which contained the lymphocytes, was carefully transferred into a second falcon tube, taking care not to remove any of Histopaque-1077 from the layer below. The lymphocytes were washed with phosphate buffered saline (PBS; 1mg /ml in distilled water). A 10 ml aliquot of PBS was added to the lymphocytes and centrifuged at 250 g for 10 minutes at room temperature. The supernatant was removed leaving the lymphocyte pellet at the base of the tube. Five ml PBS was aliquoted into the tube and the lymphocytes were gently aspirated with a Pasteur pipette, this was then spun at 250 g for 10 minutes at room temperature. The washing process was repeated twice more. After the final wash 0.5 ml PBS was added to the lymphocyte pellet at the base of the tube and gently aspirated with a Pasteur pipette. The lymphocytes in PBS were aliquoted into a cryo tube (containing 10μl BHT (1 mg/ ml ethanol), snap frozen in
liquid nitrogen and stored at -80 °C until analysis, samples were analysed within six months.

2.2.9 Isolation of lipoproteins from plasma

For the study detailed in Chapter 4 chylomicrons were isolated from plasma directly obtained from whole blood. Whereas for the studies reported in Chapters 5 and 6, lipoproteins were isolated from frozen plasma retrieved from the procedure to isolate lymphocytes (shown in figure 2.1).

2.2.9.1 Isolation of chylomicrons

The isolation of chylomicrons from plasma was performed by a method adapted from Weintraub et al. (1987b). In reusable Beckman ultracentrifuge tubes (16 × 76 mm) 4 ml plasma was overlaid with 4 ml 0.9% sodium chloride solution (density (d) = 1.006 g/ml). The samples were placed into a 70.1 Ti Beckman Coulter rotor then centrifuged at 110,000 g for 15 minutes at 21°C, max acceleration, deceleration 5 (Beckman Opima XL-1000 ultracentrifuge, USA) to float the chylomicron particles (d = 0.095 – 1.006 g/ml). The top 1 ml, chylomicron fraction, was recovered with a syringe and needle into a 1 ml volumetric flask. For the study detailed in Chapter 4 chylomicrons were isolated from fresh plasma and subsequently aliquoted equally into cryo tubes (containing 10 µl BHT (1 mg/ ml ethanol)), snap frozen in liquid nitrogen and stored at -80 °C until analysis; samples were analysed within six months. In the studies detailed in Chapters 5 and 6 the chylomicrons were isolated from frozen plasma and were analysed immediately for cholesterol, triacylglycerol, protein and vitamin E content.

2.2.9.2 Isolation of very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) by sequential ultra centrifugation

For the isolation of VLDL and IDL, LDL and HDL the chylomicron free plasma was retrieved, obtained as described in section 2.2.9.1, a steel cannula was placed (2.5 mm diameter) into the tube and 4 ml of plasma drawn up using a syringe. VLDL and IDL, LDL and HDL were subsequently isolated by sequential ultra centrifugation from this plasma at their respective hydrated densities: VLDL and IDL
(d 1.006 – 1.019 g/ ml), LDL (d 1.019 – 1.0639 g/ ml) and HDL (d 1.210 g/ ml). Density solutions (sodium bromide; NaBr 1.019 g/l and 1.182 g/ ml) were confirmed by a digital density meter (Parr Scientific, UK). Appendix II details the formula use to make density solutions.

To isolate VLDL and IDL, 4 ml chylomicron free plasma was transferred into Beckman Ultraclear centrifuge tubes (16 x 76 mm), 0.32 ml NaBr solution (1.182 g/ ml) was added to adjust density to 1.019 g/ml, the volume was made up to 11 ml with 6.68 ml NaBr solution (1.019 g/ ml). The tubes were mixed by inversion then placed into a 70.1 Ti Beckman Coulter rotor and centrifuged at 15,000 g for 20 h at 15°C, max acceleration and max deceleration (Beckman Opima XL-1000 ultracentrifuge, USA) to float the VLDL particles (d 1.006 – 1.019 g/ ml). The top 2 ml were recovered with a syringe and needle into a 2 ml volumetric flask taking care not to disturb the lipoproteins in the infranatant, a further 1 ml was discarded.

To isolate LDL, 2.94 ml NaBr solution (1.182 g/ ml) was added to the tube and the density of the solution adjusted to 1.063 g/ ml. With a glass pipette the gelatinous pellet was disrupted. The tubes were mixed by inversion then centrifuged under the same conditions used for VLDL isolation. The LDL particles (d 1.019 – 1.063 g/ ml) floated to the top. The top 2 ml was recovered with a syringe and needle into a 2 ml volumetric flask taking care not to disturb the lipoproteins infranatant.

To isolate HDL, 2.1125 g potassium bromide was added to the infranatant adjusting the density of the solution to 1.21 g/ ml. The tubes were mixed by inversion then centrifuged under the same conditions used for VLDL and LDL isolation. The top 1 ml was recovered with a syringe and needle into a 1 ml volumetric flask.

Aliquots of the isolated lipoprotein fractions were immediately analysed for cholesterol, triacylglycerol, protein content and vitamin E.

2.2.9.3 Separation of LDL subclasses by self generated gradient of iodixanol

A method described by Davies et al. (2003) was used for the separation of LDL subclasses from plasma. According to this method 7.8 ml 9 % iodixanol/ PBS solution was transferred into a Beckman Optiseal™ centrifuge tube (16 x 67 mm). In a separate tube 2.8 ml plasma and 0.7 ml Optiprep (60 % Iodixanol w/v) were mixed by inversion. Using a steel cannula (2.5 mm diameter) and syringe, 3 ml of plasma/iodixanol solution was under-layered into the centrifuge tubes containing the
9% iodixanol solution, care was taken not to introduce air bubbles. Optiseal plugs were placed on the tubes and secured using the Beckman plug sealer. The tubes were left to settle for 30 minutes before being placed in the Beckman NVT65 near-vertical rotor. Bronze spacers were placed over the tubes and tightened to a torque of 180 inch pounds. The samples were centrifuged at 341,000 g for 3 hours at 16 °C, acceleration and deceleration programs 5 (Beckman Opima XL-1000 ultracentrifuge, USA).

Tubes were removed from the rotor, taking care not to disturb the gradient. The top 500 µl was discarded before fractionation to minimize the contamination from chylomicrons or VLDL. The tubes were placed in a Beckman Fraction Recovery System and the tubes were pierced by a needle assembly attached to an infusion pump. The gradient was upwardly displaced by a dense, hydrophobic displacement fluid (Perfluorodecalin), and 300 µl fractions were collected using RediFrac fraction collector (Pharmacia Biotech). The fractions were frozen and stored at −80 °C until analysis, the samples were analysed within six months.

The cholesterol content of the LDL sub fractions were measured as described in section 2.2.11. The cholesterol values were plotted on a graph to determine the individual profiles and adjacent fractions were pooled together for vitamin E analysis, as shown in appendix III.

2.2.10 Determination of plasma and lipoprotein triacylglycerol

Plasma and lipoprotein triacylglycerol concentrations were measured automatically by the SPACE biochemical analyser (Alfa Wasserman, The Netherlands), using ACE™ Triglycerides reagent. The reactions involved are outlined below:

\[
\text{Triacylglycerol} \xrightarrow{\text{lipases}} \text{glycerol + fatty acids}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate + H}_2\text{O}_2
\]

\[
\text{Glycerol-3-phosphate} \xrightarrow{\text{oxidase}} \text{dihydroxyacetone phosphate + H}_2\text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 + 4 \text{Aminoantipyrin + 4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine + HCl + 4 H}_2\text{O}
\]
Chapter 2

The end product, quinoneimine was measured at 505 nm/ 692 nm and its concentration was directly proportional to triacylglycerol concentration. The range of quantitation of the assay was 0.02 – 11.4 mmol/ L. Quality control samples were inserted at the beginning of every assay; if the value fell outside 2 standard deviations of the mean the assay was repeated. The inter-assay coefficient of variation (CV) for QCs was less than 2 %.

2.2.11 Determination of plasma and lipoprotein cholesterol

Plasma and lipoprotein cholesterol concentrations were measured automatically by the SPACE biochemical analyser (Alfa Wasserman, The Netherlands), using ACE™ Cholesterol reagent. The reactions involved are outlined below:

\[
\text{Cholesterol esterase} \quad \text{Cholesterol ester} + H_2O_2 \rightarrow \text{cholesterol} + \text{fatty acids}
\]

\[
\text{Cholesterol oxidase} \quad \text{Cholesterol} + O_2 \rightarrow \text{cholestene-3-one} + H_2O_2
\]

\[
2H_2O_2 + \text{phenol} + \text{4- Aminoantipyrin} \rightarrow \text{quinoneimine} + H_2O
\]

The end product, quinoneimine was measured at 505 nm/ 692 nm and was directly proportional to cholesterol concentration. The limit of quantitation for the assay is 19.3 mmol/ l. Quality control samples were inserted at the beginning of every assay; if the value fell outside 2 standard deviations of the mean the assay was repeated. The inter-assay coefficient of variation (CV) for QCs was less than 2 %.

2.2.12 Determination of low-density lipoprotein (LDL) cholesterol

Plasma LDL cholesterol were measured automatically by the SPACE biochemical analyser (Alfa Wasserman, The Netherlands), using ACE™ LDL cholesterol reagents.

In the initial reaction eliminated chylomicrons, VLDL and HDL

\[
\text{Cholesterol esterase} \quad \text{Cholesterol ester} + H_2O_2 \rightarrow \text{cholesterol} + \text{fatty acids}
\]

\[
\text{Cholesterol oxidase} \quad \text{Cholesterol} + O_2 \rightarrow \text{cholestene-3-one} + H_2O_2
\]
\[ 2 \text{H}_2\text{O}_2 \xrightarrow{\text{cholesterol oxidase}} 2 \text{H}_2\text{O} + \text{O}_2 \]

Then LDL cholesterol was released by detergents in reagent 2

\[ \text{cholesterol esterase} \]
\[ \text{Cholesterol ester} + \text{H}_2\text{O}_2 \xrightarrow{} \text{cholesterol} + \text{fatty acids} \]

\[ \text{cholesterol oxidase} \]
\[ \text{Cholesterol} + \text{O}_2 \xrightarrow{} \text{cholestene-3-one} + \text{H}_2\text{O}_2 \]

\[ 2 \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{TOOS} \xrightarrow{\text{peroxidase}} \text{quinonine pigment} + 4 \text{H}_2\text{O} \]

TOOS; N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline

The intensity of the quinonine pigment was directly proportional to cholesterol concentration at 600nm. Quality control samples were inserted at the beginning of every assay; if the value fell outside 2 standard deviations of the mean the assay was repeated.

2.2.13 Determination of high-density lipoprotein (HDL) cholesterol

Plasma HDL cholesterol were measured automatically by the SPACE biochemical analyser (Alfa Wasserman, The Netherlands), using ACE™ HDL cholesterol reagents.

In the initial reaction eliminated chylomicrons, VLDL and LDL

\[ \text{cholesterol esterase} \]
\[ \text{Cholesterol ester} + \text{H}_2\text{O}_2 \xrightarrow{} \text{cholesterol} + \text{fatty acids} \]

\[ \text{cholesterol oxidase} \]
\[ \text{Cholesterol} + \text{O}_2 \xrightarrow{} \text{cholestene-3-one} + \text{H}_2\text{O}_2 \]

\[ 2 \text{H}_2\text{O}_2 \xrightarrow{\text{cholesterol oxidase}} 2 \text{H}_2\text{O} + \text{O}_2 \]
Then HDL cholesterol was released by detergents in reagent 2

\[ \text{Cholesterol ester} + \text{H}_2\text{O}_2 \to \text{cholesterol} + \text{fatty acids} \]

\[ \text{cholesterol esterase} \]

\[ \text{Cholesterol} + \text{O}_2 \to \text{cholestene-3-one} + \text{H}_2\text{O}_2 \]

\[ \text{cholesterol oxidase} \]

\[ 2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{HDAOS} \to \text{quinonine pigment} + 4\text{H}_2\text{O} \]

\[ \text{peroxidase} \]

HDAOS; N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline

The intensity of the quinonine pigment was directly proportional to cholesterol concentration at 600nm. Quality control samples were inserted at the beginning of every assay, if the value fell outside 2 standard deviations of the mean the assay was repeated.

2.2.14 Determination of protein in platelets and lymphocytes

The isolated platelets and lymphocytes (isolation procedure detailed in sections 2.2.6 and 2.2.8) were defrosted at room temperature then sonicated for 3 minutes to ensure a homogenous sample. Lymphocytes were diluted 1 in 3 with PBS before analysis. Samples were analysed in duplicate and the mean values was used.

The protein content in platelets and lymphocytes was determined automatically by SPACE biochemical analyser (Alfa Wasserman, The Netherlands) using a colorimetric urinary protein kit (validated for use in platelets and lymphocytes as described in section 2.2.14.1). The method is based on the formation of pyrogallol red complexes with proteins in an acid environment containing molbdate ions, whose optical density at 600nm is directly proportional to the protein concentration of the samples.

2.2.14.1 Validation of urinary protein kit with the modified Lowry protein assay

The urinary protein (UP) kit was designed to measure protein in urine, however, due to the high sensitivity of the assay it was used to measure the protein content in the isolated platelets and lymphocytes in the investigations described in this thesis. The method was validated as the protein in platelets is suspended in a Tris buffer and the lymphocytes in PBS. The modified Lowry protein assay was used to
validate the protein measurement by the urinary protein kit as it is a sensitive assay (Lowry & Rosebrough, 1951).

The modified Lowry protein assay was carried out as follows; 2 ml Biuret reagent (100 ml 2 % Na₂CO₃ in 0.1 M NaOH w/v solution, 1 ml 2 % NaK Tartrate w/v solution and 1 ml 1 % CuSO₄ w/v solution) was added to 400 μl standards, quality controls and samples, then vortexed and left to stand for 10 minutes. Folin Coicalteu reagent was diluted 1:1 with distilled water, 200 μl was then added to the standards, QCs and samples and immediately vortexed. They were left to stand for 30 minutes and the absorbance at 750 nm was then measured on a spectrophotometer (Beckman Du 650, USA). A standard curve was generated from standards, protein concentration for QCs and samples were calculated from the equation of the standard curve. Standards were prepared from a 1 mg/ ml human albumin stock solution, provided with the urinary protein kit, diluted with distilled water to range from 0 – 50 μg protein.

The validation of the urinary protein kit to accurately measure protein in platelets and lymphocytes was performed by measuring the protein content in six platelet and six lymphocyte samples (n = 12), using the UP kit and the modified Lowry protein assay. A close correlation was observed between the two methods (r² = 0.94). The Bland and Altman test was used to assess the agreement between methods; the mean difference was 0.017 g protein with 95 % confidence interval of + 0.38 or - 0.18 mg/ ml protein (Bland & Altman, 1986).

The reproducibility of the UP kit to measure protein in platelets and lymphocytes was determined by the coefficient of variation, with an n of 12 over the course of 3 days. The coefficient of variation for platelet and lymphocyte protein was 4.5 and 5.3 %, respectively.

### 2.2.15 Determination of protein in lipoproteins

The protein content of lipoproteins were measured using the method described by Bradford (1976). Bio-Rad protein assay dye reagent concentrate was diluted 1 : 5 with distilled DDI water then filtered through a nylon membrane filter 0.2 μm (47 mm Whatman, Maidstone, UK). Standard protein solutions were diluted from stock solution of 1 mg/ ml human albumin serum with PBS, to a range from 0.05 to
0.5 mg/ml. Into a microtiter 96 well plate 10 μl of standards and samples were dispensed in triplicate. To each well 200 μl of diluted dye reagent was added, then mixed in microplate mixer for 10 seconds. Samples were left to incubate at room temperature for 5 minutes. Absorbance was measured at 620 nm on a spectrophotometer equipped with Genesis II software (Windows™ based, version 3.05 Labsystems Multiskan, Biochromatic). A standard curve was generated in Microsoft Excel and the linear equation was used to calculate the concentration of samples.

2.2.16 Ascorbic acid extraction from plasma

A method described by (Omaye et al., 1987) was used for the isolation of ascorbic acid was isolated from fresh plasma by the addition of 1 ml 5 % metaphosphoric acid in 1 mM diethylenetriaminepentacetic acid (made fresh daily) to 1 ml plasma, mixed by inversion and spun at 700 g for 5 minutes at room temperature. The supernatant was equally aliquoted into 0.5 ml tubes. The samples were stored at -80°C until analysis. Samples were analysed within six months.

2.2.17 Ascorbic acid quantification with HPLC with electrochemical detection

A method described by Omaye et al. (1987) was used for HPLC quantification of ascorbate. The ascorbic acid samples were defrosted at room temperature. To 10 μl sample, 10 μl 1 mM DTPA and 74 μl mobile phase (see below for composition) were added together and the mixture was vortexed. A 20 μl aliquot was analysed by HPLC with electrochemical detection (ECD). The HPLC system was a 2690 Waters Alliance System (Waters Ltd, Elstree, UK) comprising a solvent delivery system, online degasser, peltier-cooled autosampler (set at 4 °C), controller and column oven (set at 25°C) in conjunction with an LC-4C amperometric ECD (Bioanalytical Systems, Lafayette, USA) equipped with a glassy-carbon working electrode and a Ag/AgCl/gel reference electrode operating with an applied potential of ± 0.5 V. The mobile phase consisted of 40 mM sodium acetate, 0.51 mM DTPA, 1.5 mM 1-dodecane sulfonic acid sodium salt for ECD and 7.5 % (v/v) methanol adjusted to pH 4.75 with glacial acetic acid, flow rate was delivered at 1 ml/ min. The ascorbic acid was separated using a Supelcosil™ LC-8 column (150 x 4.6 mm 5μm) (Supelco, USA).
Chromatogram peaks were processed using Waters Millennium ®32 software and quantitated using an external calibration curve prepared with ascorbic acid standards. Calibration curve consisted of 0.25, 0.5, 0.75 and 1.0 nmoles generated from different injection volumes of a10 μM ascorbic acid stock solution.

2.2.18 Vitamin E extraction from blood components by solvent extraction

All vitamin E extractions from biological samples were carried out by the following method adapted from Burton et al. (1985). For analysis by LC/MS plasma was diluted 1 : 5 with PBS prior to vitamin E extraction. Platelet and lymphocyte samples were sonicated for 3 minutes prior to vitamin E extraction to ensure homogeneity of the sample.

Into a glass screw top tube, 5 μl internal standard (500 μM δ-tocopherol for HPLC analysis, 10 μM d3 all rac-α-tocopherol for LC/MS analysis) was added at the start of each extraction, followed by 25 μl BHT (1 mg/ml ethanol), 100μl sample and 900 μl 0.1 M sodium dodecylsulphate. Samples were vortexed, then 2 ml absolute ethanol was added. Samples were vortexed, then 4.5 ml hexane was added and subsequently vortexed. The samples were centrifuged at 700 g for 3 minutes at 5 °C. Three ml of the hexane upper layer was aliquoted from each sample into a new glass tube using a displacement pipette. The hexane was dried down completely under nitrogen. For analysis by HPLC, 100 μl absolute ethanol was immediately added to the dried sample. For analysis by LC/MS, 500 μl absolute ethanol was immediately added to the dried plasma sample while 200 μl absolute ethanol was added for all the other dried blood component samples. Samples were extracted in duplicate and quality controls were extracted with each extraction. Fifty μl reconstituted sample was placed into Waters vials for analysis, kept at 4 °C until analysis which took place within 24 hours of extraction.

2.2.19 Vitamin E extraction by saponification

The vitamin E content of the vitamin E capsules used in the studies detailed in Chapters 3, 4, 5, and 6 were analysed by the following method adapted from Kayden et al. (1983).
Approximately 10 mg oil was placed into a glass screw top tube, 900 µl water (1 % ascorbic acid) and 2 ml ethanol (1 % ascorbic acid) were added and the tube was vortexed. Subsequently 0.3 ml saturated KOH was added and vortexed. The samples were incubated at 70 °C for 30 minutes, then rapidly cooled on ice. After cooling, 25 µl BHT and 1 ml water (1 % ascorbic acid) were added to the samples and vortexed. Four ml hexane was added, samples were vortexed then allowed to separate. The top 3 ml of the hexane layer was transferred to a new glass tube using a displacement pipette. The hexane was completely dried down under nitrogen. Approximately 10 ml absolute ethanol was used to reconstitute the oils extracted from the vitamin E capsules in order to perform HPLC analysis. Approximately 5 ml absolute ethanol was used to reconstitute the hydrolysed d6 RRR-α-tocopherol and d3 all rac-α-tocopherol standards for the preparation of stock solutions.

2.2.20 Vitamin E quantification using HPLC with electrochemical detection

Vitamin E was quantified by HPLC with electrochemical detection (ECD) using a method adapted from Podda et al. (1996). The vitamin E concentration of plasma, erythrocytes, platelets and lymphocytes isolated for the studies detailed in Chapters 3 and 7 were quantified using the following method.

The HPLC system used was the same as described in section 2.2.17. The mobile phase consisted of 99 % methanol, 1% water and 0.1 % lithium perchlorate filtered through 0.2 µm pore size nylon membrane filters (47 mm Whatman Maidstone, UK). The tocopherols were separated using a reverse phase Waters Spherisorb® ODS-2 column (250 mm × 4.6 mm, C18, 5µm particle size) at a flow rate of 1.5 ml/ min. Tocopherols peaks were identified from retention times, δ-, γ- and α-tocopherol eluted at 9.2, 10.9 and 12.9 minutes respectively. Chromatogram peaks were identified and quantified using internal standard (6-tocopherol) with Waters Millennium ©32 software. Quality control samples were inserted at the beginning of every assay, and the inter-assay coefficient of variation (CV) for the assay was 12 %.

α-, γ- and δ-Tocopherol standards were made up from pure oils (α-, γ-tocopherol > 99%, δ-tocopherol > 90%) diluted in absolute ethanol. The exact concentrations were determined by measuring their absorbance on a spectrophotometer and their concentrations were calculated using Beer-Lamberts law.
(absorption/ extinction coefficient = concentration), wavelength and extinction coefficients were ascertained from Kofler et al. (1962) and are reported in table 2.1. Standard tocopherol solutions were stored at -20 °C and their concentrations were regularly checked.

**Table 2.1 Physiochemical data for α-, γ- and δ-tocopherol**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Molecular weight</th>
<th>Wavelength λ max (nm)</th>
<th>Extinction coefficient (e) for ethanol solutions (M⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol</td>
<td>430.7</td>
<td>292</td>
<td>3270</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>416.7</td>
<td>298</td>
<td>3810</td>
</tr>
<tr>
<td>δ-Tocopherol</td>
<td>402.7</td>
<td>298</td>
<td>3520</td>
</tr>
</tbody>
</table>

From Kofler et al. (1962)

2.2.20.1 Validation of δ-tocopherol as an appropriate internal standard

Previous researchers have quantified tocopherols using an external calibration curve (Podda et al., 1996). Quantification using an external calibration curve cannot take into account losses incurred during the extraction process, hence an internal standard method was developed to quantify α- and γ-tocopherol. δ-Tocopherol was validated as an appropriate internal standard; its retention time was at least one minute less then α- and γ-tocopherol, the compounds of interest, therefore the peaks did not overlap, as was the case with β-tocopherol.

Response calibration curves were linear from 0.2 to 60 µM tocopherol in sample (equivalent to 0.002 to 0.40 pmoles injected) illustrated in figure 2.2. Recovery of α-, γ- and δ-tocopherol was 99, 98 and 95 % respectively.

![Figure 2.2 Response calibration curves for α- and γ-tocopherol in ethanol](image-url)
The comparison between quantification within an internal standard and quantification using an external calibration curve, was carried out simultaneously in 20 plasma samples. There was a close correlation between the two methods ($r^2 = 0.86$). The Bland and Altman test was used to assess the agreement between methods; the mean difference was 0.8 μM α-tocopherol with 95% confidence interval of $+10.0$ or $-8.4$ μM α-tocopherol (Bland & Altman, 1986). Although the agreement was poor, the coefficient of variation between duplicates was consistently larger when measured by external curve compared with the internal standard quantification, 5.6% and 3.2% respectively (20 duplicate samples). Therefore, the external curve was less accurate and less precise.

2.2.21 LC/MS quantification of vitamin E

LC/MS quantification of vitamin E was carried out by the method described by Hall et al. (2003). Deuterium labelled (d6 RRR-α-tocopherol) and unlabelled (d0) α-tocopherol were quantified by LC/MS in the studies detailed in Chapters 4, 5 and 6. d0 and d6 α-Tocopherol were present in the biological samples after supplementation, d3 all rac α-tocopherol was the internal standard.

The HPLC system was a 2695 Waters Alliance System (Waters Ltd, Elstree, UK) comprising a solvent delivery system, online degasser, peltier-cooled autosampler (set at 4 °C), controller and column over (set at 25 °C). The mobile phase consisted of 100% LC/MS grade methanol. The tocopherols were separated using a reverse phase Waters Symmetry® column (2.1 mm x 50 mm, C18, 2.5 μm particle size) at a flow rate of 0.3 ml/ min. Tocopherols eluted at 2.2 minutes.

The HPLC system was coupled with a Micromass LCT™ time-of-flight (TOF) mass spectrometer (Micromass UK Ltd, Manchester, UK). The LCT™ consisted of an orthogonal ion extraction (Z-Spray) source equipped with an electrospray inlet. The ions are transferred via a dual hexapole pusher region where the ions are deflected into the analyser flight tube at a rate of 20 KHz (50 μs flight time). Ion arrival times are recorded using a multichannel plate detector linked to a 3.6 GHz time to digital converter (TDC). RF lenses transfer ions from source to the TOF mass analyser. Ionisation parameters were as follows; negative ion electrospray ionisation (ESI-), desolvation gas temperature 250 °C, source temperature 120 °C, capillary voltage,
2800 V, sample cone voltage −35 V, extraction cone voltage 3 V, RF lens 450. TDC: start mV 800 and stop mV 181. Ions in the range of 350 – 500 m/z were collected from 1.5 to 3.5 minutes following injection and acquired at 4000 FWHM resolution. A 0.95 ms scan time was used with a 0.05 ms inter-scan delay. Ions generated by d0, d3 and d6 α-tocopherol were 429.37, 432.39 and 435.41 m/z respectively. The identified peaks were integrated and amounts calculated from response curves generated from a range of standards using Mass Lynx 4© software.

The standards underwent the same extraction procedure as the samples, differing amounts of d0 and d6 α-tocopherol were aliquoted in duplicate, 5 µl of 10 µM d3 all rac-α-tocopherol (internal standard) and 100 µl PBS were added and the extraction procedure was carried out as detailed in section 2.2.18. Reconstituted ethanol standards were stored at −80 °C for a maximum of two months.

A d6 α-tocopherol standard curve extracted from PBS did not agree with one extracted from erythrocytes, therefore was not used. A consequence of this was d0 α-tocopherol could not be quantified in the samples.

d3 and d6 α-Tocopherol standards (> 98 % isotopically pure) were prepared from their tocopheryl acetates as described in section 2.2.19, d0 was prepared from pure α-tocopherol oil (> 99% purity); standards were diluted in absolute ethanol. The exact concentrations were determined by measuring their absorbance on a spectrophotometer and calculating their concentration using beer-Lamberts law (absorption at 298nm / extinction coefficient ε 3270 = concentration), wavelength and extinction coefficients were ascertained from Kofler et al. (1962). Standard tocopherol solutions were stored at −20 °C, their concentrations were regularly checked.

2.2.21.1 Development of liquid chromatography time-of-flight mass spectrometric (LC TOF MS) method to detect deuterium labelled tocopherols

A LC TOF MS method was developed to quantitatively measure deuterium labelled tocopherols in biological samples (Hall et al., 2003). Previous methods have been published for gas chromatography MS (Ingold et al., 1987b) and LC MS utilising either single or triple quadrupole instruments (Kalman et al., 2003; Lauridsen et al., 2001; Mottier et al., 2002). TOF Mass spectrometers are very sensitive with high mass accuracy.
The LC TOF MS instruments and procedure are detailed in section 2.2.21. To optimise conditions the TOF MS was tuned by continuous infusion of a 10 μM d0 α-tocopherol into the spectrometer. Ionisation was tested in both positive and negative ion mode of electrospray and atmospheric pressure chemical ionisation, negative ion electrospray produced the maximal signal and was therefore used. Optimum conditions are described in section 2.2.21. TOF MS produces a single ion of exact mass, the mass for d0 (429.3742) corresponds almost exactly to the [M - H]⁻ calculated mass of α-tocopherol (429.3733). Figure 2.3 shows the typical total ion chromatogram and m/z scans following the injection of 0.5 pmoles of d0, d3 or d6 α-tocopherol.

![Figure 2.3 Typical total ion chromatogram and m/z scans following the injection of 0.5 pmoles of d0, d3 or d6 α-tocopherol](image)

A linear response was observed up to 15 pmoles of d0, d3 and d6 α-tocopherol pure standards in ethanol. Figure 2.4 shows the calibration curves for peak area verses concentration. The d6 α-tocopherol response is considerably lower than d0 and d3 α-tocopherol. Injections of 150 pmoles or greater α-tocopherol resulted in saturation of
the detector, resulting in a non-linearity. The TOF MS has a low dynamic range (approximately 3 orders of magnitude), therefore the extracted biological samples were diluted to be within the range of the instrument, so preventing saturation by d0 \( \alpha \)-tocopherol.

![Calibration curves for peak area versus concentration of d0, d3 and d6 \( \alpha \)-tocopherol](image)

**Figure 2.4 Calibration curves for peak area versus concentration of d0, d3 and d6 \( \alpha \)-tocopherol**

An internal standard, d3 \emph{all rac-}\( \alpha \)-tocopherol, was used for the quantification of d0 and d6 \emph{RRR-}\( \alpha \)-tocopherol in biological samples. A matrix ion and an ion suppression effect from the extraction procedure was observed when spiked samples were compared with ethanol standards. To overcome this, standards underwent identical extraction procedure as the biological samples (detailed in section 2.2.21). The identified peaks were integrated and amounts calculated from response curves generated from the range of standards using Mass Lynx 4© software. Figure 2.5 illustrates the response curve for d0 and d6 \( \alpha \)-tocopherol, the curves are non-linear and fit a polynomial equation for quantitation. Response for d0 and d6 \( \alpha \)-tocopherol were calculated by the equation below whereby the unknown was replaced by either d0 or d6 \( \alpha \)-tocopherol.

\[
\text{Response} = \text{Area}_{\text{unknown}} \times \left( \frac{\text{d3}_{\text{concentration}}}{\text{d3}_{\text{peak area}}} \right)
\]
The limits of detection and quantification were calculated using pure ethanol standards and blood components spiked with varying concentrations of d6 α-tocopherol prior to extraction. The limit of detection (LOD) was determined from the amount of tocopherol injected with a signal to noise ratio of 5:1. For ethanol standards LOD was 5 fmols, whereas for plasma, erythrocytes and platelets it was 15, 30 and 15 fmols respectively. The limit of quantification (LOQ) was determined from the lowest point in the standard response curve in which an amount can be accurately and reproducibly determined. The LOQ for plasma, erythrocytes and platelets was 50, 100 and 50 fmols respectively.

To assess the precision of the TOF MS plasma, erythrocytes and platelet tocopherol extractions were analysed for within day and between day variation. Within day variation was determined by tocopherol extractions measured eight times, coefficient of variation ranged from 3 to 7 % for d0 and d6 α-tocopherol. Between day variation was determined by tocopherol extractions measure eight times over a period of 4 weeks, coefficient of variation ranged from 3 to 10 % for d0 and d6 α-tocopherol.

The accuracy of the TOF MS to quantify tocopherols was determined by measuring plasma extractions spiked with d6 α-tocopherol ranging from 1 –15 μM in 12 samples. The accuracy was found to be 98 ± 8 % of the calculated concentration. The TOF MS quantification of tocopherols was validated with HPLC-ECD analysis of
d0 \( \alpha \)-tocopherol. HPLC-ECD is unable to distinguish between the different species of \( \alpha \)-tocopherol. Plasma and platelet samples were extracted separately, \( \delta \)-tocopherol was added as an internal standard for HPLC-ECD analysis, whereas \( \delta^3 \) all rac-\( \alpha \)-tocopherol was added as an internal standard for TOF MS analysis. The concentrations from the HPLC-ECD and TOF MS closely correlated \((r^2 = 0.94)\). The Bland and Altman test was used to assess the agreement between methods; the mean difference was +3.6 \( \mu \)M \( \alpha \)-tocopherol with 95 \% confidence interval of +8.2 or -2.1 \( \mu \)M \( \alpha \)-tocopherol (Bland & Altman, 1986).

2.2.22 Quantification of vitamin E urinary metabolites

Urinary metabolites of \( \alpha \)- and \( \gamma \)-tocopherol, 2,5,7,8-tetramethyl-2 \((2'-\text{carboxyethyl})-6\)-hydroxycroman (\( \alpha \)-CEHC) and 2,7,8-trimethyl-2- \((2'-\text{carboxyethyl})-6\)-hydroxycroman (\( \gamma \)-CEHC) were measured by HPLC with electrochemical detection (ECD), as described by Lodge et al. (2000).

The urine samples were defrosted at room temperature. To a 5 ml aliquot of urine, 400 \( \mu \)l of freshly made enzyme solution (4mg \( \beta \)-glucuronidase in 450 \( \mu \)l 0.1 M acetate buffer with enzyme activity;1850 units) and 10 \( \mu \)l Trolox (1 mg/ ml ethanol as internal standard) was added and then the mixture was gently shaken. The samples were incubated at 37 \( ^\circ \)C for 4 hours, then cooled on ice. Once cooled 50 \( \mu \)l concentrated HCl was added and samples gently shaken. Using an automated glass volumetric pipette 15 ml diethyl ether was added to the samples. Samples were then vortexed and mixed by inversion. The samples were centrifuged at 700 g for 1 minute at 4 \( ^\circ \)C. Ten ml diethyl ether was removed with an automated glass volumetric pipette into a separate tube. The diethyl ether was dried down completely with nitrogen, the samples were re-suspended with 100 \( \mu \)l ethanol for HPLC analysis.

The HPLC system was a 2690 Waters Alliance System (Waters Ltd, Elstree, UK) as described in section 2.2.2.17 in conjunction with an LC-4C amperometric ECD (Bioanalytical Systems, Lafayette, USA). The mobile phase was delivered at a flow rate of 0.4 ml/ min and consisted of a gradient with McIlvaine buffer (0.01 M citric acid, 0.02 M diammonia hydrogen orthophosphate, 0.1 \% lithium perchlorate in distilled water and adjusted to pH 4.15 with 12 M HCl (0.2 g of preservative sodium
azide was added) and acetonitrile (ACN) with 0.1 % lithium perchlorate, gradient
detailed in table 2.2.

Table 2.2 Mobile phase gradient used to separate urinary metabolites and trolox

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>McIlvaine buffer</th>
<th>ACN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6.5</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>1 minutes to change</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>7.5-19.5</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>23.5-30</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>1 minutes to change</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

The metabolites and trolox were separated using a reverse phase Sepserv
ES1000™, column (250 mm x 4 mm, C18, 5μm particle size). Peaks were identified
from retention times: Trolox, α-CEHC and γ-CEHC eluted at 21, 24.5 and 28 min,
respectively.

The peaks were identified using Waters Millennium® software then
quantitated using the peak area of trolox. The area of trolox peak was known to equate
to 0.01 mg therefore the concentration of α-CEHC and γ-CEHC could subsequently be
calculated; 

\[
\frac{0.01}{(\text{Trolox peak area/ CEHC peak area})} \times \text{trolox response factor} = \text{mg CEHC in original 5 ml urine sample. The response factor to trolox was approximately 0.9 for γ-CEHC and 0.7 for α-CEHC calculated from calibration curves.}
\]

Samples were extracted in duplicate and quality controls were extracted with
each extraction. Quality control samples were inserted at the beginning of every assay,
and the inter-assay coefficient of variation (CV) for the assay was 16 %.

2.2.23 Determination of urinary creatinine

Urinary creatinine was measured automatically by the SPACE biochemical
analyzer (Alfa Wasserman, The Netherlands), using a colorimetric kit supplied by
Randox (County Antrim, UK). The urine samples were diluted 1 in 50 with distilled
water before analysis. The creatinine in alkaline solution reacts with picric acid to
form a coloured complex. The amount of the complex formed was directly
proportional to the creatinine concentration.
2.2.24 Cotinine measurement by HPLC with UV detection

Urinary cotinine (a nicotine metabolite) was measured by HPLC with UV detection by a method adapted from Greaves et al. (2001). Urinary cotinine was measured to confirm cigarette smoking status in Chapter 7. The urine samples were defrosted at room temperature; a 2 ml aliquot of urine was transferred into a glass screw top tube. Fifty µl 150 % NaOH was added to the urine and shaken to mix. Using a glass volumetric pipette, 8 ml dichloromethane was added, vortexed then mixed by inversion. The samples were centrifuged at 1,100 g for 2 minutes at room temperature. With care, 4 ml of the infranatant was transferred to a new tube using a glass volumetric pipette. This was then dried under nitrogen and reconstituted in 100 µl 20 % methanol 80 % water solution.

The HPLC system was a 2690 Waters Alliance System (Waters Ltd, Elstree, UK) as described in section 2.00 in conjunction with a Waters 2487 dual λ absorbance UV detector (Waters Ltd, Elstree, UK). The mobile phase was delivered at a flow rate of 1 ml/min and consisted of 64 % water, 20% methanol, 12 % 0.4 mM sodium acetate (pH 4.3), 4 % ACN. A C18, Discovery™® column was used to separate the cotinine. Absorbance was taken at 260 nm, peaks were identified from the retention time, cotinine eluted at 11.5 minutes. The peaks were identified using Waters Millennium® software then quantitated using an external calibration curve. Cotinine standards were made up from pure cotinine. An external calibration curve, which ranged from 0.2 to 1.0 µg, was used. Limit of detection of assay is reported to be 10 µg/ L with a signal to noise ration of 5 (Greaves et al., 2001).
2.3 Statistical analysis

Results were analysed using GraphPad InStat® (version 3.01, 32 bit for Windows 95/ NT (1998) San Diego, USA) and STATISTICA (version 5.1 for Windows, Statsoft Inc (1997) Tulsa, OK, USA). Pearson’s correlation coefficient as used to test the association between two sets of data, expressed as \( r^2 \) the product moment coefficient; Spearman rank order correlation was used if the data was not normally distributed. Basic descriptive statistics, unpaired and paired t tests, one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA were performed as appropriate. Post hoc analysis of effects was carried out using Tukey’s Honestly Significant Difference test as required. Statistical significance was assumed if \( p < 0.05 \) and a trend towards significance if \( p < 0.10 \) and \( > 0.05 \).

In Chapters 3, 5 and 6 area under the curve (AUC) was calculated by trapezoid method and for Chapter 6 power calculations were conducted using an internet based power calculator at www.health.ucalgary.ca/~rollin/stats/ssize.
Chapter 3

Urinary $\alpha$- and $\gamma$-CEHC excretion and plasma, erythrocyte, platelet and lymphocyte $\alpha$- and $\gamma$-tocopherol: Response to varying single doses of vitamin E
3.1 Introduction

Vitamin E is essential to health, it acts as an antioxidant maintaining the integrity of biological membranes and there are important non-antioxidant roles emerging for α-tocopherol (Azzi et al., 2003; Wang & Quinn, 1999). This chapter investigates the uptake of vitamin E within plasma, erythrocytes, platelets and lymphocytes and the urinary excretion of vitamin E metabolites, in response to varying single doses of α-tocopheryl acetate. Investigations into a possible dose-response relationship between vitamin E in blood components (other than plasma) and urinary vitamin E metabolites (CEHC) have not previously been undertaken.

Presently, there is no universally accepted biomarker for reliable and accurate measurement of vitamin E status (Morrissey & Sheehy, 1999). A number of steady-state and functional biomarkers have been suggested, these include vitamin E concentration in erythrocytes, platelets, lymphocytes and urinary CEHC excretion (Lehmann et al., 1988; Schultz et al., 1995; Vatassery et al., 1983). A better understanding of the response in blood components and urinary metabolites to vitamin E supplementation within healthy people is required to further elucidate vitamin E regulation.

Steady-state plasma α- and γ-tocopherol concentrations are frequently used to assess vitamin E status (Hense et al., 1993; Salonen et al., 1985; Winklhofer-Roob et al., 1997). Plasma vitamin E concentration represents the combined total of vitamin E within circulating lipoproteins (Traber, 1994). The vitamin E content of lipoproteins is predominately due to their chemical composition rather than the presence of transport proteins, as may be the case in peripheral tissues (Blatt et al., 2001; Perugini et al., 2000). There are several confounding factors that reduce the sensitivity of plasma as a biomarker for vitamin E status; it is a saturable system (Dimitrov et al., 1991), subjected to hepatic regulation (Traber et al., 1990a; Traber & Kayden, 1989a) and influenced by plasma lipids (Horwitt et al., 1972; Thurnham et al., 1986). Vitamin E within erythrocytes is membrane bound and these cells possess tocopherol binding proteins (Kitabchi & Wimalasena, 1982b), it has therefore been proposed that vitamin E concentration within erythrocytes is a better indicator of tissue vitamin E status than plasma (Simon et al., 2001). Lymphocytes have LDL receptors and are thought to obtain their vitamin E by internalisation of LDL. It is currently unknown how platelets obtain their vitamin E. Both platelets and lymphocytes are functionally affected by
vitamin E and therefore vitamin E concentration within these cells may represent a function biomarker of vitamin E. Vitamin E regulation and distribution between the aforementioned blood components is poorly understood.

Within the liver, α- and γ-tocopherol are metabolised to 2,5,7,8-tetramethyl-2-(2' -carboxyethyl)-6-hydroxychroman (α-CEHC) and 2,7,8-trimethyl-2-(2' -carboxyethyl)-6-hydroxychroman (γ-CEHC) respectively. The hepatic α-tocopherol transfer protein selects RRR-α-tocopherol for secretion within VLDL, while the other forms of vitamin E are presumed to be metabolised and excreted into bile (Hattori et al., 2000; Mustacich et al., 1998). The vitamin E metabolites present in plasma (Galli et al., 2002; Stahl et al., 1999) are thought to be reabsorbed from the intestine into the systemic circulation. The proportion of vitamin E metabolites not reabsorbed by this route and excreted into the faeces is currently unknown. Vitamin E metabolites within plasma are excreted in the urine after kidney filtration. It has been proposed that urinary vitamin E metabolites might reflect vitamin E regulation within the liver (Lodge et al., 2001).
3.2 Aim and hypothesis

At present there is no adequate biomarker to reliably and accurately assess vitamin E status. This is, in part, due to the limited information available about the regulation of vitamin E and the extent of $\alpha$-tocopherol metabolism to $\alpha$-CEHC. $\alpha$-CEHC has been proposed as a biomarker for adequate vitamin E status, however, relatively little is known about $\alpha$-CEHC formation and excretion in response to vitamin E supplementation, and therefore further investigation into factors affecting its excretion are needed. Plasma is frequently used to assess vitamin E status. Vitamin E levels within erythrocytes, platelets and lymphocytes have been proposed as potential biomarkers for vitamin E status. Limited information exists on dose-response uptake of $\alpha$-tocopherol into blood components and the extent of $\alpha$-tocopherol metabolism to $\alpha$-CEHC.

Instead of plasma, vitamin E levels within erythrocytes, platelets, lymphocytes and urinary CEHC excretion, could potentially be used as biomarkers of vitamin E status. However, before they can be used as biomarkers, an understanding of the relationship between supplementation and vitamin E concentration and formation urinary CEHC excretion must be established.

The current study aims to;

1. Determine the response of vitamin E concentration within plasma, erythrocytes, platelets and lymphocytes, and the urinary excretion of CEHC metabolites to varying single doses of $\alpha$-tocopheryl acetate.
2. Establish whether there is a relationship between vitamin E levels in plasma, erythrocytes, platelets and lymphocytes and the urinary excretion of CEHC metabolites.
3. Determine the percentage of $\alpha$-tocopheryl acetate dose excreted as urinary $\alpha$-CEHC.

It is hypothesised that a dose-dependent increase in $\alpha$-tocopherol within plasma, erythrocytes, platelets and lymphocytes, and urinary $\alpha$-CEHC will be observed. It is also hypothesise that a peak in urinary $\alpha$-CEHC will be observed on the same day of $\alpha$-tocopheryl acetate ingestion, as reported for other forms of vitamin E.
3.3 Study design

Twenty healthy subjects (7 males and 13 females) were recruited from the university and advertisements in local newspapers. Selection criteria stated subjects must not have been taking dietary supplements (6 months prior to the study), were non-smokers and had no gastrointestinal disorders as determined by a written questionnaire. Subjects with blood lipid abnormalities were excluded (selection criteria was cholesterol < 6 mmol/l and triglycerides < 1.5 mmol/l).

The vitamin E doses given to the subjects were 130, 195, 390 and 780 α-tocopheryl acetate. Five subjects were randomly assigned to each α-tocopheryl acetate dosage.

Figure 3.1 illustrates the study protocol. Subjects were requested to complete a 7-day food diary finishing on day 5 of the study. The subjects collected 24 h urine collections for two days prior to ingesting the α-tocopheryl acetate capsule, the day the capsule was consumed and the subsequent two days. The subjects were instructed to consume the α-tocopheryl acetate capsule with a fat containing breakfast on day 3, but otherwise the subjects ate ad libitum. A fasted 20ml blood sample was taken 24 hours prior to and 24 hours following ingestion of the α-tocopheryl acetate capsule.

Figure 3.1 Schematic figure of study protocol
The subjects were requested to complete a food diary during the study period. The subjects collected 24 h urine collections for five days, on days 2 and 4 a fasted blood sample was taken. On day 3 the subjects ingested the α-tocopheryl acetate capsule (either 130, 195, 390 and 780 mg α-tocopheryl acetate)
The completed food diaries were analysed for the average daily nutrient intake as detailed in section 2.2.2. Plasma, erythrocytes, platelets and lymphocytes were isolated from the fasted blood samples and α- and γ-tocopherol were subsequently measured, as detailed in sections 2.2.4 – 2.2.8. Plasma cholesterol and triacylglycerol were measured as detailed in section 2.2.10 and 2.2.11. Creatinine and α- and γ-CEHC were measured from aliquots of the 24 hour urine collections as detailed in sections 2.2.3, 2.2.22 and 2.2.23. The mean value from day 1 and 2 urine collections were used for the baseline value.
3.4 **Results**

The vitamin E content of the ‘Holland and Barrett’ α-tocopheryl acetate capsules was analysed, after saponification (method detailed in section 2.2.19). The oil contained 97% α-tocopherol and 3% γ-tocopherol, as a consequence the precise doses given to the subjects were 130 and 4 mg, 195 and 6 mg, 390 and 12 mg, 780 and 24 mg α-tocopheryl acetate and γ-tocopheryl acetate respectively. From here onwards the doses of vitamin E will be expressed as mg α-tocopheryl acetate only for clarity.

3.4.1 **Subject characteristics**

Twenty healthy normolipidemic subjects participated in the study, there were five subjects in each of the four α-tocopheryl acetate dosage groups. During the study period no one reported any side effects or withdrew from the study. Table 3.1 shows the subject characteristics of each group and mean values for all twenty subjects. Age, body mass index, plasma cholesterol and triacylglycerol concentrations, and habitual dietary vitamin E intakes were not significantly different between groups as analysed by one-way ANOVA (Table 3.1). None of the subjects consumed greater than 10 mg vitamin E a day from their habitual diet during the study period.

<table>
<thead>
<tr>
<th>Group α-TA</th>
<th>Gender (M/F)</th>
<th>Age (Years)</th>
<th>BMI (kg/m²)</th>
<th>Cholesterol (mmol/l)</th>
<th>TAG (mmol/l)</th>
<th>Vitamin E intake (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 mg</td>
<td>1/4</td>
<td>42 ± 6</td>
<td>24.5 ± 1.3</td>
<td>4.5 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>6.65 ± 1.04</td>
</tr>
<tr>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>195 mg</td>
<td>2/3</td>
<td>34 ± 5</td>
<td>22.8 ± 1.2</td>
<td>4.6 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>5.26 ± 0.62</td>
</tr>
<tr>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>390 mg</td>
<td>3/2</td>
<td>39 ± 6</td>
<td>25.2 ± 2.2</td>
<td>4.7 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>6.77 ± 0.81</td>
</tr>
<tr>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>780 mg</td>
<td>1/4</td>
<td>40 ± 5</td>
<td>24.2 ± 1.0</td>
<td>4.6 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td>5.27 ± 0.31</td>
</tr>
<tr>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>7/13</td>
<td>39 ± 3</td>
<td>24.2 ± 0.7</td>
<td>4.6 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>6.00 ± 0.48</td>
</tr>
<tr>
<td>n = 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. α-TA: mg of α-tocopheryl acetate in each dose, M: male, F: female, BMI: body mass index, TAG: triacylglycerol
3.4.2 Baseline values

Table 3.2 shows the mean baseline α- and γ-tocopherol concentrations in plasma, erythrocytes, platelets and lymphocytes for each of the four dosage groups and the combined subject total. The mean baseline α-tocopherol concentration in plasma was 23.1 μM (range 16.8-31.1 μM) for all subjects. There was significantly greater α-tocopherol in each blood component compared with γ-tocopherol as analysed by paired t-test (p < 0.0001). Baseline α- and γ-tocopherol concentration in plasma, erythrocytes, platelets and lymphocytes were similar in each of the four groups of five subjects. Baseline values for the seven males and thirteen females were not significantly different from each other (data not shown).

Table 3.2 Mean baseline α- and γ-tocopherol concentrations in blood components for each group and total

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma α-TA</th>
<th>Plasma α</th>
<th>Erythrocytes</th>
<th>Platelets</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/l</td>
<td>μmol/ mmol C</td>
<td>μmol/ PCV</td>
<td>μmol/ g protein</td>
<td>μmol/ g protein</td>
</tr>
<tr>
<td>130 mg α-T</td>
<td>23.1 ± 3.3</td>
<td>5.1 ± 0.4</td>
<td>4.0 ± 0.3</td>
<td>1.29 ± 0.24</td>
<td>2.46 ± 0.30</td>
</tr>
<tr>
<td>γ-T</td>
<td>1.57 ± 0.28</td>
<td>0.34 ± 0.04</td>
<td>0.37 ± 0.05</td>
<td>0.14 ± 0.03</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>195 mg α-T</td>
<td>22.3 ± 1.4</td>
<td>5.1 ± 0.5</td>
<td>3.5 ± 0.2</td>
<td>1.09 ± 0.23</td>
<td>2.17 ± 0.17</td>
</tr>
<tr>
<td>γ-T</td>
<td>1.55 ± 0.16</td>
<td>0.33 ± 0.02</td>
<td>0.29 ± 0.04</td>
<td>0.09 ± 0.03</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>390 mg α-T</td>
<td>22.9 ± 1.5</td>
<td>4.9 ± 0.4</td>
<td>3.7 ± 0.4</td>
<td>1.24 ± 0.25</td>
<td>2.34 ± 0.16</td>
</tr>
<tr>
<td>γ-T</td>
<td>1.55 ± 0.05</td>
<td>0.34 ± 0.03</td>
<td>0.33 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>780 mg α-T</td>
<td>25.5 ± 0.7</td>
<td>4.7 ± 0.5</td>
<td>3.7 ± 0.4</td>
<td>1.10 ± 0.21</td>
<td>2.32 ± 0.54</td>
</tr>
<tr>
<td>γ-T</td>
<td>1.35 ± 0.07</td>
<td>0.31 ± 0.03</td>
<td>0.27 ± 0.03</td>
<td>0.08 ± 0.02</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>Total α-T</td>
<td>23.1 ± 1.3</td>
<td>5.0 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>1.18 ± 0.11</td>
<td>2.32 ± 0.15</td>
</tr>
<tr>
<td>γ-T</td>
<td>1.51 ± 0.08</td>
<td>0.33 ± 0.07</td>
<td>0.31 ± 0.09</td>
<td>0.10 ± 0.06</td>
<td>0.26 ± 0.08</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. α-TA: mg of α-tocopheryl acetate in each dose, α-T: α-tocopherol, γ-T: γ-tocopherol, C: cholesterol, PCV: packed cell volume.

Table 3.3 shows the mean baseline urinary excretion of α- and γ-CEHC over 24 hours and corrected for creatinine for each group and the combined subject total. Urinary excretion of γ-CEHC was significantly greater than α-CEHC in all subjects as analysed by paired t-test (p < 0.001). Baseline urinary excretion of α- and γ-CEHC were similar in each of the four groups. Baseline values for the seven males and thirteen females were not significantly different from each other (data not shown).
Table 3.3 Mean baseline urinary α- and γ-CEHC excretion for each group and total

<table>
<thead>
<tr>
<th>Group</th>
<th>α-CEHC µg/24 h</th>
<th>α-CEHC mg/g creatinine</th>
<th>γ-CEHC µg/24 h</th>
<th>γ-CEHC mg/g creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 mg</td>
<td>349 ± 80</td>
<td>0.35 ± 0.12</td>
<td>652 ± 130</td>
<td>0.63 ± 0.15</td>
</tr>
<tr>
<td>195 mg</td>
<td>349 ± 50</td>
<td>0.37 ± 0.10</td>
<td>471 ± 61</td>
<td>0.51 ± 0.16</td>
</tr>
<tr>
<td>390 mg</td>
<td>205 ± 60</td>
<td>0.19 ± 0.07</td>
<td>380 ± 97</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>780 mg</td>
<td>339 ± 91</td>
<td>0.30 ± 0.03</td>
<td>448 ± 70</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>Total</td>
<td>302 ± 33</td>
<td>0.30 ± 0.04</td>
<td>479 ± 48</td>
<td>0.48 ± 0.06</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. α-TA: mg of α-tocopheryl acetate in each dose group

*a Baseline 24 h urinary α- and γ-CEHC excretion were the mean from day 1 and 2 collections

3.4.2.1 Baseline correlations

Table 3.4 shows a correlation matrix for baseline α-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes and baseline urinary excretion of α-CEHC. There were no correlations between α-tocopherol concentration in plasma with α-tocopherol concentration in erythrocytes, platelets or lymphocytes or urinary excretion of α-CEHC. There was a negative correlation between α-tocopherol in erythrocytes and urinary α-CEHC excretion and a positive correlation between α-tocopherol in erythrocytes and platelets.

Table 3.4 Correlation matrix for baseline α-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes and urinary α-CEHC excretion (n = 20)

<table>
<thead>
<tr>
<th></th>
<th>Plasma α-T/ mmol C</th>
<th>Erythrocytes α-T/ PCV</th>
<th>Platelets α-T/ g pro.</th>
<th>Lymphocytes α-T/ g pro.</th>
<th>α-CEHC/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma α-T/ mmol C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythrocytes α-T/ PCV</td>
<td>r² = 0.01</td>
<td>r² = 0.25</td>
<td>r² = 0.00</td>
<td>r² = 0.24</td>
<td>r² = 0.00</td>
</tr>
<tr>
<td>r² = 0.01</td>
<td>p = 0.67</td>
<td>p = 0.03</td>
<td>p = 0.90</td>
<td>p = 0.57</td>
<td>p = 0.81</td>
</tr>
<tr>
<td>p = 0.90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets α-T/ g pro.</td>
<td>r² = 0.00</td>
<td>r² = 0.24</td>
<td>r² = 0.00</td>
<td>r² = 0.00</td>
<td>r² = 0.00</td>
</tr>
<tr>
<td>r² = 0.00</td>
<td>p = 0.81</td>
<td>p = 0.03</td>
<td>p = 0.57</td>
<td>p = 0.80</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes α-T/ g pro.</td>
<td>r² = 0.01</td>
<td>r² = 0.06</td>
<td>r² = 0.02</td>
<td>p = 0.57</td>
<td></td>
</tr>
<tr>
<td>r² = 0.01</td>
<td>p = 0.57</td>
<td>p = 0.57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-CEHC/24 h</td>
<td>r² = 0.00</td>
<td>r² = 0.00</td>
<td>r² = 0.00</td>
<td>p = 0.80</td>
<td></td>
</tr>
<tr>
<td>r² = 0.00</td>
<td>p = 0.03</td>
<td>p = 0.89</td>
<td>p = 0.80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

α-T: α-tocopherol, C: cholesterol, PCV: packed cell volume. pro: protein
Table 3.5 shows a correlation matrix for baseline \( \gamma \)-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes and baseline urinary excretion of \( \gamma \)-CEHC. There was a strong positive correlation between \( \gamma \)-tocopherol concentration in plasma and erythrocytes. Urinary \( \gamma \)-CEHC excretion did not correlate with any of the blood components measured.

### Table 3.5 Correlation matrix for baseline \( \gamma \)-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes and urinary \( \gamma \)-CEHC excretion (n = 20)

<table>
<thead>
<tr>
<th></th>
<th>Plasma ( \gamma )-T/ mmol C</th>
<th>Erythrocytes ( \gamma )-T/ PCV</th>
<th>Platelets ( \gamma )-T/ g pro.</th>
<th>Lymphocytes ( \gamma )-T/ g pro.</th>
<th>( \gamma )-CEHC/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ( \gamma )-T/ mmol C</td>
<td>(-)</td>
<td>( r^2 = 0.68 ) ( p &lt; 0.01 )</td>
<td>( r^2 = 0.07 ) ( p = 0.26 )</td>
<td>( r^2 = 0.11 ) ( p = 0.15 )</td>
<td>( r^2 = 0.00 ) ( p = 0.81 )</td>
</tr>
<tr>
<td>Erythrocytes ( \gamma )-T/ PCV</td>
<td>( r^2 = 0.68 ) ( p &lt; 0.01 )</td>
<td>(-)</td>
<td>( r^2 = 0.14 ) ( p = 0.11 )</td>
<td>( r^2 = 0.18 ) ( p = 0.07 )</td>
<td>( r^2 = 0.05 ) ( p = 0.37 )</td>
</tr>
<tr>
<td>Platelets ( \gamma )-T/ g pro.</td>
<td>( r^2 = 0.07 ) ( p = 0.26 )</td>
<td>( r^2 = 0.14 ) ( p = 0.11 )</td>
<td>(-)</td>
<td>( r^2 = 0.07 ) ( p = 0.29 )</td>
<td>( r^2 = 0.05 ) ( p = 0.39 )</td>
</tr>
<tr>
<td>Lymphocytes ( \gamma )-T/ g pro.</td>
<td>( r^2 = 0.11 ) ( p = 0.15 )</td>
<td>( r^2 = 0.18 ) ( p = 0.07 )</td>
<td>( r^2 = 0.07 ) ( p = 0.29 )</td>
<td>(-)</td>
<td>( r^2 = 0.04 ) ( p = 0.43 )</td>
</tr>
<tr>
<td>( \gamma )-CEHC/24 h</td>
<td>( r^2 = 0.00 ) ( p = 0.81 )</td>
<td>( r^2 = 0.05 ) ( p = 0.37 )</td>
<td>( r^2 = 0.05 ) ( p = 0.39 )</td>
<td>( r^2 = 0.04 ) ( p = 0.43 )</td>
<td>(-)</td>
</tr>
</tbody>
</table>

\( \gamma \)-T: \( \alpha \)-tocopherol, C: cholesterol, PCV: packed cell volume, pro: protein

3.4.3 \( \alpha \) and \( \gamma \)-Tocopherol concentration 24 h post ingestion of differing single doses of \( \alpha \)-tocopheryl acetate

Table 3.6 shows the mean \( \alpha \)-tocopherol concentrations in plasma, erythrocytes, platelets and lymphocytes at baseline and 24 h post ingestion of varying single doses of \( \alpha \)-tocopheryl acetate. Plasma and erythrocyte \( \alpha \)-tocopherol concentrations significantly increased 24 h after ingestion of 195, 390 and 780 mg \( \alpha \)-tocopheryl acetate. A non-significant increase was observed in \( \alpha \)-tocopherol concentration within platelets and lymphocytes 24 h after the varying single doses of \( \alpha \)-tocopheryl acetate.

Table 3.7 shows the mean \( \gamma \)-tocopherol concentrations in plasma, erythrocytes, platelets and lymphocytes at baseline and 24 h post ingestion of varying single doses of \( \alpha \)-tocopheryl acetate. \( \gamma \)-Tocopherol concentration decreased in all blood components, although not significantly.
Table 3.6 α-Tocopherol concentration at baseline and 24 h post ingestion of varying single doses of α-tocopheryl acetate

<table>
<thead>
<tr>
<th>α-Tocopheryl acetate dose</th>
<th>Plasma µmol/ mmol C</th>
<th>Erythrocytes µmol/ PCV</th>
<th>Platelets µmol/ g protein</th>
<th>Lymphocytes µmol/ g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 mg Pre</td>
<td>5.10 ± 0.38</td>
<td>4.01 ± 0.28</td>
<td>1.29 ± 0.24</td>
<td>2.46 ± 0.30</td>
</tr>
<tr>
<td>Post</td>
<td>6.05 ± 1.04</td>
<td>4.67 ± 0.55</td>
<td>1.15 ± 0.22</td>
<td>3.05 ± 0.30</td>
</tr>
<tr>
<td>195 mg Pre</td>
<td>5.40 ± 0.51</td>
<td>3.51 ± 0.23</td>
<td>1.09 ± 0.23</td>
<td>2.17 ± 0.17</td>
</tr>
<tr>
<td>Post</td>
<td>6.89 ± 0.46*</td>
<td>5.42 ± 0.45*</td>
<td>1.13 ± 0.20</td>
<td>2.42 ± 0.26</td>
</tr>
<tr>
<td>390 mg Pre</td>
<td>4.92 ± 0.41</td>
<td>3.70 ± 0.41</td>
<td>1.24 ± 0.25</td>
<td>2.34 ± 0.16</td>
</tr>
<tr>
<td>Post</td>
<td>6.94 ± 0.97*</td>
<td>4.68 ± 0.47#</td>
<td>1.38 ± 0.36</td>
<td>2.23 ± 0.37</td>
</tr>
<tr>
<td>780 mg Pre</td>
<td>4.71 ± 0.49</td>
<td>3.72 ± 0.36</td>
<td>1.10 ± 0.21</td>
<td>2.75 ± 0.38</td>
</tr>
<tr>
<td>Post</td>
<td>7.42 ± 0.87#</td>
<td>5.72 ± 0.55*</td>
<td>1.40 ± 0.29</td>
<td>3.09 ± 0.35</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM * Significantly different from baseline concentration, analysed by paired t-test (p < 0.05), # p = 0.06 C: cholesterol, PCV: packed cell volume n = 5 in each group except lymphocytes n = 4 due to insufficient sample

Table 3.7 γ-Tocopherol concentration at baseline and 24 h post ingestion of varying single doses of α-tocopheryl acetate

<table>
<thead>
<tr>
<th>α-Tocopheryl acetate dose</th>
<th>Plasma µmol/ mmol C</th>
<th>Erythrocytes µmol/ PCV</th>
<th>Platelets µmol/ g protein</th>
<th>Lymphocytes µmol/ g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 mg Pre</td>
<td>0.34 ± 0.04</td>
<td>0.37 ± 0.05</td>
<td>0.14 ± 0.03</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>Post</td>
<td>0.30 ± 0.03</td>
<td>0.25 ± 0.04</td>
<td>0.14 ± 0.05</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>195 mg Pre</td>
<td>0.33 ± 0.02</td>
<td>0.29 ± 0.04</td>
<td>0.09 ± 0.03</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Post</td>
<td>0.20 ± 0.02*</td>
<td>0.19 ± 0.04</td>
<td>0.06 ± 0.02</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>390 mg Pre</td>
<td>0.34 ± 0.03</td>
<td>0.33 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>Post</td>
<td>0.32 ± 0.04</td>
<td>0.25 ± 0.03*</td>
<td>0.10 ± 0.03</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>780 mg Pre</td>
<td>0.31 ± 0.03</td>
<td>0.27 ± 0.03</td>
<td>0.08 ± 0.02</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>Post</td>
<td>0.27 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>0.21 ± 0.02</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM * Significantly different from baseline concentration, analysed by paired t-test (p < 0.05) C: cholesterol, PCV: packed cell volume n = 5 in each group except lymphocytes n = 4 due to insufficient sample
Figure 3.2 illustrates the mean percentage increases in α-tocopherol concentration 24 h following ingestion of varying single doses of α-tocopheryl acetate in plasma, erythrocytes, platelets and lymphocytes. A larger percentage increase in α-tocopherol concentration was observed in plasma and erythrocytes compared with platelets and lymphocytes. A dose-response increase in α-tocopherol was not observed in any of the blood components. There was large inter-individual variation in the percentage change in α-tocopherol concentration, as illustrated by the large standard error bars in figure 3.2.

The percentage decrease in γ-tocopherol did not correlate with the percentage increase in α-tocopherol concentration.

Figure 3.2 Mean percentage changes in α-tocopherol concentrations within blood components between baseline and 24 h post ingestion of varying single doses of α-tocopheryl acetate. Values expressed as mean ± SEM. No dose-response observed in percentage increase of α-tocopherol in plasma, erythrocytes, platelets or lymphocytes after varying single doses of α-tocopheryl acetate. a-TA: α-tocopheryl acetate dose ingested.
3.4.4 Urinary α- and γ-CEHC levels after varying single-doses of α-tocopheryl acetate

Individual urinary α-CEHC excretion profiles are shown in the following section to clearly illustrate the inter-individual differences in the excretion profiles.

Figure 3.3 illustrates the five individual and the mean urinary α-CEHC excretion profiles over five days when 130 mg α-tocopheryl acetate was ingested on day 3. There was no significant difference in α-CEHC excretion over time.

![Graph](image)

**Figure 3.3** Urinary excretion of α-CEHC over 5 days when 130 mg α-tocopheryl acetate was ingested on day 3. There was no significant difference in α-CEHC excretion over time, analysed by repeated measures ANOVA

Figure 3.4 shows the five individual and mean urinary α-CEHC excretion profiles over five days when 195 mg α-tocopheryl acetate was ingested on day 3. There was a significant time effect in α-CEHC excretion (p = 0.024), with greater α-CEHC excretion on day 3 compared with days 1 and 2. One individual excreted a large amount of α-CEHC on days 3 and 4 as shown in figure 3.4.
Figure 3.4 Urinary excretion of α-CEHC over 5 days when 195 mg α-tocopheryl acetate was ingested on day 3.
There was a significant time effect in α-CEHC excretion ($p = 0.024$) analysed by repeated measures ANOVA. * Post hoc analysis showed greater α-CEHC excretion on day 3 compared with days 1 and 2.

Figure 3.5 illustrates the five individual and mean urinary α-CEHC excretion profiles over five days when 390 mg α-tocopheryl acetate was ingested on day 3. There was no significant difference in α-CEHC excretion over time.

Figure 3.5 Urinary excretion of α-CEHC over 5 days when 390 mg α-tocopheryl acetate was ingested on day 3.
There was no significant difference in α-CEHC excretion over time, analysed by repeated measures ANOVA.
Urinary α-CEHC excretion was lower after the 390 mg dose compared with the 195 mg dose. There were some subjects that did not excrete any more α-CEHC above baseline; this is illustrated by two flat profiles in both figures 3.3 and 3.5.

Figure 3.6 shows the five individual and mean urinary α-CEHC excretion profiles over five days when 780 mg α-tocopheryl acetate was ingested on day 3. There was a trend towards a significant time effect in α-CEHC excretion ($p = 0.068$). One individual excreted a large amount of α-CEHC on day 3 compared with the other subjects.

Overall there was a large inter-individual variation in the magnitude and pattern of urinary α-CEHC excretion in response to varying single doses of α-tocopheryl acetate.

There was no significant difference in the excretion of γ-CEHC compared with baseline values post ingestion of the varying single doses of α-tocopheryl acetate.

Figure 3.7 illustrates the collective amount of α-CEHC excreted over baseline over 72 hours after ingestion of the varying single doses of α-tocopheryl acetate. There was large inter-individual variation in amount of α-CEHC excreted, illustrated...
by the very large standard error bars in figure 3.7. No dose-dependant increase in urinary α-CEHC excretion was observed.

![Figure 3.7](image)

**Figure 3.7** Collective urinary excretion of α-CEHC above baseline from days 3, 4 and 5. There was no dose-dependant increase in urinary α-CEHC excretion

Table 3.8 shows the mean α-CEHC excreted above baseline and the calculated amount this would equate to as percentage of dose ingested. The calculated percentage dose of α-tocopheryl acetate excreted as α-CEHC was less than 1% after each dose.

**Table 3.8** Urinary α-CEHC excretion up to 72 h after ingestion of varying doses of α-tocopheryl acetate

<table>
<thead>
<tr>
<th>Group</th>
<th>α-CEHC mg over baseline</th>
<th>% dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SEM</td>
<td>range</td>
</tr>
<tr>
<td>130 mg</td>
<td>0.42 ± 0.34</td>
<td>-0.30 - 1.29</td>
</tr>
<tr>
<td>195 mg</td>
<td>1.63 ± 0.65</td>
<td>0.47 - 4.12</td>
</tr>
<tr>
<td>390 mg</td>
<td>0.51 ± 0.12</td>
<td>0.22 - 0.85</td>
</tr>
<tr>
<td>780 mg</td>
<td>1.34 ± 0.50</td>
<td>0.35 - 3.94</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. % dose = (100/ mg dose) \times mg excreted over baseline
### 3.4.4.1 Correlations after varying single doses of α-tocopheryl acetate

Table 3.9 shows a correlation matrix for α-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes 24 h post ingestion of varying doses of α-tocopheryl acetate and incremental AUC (iAUC) urinary α-CEHC excretion up to 72 h post ingestion of α-tocopheryl acetate. There was a positive correlation between concentration of α-tocopherol in plasma and erythrocytes post 24 h with iAUC of α-CEHC excretion over baseline, and between platelets and erythrocytes. There were no correlations between platelets or lymphocytes with plasma or urinary α-CEHC excretion.

#### Table 3.9 Correlation matrix for α-tocopherol concentration in plasma, erythrocytes, platelets, lymphocytes 24 h following ingestion of varying single doses of α-tocopheryl acetate and incremental AUC for urinary α-CEHC excretion up to 72 h post ingestion of α-tocopheryl acetate (n = 20)

<table>
<thead>
<tr>
<th>Plasma α-T/ mmol C</th>
<th>Erythrocytes α-T/ PCV</th>
<th>Platelets α-T/ g pro.</th>
<th>Lymphocytes α-T/ g pro.</th>
<th>α-CEHC iAUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma α-T/ mmol C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythrocytes α-T/ PCV</td>
<td>r² = 0.18 p = 0.06</td>
<td>r² = 0.09 p = 0.21</td>
<td>r² = 0.36 p &lt; 0.01</td>
<td>r² = 0.36 p &lt; 0.01</td>
</tr>
<tr>
<td>Platelets α-T/ g pro.</td>
<td>r² = 0.00 p = 0.80</td>
<td>r² = 0.36 p &lt; 0.01</td>
<td>r² = 0.00 p = 0.91</td>
<td>r² = 0.00 p = 0.17</td>
</tr>
<tr>
<td>Lymphocytes α-T/ g pro.</td>
<td>r² = 0.09 p = 0.21</td>
<td>r² = 0.01 p = 0.83</td>
<td>r² = 0.10 p = 0.91</td>
<td>r² = 0.02 p = 0.52</td>
</tr>
<tr>
<td>α-CEHC iAUC</td>
<td>r² = 0.36 p &lt; 0.01</td>
<td>r² = 0.36 p &lt; 0.01</td>
<td>r² = 0.10 p = 0.17</td>
<td>r² = 0.02 p = 0.52</td>
</tr>
</tbody>
</table>

α-T: α-tocopherol, C: cholesterol, PCV: packed cell volume, pro: protein, iAUC: incremental AUC up to 72 h post ingestion of α-tocopheryl acetate.
3.5 Discussion

Vitamin E is an important micronutrient, however, presently there is no adequate biomarker to assess vitamin E status. The uptake of vitamin E into blood cells and factors influencing the production of urinary α-CEHC are poorly understood. This study aimed to investigate the response of vitamin E concentration within plasma, erythrocyte, platelets and lymphocytes, and urinary excretion of α-CEHC to varying single doses of α-tocopheryl acetate.

Key findings:
1. There was an increase in α-tocopherol concentration in plasma and erythrocytes, and a non-significant increase in platelets and lymphocytes 24 hours post varying single doses of α-tocopheryl acetate. There was no dose-response increase in α-tocopherol concentration in plasma, erythrocytes, platelets or lymphocytes.
2. A transient increase in urinary α-CEHC excretion was observed after the 195 mg and 780 mg α-tocopheryl acetate doses. There was no dose-response in α-CEHC excretion.
3. Less than 1 % of α-tocopheryl acetate dose was excreted as urinary α-CEHC.
4. There was a positive correlation between percentage increase in α-tocopherol concentration in plasma and erythrocyte and mg α-CEHC excreted over baseline.

3.5.1 Baseline α- and γ-tocopherol concentration in plasma, erythrocytes, platelets and lymphocytes, and α- and γ-CEHC urinary excretion

Plasma vitamin E levels are often used as a biomarker for vitamin E status, however there are several confounding factors that cumulatively reduce the reliability of plasma vitamin E to predict vitamin E status. The current study utilises vitamin E concentration within a number of blood components to assess vitamin E response to supplemental α-tocopheryl acetate.

The baseline α- and γ-tocopherol values for plasma are similar to previously published values (Galli et al., 2002; Galli et al., 2003; Traber et al., 1998a). Also, α- and γ-tocopherol concentrations within erythrocytes were similar to those reported by Simon et al. (1997) and in the lower range of those reported Roxborough et al. (2000). α- and γ-Tocopherol concentration in platelets reported in the current study were
slightly greater than those previously published (Lehmann et al., 1988; Saito et al., 1992; Vatassery et al., 1983). Vitamin E within lymphocytes is less well documented. Lymphocyte α-tocopherol concentrations reported here are greater than those reported by Lehmann et al, (1988) and Lenton et al. (2000).

Baseline urinary α- and γ-CEHC excretion were similar to published values by Lodge et al. (2000), Schuelke et al. (2000) and Traber et al. (1998a). However, this does not agree with the findings reported by Galli et al. (2002, 2003) whereby greater urinary excretion of α-CEHC compared with γ-CEHC was observed. Urinary α- and γ-CEHC excretion depends on the habitual diet of the population used in a study (Jiang et al., 2001). α- and γ-CEHC are specific metabolites formed from the metabolism of α- and γ tocopherols (Schönfeld et al., 1993; Wechter et al., 1996) and tocotrienols (Lodge et al., 2001) respectively. It is hypothesised that they are excreted into bile and reabsorbed via the intestine into the systemic circulation before urinary excretion (Hattori et al., 2000; Lodge et al., 2001).

3.5.1.1 Baseline correlations

Basal plasma α-tocopherol per mmol cholesterol did not correlate with α-tocopherol concentration in erythrocytes, platelets nor lymphocytes. It can be inferred from this finding that blood cell α-tocopherol concentrations are independent of plasma concentration, and that presumably active transfer processes regulate α-tocopherol concentration within these cells rather than passive diffusion. The lack of correlation also implies that plasma α-tocopherol is unable to reflect α-tocopherol concentration within platelets or lymphocytes and thus their functional status.

There have been conflicting findings on a correlation between plasma α-tocopherol corrected for lipids and platelets. Vatassery et al. (1983) found a positive correlation whereas Lehmann et al. (1988) found no correlation. The current study did not observe a correlation.

However, there was a positive correlation between γ-tocopherol concentration in plasma and erythrocytes. This could be as a result of passive diffusion, whereas α-tocopherol binding activity in erythrocytes may redistribute α-tocopherol (Wimalasena et al., 1982).
There were no correlations between baseline urinary α-CEHC excretion with α-tocopherol concentration in plasma, platelets or lymphocytes. Interestingly, there was a negative correlation between α-tocopherol concentration in erythrocytes and urinary α-CEHC excretion. However, a physiological explanation for this finding could not be found.

The lack of correlation between plasma α-tocopherol concentration and urinary α-CEHC excretion implies that urinary α-CEHC excretion does not reflect α-tocopherol concentration within the systemic circulation. This is in agreement with the findings by Schuelke et al. (2000) who reported that patients with a defective α-TTP excreted large amounts of α-CEHC despite low plasma levels of α-tocopherol. Small amounts of α-tocopherol are metabolised to α-CEHC from vitamin E within the habitual diet, it is hypothesised that this is due to the non-specific catabolism of RRR-α-tocopherol and thus some RRR-α-tocopherol being inevitably metabolised to α-CEHC. α-TTP is believed to selectively remove ftR/Ga-tocopherol from the metabolic pathway within the liver. Urinary α-CEHC may reflect the inter individual variation in processes involved in the catabolism of vitamin E and excess from supplementation rather than vitamin E status per se.

3.5.2 Response to varying single-doses of α-tocopheryl acetate in plasma, erythrocytes, platelets and lymphocytes

α-Tocopherol concentration increased in plasma and erythrocytes 24 hours after 195, 390 and 780 mg α-tocopheryl acetate, indicating that plasma and erythrocytes are responsive to, and therefore reflective of, short-term dietary intake. α-Tocopherol concentration increased non-significantly in platelets and lymphocytes. It is possible that platelets and lymphocytes only take up a small amount of newly absorbed α-tocopherol or have a slower uptake whereby at 24 h maximal uptake is not yet achieved. The uptake of newly absorbed α-tocopherol has not been investigated in platelets and lymphocytes; this information may provide an insight into the low uptake of α-tocopherol observed in the current study.

It was hypothesised that a dose-responsive increase in α-tocopherol would be observed in the blood components measured, however, this was not the case. The large
inter-individual variation in response to supplemental vitamin E is well documented (Dimitrov et al., 1991; Traber et al., 1998b) and this may have contributed to the lack of dose-response observed. A within subject study designed would have reduced the confounding variation between dose groups as Roxborough et al. (2000) reported lower intra-individual variation. Dimitrov et al. (1991) measured plasma α-tocopherol response in three individuals after a single dose of 440, 880, and 1320 mg all rac-α-tocopherol using a within subject study design. They reported a dose-response in two of the three subjects.

In the current study and the one by Dimitrov et al. (1991) it was not possible to distinguish between newly absorbed α-tocopherol from the supplement and pre-existing α-tocopherol within the body. It has previously been shown that newly absorbed α-tocopherol displaces pre-existing tocopherol in plasma (Burton et al., 1998; Traber et al., 1994). It has not previously been investigated whether newly absorbed α-tocopherol also displaces pre-existing tocopherol in erythrocytes, platelets and lymphocytes, although it is probable. It is also possible that there was a dose-responsive appearance of newly absorbed α-tocopherol into the blood components, but it was masked by a decrease in pre-existing vitamin E. Traber et al. (1998b) administered volunteers with 15, 75 and 150 mg deuterium labelled RRR-α-tocopheryl acetate and they reported a dose-responsive increase in deuterium labelled α-tocopherol within plasma, but total tocopherol remained unchanged. Deuterium labelled α-tocopherol was not available when this study was conducted.

A multiple dose-response study conducted by Lehmann et al. (1988) reported a dose responsive increase in α-tocopherol concentration within plasma, erythrocytes, platelets and lymphocytes. However, due to the large inter-individual variation in α-tocopherol concentration it is unlikely that the significant dose-response reported would remain when the appropriate statistical test is applied (one-way ANOVA rather than multiple t-tests).

Twenty-four hours after varying single doses of α-tocopheryl acetate, γ-tocopherol concentration was reduced in plasma and erythrocytes, but significantly only after 195 and 390 mg doses. Previously published studies have shown α-tocopherol supplementation to reduce γ-tocopherol concentration in plasma due to newly absorbed α-tocopherol replacing pre-existing tocopherol (Baker et al., 1986;
Handelman et al., 1985; Traber et al., 1992a). The \( \alpha \)-tocopheryl acetate capsules also contained \( \gamma \)-tocopherol, presumably originating from the soya bean oil used which contains 80mg \( \gamma \)-tocopherol per 100 g oil (Sheppard et al., 1993). The decrease in \( \gamma \)-tocopherol concentration was observed despite 4, 6, 12 and 24 mg \( \gamma \)-tocopherol being administered with the 130, 195, 390 and 780 mg \( \alpha \)-tocopheryl acetate doses, respectively. \( \gamma \)-Tocopherol is rapidly metabolised to \( \gamma \)-CEHC within the liver (Galli et al., 2001; Galli et al., 2003), therefore, at 24 h post ingestion of the comparatively small amount of \( \gamma \)-tocopherol would probably have returned to baseline, as shown by Galli et al. (2003) after a 100 mg dose of deuterium labelled \( \gamma \)-tocopheryl acetate (Galli et al., 2003).

3.5.3 Urinary \( \alpha \)-CEHC excretion in response to varying single-doses of \( \alpha \)-tocopheryl acetate

There was a transient increase in urinary \( \alpha \)-CEHC excretion after ingestion of varying single doses of \( \alpha \)-tocopheryl acetate (significant increase after the 390 mg dose only). This is in agreement with Himmelfarb et al. (2003) who reported a non-significant increase in serum \( \alpha \)-CEHC after a single dose of 600 mg \( \text{RRR} \) \( \alpha \)-tocopherol. A dose-response increase in \( \alpha \)-CEHC excretion was not observed after varying single doses of \( \alpha \)-tocopheryl acetate. This could be due to selective retention of \( \alpha \)-tocopherol within the liver and the large inter-individual variation.

Extensive dose responsive \( \text{RRR} \)-\( \alpha \)-tocopherol metabolism may only occur once \( \alpha \)-TTP is saturated. Schultz et al. (1995) administered increasing doses of \( \alpha \)-tocopheryl acetate (0 - 800 mg) over four weeks, they reported a dose response increase once plasma \( \alpha \)-tocopherol concentration exceeded 7 - 9 \( \mu \)M/ g total lipid following multiple \( \alpha \)-tocopherol supplementation.

A large inter-individual variation was observed in the transient increase in urinary \( \alpha \)-CEHC excretion up to 72 h after varying single doses of \( \alpha \)-tocopheryl acetate. This was clearly shown by the range of mg \( \alpha \)-CEHC excreted above baseline for each dose (Table 3.8). Some individuals did not increase their excretion of \( \alpha \)-CEHC over baseline, whereas others excreted up to ten-fold the baseline values. Hepatic \( \alpha \)-TTP is believed to remove \( \text{RRR} \)-\( \alpha \)-tocopherol from the metabolic pathway.
Hence the increase in urinary α-CEHC excretion observed maybe due to an excess of α-tocopherol greater than the capacity of α-TTP to transfer all the RRR-α-tocopherol away from the metabolic pathway. It is likely that the α-TTP capacity to transfer α-tocopherol differs in individuals and therefore potentially resulting in the observed inter-individual variation in urinary α-CEHC excretion. α-CEHC is excreted into bile (Hattori et al., 2000) and it is hypothesised that a proportion of the α-CEHC is reabsorbed into the systemic circulation from the intestine. The extent of reabsorption is also likely to vary between individuals, contributing to the inter-individual variation observed.

The absorption of vitamin E has been reported to be between 20 - 80 % (Blomstrand & Forsgren, 1968; Kelleher & Losowsky, 1970). The amount of α-tocopheryl acetate absorbed was not measured in the current study, however, α-tocopherol concentration at 24 h post ingestion of α-tocopheryl acetate positively correlated with urinary α-CEHC incremental AUC over 72 h. This suggests a possible relationship between the amount of α-tocopheryl acetate absorbed and the extent of urinary α-CEHC excretion.

Relatively recent publications reporting plasma or serum α- and γ-CEHC, illustrate the potential of measuring metabolites in plasma rather than urine (Galli et al., 2002; Radosavac et al., 2002). The process of urine collection can deter people from volunteering for a study and there is the possibility of incomplete collections. Measurements of urinary metabolites also include a degree of variation in individuals' kidney function, partly eliminated by correcting for creatinine values. The measurement of plasma α-CEHC may have provided further insight to the extent of α-tocopherol conversion to α-CEHC, however, the required methodology was not available to measure plasma α-CEHC in the current study.

The percentage of α-tocopheryl acetate dose excreted, as urinary α-CEHC was very low (< 1%) (calculated by mg α-CEHC excreted over baseline). This extremely low excretion of α-CEHC is in agreement with the low excretion of deuterium labelled α-CEHC following ingestion of 150 mg deuterium labelled RRR α-tocopherol reported by Traber et al. (1998a). Whereas, approximately 2 –6 % of a dose (125 or 500 mg) of α- or γ-tocotrienol was recovered as the respective urinary CEHCs (Lodge
Chapter 3

et al.,), the synthetic form of \( \alpha \)-tocopherol was metabolised 2 fold greater than the \( RRR \)-form when ingested together (150 mg of each) (Traber et al., 1998a). \( \gamma \)-Tocopherol has been found to undergo rapid metabolism with approximately 7 % of a dose (100 mg) of \( \gamma \)-tocopherol recovered as urinary \( \gamma \)-CEHC (Galli et al., 2003). The low urinary \( \alpha \)-CEHC excretion was probably due to the preferential retention of \( RRR \)-\( \alpha \)-tocopherol in the body rather than its metabolism. The current study shows that the body appears to be able to retain a single dose up to 780 mg \( RRR \)-\( \alpha \)-tocopherol without substantial metabolism to \( \alpha \)-CEHC. This agrees with the findings from Radosavac et al. (2002) whereby there was only a small increase in plasma \( \alpha \)-CEHC after a single dose of 306 mg \( RRR \) \( \alpha \)-tocopheryl acetate.

There are several factors involved in the production of urinary \( \alpha \)-CEHC that are currently unknown; these includes the proportion of the \( \alpha \)-tocopheryl acetate dose absorbed into the body, actual amount of \( \alpha \)-tocopherol converted to \( \alpha \)-CEHC and the fraction of \( \alpha \)-CEHC reabsorbed from the intestine. These are important considerations when attempting to understand the significance of urinary \( \alpha \)-CEHC excretion.

A positive correlation between plasma and erythrocyte \( \alpha \)-tocopherol concentration 24 hour after ingestion of the varying single doses of \( \alpha \)-tocopherol and urinary \( \alpha \)-CEHC excretion above baseline was observed. This implies a relationship between plasma and erythrocyte \( \alpha \)-tocopherol with \( \alpha \)-CEHC excretion rather than dose given. Schultz et al. also reported a correlation between plasma \( \alpha \)-tocopherol after supplementation and urinary \( \alpha \)-CEHC excretion (Schultz et al., 1995). This correlation only exists after \( \alpha \)-tocopherol doses substantially greater than that obtainable form the diet are ingested. Hence, the role of urinary \( \alpha \)-CEHC excretion in predicating vitamin E status appears to be limited.

3.5.4 Conclusions

The inter-individual variation in uptake of vitamin E into plasma could, in part, be due to differing amounts and rates of absorption from the intestine. The subjects were requested to consume a breakfast containing fat rather than being provided with a standard breakfast, the extent of variation introduced is not known, the food diaries indicated that the meals ranged from approximately 2.7 to 17.5 g fat (as analysed from
The blood sample was taken 24 hours post ingestion of the α-tocopheryl acetate capsules, in order for the newly absorbed α-tocopherol to enter the blood components and for it be comparable to the 24 h collection of urine. The measurements were taken after the peak α-tocopherol concentration in plasma. Several blood samples, taken over a number of time-points to calculate area under the curve, would be a preferable measure of α-tocopherol uptake into the blood components.

In conclusion, following varying single doses of α-tocopheryl acetate up to 780 mg, α-tocopherol is preferentially retained within the body with less than 1% of the dose recovered as α-CEHC in the urine. A dose responsive increase in α-tocopherol was not observed in blood components or urinary α-CEHC excretion.

The extent of inter-individual variation in absorption of α-tocopheryl acetate dose is an important area for future research as is the amount of fat required for maximal vitamin E absorption. Another important area of research is the uptake of α-tocopherol into platelets and lymphocytes, as both cell types are functionally affected by α-tocopherol. However, there is extremely limited information on how they acquire and regulate vitamin E.
Chapter 4

Absorption of labelled vitamin E following four test meals
4.1 Introduction

This chapter will investigate the effect of fat content and physical properties of food on vitamin E absorption. Vitamin E is believed to enter the enterocytes by passive diffusion and is reliant on processes involved in lipid emulsification, hydrolysis and enterocyte uptake (Hollander et al., 1975; Muralidhara & Hollander, 1977; Traber et al., 1993). Once within the enterocytes vitamin E is incorporated into chylomicrons with lipids and released into the systemic circulation via the thoracic duct (Bisgaier & Glickman, 1983).

It is widely accepted that dietary fats enhance vitamin E absorption (Traber et al., 1990b). Cohn et al. (1992b) demonstrated that mixed micelles are more efficient at incorporating α-tocopherol than pure bile salt micelles not containing lipid products, in vitro. Furthermore, Cohn et al. (1997) reported that α-tocopherol absorption required the hydrophobic molecules of lipid hydrolysis at the brush border membrane to allow the diffusion of α-tocopherol. There are few human studies that have investigated the effect of different fat quantities on vitamin E absorption. Dimitrov et al. (1991) reported significantly greater plasma α-tocopherol levels in humans after 5 days when given 800 mg all rac α-tocopherol with a high fat diet compared to a low fat diet. There are a few contradictory studies in rats, Brink et al. (1996) and Tijburg et al. (1997) both reported plasma α-tocopherol levels were similar after feeding vitamin E, irrespective of the fat content of the diet they were fed over a number of weeks. It has been proposed that the low fat diets contained sufficient fat for absorption of vitamin E (the low fat diets were 7 g fat/ kg feed or 15% energy). The amount of fat necessary for maximal vitamin E absorption is currently undetermined (Parks & Traber, 2000).

Despite the large number of vitamin E supplementation studies, the effect of different meals on the rate and magnitude of vitamin E absorption has not yet been investigated. This is the first post-prandial study to compare the uptake of α-tocopherol into plasma and chylomicrons after ingestion of α-tocopheryl acetate with meals containing different amounts of fat. The physical properties of food is known to affect gastric emptying, for example foods that are high in fibre, have high viscosity and the protein content protein (Low, 1990). These could in turn affect the rate of vitamin E uptake into enterocytes
4.2 Aim and hypothesis

A large degree of variation was observed in the magnitude of vitamin E uptake into plasma in Chapter 3. In the study, volunteers were instructed to consume a breakfast containing fat with varying single doses of α-tocopheryl acetate. Food diary analysis indicated that breakfasts ranged from cereal and semi-skimmed milk to toast and butter, or both. It was hypothesised that some of the variation observed was due to the subjects consuming an un-standardised meal with their vitamin E capsules.

The current study aims to;
1. Determine the extent of variation in α-tocopherol absorption when α-tocopheryl acetate is ingested with semi-skimmed milk and cereal compared with toast and butter.
2. Establish whether the rate and magnitude of α-tocopherol absorption following α-tocopheryl acetate ingestion is affected by the fat content of the meal.
3. Discover if the physical properties of a meal, independently of its fat content, affect α-tocopherol absorption.
4.3 Study design

Eight healthy volunteers were recruited from within the university. Selection criteria stated subjects must be non-smoking, not taking dietary supplements and with no gastrointestinal disorders as determined from a written questionnaire. Subjects with blood lipid abnormalities were excluded (selection criteria was cholesterol < 6 mmol/l and triacylglycerol < 1.5 mmol/l).

A within-subject, repeated measures study design was used, with each subject serving as his or her own control. Each subject consumed 150 mg deuterium labelled (d6) RRR-α-tocopheryl acetate on four separate occasions, with each occasion 7 days apart. Each subject consumed the α-tocopherol acetate capsule with four test meals in a randomised order. The four test meals were: 1) toast and butter, 2) full fat milk, single cream and cornflakes, 3) semi-skimmed milk and cornflakes, and 4) water. Table 4.1 shows the macronutrient content of the four test meals. The toast and butter meal and the low fat cereal meal were chosen to represent standard breakfasts consumed in the study detailed in Chapter 3. The high fat cereal breakfast was designed to contain the same amount and type of fat as the cereal and toast breakfast as it has previously been shown that the type of fat can affect the rate of vitamin E absorption (Ohrall et al. 2001).

Table 4.1 Macronutrient composition of the four test meals

<table>
<thead>
<tr>
<th>Test meal</th>
<th>Protein* (g)</th>
<th>Fat* (g)</th>
<th>CHO* (g)</th>
<th>Energy* (kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 slices white toast and 20 g butter</td>
<td>6.8</td>
<td>17.5</td>
<td>41.2</td>
<td>323</td>
</tr>
<tr>
<td>40 g cornflakes, 75 g full fat milk and 75 g single cream</td>
<td>7.5</td>
<td>17.5</td>
<td>41.0</td>
<td>342</td>
</tr>
<tr>
<td>40 g cornflakes and 75 g semi-skimmed milk</td>
<td>8.1</td>
<td>2.7</td>
<td>41.9</td>
<td>213</td>
</tr>
<tr>
<td>Glass of water</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values were calculated from Holland et al. (1995)

The subjects were instructed to fast for 12 h before the study commenced. Figure 4.1 summarises the study protocol. Subjects arrived at the Clinical Investigation Unit at the University of Surrey between 07:15 and 07:45 h. A trained doctor inserted an intravenous cannula into an antecubital vein in the forearm under local anaesthetic. A 10 ml baseline blood sample was taken, before the subjects
Subjects were only allowed water during the study period, and they consumed a standard lunch following the 6-hour blood sample. The standard lunch consisted of a sandwich and low fat yoghurt containing 23 g protein, 2.5 g fat, 59 g carbohydrate for a total energy content of 340 kcal.

![Figure 4.1 Schematic figure of study protocol](image)

10 ml blood samples were taken at baseline before ingestion of the \( \alpha \)-tocopheryl acetate capsule, then 0.5, 1, 1.5, 2, 3, 6 and 9 hours after ingestion of the capsule and test meal. Plasma was separated from the blood samples at each time point. Chylomicrons were then isolated from the plasma at the 0.5, 1, 1.5, 2, 3 and 6 h time points (the methods are described in sections 2.2.5 and 2.2.9.1). Plasma cholesterol and triacylglycerol, and chylomicron triacylglycerol and protein concentration were measured (as detailed in sections 2.2.10, 2.2.11 and 2.2.15). Deuterium labelled (d6) \( \alpha \)-tocopherol was measured in plasma and chylomicron samples by LC/MS (as detailed in sections 2.2.19 and 2.2.21).
4.4 Results

4.4.1 Subject characteristics

Eight healthy normolipidemic volunteers (5 female and 3 male) participated in the study. Table 4.2 shows the subject characteristics. During the study period no one reported any side effects or withdrew from the study.

Table 4.2 Subject characteristics (n = 8)

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>BMI (kgm(^2))</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>d6 α-TA/ kg body weight (mg/ kg)</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Cholesterol (mmol/ L)</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/ L)</td>
<td>0.95 ± 0.2</td>
</tr>
<tr>
<td>Plasma α-tocopherol (µmol/ L)</td>
<td>23.6 ± 2.2</td>
</tr>
<tr>
<td>Plasma α-T/ cholesterol (µmol/ mmol)</td>
<td>5.6 ± 0.3</td>
</tr>
</tbody>
</table>

BMI: body mass index, α-T: α-tocopherol α-TA: d6 α-tocopheryl acetate

4.4.2 Plasma and chylomicron cholesterol and triacylglycerol concentrations following four test meals

The concentration of plasma cholesterol and triacylglycerol (TAG) did not differ significantly during the study period (see graph in appendix IV).

Figure 4.2 illustrates the concentration of TAG in chylomicrons over 6 hours following four test meals. There was a significant difference over time in the TAG concentration of chylomicrons (p = 0.025), with a trend towards a significant difference in the TAG concentration profile between test meals (p = 0.051). Figure 4.3 illustrates the protein concentration in chylomicrons over 6 hours following four test meals. There was no significant difference in the protein content of chylomicrons over time or between test meals. A large inter-individual variation was observed in protein concentration up to 2 h, as shown by the large SEM bars in figure 4.3.
Figure 4.2 Chylomicron triacylglycerol concentration following ingestion of 150 mg d6 RRR-a-tocopheryl acetate with four test meals over 6 hours
Values expressed as mean ± SEM (n = 8). There was a significant difference over time (p = 0.025) and a trend towards a difference between test meals (p = 0.051) analysed by two way repeated measures ANOVA.

Figure 4.3 Chylomicron protein following ingestion of 150 mg d6 RRR-a-tocopheryl acetate with four test meals over 6 hours
Values expressed as mean ± SEM (n = 8). There was no significant difference over time as analysed by two way repeated measures ANOVA.
4.4.3 Plasma and chylomicron uptake of d6-RRR-α-tocopherol post ingestion of α-tocopheryl acetate with four test meals

On six occasions d6 α-tocopherol was detected (< 2 μM) in baseline plasma samples, it was present from a previous study day. The plasma d6 α-tocopherol values were corrected for baseline d6 α-tocopherol concentration and used in the analysis.

Figure 4.4 shows the plasma d6 α-tocopherol concentration over 9 hours. d6 α-Tocopherol increased from 2-3 h with the toast and butter and high fat cereal meals. There was a significant difference in d6 α-tocopherol concentration over time (p < 0.001) and between the four test meals (p < 0.001), per litre plasma or per mmol cholesterol (data not shown). There was greater d6-α-tocopherol absorption when the α-tocopheryl acetate was ingested with toast and butter compared with the low fat cereal meal and the water (p < 0.001) and there was a trend towards a greater d6 α-tocopherol absorption with toast and butter compared with the high fat cereal meal (p = 0.065). There was also significantly greater d6-α-tocopherol absorption when the α-tocopheryl acetate was ingested with the high fat cereal meal compared with the low fat cereal meal or water (p < 0.05)

Figure 4.4 Plasma d6-α-tocopherol concentration, following ingestion of 150 mg d6 RRR-α-tocopheryl acetate with 4 different meals over 6 hours
Values expressed as mean ± SEM (n = 8). There was significant difference over time and between test meals (p < 0.001) analysed by two way repeated measures ANOVA
Figures 4.5 and 4.6 show chylomicron d6 \( \alpha \)-tocopherol concentration per mmol TAG and per g protein. d6 \( \alpha \)-Tocopherol increased from 1.53 h with the toast and butter and 2 h with the high fat cereal meal. There was a significant difference in d6 \( \alpha \)-tocopherol concentration mmol TAG and per g protein over time \((p < 0.001)\) and between the four test meals \((p < 0.001)\). There was greater d6-\( \alpha \)-tocopherol absorption when the \( \alpha \)-tocopheryl acetate was ingested with toast and butter compared with the high fat cereal, low fat cereal and water meals \((p < 0.03)\).

Less inter-individual variation was observed in d6 \( \alpha \)-tocopherol per mmol TAG compared with per g protein, as illustrated by the large SEM bars in figure 4.6.

![Figure 4.5 Chylomicron d6-\( \alpha \)-tocopherol per mmol triacylglycerol (TAG) following ingestion of d6 \( \alpha \)-tocopheryl acetate with four test meals over 6 hours. Values expressed as mean ± SEM \((n = 8)\). There was significant difference over time \((p < 0.001)\) and between test meals \((p < 0.001)\) analysed by two-way repeated measures ANOVA.](image)
4.4.4 Inter-individual variation in uptake of d6-RRR-α-tocopherol

A large degree of inter-individual variation was observed in the uptake of d6 α-tocopherol in chylomicrons and plasma, after ingestion of 150 mg d6 RRR-α-tocopheryl acetate with the same test meal. Figure 4.7 shows the individual plasma d6 α-tocopherol absorption after following the ingestion of d6 α-tocopheryl acetate with the toast and butter meal. Individuals vary in the time course and magnitude of d6 α-tocopherol absorption. One individual differed from the other in that plasma d6 α-tocopherol decreased sharply at 9 h.
Figure 4.7 Individual d6 α-tocopherol plasma concentration profiles after ingestion of 150 mg d6-α-tocopheryl acetate with the toast and butter meal.
4.5 Discussion

The aim of this study was to determine if the absorption of \( \alpha \)-tocopheryl acetate was affected by the fat content and physical properties (independent of fat content) of the meal it was consumed with. Deuterium labelled \( RRR \) \( \alpha \)-tocopheryl acetate was used to trace the uptake of newly absorbed \( \alpha \)-tocopherol into plasma and chylomicrons.

Key findings;
1. The fat content of a meal (2.7 compared with 17.5 g fat) significantly influenced the rate and magnitude of \( \alpha \)-tocopherol uptake into plasma and chylomicrons over 9 hours.
2. The absorption of \( \alpha \)-tocopherol was significantly greater over 9 hours, when \( \alpha \)-tocopheryl acetate was ingested with toast and butter compared with full fat milk, single cream and cereal (both meals contained 17.5 g fat). This indicates that the physical properties of the meal affect the rate and magnitude of \( \alpha \)-tocopherol absorption.
3. A large inter-individual variation in the rate and magnitude of \( \alpha \)-tocopherol absorption was observed when \( \alpha \)-tocopheryl acetate was ingested with the same meal.

There are very few studies that have investigated the absorption of vitamin E in humans. This may be due to the inherent difficulty in measuring absorption and the requirement of labelled vitamin E in order to distinguish between existing and newly absorbed \( \alpha \)-tocopherol within the body. This study measured the uptake profiles of deuterium labelled \( \alpha \)-tocopherol in plasma and chylomicrons to compare the effects of different test meals on \( \alpha \)-tocopherol absorption. The study was not designed to measure the amount of \( \alpha \)-tocopherol absorbed from 150 mg d6 \( \alpha \)-tocopheryl acetate capsule.

The large inter-individual variation observed in Chapter 3 was hypothesised to be partially due to the fact that the vitamin E capsules were consumed with meals varying in fat content. The extent of variation this may have caused was thus investigated in the current study.
4.5.1 Absorption of d6 α-tocopheryl acetate following four test meals

The uptake of α-tocopherol into plasma and chylomicrons was determined following the ingestion of d6 α-tocopheryl acetate with two common breakfasts that subjects consumed in the study detailed in Chapter 3, these were a) semi-skimmed milk and cereal, b) and toast and butter. There was significantly greater uptake of d6 α-tocopherol, over 9 hours, when the capsule was consumed with toast and butter compared with semi-skimmed milk and cereal. The uptake of d6 α-tocopherol when ingested with the semi-skimmed milk and cereal was not statistically different to water.

The reason for the differences observed in α-tocopherol uptake into plasma and chylomicrons could be due to the greater fat content of the toast and butter compared with the semi-skimmed milk and cereal (17.5 g compared to 2.7 g respectively). However, the two test meals also differed greatly in their physical properties, which may impact on the absorption of vitamin E. In plasma there was a significantly greater d6 α-tocopherol absorption with the high fat cereal meal compared with the low fat cereal meal and a trend towards a significantly greater absorption with toast and butter compared with high fat cereal meal. This suggests that both the fat content and the physical properties of a meal influence α-tocopherol absorption.

4.5.1.1 The effect of fat content on the absorption of α-tocopherol

It is generally accepted that vitamin E requires mixed micelles and a hydrophobic environment at the brush border membrane of the enterocytes for optimal absorption (Cohn et al., 1992b; Cohn, 1997). There was significantly greater α-tocopherol uptake into plasma and chylomicrons when α-tocopheryl acetate was ingested with high fat cereal meal (17.5 g fat) compared with low fat cereal meal (2.7 g fat), over 9 hours. Therefore, the vitamin E uptake profiles observed in this study are in agreement with the current understanding of vitamin E absorption.

Previously published studies have provided different amounts of fat when investigating the effects of supplemental vitamin E (Cheeseman et al., 1995; Traber et al., 1998b). This was the first post-prandial study to investigate whether the amount of dietary fat effects vitamin E absorption in humans. It showed that α-tocopheryl acetate
consumed with 17.5 g fat was absorbed to a greater extent over 9 hours compared with 
\( \alpha \)-tocopheryl acetate consumed with 2.7 g fat (Figure 4.4).

Traber et al. (1998) gave 150 mg d3 \( \text{RRR-} \alpha \)-tocopheryl acetate with a meal 
containing 30 g fat and reported plasma d3 \( \alpha \)-tocopherol concentration to be 
approximately 7 \( \mu \)M at 9 hours. In the current study the mean d6 \( \alpha \)-tocopherol 
concentration at 9 hours was 6 and 10 \( \mu \)M, following high fat cereal and toast and 
butter meals, respectively. It is therefore possible that 17.5 g fat is sufficient for 
optimal absorption of 150 mg \( \alpha \)-tocopheryl acetate, as Traber did not report a greater 
absorption following ingestion with 30 g fat. Traber et al. (1998) also measured \( \alpha \)-
tocopherol uptake into chylomicrons, however the data cannot be compared as they 
express \( \alpha \)-tocopherol per litre plasma, whereas in the current study the \( \alpha \)-tocopherol 
within chylomicrons is expressed as per mmol chylomicron TAG or per gram 
chylomicron protein. The current study did not report \( \alpha \)-tocopherol concentration in 
lipoproteins per litre plasma due to the limitations in the methodology used to isolate 
the lipoproteins (method detailed in section 2.2.9).

Chronic \( \alpha \)-tocopherol and lipid feeding studies have investigated the influence 
of fat on plasma vitamin E levels; collectively the results are inconclusive. Brink et al. 
(1996) fed rats a high (190 g fat/ kg feed) or low fat (7 g fat/ kg feed) diet over 3 
weeks and vitamin E absorption was determined by intake minus faecal excretion. 
They reported no significant difference in the absorption of vitamin E between the two 
diets (Brink et al., 1996). This finding was replicated when Tijburg et al. (1997) fed 
rats either 15 or 30 % fat diets and concluded that the low fat diet contained sufficient 
fat for significant absorption of vitamin E. A study in humans also revealed that 
plasma \( \alpha \)-tocopherol concentration was similar following 7 days of 50 mg \( \alpha \)-
tocopherol consumed with a low fat (< 6.5 g fat) or high fat (~ 45 g fat) meal 
(Roodenburg et al., 2000). Furthermore, Hayes et al. (2001) reported that milk 
enhanced vitamin E uptake, irrespective of fat content. Their conclusions were based 
on a similar percentage increase in plasma \( \alpha \)-tocopherol after 4-week supplementation 
with fat-soluble version of vitamin E dispersed in 1 % fat milk, and a water-soluble 
form of vitamin E in skimmed milk (0.1 % fat). The 1 % fat milk used had a much 
lower fat content (~ 5 g fat ingested) compared with the high fat cereal meal used 
within the current study (17.5 g fat), therefore Hayes et al. (2001) were unable to take
into account if a higher fat content milk would increase vitamin E absorption. However, Dimitrov et al. (1991) reported a significantly greater plasma uptake of \( \alpha \)-tocopherol in humans fed a high fat diet (~ 115 g fat per day) compared to a low fat diet (~ 51 g fat per day) over a five day supplementation with 800 mg all rac \( \alpha \) -tocopheryl acetate. Likewise, Iuliano et al. (2001) reported greater plasma \( \alpha \)-tocopherol concentration after 15 days when 300 mg \( \alpha \)-tocopheryl acetate was consumed with a meal compared with an ‘empty stomach’.

It is possible that a small amount of \( \alpha \)-tocopherol is absorbed without dietary fat. In the current study there was a small increase in plasma d6 \( \alpha \)-tocopherol at 9 hours and Dimitrov et al. (1991) also reported a small increase in plasma \( \alpha \)-tocopherol after a fat free meal, the mechanisms for this are unclear. It is possible that the enterocytes contain a pool of lipid from the previous meal and this may aid chylomicron formation and vitamin E incorporation.

The current study confirms that fat is required for vitamin E absorption, however, the minimal amount of dietary fat necessary for optimal vitamin E absorption remains undetermined. Future dose-response studies to investigate the different amounts of fat are necessary to determine the minimum amount of fat required for optimal absorption. The amount of dietary fat required may also vary with the amount of \( \alpha \)-tocopherol ingested.

4.5.1.2 The effect of meal physical properties on the absorption of \( \alpha \)-tocopherol

The uptake of vitamin E was investigated after the ingestion of 150 mg d6 -RRR-\( \alpha \)-tocopheryl acetate with two meals identical in fat content (17.5 g fat) but different in physical properties; full fat milk, single cream and cereal (high fat cereal) compared with toast and butter. The physical properties of toast and butter compared to high fat milk and cereal meal produced a greater uptake profile of d6 \( \alpha \)-tocopherol in the plasma and chylomicrons over 9 and 6 hours respectively. There have been no previous suggestions within the literature that the physical properties of a meal vitamin E is consumed with affects its absorption. The physical properties of food are known to affect gastric emptying, foods that are high in fibre, viscosity and protein slow gastric emptying (Low, 1990). These could in turn affect the rate of vitamin E uptake into enterocytes.
The total amount of \( \alpha \)-tocopherol absorbed from the \( \alpha \)-tocopheryl acetate capsule was not determined in this study, therefore, it is not known whether the amount absorbed when consumed with an isocaloric cereal and milk meal is less than toast and butter or simply delayed. Further investigations would be interesting and longer sampling time may help to elucidate whether there is a difference in amount absorbed. The inherent difficulties in measuring total absorption of vitamin E, either by lymph duct cannulation or faecal excretion, means that these studies are unlikely to be carried out.

The reduced absorption of d6 \( \alpha \)-tocopherol into plasma and chylomicrons over 9 hours following a high fat cereal meal may be due to delayed gastric emptying. The high fat cereal meal had a greater volume compared with the toast and butter meal. Low et al. (1990) reported that the meal volume and proportions of solid and liquid within a meal have different gastric emptying patterns (Low, 1990). Additionally the high fat cereal meal contained casein, a milk protein, has been shown to delay gastric emptying (Low, 1990). A delayed gastric emptying of the high fat cereal meal compared with the toast and butter would have increased the time required for the lipids and \( \alpha \)-tocopherol to reach the enterocytes.

4.5.2 Inter-individual variation in the absorption of \( \alpha \)-tocopheryl acetate

A large inter-individual variation in the magnitude and time of d6-\( \alpha \)-tocopherol uptake within plasma and into chylomicrons was observed, illustrated by the large SEM bars and the individual uptake profiles after toast and butter. Roxborough et al. (2000) and Cheeseman et al. (1995) have also reported large inter-individual variation in the absorption of d6 RRR-\( \alpha \)-tocopherol in plasma up to 51 hours. Roxborough et al. (2000) reported a low intra individual variation in the uptake of d6 RRR-\( \alpha \)-tocopherol in to plasma, which may imply a genetic influence in the inter individual variation observed. This observed inter-individual variation is probably due to a number of factors including the gastrointestinal handling of lipids, processes involved in the packaging of tocopherol into chylomicrons and their release into the systemic circulation. Variation in the secretion of bile and pancreatic lipases would impact on the handling of lipids and consequently effect vitamin E uptake.
It is not known how vitamin E is packaged into chylomicrons. Arita et al. (1997) speculated that α-tocopherol is associated with VLDL after it is secreted, which may also be the case for α-tocopherol incorporation into chylomicrons. There are a number of genetic polymorphisms involved in lipoprotein regulation (Ye & Kwiterovich, 2000). For example, polymorphism in apo B or the chylomicron assembly protein, microsomal transfer protein, may influence the rate of chylomicron secretion and therefore appearance of newly absorbed α-tocopherol in the systemic circulation (Bergeron & Havel, 1997; Hussain, 2000; Peacock et al., 1995).

It is important to gain an understanding of the extent of inter-individual variation in uptake of vitamin E in healthy individuals for designing future studies investigating different sub groups within the population.

4.5.3 Study design

The time points for the current study were chosen to identify the initial stages of α-tocopherol uptake in chylomicrons and plasma. Borel et al. (1997) reported α-tocopherol within chylomicrons to be significantly greater than baseline after 1 hour. In the current study none was detected in the chylomicrons before 1.5 hours. The washout period between study dates was 7 days, however, d6 α-tocopherol was detected in some of the baseline plasma samples. Therefore, the time between the study days needed to be longer to allow the d6 α-tocopherol to reach negligible level within the plasma before administering another dose.

The current study was designed to measure the uptake profile of α-tocopherol in chylomicrons and plasma and therefore blood sampling stopped at 9 hours. If some d6 α-tocopherol remained in the enterocyte until the next meal, this would enrich the second influx of chylomicrons with d6 α-tocopherol and the study would be unable to distinguish between the first or second phase of chylomicron absorption. Additionally there would be transfer of tocopherol between lipoproteins, confounding the concentrations of d6 α-tocopherol taken up from the intestine. Further time points would have strengthened the results in determining the total amount of d6 α-tocopherol absorbed.
The evening meal of the subjects was not standardised and therefore may have influenced the postprandial response the test meals, increasing the inter individual variation observed.

4.5.4 Conclusions and future work

In conclusion this study has presented important findings in the post-prandial handling of α-tocopherol following meals containing high or low fat (17.5 or 2.7 g fat) and the influence of the physical properties of the meal. Clinical trials with vitamin E have not proved conclusive towards a benefit for vitamin E, to obtain maximal absorption vitamin E must be consumed with a certain amount of fat, this should be taken into consideration for the design of future studies investigating vitamin E uptake.

A dose-response investigation into the amount of fat required for maximal vitamin E absorption at differing vitamin E doses ingested is an important area for future research.
Chapter 5

Biokinetics of labelled $\alpha$-tocopherol in healthy individuals
5.1 Introduction

Vitamin E biokinetics within the systemic circulation is governed by plasma lipoprotein kinetics. Chylomicrons carry newly absorbed vitamin E, whilst circulating they transfer lipids and vitamin E to peripheral tissue during lipid hydrolysis (Traber et al., 1985). Excess chylomicron surface area is consequently produced and along with vitamin E it is transferred to HDL (Traber & Kayden, 1989a). The resultant chylomicron remnants are taken up into the liver and RRR α-tocopherol is packaged into VLDL and then secreted into the blood (Traber et al., 1993). VLDL is catabolised to LDL and during this process vitamin E is transferred to HDL (Kayden & Traber, 1993). Vitamin E is also taken up into peripheral tissues via LDL receptors (Traber & Kayden, 1984). Whilst lipoproteins are circulating there is a constant flux of vitamin E between them (Traber et al., 1992b).

The uptake and distribution of newly absorbed α-tocopherol has previously been reported for plasma, lipoproteins and erythrocytes, but not together within the same individuals (Cheeseman et al., 1995; Roxborough et al., 2000; Traber et al., 1998b). This chapter will focus on the uptake and distribution profiles of newly absorbed α-tocopherol into erythrocytes, platelets and lymphocytes in healthy people. The uptake and biokinetics of newly absorbed α-tocopherol into platelets and lymphocytes has previously not been investigated.

Vitamin E within erythrocytes is membrane bound and they obtain vitamin E via tocopherol binding proteins (Kitabchi & Wimalasena, 1982b). Lymphocytes have LDL receptors and are thought to obtain their vitamin E by internalisation of LDL. It is currently unknown how platelets obtain their vitamin E. Both platelets and lymphocytes are functionally affected by vitamin E and therefore vitamin E concentration within these cells may represent a functional biomarker of vitamin E. Vitamin E regulation and distribution between the aforementioned blood components is poorly understood. Investigating the biokinetics of α-tocopherol will help towards elucidating how they acquire their α-tocopherol.
5.2 Aim and hypothesis

The objective of this research was to investigate the response of vitamin E concentration within blood components to supplemental α-tocopherol in healthy subjects. The study aimed to establish the uptake and distribution profile in response to a single dose of stable isotope labelled α-tocopherol in plasma, lipoproteins, erythrocytes, platelets and lymphocytes. The uptake profile of newly absorbed α-tocopherol into platelets and lymphocytes is currently unknown.

It was hypothesised that an increase in newly absorbed α-tocopherol would be observed first in the plasma, followed by erythrocytes, platelets and lymphocytes as they acquire the newly absorbed α-tocopherol from the circulating lipoproteins. It was also hypothesised that the greater the d6 α-tocopherol uptake in to plasma the greater the uptake will be into blood cells.
5.3 Study design

Twelve healthy males were recruited from the general population by an advertisement in local newspapers. Selection criteria stated subjects must be non-smoking, not taking dietary supplements and with no gastrointestinal disorders as determined from a written questionnaire. Subjects with blood lipid abnormalities were excluded (selection criteria was cholesterol < 6 mmol/1 and triacylglycerol < 1.5 mmol/l).

Subjects arrived at the Clinical Investigation Unit at the University of Surrey at 7.45 am, having been requested to fast from 8 pm the night before the study. Figure 5.1 summarises the study protocol. The subjects consumed a standard breakfast containing 40g fat (2 croissants and 20g butter); investigators ensured the capsule, containing 150 mg deuterium labelled (d6) RRR α-tocopheryl acetate, was taken midway through the meal. The breakfast contained 40g fat to maximise vitamin E absorption. The time the capsule was consumed was taken as 0 hours and blood sampling was timed from this point. A suitable trained person drew 20 ml blood samples from the antecubital vein in the forearm at 3, 6, 9, 12, 24 and 48 h hours after ingestion of the capsule (illustrated in figure 5.1). Subjects were only allowed water and a standard meal at 12:00 until 15:00 on day 1, and then they were allowed to eat ad libitum.

From the blood samples plasma, erythrocytes, platelets and lymphocytes were freshly isolated. Chylomicrons, VLDL, LDL and HDL were isolated from frozen plasma at a later date, procedures detailed in sections 2.2.4 - 9. d0 and d6 α-tocopherol were measured in all blood component (method detailed in section 2.2.21).

![Figure 5.1 Schematic diagram of study protocol](image)

150 mg d6 RRR α-tocopheryl acetate capsule was taken with a meal containing 40 g fat. 20 ml blood was taken at 3, 6, 9, 12, 24 and 48 h after ingesting the capsule.
5.4 Results

5.4.1 Subject information

Twelve healthy normolipidemic males participated in the study. During the study period no one reported any side effects or withdrew from the study. Table 5.1 outlines the characteristics of the subjects who participated in the study. The mean body mass index was slightly higher than the recommended ideal range of 20 – 25 kgm$^{-2}$ (WHO, 1998). Dosage of d6 α-tocopheryl acetate was calculated per kg body weight (table 5.1), there was a coefficient of variation of 10% between subjects.

Table 5.1 Subject characteristics (n = 12)

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48 ± 10</td>
<td>31 - 58</td>
</tr>
<tr>
<td>BMI (kgm$^{-2}$)</td>
<td>25.6 ± 2.5</td>
<td>23.1 – 32.1</td>
</tr>
<tr>
<td>d6 α-TA/ kg body weight (mg/ kg)</td>
<td>1.9 ± 0.2</td>
<td>1.7 – 2.2</td>
</tr>
<tr>
<td>Total cholesterol (mmol/ L)</td>
<td>5.0 ± 0.6</td>
<td>3.88 – 5.84</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/ L)</td>
<td>1.2 ± 0.3</td>
<td>0.76 – 1.49</td>
</tr>
</tbody>
</table>

BMI; body mass index, α-TA; d6 α-tocopheryl acetate
5.4.2 Plasma and lipoprotein α-tocopherol profiles

Plasma cholesterol concentrations were not statistically different during the study period. However, there was a trend towards a significant difference in triacylglycerol (TAG) concentration over time (p = 0.06) (see graph in appendix IV).

Table 5.2 shows d6 α-tocopherol area under curve (AUC), maximum concentration (C max) and time of C max for plasma, chylomicrons, VLDL, LDL and HDL. These parameters give an indication of the extent of inter-individual variation in uptake of newly absorbed d6 α-tocopherol. There was a larger inter-individual variation in chylomicron d6 α-tocopherol per g protein compared with d6 α-tocopherol per mmol TAG, as determined by lower coefficient of variation for AUC and C max as shown in table 5.2.

### Table 5.2 Area under curve (AUC), maximum concentration (C max) and time of C max for d6 α-tocopherol in plasma and lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>C max</th>
<th>Time C max (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/L.</td>
<td>Mean ± SEM</td>
<td>678 ± 92</td>
<td>21.4 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(233 – 1140)</td>
<td>(7.7 – 30.9)</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>38 %</td>
<td>32 %</td>
</tr>
<tr>
<td><strong>Chylomicrons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein</td>
<td>Mean ± SEM</td>
<td>226 ± 59</td>
<td>22.5 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(64 – 602)</td>
<td>(4.2 – 60.2)</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>74 %</td>
<td>78 %</td>
</tr>
<tr>
<td><strong>Chylomicrons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ mmol TAG</td>
<td>Mean ± SEM</td>
<td>101 ± 20</td>
<td>7.7 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(36 – 220)</td>
<td>(4.1 – 13.8)</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>58 %</td>
<td>50 %</td>
</tr>
<tr>
<td><strong>VLDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein</td>
<td>Mean ± SEM</td>
<td>874 ± 96</td>
<td>28.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(260 – 1250)</td>
<td>(6.7 – 44.6)</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>31 %</td>
<td>34 %</td>
</tr>
<tr>
<td><strong>LDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein</td>
<td>Mean ± SEM</td>
<td>556 ± 96</td>
<td>17.3 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(218 – 1022)</td>
<td>(7.1 – 31.4)</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>49 %</td>
<td>51 %</td>
</tr>
<tr>
<td><strong>HDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein</td>
<td>Mean ± SEM</td>
<td>45.5 ± 4.7</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(21.5 – 63.7)</td>
<td>(0.7 – 1.9)</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>29 %</td>
<td>29 %</td>
</tr>
</tbody>
</table>

Plasma; n = 12, lipoproteins; n = 11 there was insufficient plasma to isolate lipoproteins. TAG: triacylglycerol, CV: coefficient of variation
Figure 5.2 illustrates the mean total, pre existing (d0) and newly absorbed (d6) α-tocopherol profiles in plasma over 48 h. d6 α-Tocopherol concentration increased significantly over time (p < 0.0001), there was a peak in concentration at 9 h, then it plateaued until 24 h whereby the concentration started to decline. There was a significant increase in total tocopherol up to 9 h and an initial decrease in d0 α-tocopherol concentration to 12 h (p < 0.001). The time of d6 α-tocopherol Cmax ranged from 9 to 24 h (Table 5.2). At 12 h Cmax in plasma was 18.0 ± 2.3 μmol/L, with a coefficient of variation of 37 %.

![Graph](image_url)

**Figure 5.2** Plasma total (d0 + d6), d0 and d6 α-tocopherol concentration profile over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate

Values expressed as mean ± SEM. (n = 12). Total and d6 α-tocopherol concentration increased significantly over time and d0 α-tocopherol decreased over time (p < 0.0001) as analysed by repeated measures ANOVA.
Figure 5.3 illustrates the mean uptake profile of d6 α-tocopherol into chylomicrons. The uptake of d6 α-tocopherol was significantly different over time (p < 0.0001). There was a sharp increase from 3 to 6 h and d6 α-tocopherol concentration peaked at 6 h (there was relatively little variation in the time of d6 α-tocopherol Cmax in chylomicrons shown by a coefficient of variation of only 15% shown in table 5.2). There was a large inter-individual variation in the magnitude of d6 α-tocopherol uptake into chylomicrons, as illustrated by the large SEM bars at 6 h in figure 5.3 and the coefficient of variation for Cmax was 78%, as shown in table 5.2. There was less inter-individual variation in Cmax and AUC for d6 α-tocopherol per mmol TAG in chylomicrons as shown in table 5.2. At 48 h d6 α-tocopherol concentration was only 1 μM/g protein.

![Graph showing chylomicron d6 α-tocopherol concentration over 48 h](image)

**Figure 5.3 Chylomicron d6 α-tocopherol per g protein profile over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate**

Values expressed as mean ± SEM. (n = 11; insufficient plasma to isolate lipoproteins from one subject). d6 α-Tocopherol concentration increased significantly over time (p < 0.0001) as analysed by repeated measures ANOVA.

Figures 5.4A and B illustrate the mean d6 and d0 α-tocopherol concentration in VLDL, LDL and HDL over 48 h. There was a significant increase in d6 α-tocopherol concentration over time in all lipoproteins (p < 0.0001). d6 α-Tocopherol peaked at 12 h in VLDL and then started to decline, whereas there was a plateau in d6 α-tocopherol concentration between 12 and 24 h in LDL and HDL.
There was a decrease in d0 \( \alpha \)-tocopherol concentration in VLDL, LDL and HDL up to 12 h post ingestion of the d6 \( \alpha \)-tocopheryl acetate \((p < 0.003)\). There was substantially less inter-individual variation in d6 \( \alpha \)-tocopherol AUC and Cmax in VLDL, LDL and HDL \((CV = 30 - 50\%)\) compared with d6 \( \alpha \)-tocopherol in chylomicrons \((CV > 70\%)\), as shown in table 5.2.

Figures 5.4 A. and B: VLDL, LDL and HDL d6 and d0 \( \alpha \)-tocopherol per g protein over 48 h after ingestion of 150 mg d6 RRR-\( \alpha \)-tocopheryl acetate, respectively

Values expressed as mean \( \pm \) SEM \((n = 11)\). There was a significant increase in d6 \( \alpha \)-tocopherol concentration over time in VLDL, LDL and HDL \((p < 0.0001)\) analysed by repeated measures ANOVA.
Figure 5.5 shows the d6 α-tocopherol percentage of total tocopherol in chylomicrons, VLDL, LDL and HDL over 48 h. In chylomicrons the percentage d6 α-tocopherol peaked at 6 h then declined to approximately 15 % at 48 h. Whereas, the percentage d6 α-tocopherol uptake in VLDL, LDL and HDL were very similar with a peak at 12 h and a decline from 24 h. The maximum percentage of d6 α-tocopherol within chylomicrons was 55% (range 22 - 76 %), whereas the maximum percentage of d6 α-tocopherol in VLDL, LDL and HDL was 36% (range 29 – 57 %).

Figure 5.5 Percentage d6 α-tocopherol uptake profiles for chylomicrons, VLDL, LDL and HDL over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate
Values expressed as mean ± SEM (n = 11).
5.4.3 α-Tocopherol profiles for erythrocytes, platelets and lymphocytes

Table 5.3 shows d6 α-tocopherol AUC, C max and time of C max for plasma, erythrocytes, platelets and lymphocytes. The extent of inter-individual variation in AUC and Cmax was similar for d6 α-tocopherol in plasma and erythrocytes, platelets and lymphocytes, as determined by % CV. The variation in time of C max was substantially greater in platelets and plasma compared with erythrocytes and lymphocytes as determined by coefficient of variations shown in table 5.3.

Table 5.3 Area under curve (AUC), maximum concentration (C max) and time of C max for d6 α-tocopherol in plasma and blood cells

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>C max</th>
<th>Time C max (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Mean ± SEM</td>
<td>678 ± 92</td>
<td>21.4 ± 2.4</td>
</tr>
<tr>
<td>μmol/L</td>
<td>(range)</td>
<td>(233 – 1140)</td>
<td>(7.7 – 30.9)</td>
</tr>
<tr>
<td>% CV</td>
<td>38 %</td>
<td>32 %</td>
<td>40 %</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Mean ± SEM</td>
<td>118 ± 11</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>μmol/ PCV</td>
<td>(range)</td>
<td>(58 – 190)</td>
<td>(2.6 – 7.9)</td>
</tr>
<tr>
<td>% CV</td>
<td>32 %</td>
<td>29 %</td>
<td>16 %</td>
</tr>
<tr>
<td>Platelets</td>
<td>Mean ± SEM</td>
<td>15.5 ± 1.5</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>μmol/ g protein</td>
<td>(range)</td>
<td>(6.5 – 2.4)</td>
<td>(0.23 – 0.69)</td>
</tr>
<tr>
<td>% CV</td>
<td>34 %</td>
<td>29 %</td>
<td>50 %</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Mean ± SEM</td>
<td>10.2 ± 0.9</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>μmol/ g protein</td>
<td>(range)</td>
<td>(5.7 – 18.3)</td>
<td>(0.19 – 0.52)</td>
</tr>
<tr>
<td>% CV</td>
<td>36 %</td>
<td>34 %</td>
<td>19 %</td>
</tr>
</tbody>
</table>

n = 12 apart from lymphocytes n = 11 there was insufficient plasma to isolate lymphocytes from one subject. PCV: packed cell volume, CV: coefficient of variation

Figure 5.6 illustrates the mean d6 α-tocopherol concentration in erythrocytes up to 48 h, with the plasma d6 α-tocopherol uptake profile in grey shown for comparison. Erythrocyte d6 α-tocopherol concentration significantly increased over time (p < 0.0001), it peaked at 24 hours then started to decline at 48 h. At 24 h C max in erythrocytes was 4.76 ± 0.43 μmol/ PCV, with a coefficient of variation of 30 %. There was no d0 α-tocopherol data available for erythrocytes due to methodological problems detailed in section 2.2.21.

There was a positive correlation between d6 α-tocopherol AUC in erythrocytes and HDL ($r^2 = 0.55$, $p = 0.006$) and plasma ($r^2 = 0.46$, $p = 0.03$), however, there were no correlations between d6 α-tocopherol AUC for chylomicrons, VLDL or LDL with erythrocyte d6 α-tocopherol AUC.
Figure 5.6 Erythrocyte d6 α-tocopherol corrected for packed cell volume (PCV) over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate. Plasma d6 α-tocopherol in grey shown for comparison. Values expressed as mean ± SEM (n = 12). There was a significant increase in d6 α-tocopherol concentration over time in erythrocytes (p < 0.0001) analysed by repeated measures ANOVA.

Figure 5.7 illustrates the mean total, d0 and d6 α-tocopherol concentration in platelets over 48 h. d6 α-Tocopherol concentration significantly increased over time, while d0 α-tocopherol decreased (p < 0.0001), there was no significant difference in total α-tocopherol concentration. Subjects were classified into two groups, those that had a biphasic uptake of d6 α-tocopherol and those that did not. Figure 5.8 illustrates the mean d0 and d6 α-tocopherol over 48 h in four subjects who exhibited a biphasic response in contrast to the other eight subjects. Subjects who did not have a biphasic uptake gradually incorporated d6 α-tocopherol from 3 h and plateaued between 24 and 48 h. Whereas, in the biphasic group d6 α-tocopherol peaked at 6 h then declined at 9 h, then there was a gradual uptake similar to the subjects that did not exhibit a biphasic response. d0 α-Tocopherol profiles were similar in both groups, as shown in figure 5.8. Figure 5.9 shows the initial d6 α-tocopherol peak at 6 hours, in subjects who exhibited a biphasic uptake, which coincided with the d6 α-tocopherol peak in chylomicrons.
Figure 5.7 Total (d0 + d6), d0 and d6 α-tocopherol in platelets per g protein over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate
Values expressed as mean ± SEM (n = 12). d6 α-Tocopherol concentration increased significantly over time and d0 α-tocopherol decreased over time (p < 0.0001) as analysed by repeated measures ANOVA.

Figure 5.8 d6 and d0 α-tocopherol profile in four subjects that had a biphasic uptake of d6 α-tocopherol and eight subjects who had a gradual d6 α-tocopherol uptake profile over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate
Values expressed as mean ± SEM. α-T: α-tocopherol
There was a significant correlation between d6 α-tocopherol AUC for platelets and LDL ($r^2 = 0.51$, $p = 0.04$), however, there were no correlations between d6 α-tocopherol AUC for plasma, other lipoproteins or erythrocytes. There was a significant correlation between d6 α-tocopherol AUC for platelets and lymphocytes ($r^2 = 0.57$, $p = 0.009$).

**Figure 5.9** Biphasic uptake of d6 α-tocopherol in platelets per g protein in 4 subjects (and chylomicron d6 α-tocopherol in grey shown for comparison) over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate

Values expressed as mean ± SEM
Figure 5.10 illustrates the mean total, d0 and d6 α-tocopherol concentration in lymphocytes over 48 h. Lymphocyte d6 α-tocopherol concentration significantly increased over time and d0 α-tocopherol decreased (p < 0.0001) and there was no difference in total α-tocopherol concentration. From 6 hours a gradual uptake of d6 α-tocopherol into lymphocytes was observed and d6 α-tocopherol concentration continued to increase at 48 h.

There was a trend towards a significant correlation between d6 α-tocopherol AUC in lymphocytes and LDL (r^2 = 0.49, p = 0.06), however, there were no correlations between d6 α-tocopherol AUC for plasma, other lipoproteins or erythrocytes. There was a significant correlation between d6 α-tocopherol AUC for lymphocytes and platelets (r^2 = 0.57, p = 0.009).

![Graph showing mean total, d0 and d6 α-tocopherol concentration](image)

**Figure 5.10** Lymphocytes total (d0 + d6), d0 and d6 α-tocopherol per g protein over 48 h after ingestion of 150 mg d6 RRR-α-tocopheryl acetate

Values expressed as mean ± SEM. d6 α-Tocopherol concentration increased significantly over time and d0 α-tocopherol decreased over time (p < 0.0001) as analysed by repeated measures ANOVA.
5.5 Discussion

This study aimed to determine the uptake and distribution profile of a single dose of deuterium labelled α-tocopherol into plasma, lipoproteins, erythrocytes, platelets and lymphocytes in healthy subjects. The uptake profile of newly absorbed α-tocopherol into platelets and lymphocytes had not previously been investigated.

Key findings;
1. The uptake profile of newly absorbed (d6) α-tocopherol was determined in platelets and lymphocytes. A third of subjects exhibited a biphasic uptake profile of d6 α-tocopherol in platelets. d6 α-Tocopherol concentration gradually increased from 6 h in lymphocytes up to 48 h.
2. The magnitude of d6 α-tocopherol uptake into plasma did not correlate with that in platelets or lymphocytes.
3. Pre existing (d0) α-tocopherol concentration decreased in all blood components measured

5.5.1 Uptake of newly absorbed d6 α-tocopherol in plasma and lipoproteins

Plasma α-tocopherol concentrations represent the combined total of α-tocopherol within circulating lipoproteins. Following a single dose of 150 mg d6 RRR α-tocopheryl acetate plasma d6 α-tocopherol concentration increased from 3 h, plateaued between 9 and 24 h and then started to decline, this was similar to previously published studies (Cheeseman et al., 1995; Roxborough et al., 2000; Traber et al., 1998b). These studies are outlined in table 5.4.

Chylomicron d6 α-tocopherol peaked at 6 h then decreased, this pattern of uptake was in agreement with published studies (Traber et al., 1990a; Traber et al., 1992a). Once the chylomicrons entered the systemic circulation the newly absorbed d6 α-tocopherol was rapidly incorporated into all lipoproteins. d6 α-Tocopherol uptake into VLDL peaked at 12 h then started to decline, whereas there was a plateau in d6 α-tocopherol concentration between 12 and 24 h in LDL and HDL before d6 α-tocopherol concentration started to decline. These observations were similar to previously published studies (Traber et al., 1990a; Traber et al., 1992a; Traber et al., 1998b).
Table 5.4 Studies investigating labelled α-tocopherol biokinetics

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Dosage α-TA</th>
<th>Fat *</th>
<th>Blood components measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheeseman et al. (1995)</td>
<td>14</td>
<td>100 mg</td>
<td>~17 g</td>
<td>Plasma and erythrocytes</td>
</tr>
<tr>
<td>Traber et al. (1998)</td>
<td>5</td>
<td>15, 75 and 150 mg</td>
<td>30 g</td>
<td>Plasma and lipoproteins</td>
</tr>
<tr>
<td>Roxborough et al. (2000)</td>
<td>30</td>
<td>75 mg</td>
<td>~17 g</td>
<td>Plasma and erythrocytes</td>
</tr>
<tr>
<td>Current study</td>
<td>12</td>
<td>150 mg</td>
<td>40 g</td>
<td>Plasma, lipoproteins and blood cells</td>
</tr>
</tbody>
</table>

α-TA: deuterium labelled RRR α-tocopheryl acetate. * The amount of fat α-TA was ingested with

Interestingly, chylomicrons were enriched by 54% deuterium labelled α-tocopherol (range 22 – 76%) in the current study, whereas Traber et al. (1998) reported an average enrichment of 70% after 150 mg deuterium labelled α-tocopheryl acetate, thus implying greater deuterium labelled α-tocopherol absorption. However, the current study observed a greater uptake of d6 α-tocopherol into plasma, as measured by AUC, compared with Traber et al. (1998). It should be noted that fractional absorption of dose was not measured in either study.

The current study did not report α-tocopherol concentration in lipoproteins per litre plasma due to the limitations in the methodology used to isolate the lipoproteins (method detailed in section 2.2.9). Consequently, a direct comparison between the uptake of deuterium labelled α-tocopherol into chylomicrons or other lipoproteins in the current study and the one reported by Traber et al. (1998) could not be established.

There was significantly greater uptake of d6 α-tocopherol in VLDL compared with LDL per g protein, this is consistent with previously published supplementation studies (Goulinet & Chapman, 1997; Perugini et al., 2000). VLDL and LDL would have acquired d6 α-tocopherol via hepatic regulation and via exchange from other circulating lipoproteins; it was not possible to determine the proportion of d6 α-tocopherol obtained via these mechanisms. LDL is formed from VLDL metabolism and consequently contains the same apolipoprotein B100 moiety, consequently d6 α-tocopherol per g protein in VLDL and LDL is directly comparable. The lower amount of d6 α-tocopherol within LDL was presumably due to the transfer of d6 α-tocopherol
to HDL and uptake into peripheral tissues from VLDL (Perugini et al., 2000; Traber et al., 1992b).

5.5.1.1 Inter-individual variation in the uptake of d6 α-tocopherol into plasma and lipoproteins

There was a large inter-individual variation in the magnitude of d6 α-tocopherol uptake into chylomicrons. This was presumably due to a combination of factors, including; the gastrointestinal handling of lipids whereby differing secretion of bile and pancreatic lipases would impact on the handling of lipids and consequently affect vitamin E absorption. It is not known how vitamin E is packaged into chylomicrons, however, processes involved in the packaging of tocopherol into chylomicrons and their release into the systemic circulation would impact upon vitamin E appearance in the systemic circulation. For example, genetic polymorphisms involved in chylomicron secretion such as, polymorphism in apo B or the chylomicron assembly protein, microsomal transfer protein, may influence the rate of chylomicron secretion (Bergeron & Havel, 1997; Hussain, 2000; Peacock et al., 1995).

There was greater inter-individual variation in d6 α-tocopherol Cmax in chylomicrons per g protein (CV = 78 %) compared with d6 α-tocopherol per mmol TAG (CV = 58 %). A possible explanation for this difference is the increase in fat content of chylomicrons during absorption, with relatively little difference in protein (Hussain, 2000). Vitamin E follows fat absorption (Traber et al., 1990b), therefore it is probable that there was more d6 α-tocopherol in larger chylomicrons compared with smaller chylomicrons.

Inter-individual variation was not only observed in the magnitude of d6 α-tocopherol uptake, but also in the time of maximum concentration (Cmax). There was larger inter-individual variation in plasma and lipoprotein d6 α-tocopherol Cmax compared with erythrocytes and lymphocyte, as determined by coefficient of variation (shown in Tables 5.2 and 5.3). The observed inter-individual variation in the biokinetics of d6 α-tocopherol within lipoproteins can, in part, be attributed to inter-individual variation in lipoprotein metabolism. There are several genetic polymorphisms in the apolipoproteins moiety of the lipoproteins, the lipid processing
enzymes and lipoprotein receptor uptake that are known to influence lipoprotein metabolism (Ye & Kwiterovich, 2000). For example, a polymorphism in the lipoprotein lipase gene is associated with plasma TAG and HDL concentration (Clifton & Abbey, 1997). Variation in lipoprotein metabolism would affect the uptake of d6 α-tocopherol in peripheral tissues and the liver, as well as the length of time circulating within the systemic circulation.

The studies detailed in Chapters 3 and 4 reported large inter-individual variation in response to supplemental α-tocopheryl acetate, in these studies male and female subjects participated. The current study only recruited males to reduce the external inter-individual variation. Variation in body size and hence blood volume would also have influenced the biokinetics of d6 α-tocopherol, as the amount absorbed would be diluted in blood components accordingly. The dose of d6 α-tocopheryl acetate per body weight ranged from 1.7 to 2.2 mg per kg, this is similar to the range used by Traber et al. (1998) used, but was not reported by Roxborough et al. (2000) or by Cheeseman et al. (1995).

In the current study the coefficient of variation in plasma d6 α-tocopherol concentration at 12 hours was 38 %, this was similar to the 40 % following the consumption of 100 mg dose reported by Cheeseman et al. (1995). However, Roxborough et al. (2000) reported a 60 % coefficient of variation at 12 hours. A possible reason for this inconsistency in reported inter-individual variation could be the different fat content of the meal consumed with the α-tocopherol capsules, as shown in table 5.4. In the current study the subjects were provided with 40 g fat whereas Cheeseman et al. (1995) and Roxborough et al. (2000) reported the subjects ate lightly buttered toast, which contains approximately 17 g fat (Ministry of Agriculture, 1993). The amount of fat needed for optimal absorption of vitamin E has not been established, if sufficient fat was not available for maximal vitamin E absorption, greater variation may be observed. Different populations were used in the studies and there was a larger subject group in the study carried out by Roxborough, however, the reasons for this disparity remains unclear.

The biokinetic uptake of newly absorbed α-tocopherol within lipoproteins helps in the understanding of α-tocopherol uptake into blood cells and peripheral tissues.
5.5.2 Uptake of d6 α-tocopherol into erythrocytes, platelets and lymphocytes

Erythrocyte d6 α-tocopherol concentration peaked at 24 hours then started to decline at 48 h, this was similar to that reported by Cheeseman et al. (1995) and Roxborough et al. (2000). The current study reported a coefficient of variation of 32% for d6 α-tocopherol AUC, like plasma, this was a lot lower than the 60% coefficient of variation reported by Roxborough et al. (2000).

The correlation found between d6 α-tocopherol AUC in plasma and erythrocytes agrees with the findings reported by Roxborough et al. (2000). There was also a correlation between erythrocyte and HDL d6 α-tocopherol AUC, but not with the other lipoproteins. This may be explained by the more efficient transfer of α-tocopherol from HDL to erythrocytes compared with other lipoproteins (Kayden & Bjornson, 1972; Kostner et al., 1995).

The uptake of newly absorbed d6 α-tocopherol into platelets was particularly interesting. A gradual increase in d6 α-tocopherol concentration from 3 h was observed in eight subjects, however, a distinct biphasic uptake was observed in the other four subjects (Figure 5.9). Presently it is not known how platelets acquire vitamin E. The initial peak in d6 α-tocopherol concentration in subjects with a biphasic response coincided with the d6 α-tocopherol peak in chylomicrons at 6 h. It is hypothesised that this flux of d6 α-tocopherol in and out of the platelets was through uncontrolled diffusion, and the predominant source of d6 α-tocopherol was from chylomicrons. Vitamin E is transferred between lipoproteins as they collide with each other whilst circulating, it is hypothesised that this occurred with platelets, as α-tocopherol concentrations decreased in chylomicrons the platelets released d6 α-tocopherol along the concentration gradient.

These interesting findings deserve further investigation into how platelets obtain their vitamin E and the cause of this biphasic response. For example, it would be interesting to establish if there is a threshold of α-tocopherol in plasma before platelets rapidly take up newly absorbed α-tocopherol. The gradual uptake of d6 α-tocopherol into platelets from 9 hours in all subjects suggests controlled uptake, however, the mechanisms involved are unknown. Kitabchi et al. (1982) suggested, from preliminary work, that there are no tocopherol binding proteins and Kaempf et al
(1994) stated that there are no LDL receptors in platelets. A controlled uptake of d6 α-tocopherol and the correlation between d6 α-tocopherol AUC for platelets and LDL suggests that platelets may have LDL receptors and this could be the controlled uptake mechanism.

d6 α-Tocopherol uptake into lymphocytes was gradual, appearing after 6 hours and continuing to increase up to 48 h, suggesting a controlled uptake mechanism. There was also a trend towards a significant correlation between d6 α-tocopherol AUC for lymphocytes and LDL. Lymphocytes contain LDL receptors, and therefore presumably acquire vitamin E via this mechanism.

d6 α-Tocopherol first appeared in plasma and the platelets of subjects with a biphasic pattern, then in erythrocytes and a gradual increase in lymphocytes and platelets of subjects without a biphasic response. It was hypothesised that the greater the initial absorption the greater the uptake into blood cells. However, unlike erythrocytes, the magnitude of d6 α-tocopherol uptake into platelets and lymphocytes, as determined by AUC, did not correlate with the magnitude of d6 α-tocopherol uptake in plasma. This further suggests controlled α-tocopherol uptake mechanisms into these blood cells, rather than passive diffusion.

Both platelets and lymphocytes are potentially useful functional biomarkers of vitamin E status. The results presented from this study help to elucidate the biokinetics of newly absorbed α-tocopherol into these blood cells. The current study findings of newly absorbed d6 α-tocopherol uptake profiles into blood cells have provided a useful insight into the possible mechanisms of tocopherol uptake.

5.5.3 Decrease in pre-existing unlabelled (d0) α-tocopherol in all blood components

A number of studies have shown that newly absorbed RRR α-tocopherol displaces pre existing tocopherol within plasma (Handelman et al., 1985; Radosavac et al., 2002; Traber et al., 1992a). Traber et al. (1994) calculated the fractional disappearance rate of RRR α-tocopherol to be 0.4 ± 0.1 pool per day, suggesting that tocopherol rapidly leaves plasma, but RRR α-tocopherol is returned to plasma via hepatic secretion of VLDL. A decrease in γ-tocopherol concentration after varying single doses of α-tocopheryl acetate, was also observed in erythrocytes, platelets and lymphocytes in Chapter 3, although non-significant. In addition to this, the current
study showed a significant decrease in pre-existing (d0) \( \alpha \)-tocopherol in lipoproteins, platelets and lymphocytes, similar to the known phenomenon in plasma whereby newly absorbed d6 \( \alpha \)-tocopherol replaces the existing tocopherol (Traber et al., 1994). This has previously not been shown to occur in platelets and lymphocytes. It is hypothesised that this was also the case for erythrocytes. d0 \( \alpha \)-Tocopherol was not measured in erythrocytes due to problems in methodology (detailed in section 2.2.21).

### 5.5.4 Study design

The time points for the current study were chosen to measure d6 \( \alpha \)-tocopherol uptake, peak and decline within blood components without being too invasive for the subjects, additionally they are similar to the time points chosen by Cheeseman et al. (1995) and Roxborough et al. (2000). No baseline vitamin E measurement was taken, as the focus of this study was the uptake profile of newly absorbed d6 \( \alpha \)-tocopherol. None of the subjects had previously been involved in a study involving the ingestion of deuterium labelled tocopherol.

The appearance of deuterium labelled \( \alpha \)-tocopherol metabolite, \( \alpha \)-CEHC, in plasma was not measured because the assay to measure deuterium labelled \( \alpha \)-CEHC by LC/MS had not been developed within the laboratory. It would have been of interest to measure d6 and d0 \( \alpha \)-tocopherol metabolism by the appearance of d6 and d0 \( \alpha \)-CEHC in plasma to determine the time course and extent of metabolism of newly absorbed \( \alpha \)-tocopherol compared with pre-existing tocopherol. The study detailed in Chapter 3 and Traber et al. (1998a) reported a small increase in \( \alpha \)-CEHC excretion as \( \text{RRR } \alpha \)-tocopherol is preferentially retained within the body. Radosavac et al. (2002) reported a transient increase in serum \( \alpha \)-CEHC following supplementation with 300 mg unlabelled \( \text{RRR } \alpha \)-tocopherol, however they could not distinguish \( \alpha \)-CEHC from the pre-existing \( \alpha \)-tocopherol and the newly absorbed \( \alpha \)-tocopherol. Whereas, when Galli et al. (2003) gave subjects 100 mg deuterium labelled (d2) \( \gamma \)-tocopherol, there was a sharp increase in plasma d2 \( \gamma \)-CEHC at 9 h and a smaller increase in d0 \( \gamma \)-CEHC. It is hypothesised that a transient increase in d0 \( \alpha \)-CEHC and d6 \( \alpha \)-CEHC excretion would be observed, following ingestion of d6 \( \alpha \)-TA, d0 \( \alpha \)-CEHC formed initially from the displacement of tocopherol from blood components. The extent of
d6 α-CEHC excretion would also provide an indication to the amount of dose retained within the body.

5.5.5 Conclusions and future work

The study detailed in Chapter 3 only reported a significant increase in total α-tocopherol concentration in plasma after varying single doses of unlabelled α-tocopheryl acetate. The replacement of existing α-tocopherol with newly absorbed d6 α-tocopherol in all blood components whilst total α-tocopherol concentration did not change significantly, as observed in the current study, in part explains the lack of significance in Chapter 3.

The uptake of α-tocopherol into platelets could be further investigated *ex vivo* whereby d6 α-tocopherol enriched chylomicrons are placed with platelets; the uptake of d6 α-tocopherol into platelets could then be measured. Also the vitamin E status of platelets may affect their ability to take up α-tocopherol.

In conclusion this study has revealed the uptake profile of newly absorbed α-tocopherol into platelets and lymphocytes to be strikingly different to plasma and erythrocytes, implicating different uptake mechanisms. The α-tocopherol uptake mechanism(s) for platelets and lymphocytes are hypothesised to include LDL receptors and requires further investigation. A better understanding of α-tocopherol uptake and regulation within blood cells and peripheral tissues is needed to further elucidate better ways to assess vitamin E status.

The extent of inter-individual variation in the uptake and distribution of newly absorbed α-tocopherol is important to ascertain for the design of future studies comparing healthy individuals with those who may have an altered vitamin E status. Interestingly Roxborough *et al.* (2000) showed that intra individual variation in the uptake of newly absorbed labelled α-tocopherol was relatively consistent.
Chapter 6

Biokinetics of labelled \(\alpha\)-tocopherol in normolipidemic and hypercholesterolemic individuals
6.1 Introduction

This chapter investigates the biokinetics of α-tocopherol in the blood components of normolipidemic people compared with hyperlipidemic people. It is currently unknown whether vitamin E status in people with raised lipids differs to normolipidemic people.

Raised plasma low-density lipoprotein (LDL) cholesterol is a risk factor for Cardiovascular disease (CVD) (Martin & et al., 1986; Rose & Shipley, 1986), a suggested ideal level for LDL is < 3.0 mmol per litre plasma (Wood et al., 1998). Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the Western world. CVD is a chronic, multi-factorial disease and the aetiology can be attributed to the accumulation of risk factors.

LDL, which may be oxidatively modified, is an important factor in inducing endothelial dysfunction, which occurs in the early stages of atherosclerosis (Griendling & Alexander, 1997; Stokes et al., 2002b). Furthermore, LDL can become trapped within the arterial wall, whereby oxidatively modified LDL are internalised into macrophages leading to the formation of foam cells (Steinberg et al., 1989). LDL are heterogeneous, and have been classified into sub populations; a predominance of small dense LDL is considered more atherogenic than large, buoyant LDL (Berneis & Krauss, 2002; Griffin et al., 1994).

Hypercholesterolemia may result from an increased production of LDL cholesterol and/or reduced clearance from the systemic circulation. Polygenic hypercholesterolemia is the most common cause of raised plasma LDL cholesterol (Wilding & Williams, 1997). There are several genetic polymorphisms in lipoprotein metabolism including the apolipoproteins moiety of the lipoproteins, the lipid processing enzymes and lipoprotein receptor uptake (Ye & Kwiterovich, 2000). For example, the Apo E phenotype influences the uptake of lipoproteins via interactions with LDL receptor and hepatic chylomicron remnant receptor (Weintraub et al., 1987a).

Vitamin E is transported within lipoproteins whilst circulating in the blood. As a consequence, individuals with raised plasma cholesterol usually have increased concentrations of plasma vitamin E (Horwitt et al., 1972; Lambert & Mourot, 1984; Simon et al., 1997; Thurnham et al., 1986). The implications of raised plasma vitamin E concentration on vitamin E status are poorly understood. It has been previously
shown that vitamin E concentration within erythrocytes is lower in hypercholesterolemic subjects, the authors speculated that there was preferential retention of α-tocopherol within the lipoproteins (Simon et al., 1997).

The antioxidant properties of vitamin E have been implicated in the prevention of CVD for a number of years (Gey et al., 1991; Steinberg, 1989), more recently vitamin E has been shown to inhibit cells crucial in the progression of atherosclerosis both directly and via the inhibition of LDL oxidation (Azzi & Stocker, 2000). An altered vitamin E status may contribute to the increased risk of CVD associated with raised plasma LDL cholesterol.

The biokinetics of newly absorbed α-tocopherol has not previously been investigated in subjects with raised plasma lipids. This chapter investigates the uptake and distribution profiles of newly absorbed labelled α-tocopherol into plasma, lipoproteins and blood cells (erythrocytes, platelets and lymphocytes) in normolipidemic and hypercholesterolemic subjects.
6.2 Aim and hypothesis

The study aims to determine whether there is a difference in the uptake and distribution biokinetics of newly absorbed α-tocopherol in plasma, lipoproteins and blood cells (erythrocytes, platelets and lymphocytes) in hypercholesterolemic subjects compared with normolipidemic subjects.

It is hypothesised that individuals with raised plasma LDL cholesterol have altered vitamin E biokinetics, more specifically, individuals with raised plasma LDL will retain more of the newly absorbed α-tocopherol within the LDL. It was also proposed that a decreased rate of disappearance from plasma and a reduced uptake into other blood components might be observed. LDL is heterogeneous; the study also investigates whether the uptake of newly absorbed α-tocopherol into LDL subfractions differs between normolipidemic and hypercholesterolemic subjects.
6.3 Study design

Eight normolipidemic males were recruited from an advertisement in local newspapers and eight asymptomatic hypercholesterolemic males were recruited from general practitioners and through a lipid clinic at Royal Surrey County hospital. Selection criteria stated subjects must be non-smoking, not taking dietary supplements and with no gastrointestinal disorders, as determined from a written questionnaire. Subjects with fasting triacylglycerol concentrations greater than 1.5 mmol/L were excluded. Additional selection criteria for normolipidemic subjects was; cholesterol < 5.7 mmol/L and LDL cholesterol < 3.5 mmol/L, and for hypercholesterolemic subjects; > 6 mmol/L and LDL cholesterol > 3.5 mmol/L.

Subjects arrived at the Clinical Investigation Unit at the University of Surrey at 7.45 am, having been requested to fast from 8 pm the night before the study. The subjects consumed a standard breakfast containing 40g fat (2 croissants and 20g butter); investigators ensured the capsule, containing 150 mg deuterium labelled (d6) RRR \( \alpha \)-tocopheryl acetate, was taken mid-way through the meal. 40g Fat was chosen to maximise vitamin E absorption. The time the capsule was consumed was taken as 0 hours and blood samplings were timed from this point. A suitable trained person drew 20 ml blood samples from the antecubital vein in the forearm at 3, 6, 9, 12, 24 and 48 h hours after ingestion of the capsule (illustrated in figure 6.1), an additional 10 ml blood was taken at 12 and 24 h. Subjects were only allowed water and a standard meal at 12:00 until 15:00 on day 1, and then they were allowed to eat ad libitum.

Plasma, erythrocytes, platelets and lymphocytes were isolated from the blood samples. LDL subclasses were isolated from plasma at 12 and 24 hours. Chylomicrons, VLDL, LDL and HDL were isolated from frozen plasma at a later date.

![Figure 6.1 Schematic diagram of study protocol](image)

150 mg \( d6 \) RRR \( \alpha \)-tocopheryl acetate capsule was taken with a meal containing 40g fat. 20 ml blood was taken at 3, 6, 9, 12, 24 and 48 h after ingestion of the capsule; an additional 10 ml blood was taken at 12 and 24 h.
6.4 Results

6.4.1 Subject information

Eight normolipidemic (NL) and eight hypercholesterolemic (HC) males participated in the study. During the study period no one reported any side effects or withdrew from the study. Table 6.1 shows the subject characteristics for NL and HC subjects, they were age matched and the dose of d6-α-tocopheryl acetate per body weight was similar for each group.

The HC subjects had significantly greater total cholesterol (p < 0.001), LDL cholesterol (p < 0.001) and HDL cholesterol (p = 0.03) compared with NL subjects (Table 6.1). Both groups had similar fasted plasma triacylglycerol levels.

<table>
<thead>
<tr>
<th></th>
<th>Normolipidemic</th>
<th>Hypercholesterolemic</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>46.8 ± 9.5</td>
<td>47.1 ± 10.2</td>
<td>0.87</td>
</tr>
<tr>
<td><strong>BMI (kgm²)</strong></td>
<td>26.1 ± 3.0</td>
<td>25.6 ± 2.4</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>d6 α-TA/ kg body wt</strong></td>
<td>1.9 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Total cholesterol (mmol/ L)</strong></td>
<td>4.8 ± 0.5</td>
<td>7.0 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>LDL cholesterol (mmol/ L)</strong></td>
<td>3.2 ± 0.5</td>
<td>4.4 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>HDL cholesterol (mmol/ L)</strong></td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Triacylglycerol (mmol/ L)</strong></td>
<td>1.2 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. BMI; body mass index, α-TA; α-tocopheryl acetate
* as analysed by unpaired t-test

6.4.2 Plasma and lipoprotein α-tocopherol profiles

Plasma cholesterol concentrations did not differ significantly over time for NL and HC subjects. However, in HC subjects there was a significant decrease in triacylglycerol (TAG) concentration over time (p <0.001), a lower concentration was observed at 24 and 48 h when some subjects were fasted (see Appendix IV).

Table 6.2 shows the area under the curve (AUC) for d6 α-tocopherol uptake into plasma and lipoproteins over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate. There was a significantly lower d6 α-tocopherol AUC in HC than NL subjects in LDL per g protein (p = 0.008).
Table 6.2 Mean d6 α-tocopherol area under curve (AUC), in plasma and lipoproteins over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate

<table>
<thead>
<tr>
<th></th>
<th>normolipidemic (n = 8)</th>
<th>hypercholesterolemic (n = 8)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/L. h</td>
<td>666 ± 86</td>
<td>724 ± 83</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ mmol cholesterol. h</td>
<td>144 ± 17</td>
<td>130 ± 12</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Chylomicrons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein. h</td>
<td>217 ± 46</td>
<td>208 ± 38</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>VLDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein. h</td>
<td>893 ± 110</td>
<td>763 ± 140</td>
<td>0.48</td>
</tr>
<tr>
<td><strong>LDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein. h</td>
<td>688 ± 89</td>
<td>400 ± 21</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>HDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µmol/ g protein. h</td>
<td>50.8 ± 3.4</td>
<td>49.8 ± 4.5</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM.* as analysed by unpaired t-test

TAG: triacylglycerol

Figure 6.2 A) shows the mean d0 and d6 α-tocopherol concentration per litre plasma in NL and HC subjects and figure 6.2 B) shows d0 and d6 α-tocopherol concentration per mmol cholesterol. There was a significant increase in the mean d6 α-tocopherol concentration over time in both groups (p<0.001), d6 α-tocopherol peaked at approximately 9 h and then declined from 12 h. There were no significant differences in d6 α-tocopherol profiles per litre plasma or per mmol cholesterol in NL and HC subjects. Percentage d6 α-tocopherol uptake profiles were also similar in NL and HC subjects (data not shown).

Pre-existing d0 α-tocopherol concentration decreased from 3 h to 24 h in both groups (p<0.001). d0 α-Tocopherol profile per litre plasma in HC was greater than in NL subjects, but lower per mmol cholesterol as shown in figures 6.2 A) and B), although neither were significantly different.
Chapter 6

Figure 6.2 (A) Plasma d0 and d6 α-tocopherol for NL and HC subjects (B) d0 and d6 α-tocopherol per mmol cholesterol for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate. Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol and decrease in d0 α-tocopherol in both groups/L plasma and per mmol cholesterol (p < 0.01), analysed by repeated measures ANOVA. There was no significant difference in d6 or d0 α-tocopherol biokinetic profiles in NL versus HC subjects.

α-T: α-tocopherol, HC: hypercholesterolemic, NL: normolipidemic

Figures 6.3 A) and B) illustrate the mean chylomicron d6 α-tocopherol concentration per g protein and per mmol TAG in NL and HC subjects over 48 h. d6 α-Tocopherol concentration peaked sharply at 6 h then quickly decreased. The uptake of d6 α-tocopherol into chylomicrons was not significantly different for NL and HC subjects per mmol TAG (Figure 6.3B). However, the uptake of d6 α-tocopherol per g protein in HC subjects appeared greater compared with NL subjects, although it was
not significant (Figure 6.3A). There was large inter-individual variation in d6 α-tocopherol per g protein in both groups; illustrated by the large SEM bars at 6 hours in figures 6.3 A. There was less inter-individual variation was observed in d6 α-tocopherol per mmol TAG as shown in figure 6.3 B.

Figure 6.3 (A) Chylomicron d6 α-tocopherol per g protein for NL and HC subjects (B) Chylomicron d6 α-tocopherol per mmol TAG for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate
Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol in both groups per g protein and mmol TAG (p < 0.01), analysed by repeated measures ANOVA. There was no significant difference in d6 α-tocopherol biokinetic profiles in NL versus HC subjects
TAG: triacylglycerol HC: hypercholesterolemic, NL: normolipidemic
Figure 6.4 illustrates VLDL d0 and d6 α-tocopherol per g protein in NL and HC subjects over 48 h. d6 α-Tocopherol concentration plateaued between 6 and 12 h then started to decline in NL and HC subjects, there was no significant difference in d6 α-tocopherol uptake profiles. However, d0 α-tocopherol concentration was consistently lower in the HC group compared with NL group over 48 h, although not significantly. There was a large inter-individual variation in d0 α-tocopherol concentration in VLDL for both groups illustrated by the large SEM bars in figure 6.4.

The d6 α-tocopherol per mmol TAG and percentage d6 α-tocopherol uptake profiles were also similar in NL and HC subjects (data not shown).

![Figure 6.4 VLDL d0 and d6 α-tocopherol per g protein in for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate](image)

Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol and decrease in d0 α-tocopherol in both groups (p < 0.01), analysed by repeated measures ANOVA. There was no significant difference in d6 or d0 α-tocopherol biokinetic profiles in NL versus HC subjects.  

α-T: α-tocopherol, HC: hypercholesterolemic, NL: normolipidemic
Figure 6.5 shows the mean LDL d0 and d6 α-tocopherol concentration per g protein in NL and HC subjects over 48 h. d6 α-Tocopherol concentration increased up to 12 h then started to decrease in both NL and HC subjects. However, there was a significantly lower uptake of d6 α-tocopherol per g protein in HC subjects compared with NL subjects (p = 0.004). The d6 α-tocopherol per g protein AUC was also lower in HC than NL subjects, as shown in table 6.2 (p = 0.008). d6 α-tocopherol per mmol cholesterol over 48 h was also lower in HC compared with NL, however not significantly. The percentage d6 α-tocopherol uptake profiles were similar in NL and HC subjects (data not shown).

d0 α-Tocopherol concentration was consistently lower in the HC group compared with NL group over 48 h (p = 0.03) as illustrated in figure 6.5.

There was a larger inter-individual variation in the uptake of d6 α-tocopherol in NL subjects compared with the HC subjects, illustrated by the larger SEM bars in figure 6.5.

![Figure 6.5 LDL d0 and d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate](image)

Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol and decrease in d0 α-tocopherol in both groups (p < 0.01), analysed by repeated measures ANOVA. Significantly less d0 and d6 α-tocopherol per g protein in HC compared with NL (p = 0.03 and 0.004, respectively).

α-T: α-tocopherol, HC: hypercholesterolemic, NL: normolipidemic
Figure 6.6 illustrates the mean HDL d0 and d6 α-tocopherol per g protein in NL and HC subjects over 48 h. d6 α-tocopherol plateaued between 9 and 24 h and then decreased at 48 h. The uptake and distribution profile of d6 α-tocopherol within HDL for HC was similar to that observed for NL and percentage d6 α-tocopherol uptake profiles were also similar in NL and HC subjects (data not shown). d0 α-tocopherol concentration was consistently higher in NL compared with HC, although not significantly.

Figure 6.6 HDL d0 and d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate
Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol and decrease in d0 α-tocopherol in both groups (p < 0.01), analysed by repeated measures ANOVA. There was no significant difference in d0 or d6 α-tocopherol biokinetic profiles in NL versus HC subjects.
α-T: α-tocopherol, HC: hypercholesterolemic, NL: normolipidemic
6.4.3 Erythrocyte, platelet and lymphocyte α-tocopherol profiles

Table 6.3 shows the mean area under the curve (AUC) for d6 α-tocopherol uptake into erythrocytes, platelets and lymphocytes over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate for NL and HC subjects. There was a trend towards a significantly lower d6 α-tocopherol AUC in HC subjects compared with NL in erythrocytes, platelets and lymphocytes.

Table 6.3 Mean d6 α-tocopherol area under curve (AUC) in erythrocytes, platelets and lymphocytes over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate

<table>
<thead>
<tr>
<th></th>
<th>normolipidemic (n = 8)</th>
<th>hypercholesterolemic (n = 8)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes μmol/ PCV</td>
<td>182 ± 15</td>
<td>149 ± 11</td>
<td>0.09</td>
</tr>
<tr>
<td>Platelets μmol/ g protein</td>
<td>18.5 ± 1.0</td>
<td>15.1 ± 1.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Lymphocytes μmol/ g protein</td>
<td>12.6 ± 0.9</td>
<td>10.6 ± 0.8</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. PCV: packed cell volume *analysed by unpaired t test
Figure 6.7 shows the mean d6 α-tocopherol concentration in erythrocytes in NL and HC subjects. d6 α-tocopherol concentration peaked at 24 h then decreased at 48 h. There was a greater uptake of d6 α-tocopherol into erythrocytes of NL subjects compared with HC subjects, although not significant (p = 0.17). There was a also trend towards a significantly larger AUC for d6 α-tocopherol/ PCV.h in NL subjects versus HC subjects as shown in table 6.3 (p = 0.09).

There was no d0 α-tocopherol data for erythrocytes, due to methodological limitations (detailed in section 2.2.21).

There was a trend towards a significant correlation between d6 α-tocopherol AUC in erythrocytes and HDL in NL group ($r^2 = 0.43$, p = 0.08), however, this was not evident in the HC group ($r^2 = 0.21$, p = 0.25).

![Figure 6.7 Erythrocyte d6 α-tocopherol corrected for PCV for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate](image)

Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol over time in both groups (p < 0.01), analysed by repeated measures ANOVA. There was a non-significantly lower d6 α-tocopherol uptake in HC compared with NL (p = 0.17). PCV: packed cell volume HC: hypercholesterolemic, NL: normolipidemic
Figure 6.8 shows the mean platelet d0 and d6 α-tocopherol concentration per g protein for NL and HC subjects over 48 h. The d6 α-tocopherol uptake profile into platelets appeared quite different in HC versus NL, although they were not significantly different. Six HC and two NL subjects exhibited a biphasic response, whereby there was a sharp increase in d6 α-tocopherol concentration at 6 h. The other subjects had a gradual increase in d6 α-tocopherol up to 48 h.

There was a lower d6 α-tocopherol concentration at 24 hours in the HC group compared with the NL group (p = 0.01) and a trend towards a lower d6 α-tocopherol uptake in HC, as measured by AUC in the HC group (p = 0.08) as shown in table 6.3.

d0 α-Tocopherol concentration was higher in NL compared with HC over 48 h, although not significantly.

![Figure 6.8 Platelets d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate](image_url)

Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol and decrease in d0 α-tocopherol in both groups (p < 0.01), analysed by repeated measures ANOVA. There was non-significant difference in d0 and d6 α-tocopherol biokinetic profiles in NL versus HC subjects. * Significantly greater concentration of d6 α-tocopherol at 24 h in NL compared with HC (p = 0.01) as analysed by unpaired t test.

α-T: α-tocopherol, HC: hypercholesterolemic, NL: normolipidemic
Figure 6.9 illustrates the mean d0 and d6 α-tocopherol per g protein in lymphocytes of NL and HC subjects over 48 h, d6 α-tocopherol is on a second axis to clarify the NL and HC profiles. There was a gradual increase in d6 α-tocopherol concentration up to 48 h, this was similar in NL and HC subjects. However, there was a slightly lower d6 α-tocopherol concentration at 24 and 48 hours in the HC group compared with the NL group, although not significant. d6 α-Tocopherol AUC was not significantly different in HC and NL subjects (table 6.3). One HC subject exhibited a biphasic response, with an initial peak at 6 hours.

d0 α-Tocopherol concentration was similar at 3 h then higher in NL compared with HC, although not significantly.

Figure 6.9 Lymphocyte d0 and d6 α-tocopherol per g protein for NL and HC subjects over 48 hours following ingestion of 150 mg RRR α-tocopheryl acetate
Values expressed as mean ± SEM, n = 8 in each group. There was a significant increase in d6 α-tocopherol and decrease in d0 α-tocopherol in both groups (p < 0.01), analysed by repeated measures ANOVA. There was non-significant difference in d0 and d6 α-tocopherol biokinetic profiles in NL versus HC subjects.
α-T: α-tocopherol, HC: hypercholesterolemic, NL: normolipidemic
6.4.4 d6 α-tocopherol uptake into LDL sub fractions

Figures 6.10 A and B illustrate the mean d6 α-tocopherol concentration in LDL sub fractions taken 12 h following 150 mg RRR α-tocopheryl acetate in NL and HC subjects. There was no difference in d6 α-tocopherol concentration or percentage uptake at 12 or 24 h (data not shown). The uptake of d6 α-tocopherol per mmol cholesterol was similar in NL and HC subjects as shown in figure 6.10 A. There was more d6 α-tocopherol in the lightest fraction per mmol cholesterol compared with the other fractions in both groups (p <0.05).

Figure 6.10 A d6 α-Tocopherol in LDL sub fractions at 12 hours for NL and HC per fraction, and 6.10 B d6 α-Tocopherol in LDL sub fractions at 12 hours for NL and HC per mmol cholesterol following ingestion of 150 mg RRR α-tocopheryl acetate
Values expressed as mean ± SEM, n = 8 in each group. There was no significant difference in d6 α-tocopherol concentration in NL versus HC. * There was significantly greater d6 α-tocopherol in the first fraction compared with the dense fractions 6, 7 and 8 per mmol cholesterol in both groups (p < 0.05). HC: hypercholesterolemic, NL: normolipidemic.
6.5 Discussion

The aim of this study was to compare the uptake and distribution biokinetics of newly absorbed α-tocopherol between hypercholesterolemic (HC) and normolipidemic (NL) subjects. It was hypothesised that raised plasma LDL cholesterol would result in altered α-tocopherol biokinetics in the HC group.

Vitamin E is beneficial in the prevention of CVD (Azzi et al., 2003) and thus an altered vitamin E status may contribute to the increased risk of CVD associated with raised plasma LDL cholesterol (Rose & Shipley, 1986; Steinberg, 1989).

Key findings;
1. The uptake of d6 α-tocopherol into plasma was similar in NL and HC subjects, however, there was a significantly lower uptake of d6 α-tocopherol in LDL per g protein in HC subjects compared with NL subjects.
2. There was a trend towards a significantly lower uptake of d6 α-tocopherol into erythrocytes, platelets and to a lesser extent lymphocytes in HC subjects.
3. d6 α-Tocopherol uptake was significantly greater in the lightest LDL fraction per mmol cholesterol compared with the denser fractions. There was no difference in d6 α-tocopherol uptake in NL and HC subjects.

6.5.1 Plasma and lipoprotein α-tocopherol profiles

The pre-existing (d0) α-tocopherol concentration, at 3h and before significant uptake of d6 α-tocopherol, was higher per litre plasma and lower per mmol cholesterol in HC subjects compared with NL subjects, although not significant. This finding was similar to published studies (Lambert & Mourot, 1984; Regnström et al., 1996; Simon et al., 1997).

In the current study HC subjects had significantly less d0 α-tocopherol per g protein in LDL compared with NL subjects, at 3 h, before significant uptake of d6 α-tocopherol. Lambert and Mourot (1984) who reported lower LDL vitamin E per litre serum in patients with raised LDL cholesterol compared to normolipidemic subjects and Regnström et al. (1996) reported significantly lower vitamin E per g protein in LDL from a heterogeneous group of hyperlipidemic men who had survived a myocardial infarction.
There was similar d0 α-tocopherol concentration per g protein in HDL for NL and HC subjects, at 3 h, before significant uptake of d6 α-tocopherol. This is in agreement with the findings of Lambert and Mourot (1984) reported similar HDL vitamin E per litre serum in patients with raised LDL cholesterol and Simon et al. (2000) who reported similar steady-state vitamin E per mmol HDL in NL and hyperlipidemic subjects.

6.5.1.1 Uptake profile of newly absorbed (d6) α-tocopherol

Following a single dose of 150 mg d6 RRR α-tocopheryl acetate plasma d6 α-tocopherol concentration increased from 3 h and plateaued between 9 and 12 h before decreasing in NL and HC subjects, this profile was similar to previously published studies in NL subjects (Cheeseman et al., 1995; Roxborough et al., 2000; Traber et al., 1998b). The magnitude of d6 α-tocopherol uptake into plasma was similar in NL and HC subjects, this suggests that there was no difference in d6 α-tocopherol absorption (illustrated in figure 6.2). The study did not measure the amount of α-tocopherol absorbed from 150 mg d6 α-tocopheryl acetate capsule nor could it report α-tocopherol concentration in lipoproteins per litre plasma due to the limitations in the methodology used to isolate the lipoproteins (method detailed in section 2.2.9).

The uptake of d6 α-tocopherol into chylomicrons was similar in NL and HC subjects per mmol TAG. However, there appeared to be greater uptake of d6 α-tocopherol per g protein in HC subjects, although not significant. This may suggest that the chylomicrons formed are larger and more TAG rich in HC subjects compared with NL subjects. No studies were found that explored whether there are differences in chylomicron formation in hypercholesterolemia, as suggested in the current study.

The uptake of d6 α-tocopherol into VLDL plateaued between 6 and 12 h in NL and HC subjects and to a similar magnitude. Therefore, HC subjects did not differ in their ability acquire newly absorbed α-tocopherol per g protein or per mmol TAG in VLDL. VLDL are enriched with α-tocopherol within the liver and from other lipoproteins whilst circulating, the current study suggests that neither of these process are impaired in hypercholesterolemia. Pre-existing (d0) α-tocopherol concentration appeared lower in HC over 48 h (Figure 6.4), however there was large inter-individual variation and the difference was not significant.
d6 α-Tocopherol increased up to 12 h in LDL, then started to decrease from 24 h in both NL and HC subjects. There was a significantly lower uptake of d6 α-tocopherol per g protein of LDL in HC subjects compared with NL subjects. This presumably was due to the greater volume of LDL in the plasma of HC subjects and as a consequence the d6 α-tocopherol available per particle was less compared with NL (as similar d6 α-tocopherol absorption was observed). The pre-existing d0 α-tocopherol was also significantly lower in HC subjects (illustrated in figure 6.5). LDL is formed from VLDL metabolism, in the current study d6 α-tocopherol in VLDL was similar in NL and HC over 48 h. This suggests that the difference observed in LDL occurs within the systemic circulation, after hepatic secretion. It is possible that in hypercholesterolemia, LDL is circulating for a longer time and therefore more α-tocopherol may be transferred to peripheral tissues. Interestingly the percentage uptake of d6 α-tocopherol in LDL was similar in NL and HC subjects.

There was no difference in the d6 α-tocopherol uptake profile for HDL between NL and HC subjects. This implies that high circulating LDL levels do not impair the transfer of newly absorbed α-tocopherol to HDL. Simon et al. (2000) suggested that α-tocopherol in HDL was retained to a greater extent in HC subjects, however, in the current study the disappearance rate of d6 α-tocopherol also appeared similar in NL and HC groups.

The total amount of d6 α-tocopherol circulating within the plasma was similar in NL and HC subjects, therefore, any differences observed in the uptake of d6 α-tocopherol into blood cells may be attributed to the differing d6 α-tocopherol in lipoproteins between NL and HC groups.

It was hypothesised that HC subjects would retain a greater amount of newly absorbed d6 α-tocopherol within lipoproteins, illustrated by a slower disappearance of d6 α-tocopherol from lipoproteins. In hindsight more frequent time points were needed in the current study to ascertain the disappearance rate of d6 α-tocopherol from plasma and lipoproteins.
tocopherol into blood cells may be attributed to the differing d6 α-tocopherol in lipoproteins between groups.

6.5.2 Uptake of d6 α-tocopherol into erythrocytes, platelets and lymphocytes

Erythrocyte d6 α-tocopherol concentration peaked at 24 hours then started to decline at 48 h in NL and HC subjects, which was similar to previously published studies in normolipidemic subjects (Cheeseman et al., 1995; Roxborough et al., 2000). There was a trend towards a lower uptake of d6 α-tocopherol into erythrocytes of HC subjects compared with NL subjects, as measured by d6 α-tocopherol AUC over 48 h. Erythrocyte d0 α-tocopherol concentration was not measured in the current study due to methodological problems, however, previous studies have reported lower steady-state vitamin E levels in erythrocytes of hyperlipidemic rats and humans compared to NL controls (Nishida et al., 1982; Simon et al., 1997). The reduced vitamin E concentration in erythrocytes has physiological consequences; Simon et al. (1998) showed that erythrocytes from HC subjects were more susceptible to oxidation ex vivo compared with those from NL subjects.

Chapter 5 investigated α-tocopherol biokinetics in healthy subjects and reported a strong correlation between the d6 α-tocopherol AUC of erythrocytes and HDL and in the current study there was a trend towards a significant correlation between d6 α-tocopherol AUC of erythrocytes and HDL in NL subjects. However, there was no correlation between d6 α-tocopherol AUC of erythrocytes and HDL in HC subjects. The transfer of α-tocopherol to erythrocytes from HDL is more efficient compared with other lipoproteins (Kayden & Bjornson, 1972; Kostner et al., 1995). Simon et al. (2000) reported that erythrocytes from hyperlipidemic and NL subjects were equally able to accept α-tocopherol from donor HDL ex vivo and suggested that HDL may preferentially transfer α-tocopherol to LDL rather than erythrocytes. It is possible that if erythrocytes have lower vitamin E concentration in HC compared with NL people there may also be less vitamin E distributed to other blood cells and peripheral tissues.
A distinct biphasic uptake in d6 α-tocopherol was observed in a proportion of both NL and HC, whereby d6 α-tocopherol concentration sharply increased from 3 h to 6 h, then a gradual uptake from 9 h, this was similar to what was observed in healthy subjects Chapter 5. A gradual increase in d6 α-tocopherol concentration from 3 h was observed in the other NL and HC subjects. A greater percentage of HC subjects exhibited a biphasic uptake of d6 α-tocopherol into platelets compared with NL subjects. However, as discussed in Chapter 5, the reasons for the biphasic uptake of α-tocopherol are not clear. The NL and HC biphasic and non-biphasic subjects were not compared due to the low subject numbers in each group. Whether or not lipid status affects the uptake of α-tocopherol into platelets remains to be elucidated. Very little is known regarding the mechanisms for α-tocopherol uptake and regulation within platelets. It was hypothesised in Chapter 5 that there is passive diffusion and controlled uptake of α-tocopherol into platelets, possibly via LDL receptors, whilst circulating within the blood.

Some hypercholesterolemic subjects have reduced binding activity of LDL receptors (Ye & Kwiterovich, 2000), in these individuals there may be a lower α-tocopherol uptake in cells that acquire vitamin E via LDL receptors. There was a trend towards a significantly lower uptake of d6 α-tocopherol into platelets of HC subjects compared with NL subjects, as measured by d6 α-tocopherol AUC. Additionally d0 α-tocopherol concentration was lower in HC subjects at 3 h compared with NL subjects, albeit non significantly (illustrated in figure 6.8).

Platelet adhesion and aggregation is a key factor in thrombosis and CVD pathogenesis (Handin, 1996), and it has been shown that there is greater platelet-endothelial adhesion in hypercholesterolemic animals and humans (Davi et al., 1997; Scalia et al., 1998). Additionally α-tocopherol has been shown to modulate platelet adhesion and aggregation in supplementation studies and ex vivo experiments (Calzada et al., 1997; Steiner, 1991; Steiner, 1999). Therefore, if HC subjects had less α-tocopherol within their platelets, as suggested by the current study, this could contribute to the altered platelet function observed in HC subjects.

d6 α-Tocopherol uptake into lymphocytes was gradual, appearing after 6 hours and continuing to increase up to 48 h in NL and HC subjects, this was similar to what was observed healthy subjects in Chapter 5. Chapter 5 suggested a controlled uptake
of newly absorbed \(\alpha\)-tocopherol into lymphocytes. Since lymphocytes have LDL receptors, they are thought to obtain vitamin E via this process (Kaempf \textit{et al.}, 1994). As hypothesised with the uptake of tocopherol in platelets, if there is reduced activity of LDL receptors in HC you would predict a lower uptake of LDL and therefore \(\alpha\)-tocopherol.

Peripheral lymphocytes are involved in the pathogenesis of atherosclerosis (Bach \textit{et al.}, 1997; Hansson, 1997; Song \textit{et al.}, 2001) and lymphocyte function in HC animals has been shown to be different compared with NL animals (Stokes \textit{et al.}, 2002b; Stokes \textit{et al.}, 2002a). \(\alpha\)-Tocopherol has been shown to enhance lymphocyte differentiation and proliferation \textit{in vitro and ex vivo} (Meydani \textit{et al.}, 1990; Meydani \textit{et al.}, 1986). Therefore, if HC subject have less \(\alpha\)-tocopherol within their lymphocytes, as hypothesised from the findings in the current study, this could contribute to the altered lymphocytes function observed in HC subjects.

The current study findings of newly absorbed d6 \(\alpha\)-tocopherol uptake profiles into blood cells of HC subjects has provided a useful insight into the possibility that \(\alpha\)-tocopherol regulation is different to that in NL subjects and that the delivery of \(\alpha\)-tocopherol to peripheral tissues may also be impaired in HC subjects.

\textbf{6.5.3 Uptake of d6 \(\alpha\)-tocopherol into LDL sub fractions}

LDL are heterogeneous, and have been classified into sub populations; large buoyant, intermediate or small, dense LDL. A predominance of small dense LDL is considered more atherogenic than large, buoyant LDL (Berneis & Krauss, 2002; Griffin \textit{et al.}, 1994).

Previous studies have reported less \(\alpha\)-tocopherol in small dense LDL compared with large buoyant LDL (Chancharme \textit{et al.}, 2002; Goulinet & Chapman, 1997; Tribble \textit{et al.}, 1995). The current study observed significantly greater d6 \(\alpha\)-tocopherol uptake per mmol cholesterol in the lightest LDL sub fraction compared with the denser fractions, this may help towards explaining the lower \(\alpha\)-tocopherol levels in small dense LDL. There was no difference in d6 \(\alpha\)-tocopherol uptake per mmol cholesterol between NL and HC subjects, however there was a lower, albeit non-significant, d6 \(\alpha\)-tocopherol uptake in total LDL per mmol cholesterol in HC.
The uptake of newly absorbed d6 α-tocopherol into LDL sub fractions was similar at 12 and 24 hours, this agrees with the plateau in d6 α-tocopherol per g protein in total LDL, shown in figure 6.5. The current study did not measure uptake of d6 α-tocopherol per g protein in LDL sub fractions, and therefore cannot conclude whether there is a difference in uptake per LDL molecule. d6 α-Tocopherol into total LDL was significantly greater at 12 and 24 hours in NL subjects compared with HC subjects per g protein.

6.5.4 Conclusions and future work

The initial study protocol estimated fourteen subjects in each group would provide a reasonable chance of observing a true difference between biokinetics of newly absorbed α-tocopherol in HC and NL subjects. However, there was great difficulty in recruiting HC subjects, which resulted in only eight subjects in each group completing the study. Power calculation from the current study revealed that thirty subjects within each group would be required to find a significant difference in the uptake of d6 α-tocopherol into blood cells.

No baseline vitamin E measurement was taken, as the focus of this study was the uptake profile of newly absorbed d6 α-tocopherol. However, a steady-state d0 α-tocopherol concentration for lipoproteins or blood cells would have been beneficial as there is very limited data on vitamin E status in dyslipidemia within the literature.

In conclusion HC and NL subjects absorbed d6 α-tocopherol to a similar extent, however, HC had a reduced uptake of newly absorbed d6 α-tocopherol per g LDL. This may impact upon the uptake of α-tocopherol into blood cells and peripheral tissues, as shown by the trend towards significantly lower uptake of newly absorbed d6 α-tocopherol into erythrocytes, platelets and lymphocytes. Hypercholesterolemia is a risk factor for CVD; the current study reveals that it may also contribute to an altered vitamin E regulation that may further predispose HC people to a higher risk of CVD.
Future work to investigate the biokinetics of α-tocopherol in people with an atherogenic lipoprotein phenotype, with raised plasma TAG and a predominance of small dense LDL, would be of interest to determine whether vitamin E regulation differs. It is hypothesised that they may retain more newly absorbed α-tocopherol within VLDL and subsequently reduce the transfer of newly absorbed α-tocopherol to HDL and peripheral tissues.
Chapter 7

Vitamin E status in cigarette smokers and non-smokers
7.1 Introduction

This chapter investigates the steady-state vitamin E status in cigarette smokers compared with non-smokers. Cigarette smoking has been identified as an independent risk factor for carcinogenesis, pulmonary and cardiovascular diseases (Chow, 1993; Doll et al., 1994; Lykkesfeldt et al., 1997; Peto et al., 1992). Cigarette smokers are exposed to sustained free radical attack both from those present in the cigarette smoke and those formed endogenously through the inflammatory-immune response (Alberg, 2002; Cross & Traber, 1997). Cigarette smoke contains alkenes, nitrosamines, aromatic and heterocyclic carbons and amines (Church & Pryor, 1985), these compounds are a known source of reactive oxygen species. Cigarette smoking causes an acute inflammatory reaction characterised by the accumulation of neutrophils and macrophages within the lungs, that generate an increased number of reactive oxygen species (Duthie et al., 1991; Jay et al., 1986; Kalra et al., 1991; Pryor, 1993; van Antwerpen et al., 1995).

Cigarette smokers are exposed to greater oxidative stress, this is characterised by greater lipid peroxidation of membranes and lipoproteins and oxidative DNA damage in smokers (Frei et al., 1991; Harats et al., 1990; Husgafvel-Pursiainen et al., 1984; Loft et al., 1992; Miro et al., 1999; Morrow et al., 1995). Antioxidants are crucial in the body's defence against oxidative damage from cigarette smoke (Alberg, 2002). Cigarette smoke has been shown to deplete plasma carotenoids, ascorbic acid and vitamin E in vitro (Eiserich et al., 1995; Handelman et al., 1996), and several studies have shown cigarette smokers have lower plasma ascorbic acid and carotenoids levels in vivo (Marangon et al., 1998; Mezzetti et al., 1995; Stryker et al., 1988). A review by Alberg (2002) clearly demonstrated that unlike ascorbic acid and carotenoids, measurements of plasma vitamin E are, in general, not lower in cigarette smokers compared with non-smokers.

The lower plasma antioxidants of cigarette smokers have been attributed to both increased oxidative stress and a lower dietary intake of antioxidants (Dietrich et al., 2003). Cigarette smokers tend to have a lower dietary intake of fruit and vegetables, which results in a lower intake of antioxidant micronutrients (Dallongeville et al., 1998; Ma et al., 2000; Wei et al., 2001).

It has however been reported that cigarette smokers have a lower concentration of vitamin E in alveolar fluid (Pacht et al., 1986) and a higher concentration in arterial
tissue (Mezzetti et al., 1995), compared with non-smokers. Increased levels of vitamin E were observed in the lungs of cigarette-smoked animals (Airriess et al., 1988; Chow et al., 1989). Furthermore, it has been proposed that vitamin E is mobilised to the lung tissue of cigarette smokers via ‘protein oxidation-generating bioactive molecules’ (Elsayed, 2001). These bioactive molecules can activate signal transduction pathways to mobilise vitamin E towards the site of increased oxidative stress (Suzuki et al., 1997). Biokinetic studies in humans have also indicated that cigarette smokers have an altered vitamin E uptake and utilization in plasma (Munro et al., 1997; Traber et al., 2001). Together, these studies put a strong case forward for the existence of an altered vitamin E status in cigarette smokers.
7.2 **Aim and hypothesis**

Cigarette smokers are exposed to higher levels of free radicals and oxidative stress compared with non-smokers. It is conceivable that cigarette smokers have a higher requirement for vitamin E and therefore they may have an altered vitamin E status compared with non-smokers. This study aims to report steady-state levels of vitamin E status within a number of blood components and the urinary excretion of α- and γ-tocopherol metabolites (α- and γ-CEHC) in cigarette smokers compared with non-smokers. A combination of vitamin E biomarkers are utilised since vitamin E regulation and distribution between cells is not fully understood and plasma steady-state levels are inadequate, as detailed in section 1.9.

It is hypothesised that cigarette smokers may have different levels of α- or γ-tocopherol within blood cells due to exposure to more free radicals and hence greater oxidative stress. Platelet and lymphocyte functions are affected by vitamin E status and therefore may represent a functional biomarker of vitamin E status. Previous to this investigation the vitamin E status in platelets and lymphocytes have not been measured in cigarette smokers.

It is also hypothesised that reduced urinary excretion of α-CEHC would be observed in cigarette smokers, compared with non-smokers, due to an increased vitamin E utilisation.
7.3 Study design

Thirty volunteers (15 cigarette smokers and 15 non-smokers) were recruited. Selection criteria stated subjects must not be taking dietary supplements and not have gastrointestinal disorders as determined from a written questionnaire. Subjects with blood lipid abnormalities were excluded (selection criteria was cholesterol < 6 mmol/l and triacylglycerol < 1.5 mmol/l). Additionally, non-smokers could not have previously smoked and cigarette smokers must have smoked at least 10 cigarettes a day, for greater than 5 years. Cigarette smokers were instructed to maintain their habitual smoking pattern during the study period.

Volunteers completed a 7-day food diary and collected two 24 h urine production. The 7-day food diaries were analysed for average nutrient intake per day, as detailed in section 2.2.2. The urine samples were utilised for the measurement of α- and γ-CEHC, creatinine and cotinine; the methods are detailed in sections 2.2.3 and 2.2.22 - 24. A fasted 20 ml blood sample was taken by a suitably trained person. Blood samples were used for the measurement of cholesterol, triacylglycerol, ascorbic acid and vitamin E in plasma, erythrocytes, platelets and lymphocytes; the methods are detailed in sections 2.24 - 8.
7.4 Results

7.4.1 Subject characteristics and dietary intake

Table 7.1 shows the subject characteristics of cigarette smokers and non-smokers that participate in the study. The fifteen cigarette smokers and non-smokers were of similar age, body mass index (BMI) and plasma cholesterol and triglyceride concentrations. Self-reported smoking status, expressed as pack-years, was confirmed by detection of urinary cotinine in cigarette smokers, whereas cotinine was not detected in non-smokers (limit of detection of 10 μg/ L; (Greaves et al. 2001). Cigarette smokers were relatively young and had not been smoking for longer than 15 years.

Table 7.1 Subject characteristics for cigarette smokers and non-smokers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cigarette smokers (n = 15)</th>
<th>Non-smokers (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/ female)</td>
<td>8/7</td>
<td>8/7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.5 ± 2.0</td>
<td>30.9 ± 1.9</td>
</tr>
<tr>
<td>BMI (kgm^2)</td>
<td>24.4 ± 1.0</td>
<td>23.0 ± 0.9</td>
</tr>
<tr>
<td>Cholesterol (mmol/ l)</td>
<td>4.3 ± 0.2</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/ l)</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Pack-years^# (range)</td>
<td>6.1 ± 3.4 (2.5–15.0)</td>
<td>-</td>
</tr>
<tr>
<td>Urinary cotinine (ng/ mg creatinine)</td>
<td>1144 ± 162</td>
<td>non detected</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. BMI; body mass index ^ Pack years = packs of cigarettes smoked per day × number of years smoked * Analysed by two-tailed unpaired t-test

There were no significant differences between α- or γ-tocopherol concentrations in males and female cigarette smokers and non-smokers, therefore, the collated data are presented throughout the chapter.
Table 7.2 shows the average nutrient intake per day for cigarette smokers compared with non-smokers. Analysis of habitual dietary intake revealed the cigarette smokers and non-smokers have a similar intake of energy and macronutrients. Cigarette smokers reported a significantly greater intake of alcohol compared with non-smokers (p = 0.035). Dietary intake of antioxidants vitamins A, C and E and selenium, were not significantly different between cigarette smokers and non-smokers.

Table 7.2 Average nutrient intake per day for cigarette smokers and non-smokers

<table>
<thead>
<tr>
<th>Average nutrient intake/ day</th>
<th>Cigarette smokers (n = 15)</th>
<th>Non-smokers (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2232 ± 165</td>
<td>2158 ± 106</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>79.2 ± 7.4</td>
<td>82.8 ± 6.7</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>14.5 ± 1.5</td>
<td>15.5 ± 1.7</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>255.5 ± 33.0</td>
<td>270.1 ± 18.4</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>68.7 ± 3.9</td>
<td>73.8 ± 4.2</td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>28.1 ± 4.1</td>
<td>15.1 ± 4.1*</td>
</tr>
<tr>
<td>Vitamin A intake (mg)</td>
<td>650 ± 98</td>
<td>766 ± 68</td>
</tr>
<tr>
<td>Vitamin C intake (mg)</td>
<td>90.5 ± 24.2</td>
<td>104.0 ± 16.2</td>
</tr>
<tr>
<td>Vitamin E intake (mg)</td>
<td>5.9 ± 0.7</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>Selenium (µg)</td>
<td>57.4 ± 4.9</td>
<td>64.3 ± 11.1</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. * p = 0.035 analysed by unpaired t-test

PUFA; polyunsaturated fatty acids
7.4.2 Plasma ascorbic acid and vitamin E concentration in plasma, erythrocytes, platelets and lymphocytes

Plasma ascorbic acid concentrations were lower, but not significantly, in cigarette smokers compared with non-smokers; mean ± SEM were 36.9 ± 4.8 and 42.9 ± 4.0 μM respectively (p = 0.36).

Figure 7.1 illustrates the mean plasma α- and γ-tocopherol concentrations per mmol cholesterol in cigarette smokers and non-smokers. α- and γ-Tocopherol plasma concentrations were similar in cigarette smokers and non-smokers. However, α-tocopherol per litre plasma was significantly lower in cigarette smokers compared with non-smokers (22.1 ± 1.0 and 25.2 ± 1.0 μM respectively; p = 0.03).

The ratio of α-tocopherol : γ-tocopherol in plasma of cigarette smokers and non-smokers were similar (mean ± SEM; 15.9 ± 1.4 and 16.1 ± 0.6).

![Figure 7.1 Plasma α- and γ-tocopherol concentration per mmol cholesterol in cigarette smokers and non-smokers](image_url)

Values expressed as mean ± SEM (n = 15 in each group). α- and γ-Tocopherol concentration did not differ significantly between cigarette smokers and non-smokers. Analysed unpaired t test.
Figure 7.2 shows the mean $\alpha$- and $\gamma$-tocopherol concentration in erythrocytes corrected for packed cell volume in cigarette smokers and non-smokers. $\alpha$-Tocopherol concentration was slightly lower in the erythrocytes of cigarette smokers compared with non-smokers, however, this difference was not significant ($p = 0.10$). $\gamma$-Tocopherol concentration did not differ between cigarette smokers and non-smokers ($1.59 \pm 0.18$ and $1.50 \pm 0.07 \mu$M/l packed cell volume respectively).

The ratio of $\alpha$-tocopherol : $\gamma$-tocopherol in erythrocytes of cigarette smokers and non smokers were similar (mean $\pm$ SEM; $10.5 \pm 1.1$ and $12.5 \pm 0.7$).

![Figure 7.2 Erythrocyte $\alpha$- and $\gamma$-tocopherol concentration corrected for packed cell volume in cigarette smokers and non-smokers](image)

Values expressed as mean $\pm$ SEM ($n = 15$ in each group). $\alpha$-Tocopherol concentration was slightly lower in the erythrocytes of cigarette smokers compared with non-smokers, however, this difference was not significant ($p = 0.10$). $\gamma$-Tocopherol concentration did not differ significantly between cigarette smokers and non-smokers. Analysed using unpaired t test.
Figure 7.3 illustrates the mean α- and γ-tocopherol concentration in platelets per g protein in cigarette smokers and non-smokers. Platelet α-tocopherol concentration was significantly lower in cigarette smokers compared with non-smokers (p = 0.014), whereas γ-tocopherol concentration was similar in cigarette smokers and non-smokers.

The ratio of α-tocopherol : γ-tocopherol in platelets was significantly lower in cigarette smokers compared with non-smokers (mean ± SEM; 7.4 ± 0.6 and 13.0 ± 2.1, p = 0.02).

Figure 7.3 Platelet α- and γ-tocopherol concentration per g protein in cigarette smokers and non-smokers
Values expressed as mean ± SEM (cigarette smokers; n = 15, non smokers n = 14, one sample had to be discarded). α-Tocopherol concentration was significantly lower in the platelets from cigarette smokers compared with non-smokers (p = 0.014). γ-Tocopherol concentration in platelets was similar in cigarette smokers and non-smokers. Analysed using unpaired t test. * Significantly lower than to non-smokers.
Figure 7.4 shows the mean lymphocyte $\alpha$- and $\gamma$-tocopherol per g protein in cigarette smokers and non-smokers. There was a significantly lower $\alpha$- and $\gamma$-tocopherol concentration within lymphocytes from cigarette smokers compared with non-smokers ($p = 0.002$ and $p = 0.031$, respectively).

The ratio of $\alpha$-tocopherol : $\gamma$-tocopherol in lymphocytes of cigarette smokers and non smokers were similar (mean $\pm$ SEM; 7.0 $\pm$ 0.5 and 7.6 $\pm$ 1.0).

Figure 7.4 Lymphocyte $\alpha$- and $\gamma$-tocopherol concentration per g protein in cigarette smokers and non-smokers
Values expressed as mean $\pm$ SEM (n = 15 in each group). $\alpha$- and $\gamma$-Tocopherol concentration were significantly lower in the platelets from cigarette smokers compared with non-smokers ($p = 0.002$ and $p = 0.031$ respectively). Analysed using unpaired t test. * Significantly lower than non-smokers.
7.4.3 Urinary excretion of α- and γ-CEHC

Figure 7.5 illustrates the mean urinary excretion of α- and γ-CEHC in cigarette smokers and non-smokers. Urinary excretion of α-CEHC did not differ between cigarette smokers and non-smokers. However, γ-CEHC excretion was significantly greater in cigarette smokers compared with non-smokers (p = 0.036). No correlation was observed between α- or γ-CEHC excretion and cotinine.

The ratio of α-CEHC : γ-CEHC urinary excretion was significantly lower in cigarette smokers compared with non smokers (mean ± SEM; 0.40 ± 0.04 and 0.84 ± 0.14, p = 0.007).

![Graph showing urinary excretion of α- and γ-CEHC excretion corrected for creatinine in cigarette smokers and non-smokers.](image)

Figure 7.5 Urinary excretion of α- and γ-CEHC excretion corrected for creatinine in cigarette smokers and non-smokers. Values expressed as mean ± SEM (n = 15 in each group). Urinary excretion of γ-CEHC was significantly greater in cigarette smokers compared with non-smokers (p = 0.036). α-CEHC excretion was similar in cigarette smokers and non-smokers. Analysed using unpaired t test. * Significantly higher than non-smokers.
7.5 Discussion

Cigarette smoking causes severe oxidative stress within the lungs, disruption of the antioxidant homeostasis and a probable increased requirement of antioxidants (Alberg, 2002; van Antwerpen et al., 1995). The aim of this investigation was to determine whether cigarette smokers have an altered vitamin E status compared with non-smokers by measuring a number of steady-state biomarkers. Previous studies have reported inconsistent values for plasma and erythrocyte vitamin E concentrations in cigarette smokers and non-smokers (Brown et al., 1998; Liu et al., 1998). Cigarette smokers are likely to have an altered vitamin E status due to the greater oxidative stress and therefore higher utilisation of vitamin E acting as an antioxidant.

Key Findings;
1. Plasma α- and γ-tocopherol concentrations corrected for cholesterol and erythrocyte α- and γ-tocopherol concentrations were similar in cigarette smokers and non-smokers
2. Cigarette smokers had a lower concentration of α-tocopherol in their platelets and lymphocytes, compared with non-smokers
3. Cigarette smokers also had a lower concentration of γ-tocopherol in their lymphocytes, compared with non-smokers
4. The urinary excretion of α-CEHC was similar in cigarette smokers and non-smokers. However, cigarette smokers excreted a greater amount of urinary γ-CEHC compared with non-smokers.

7.5.1 α- and γ-Tocopherol within blood components

This study observed a similar concentration of α- and γ-tocopherol per mmol cholesterol in the plasma of cigarette smokers and non-smokers. This agrees with the majority of studies which have compared vitamin E levels in cigarette smokers with non-smokers (Dietrich et al., 2003; Duthie, 1993; Marangon et al., 1998; Mezzetti et al., 1995; Ross et al., 1995). Liu et al. (1998) reported significantly lower plasma α-tocopherol (corrected for total lipid) in volunteers older than 35 years, but not in younger volunteers. A small study conducted by Munro et al. (1997) also reported significantly lower plasma α-tocopherol (corrected for cholesterol). Interestingly a
limited number of studies have reported higher \( \gamma \)-tocopherol concentration in cigarette smokers' plasma although the reasons for these observations remain unclear (Dietrich et al., 2003; Lykkesfeldt et al., 2000). From this information, it implies that there is little difference in vitamin E status between cigarette smokers and non-smokers.

A limited number of studies have investigated the plasma biokinetics of newly absorbed \( \alpha \)-tocopherol in cigarette smokers compared with non-smokers using deuterium labelled \( \alpha \)-tocopherol. Munro et al. (1997) reported a lower uptake of newly absorbed \( \alpha \)-tocopherol into plasma in cigarette smokers compared with non-smokers and Traber et al. (2001) demonstrated a trend towards a faster removal of \( \alpha \)-tocopherol from plasma in cigarette smokers compared with non-smokers. These studies demonstrate that cigarette smokers probably utilise vitamin E differently compared with non-smokers and this difference cannot be detected in steady-state plasma vitamin E levels. These differences may not be detected because plasma vitamin E is strongly associated with lipoprotein concentration and is homeostatically regulated (Horwitt et al., 1972; Traber, 1994).

Erythrocytes from cigarette smokers are more susceptible to oxidation \textit{ex vivo} compared with non-smokers (Brown et al., 1997; Codandabany, 2000). However, studies that have compared vitamin E concentration within erythrocytes have concluded that there is little, if any, difference between cigarette smokers and non-smokers (Brown et al., 1997; Brown et al., 1998; Duthie et al., 1991). Bellizzi et al. (1996) reported lower \( \alpha \)-tocopherol levels within erythrocytes from cigarette smokers. The current study found a non-significant lower erythrocyte \( \alpha \)-tocopherol concentration from cigarette smokers compared with non-smokers. However, retrospective power calculations revealed that at least 35 subjects were required within each group to statistically establish whether there was a difference. Erythrocytes have tocopherol binding proteins (Kitabchi & Wimalasena, 1982a), and Bellizzi et al. (1996) showed a reduced binding activity in the erythrocytes of cigarette smokers compared with those from non-smokers, although there was no correlation between uptake activity and vitamin E concentration within the erythrocytes.

Platelet adhesion and aggregation are influenced by vitamin E (Calzada et al., 1997; Steiner, 1991). These are important processes in the pathology of cardiovascular disease. The concentration of vitamin E within platelets may represent a functional
biomarker to detect sub optimal vitamin E status. Platelet vitamin E concentration has not previously been investigated in cigarette smokers. This study showed that steady-state α-tocopherol concentrations are lower in platelets from cigarette smokers compared with non-smokers, γ-tocopherol concentrations were similar. A number of researchers have shown that cigarette smoking increased platelet aggregation (Bierenbaum et al., 1978; Lehr et al., 1997; Rival et al., 1987). There are several proposed mechanisms which include; reduced platelet-derived nitric oxide formation (Ichiki et al., 1996), increased surface density of thromboxane receptors on platelets of smokers (Rangemark & Wennmalm, 1996), increased response to external stimuli and formation of platelet activating factor-like lipid(s) in smokers (Imaizumi et al., 1991).

The lower levels of α-tocopherol may contribute towards the increased aggregation of platelets that has been shown in cigarette smokers. A possible reason for reduced α-tocopherol concentration in platelets could be due to greater activation and therefore greater oxidative stress.

The current study showed that cigarette smokers also had lower α- and γ-tocopherol concentration within their lymphocytes compared with non-smokers. This was the first study to report the vitamin E concentration of lymphocytes in cigarette smokers. Several researchers have observed increased lipid peroxidation and oxidative DNA damage within lymphocytes from cigarette smokers compared with non-smokers (Asami et al., 1996; Miro et al., 1999; Piperakis et al., 1998; Schneider et al., 2001). Cigarette smoking has also been associated with altered lymphocyte responsiveness and function (Ekberg-Jansson A et al., 2000; Moszczynski et al., 2001; Schaberg et al., 1997). Vitamin E has been shown to protect lymphocytes from oxidative damage (Brennan et al., 2000; Schneider et al., 2001) and enhance lymphocyte differentiation and proliferation ex vivo (Beharka et al., 1997; Brennan et al., 2000; Meydani et al., 1990). It is, therefore, possible that in cigarette smokers there is greater oxidation of vitamin E within the lymphocytes, which may result from chronic inflammation known to occur within cigarette smokers (Hunninghake et al., 1979). It would have been of interest to measure tocopherylquinone in plasma, erythrocytes, platelets and lymphocytes as a measure of tocopherol oxidation. It is probable that the α-tocopheryl radical is regenerated to α-tocopherol by ascorbic acid in lymphocytes. Due to the small amount of sample collected it was not possible to
measure ascorbic acid content of lymphocytes. Such measurements may have helped towards the understanding of why cigarette smokers have lower vitamin E levels within their lymphocytes.

It is well documented that cigarette smokers have lower plasma ascorbic acid concentrations (Chow et al., 1986; Pelletier, 1970; Ross et al., 1995; Van Resnburg et al., 1989). It is also generally accepted that ascorbic acid is important in the regeneration of vitamin E in vivo (Halliwell & Gutteridge, 1999; Hamilton et al., 2000). Therefore, it is conceivable that the reduced ascorbic acid levels could limit the regeneration of the tocopheroxyl radical to tocopherol within platelets and lymphocytes. However, in the current study, the plasma ascorbic acid concentration of cigarette smokers was not significantly lower than non-smokers.

The lower γ-tocopherol concentration found in lymphocytes from cigarette smokers compared with non-smokers, could have been due to an increased utilisation in neutralising reactive oxygen and nitrogen species. Cigarette smoke produces reactive nitrogen species (Norman & Keith, 1965) and γ-tocopherol is superior to α-tocopherol at neutralising the compounds (Cooney et al., 1993). Additionally Leonard et al. (2003) reported increased levels of 5-nitro-γ-tocopherol in plasma from cigarette smokers compared with non-smokers.

7.5.2 Urinary excretion of α- and γ-CEHC

Urinary α-CEHC excretion has been suggested as a useful biomarker to determine adequate vitamin E status (Schuelke et al., 2000; Schultz et al., 1995). The regulation of α-CEHC production is not fully understood, it is hypothesised that the extent of α-CEHC metabolism, in people with functioning α-tocopherol transfer protein (α-TTP), may represent the amount of vitamin E exceeding the requirements of the body. Baseline vitamin E urinary metabolites, α- and γ-CEHC, have not previously been measured in cigarette smokers. It was hypothesised that if there were increased utilisation of α-tocopherol in cigarette smokers, regulation pathways would ensure the retention of α-tocopherol at the expense of metabolism to α-CEHC. However, this study found urinary α-CEHC excretion to be similar in cigarette smokers and non-smokers. It may be questionable as to how sensitive a biomarker urinary excretion of α-CEHC is at baseline values. Plasma α- and γ-CEHC was not
measured in the current study, these measurements may be more sensitive as the inter­
individual variation in kidney function would have been removed (Galli et al., 2002).

Radosavac et al. (2002) observed that the concentration of α-CEHC in plasma
of cigarette smokers was higher than non-smokers after α-tocopherol supplementation.
Whereas Bruno et al. (2003) reported lower urinary excretion of α-CEHC in cigarette
smokers after α-tocopherol supplementation. The contradictory results emphasise the
gaps in knowledge in α-CEHC regulation.

Interestingly urinary γ-CEHC excretion was greater in cigarette smokers
compared to non-smokers. γ-Tocopherol metabolism to γ-CEHC is believed to be the
major route of elimination for γ-tocopherol (Galli et al., 2003; Swanson et al., 1999),
its metabolism involves one or more P450 cytochrome enzymes within the liver
believed to include CYP 4F2 (Songtag & Parker, 2002). It has been widely reported
that induction of one P450 cytochrome enzyme can affect induction of others and it is
possible this is the case in cigarette smokers (Pelkonen et al., 1998). Cigarette
smoking induces CYP1A1, an effect which has been attributed to the polycyclic
aromatic hydrocarbon class of compounds (Pelkonen et al., 1998). In the current study
cigarette smokers also had greater alcohol consumption compared with non-smokers
(Table 7.2), in accordance with reports by other researchers (Dallongeville et al.,
1998) ethanol has been shown to induce cytochrome P450 enzyme CYP2E1 (Fraser,
1997). Thus, in addition to the possible effect of smoking it is plausible that induction
of enzymes by ethanol could have influenced vitamin E metabolism as well. However,
if this were the case an increased urinary α-CEHC excretion would have been
observed as well.

γ-CEHC has been described as a powerful natriuretic factor (Wechter et al.,
1996) and it is able to inhibit COX-2 in macrophages (Jiang et al., 2000), implying a
specific role for this metabolite. The increase in urinary γ-CEHC excretion may have a
specific role in cigarette smokers rather than merely a consequence of γ-tocopherol
metabolism. More research is required in the area of γ-CEHC regulation in order to
fully understand the observed increased γ-CEHC excretion in cigarette smokers.

It is also possible that the increase γ-CEHC excretion was in part due to a
higher dietary intake of γ-tocopherol in cigarette smokers; dietary analysis showed
similar vitamin E intake but could not distinguish between different vitamin E forms.
7.5.3 Conclusions and future work

In the current study the cigarette smokers were relatively light smokers, thus highlighting the particularly detrimental effects cigarette smoking may have on vitamin E status. These data suggest that in smokers platelet and lymphocyte α-tocopherol are useful biomarkers, showing an affect prior to changes in other oxidative stress markers, such as plasma ascorbic acid.

Further investigation into vitamin E status in older volunteers that have smoked for a longer period of time is needed to understand if these findings underestimate the extent of changes in vitamin E metabolism and status. Cigarette smoking is a risk factor for CVD, the altered vitamin E status in platelets and lymphocytes may contribute to the early stage of the disease.

Measurement of tocopherylquinone would have been of particular interest within the blood cells to establish whether there is greater free radical scavenging in the blood components of cigarette smokers and therefore conversion of α-tocopherol to tocopherylquinone.

The current study demonstrates the importance of measuring vitamin E concentration in multiple blood cells to assess vitamin E status. Using steady-state plasma vitamin E concentrations may lead to the misinterpretation of the involvement of vitamin E in disease states. Further work is needed to determine the biokinetic profile of α- and γ-tocopherol in erythrocytes, platelets and lymphocytes and whether uptake and turnover differ in cigarette smokers compared with non-smokers.

The current study has demonstrated that steady-state vitamin E status is altered in cigarette smokers, although the case for increased requirements has not been established. More research is required into how cigarette smokers utilise their vitamin E and whether combined supplementation of vitamin E and C can provide optimal protection against reactive oxygen and nitrogen species.
Chapter 8

General discussion and conclusions
8.1 Research problem

Vitamin E is involved in preventing the pathogenesis of cardiovascular disease (CVD), both as an antioxidant (Steinberg, 1997) and at a molecular level (Azzi & Stocker, 2000). The previous chapters detail studies that have contributed to the understanding of vitamin E status in healthy people and those with an increased risk of CVD. The studies have also demonstrated the value of using a combination of biomarkers to gain a more accurate assessment of vitamin E status.

It is important to understand and be able to assess vitamin E status when investigating possible factors relating to disease risk. At present there is no adequate biomarker to reliably and accurately assess vitamin E status. This is, in part, due to the lack of information about the mechanisms that regulate the transfer of α-tocopherol both within and between cells and tissues. Steady-state plasma α- and γ-tocopherol concentrations are frequently used to assess vitamin E status, however, plasma vitamin E is hepatically regulated (Traber et al., 1990a; Traber & Kayden, 1989a), saturable (Dimitrov et al., 1991; Jialal et al., 1995), influenced by plasma lipids (Horwitt et al., 1972; Thurnham et al., 1986) and there is limited evidence to show that it is not sensitive to altered vitamin E concentrations within cells or tissues (Mezzetti et al., 1995; Simon et al., 1997). Additionally the relationship between plasma concentration and tissue levels is not fully understood. A number of other steady-state and functional biomarkers have been suggested (Morrissey & Sheehy, 1999), although these have not been properly standardised and are not universally accepted.

8.1.1 The response of vitamin E concentration within blood components and urinary excretion of vitamin E metabolites to supplemental α-tocopherol in healthy volunteers

Vitamin E status was assessed, using a variety of biomarkers, following supplementation with α-tocopheryl acetate. Measurement of plasma, erythrocyte, platelet and lymphocyte E concentrations were chosen for a number of reasons, plasma vitamin E concentration is the combined total of vitamin E within lipoproteins and therefore reflects vitamin E transport within the systemic circulation. Erythrocytes have been proposed as a possible biomarker of vitamin E status and the oxidative susceptibility of their membranes is directly related to vitamin E status (Miyake et al., 1991; Simon et al., 1997). Platelets and lymphocytes are functionally affected by vitamin E (Lehr et al., 1998; Moriguchi & Muraga, 2000; Steiner, 1999), it was
therefore proposed that vitamin E concentration may represent a functional biomarker of vitamin E status. Lymphocytes are known to have LDL receptors, a mechanism by which vitamin E is taken up into peripheral tissues (Traber & Kayden, 1984), and therefore may reflect peripheral tissues. Urinary excretion of α- CEHC has been proposed as an indicator of adequate vitamin E status in people with a functioning α-tocopherol transfer protein (α-TTP) (Schuelke et al., 2000; Schultz et al., 1995).

The single dose-response study detailed in Chapter 3 found no clear dose-response in α-tocopherol concentration in plasma, erythrocytes, platelets or lymphocytes, 24 h after ingestion of varying single doses of α-tocopheryl acetate up to 780 mg. This was, in part, the inability to distinguish between newly absorbed α-tocopherol and α-tocopherol already within the blood components and it has previously been shown that newly absorbed α-tocopherol replaces the existing tocopherol in plasma, which results in no significant difference in total tocopherol concentration (Traber et al., 1994; Traber et al., 1998b). Therefore, it is conceivable that a similar displacement process occurred within the erythrocytes, platelets and lymphocytes. The biokinetic study detailed in Chapter 5 described a decrease in d0 α-tocopherol within these blood components after a single dose of 150 mg d6 RRR-α-tocopheryl acetate, thus supporting the displacement hypothesis. The observations of α-tocopherol uptake and the displacement of existing α-tocopherol in platelets and lymphocytes within healthy individuals is a step towards a better understanding of α-tocopherol uptake and regulation in these important cells that are functionally affected by vitamin E.

The results of the single dose-response study contribute to the understanding of the specific vitamin E urinary metabolites α- and γ-CEHC in relation to α- and γ-tocopherol concentration in plasma, erythrocytes, platelets and lymphocytes. Baseline urinary α- and γ-CEHC excretion did not correlate with α- or γ-tocopherol concentrations in plasma, erythrocytes, platelets or lymphocytes. This therefore questions whether urinary α- and γ-CEHC excretion accurately reflects vitamin E status within the body. There was a correlation between plasma α-tocopherol, corrected for cholesterol, and erythrocyte α-tocopherol at 24 h with IAUC of urinary α-CEHC excreted up to 72 h after varying single doses of α-tocopheryl acetate. If α-CEHC is only excreted in large quantities when α-TTP is saturated the results from
Chapter 3 suggest that urinary $\alpha$-CEHC excretion reflects $\alpha$-TTP capacity rather than vitamin E status.

Schultz et al. (1995) proposed urinary $\alpha$-CEHC excretion as a potential biomarker for vitamin E status. However, there is limited information on the exact metabolic pathways and details on the proportion of ingested $\alpha$-tocopherol converted to $\alpha$-CEHC are unknown. The single dose-response study reported in Chapter 3 showed no clear dose-response in urinary excretion of $\alpha$-CEHC of up to 781 mg $\alpha$-tocopheryl acetate ingested. $\alpha$-TTP is thought to selectively remove $RRR-\alpha$-tocopherol from the metabolic pathway. It is hypothesised that the metabolism of $\alpha$-tocopherol to $\alpha$-CEHC may only be observed in a dose dependant manner when $\alpha$-TTP is saturated. Less than 1% of the ingested $RRR \alpha$-tocopheryl acetate doses were excreted as urinary $\alpha$-CEHC, which was lower than that observed when other vitamin E forms are ingested (Galli et al., 2003; Lodge et al., 2001; Traber et al., 1998a), suggesting selective retention within the body. It is therefore important to consider the uptake and distribution of the newly absorbed $\alpha$-tocopherol into the blood and subsequent tissues and other routes of metabolite excretion.

The uptake profile of newly absorbed deuterium labelled $\alpha$-tocopherol has previously been reported in plasma and erythrocytes (Cheeseman et al., 1995; Roxborough et al., 2000). However, the study reported in Chapter 5 was novel in describing the uptake of newly absorbed deuterium labelled $\alpha$-tocopherol into platelets and lymphocytes. Interestingly in platelets a biphasic uptake of newly absorbed $\alpha$-tocopherol was observed in some individuals, the initial peak in $\alpha$-tocopherol uptake was suggestive of passive diffusion, however the more gradual uptake observed after 9 h suggested a more controlled uptake process. The subjects who did not exhibit a biphasic uptake had a gradual increase in newly absorbed $\alpha$-tocopherol after 3 h, it remains unknown why individuals differ in their uptake of $\alpha$-tocopherol into platelets. In lymphocytes there was also a gradual uptake of newly absorbed $\alpha$-tocopherol. It was probable that the controlled uptake of $\alpha$-tocopherol was by LDL internalisation via LDL receptors in lymphocytes and may be platelets too, although the possible presence of tocopherol binding proteins has not been properly investigated. Kitabchi and Wimalasena (1982a) reported from their preliminary studies that platelets do not have tocopherol binding proteins.
A large inter-individual variation was observed in response to the varying single doses of vitamin E in the study detailed in Chapter 3. External variation was introduced in the study protocol, which could, in part, be attributed to the subjects consuming a non-standardised breakfast with their vitamin E capsules. The results shown in Chapter 4 indicate that the fat content and physical properties of the meal with which supplemental α-tocopheryl acetate is consumed affect the rate and magnitude of α-tocopherol appearance in plasma. This may have important implications in the design of future studies when trying to provide a standard vitamin E dose. The studies detailed in Chapters 4 and 5 illustrate the large inter-individual variation in the magnitude and rate of deuterium labelled α-tocopherol entering the systemic circulation when external confounding factors are minimised.

8.1.2 Vitamin E concentration in blood components of subjects with increased risk of cardiovascular disease

A research objective was to investigate the biokinetics of newly absorbed α-tocopherol in hypercholesterolemic volunteers compared with normolipidemic volunteers, which to date has previously been unexplored. Raised plasma cholesterol is a risk factor for CVD and may also impact upon vitamin E regulation as it is transported within lipoproteins. Vitamin E has also been shown to be important in the prevention of CVD.

The biokinetic study detailed in Chapter 6 investigated the uptake and distribution profiles of newly absorbed deuterium labelled α-tocopherol within a number of blood components. Lipoproteins were separated to observe the possible difference in α-tocopherol transport within plasma that may have otherwise been disguised if plasma concentrations alone were measured. Newly absorbed α-tocopherol uptake into plasma was similar in normolipidemic and hypercholesterolemic subjects, but there was there was a lower uptake of deuterium labelled α-tocopherol into LDL, and non-significant lower uptake into erythrocytes and lymphocytes in hypercholesterolemic subjects compared with normolipidemic subjects. These data suggest that hypercholesterolemics do not incorporate α-tocopherol into blood cells as efficiently as normolipidemics. A greater number of hypercholesterolemic subjects than normolipidemic subjects exhibited a biphasic
profile in the uptake of d6 α-tocopherol into platelets, the significance of which is unknown, but may be a worthwhile area of future investigation. Hypercholesterolemia is a risk factor for CVD and the study detailed revealed that it also contributes to an altered vitamin E regulation, which may further predispose hypercholesterolemic people to a higher risk of CVD.

Cigarette smoking is also considered a risk factor for CVD and cigarette smokers are exposed to sustained increased oxidative stress. Similar steady-state plasma and erythrocyte vitamin E concentrations have previously been reported in cigarette smokers and non-smokers. However, the study detailed in Chapter 7 demonstrated that cigarette smokers had a lower concentration of α-tocopherol in their platelets and lymphocytes compared with non-smokers and cigarette smokers also had a lower concentration of γ-tocopherol in their lymphocytes, compared with non-smokers. The lower vitamin E levels may contribute towards the increased platelet aggregation that has been observed in cigarette smokers (Lehr et al., 1997). These findings imply that cigarette smokers have an altered vitamin E status, but the implications of the lower vitamin E levels remain to be understood. Additionally cigarette smokers excreted more urinary γ-CEHC compared with non-smokers, whilst α-CEHC excretion was similar. α- and γ-Tocopherol metabolism to α- and γ-CEHC involves hepatic cytochrome P450 enzymes, the activity of these can be influenced by smoking, however more research is required in the area of γ-CEHC regulation in order to understand the observed increase in γ-CEHC excretion. Tocopherylquinone levels were not measured, this would have been beneficial to assess the extent of vitamin E utilisation in the cigarette smokers. Likewise, ascorbic acid levels within platelets and lymphocytes may have been of interest as its capacity to recycle the α-tocopheroxyl radical to α-tocopherol may influence vitamin E concentration within the cells.

The alterations observed in vitamin E regulation in cigarette smokers is of particular interest as the subjects were relatively young and not heavy smokers, consequently the data suggest that in smokers platelet and lymphocyte α-tocopherol are useful biomarkers, showing an affect prior to changes in other oxidative stress markers, such as plasma ascorbic acid.
8.1.3 Limitations of research detailed in thesis

There was large inter-individual variation in response to supplemental vitamin E observed throughout the research described, this is in agreement with previously published studies (Dimitrov et al., 1991; Traber et al., 1998a; Traber et al., 1998b). The inter-individual variation observed increased the calculated number of subjects required for each study to provide significance. The studies detailed in this thesis however, involved relatively small subject numbers and the power of the results discussed would be increased with larger subject numbers. An ongoing problem throughout the studies was the difficulty in recruiting subjects, in particular those with altered lipid status and cigarette smokers and as a consequence some results are not conclusive. The studies have however provided some interesting avenues for future work.

The benefits of using deuterium labelled vitamin E are well recognised and it is evident in the study detailed in Chapter 3 that the use of non-labelled vitamin E is limited. However, deuterated tocopherols are not commercially available and are very expensive thereby potentially limiting their availability for usage in studies.

Method development to analyse plasma α- and γ-CEHC, both deuterated and non-deuterated, was not possible in the timescale available for the current research project. It would have been interesting to assess plasma α- and γ-CEHC to observe the extent and rate of production of newly absorbed α-tocopherol in Chapters 5 and 6.
8.2 **Recommendations for future work**

The work that has been carried out for this thesis has provided some important findings and in the process has prompted further questions. These questions may form the basis of future research and suggestions for further studies are outlined below.

1. The uptake of newly absorbed α-tocopherol into platelets exhibited a biphasic response in some individuals, with a greater proportion in the hypercholesterolemic population. The α-tocopherol uptake and retention mechanisms into platelets are unknown, but it has been proposed in this thesis that passive diffusion and active transport may be involved. Experiments to investigate the possible processes for α-tocopherol uptake into platelets and to investigate if different lipoproteins are better donors than others are needed.

2. This thesis illustrated that vitamin E status was altered in cigarette smokers compared with non-smokers and it was hypothesised that these differences were due to a greater utilisation of vitamin E in the cigarette smokers. To investigate the formation of metabolites, measurement of both CEHC and tocopherylquinone are required to assess the turnover of vitamin E in a biokinetic study with deuterium labelled α-tocopherol and γ-tocopherol. A study investigating the effect of vitamin C status on vitamin E levels within platelets and lymphocytes with respect to its ability to recycle tocopherylquinone to tocopherol would be of particular interest.

3. A large degree of inter-individual variation was observed in the response to vitamin E supplementation throughout the studies documented. Reasons for this variation point towards variation in absorption, genetic polymorphisms that influence lipoprotein metabolism and the uptake of vitamin E into peripheral tissues. These are important areas for future research, a better understanding of the sources of variation and the magnitude of effect will help towards designing studies to investigate vitamin E status in subpopulations with altered vitamin E regulation.
References
References


Bruno, R., Ramakrishnan, R., Bray, T., & Traber, M. G. (2003) Vitamin E kinetics in cigarette smokers are related to vitamin C status. *Free Radical Biology and Medicine*. S33-


protein kinase C activity by tocopherols and tocotrienols. *Biochimica et Biophysica Acta.* **1176**; 83-89.


Dietrich, M., Block, G., Norkus, E. P., Traber, M. G., Cross, C. E., & Packer L (2003) Smoking and exposure to environmental tobacco smoke decrease some plasma


Goti, D., Hrzenjak, A., & Levak-Frank, S. (2001) Scavenger receptor class B, type 1 is expressed in porcine brain capillary endothelial cells and contributes to
selective uptake of HDL-associated vitamin E. *Journal of Neurochemistry*. 76; 498-508.


Heart Protection Study Collaborative Group (2002) MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20, 536 high-risk individuals: a randomised placebo-controlled trial. *The Lancet. 360;* 33-


Ministry of Agriculture, Fisheries and Food (1993) Food portion sizes. 2nd Ed.


Munro, L. H., Burton, G., & Kelly, F. J. (1997) Plasma RRR-α-tocopherol concentrations are lower in smokers than in non-smokers after ingestion of a similar oral load of this antioxidant vitamin. *Clinical Science*. **92**; 87-93.


Ohrvall, M., Sundlof, G., & Vessby, B. (1996) γ, but not α, tocopherol levels in serum are reduced in coronary heart disease patients. Journal of Internal Medicine. 239; 111-117.


Antioxidant supplementation in atherosclerosis prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis. *Journal of Internal Medicine.* 248; 377-386.


hydroxychroman, as an indicator of an adequate vitamin E supply? *American Journal of Clinical Nutrition.* 62; 1527S-1534S.


Traber, M. G., Sokol, R., Burton, G. W., Ingold, K., & Papa, S. (1990c) Impaired ability of patients with familial isolated vitamin E deficiency to incorporate α-tocopherol into lipoproteins secreted by the liver. *Journal of Clinical Investigations.* 85; 397-407.


Weintraub, M. S., Eisenberg, S., & Breslow, J. L. (1987b) Different patterns of postprandial lipoprotein metabolism in normal, Type IIa, Type III and Type IV hyperlipoproteinemic individuals. *Journal of Clinical Investigations.* 79; 1110-1119.

Weiser, H., Vecchi, M., & Schlachter, M. (1986) Stereoisomers of \( \alpha \)-tocopheryl acetate. IV. USP units and \( \alpha \)-tocopherol equivalents of all-rac-, 2-ambo- and RRR-\( \alpha \)-tocopherol evaluated by simultaneous determination of resorption-gestation, myopathy and liver storage capacity in rats. *International Journal of Vitamin Nutrition Research.* 56; 45-56.


**APPENDIX I**

**Personal Information Sheet**

Please complete all sections.

All information given will be held in the strictest confidence.

<table>
<thead>
<tr>
<th>Surname:</th>
<th>Contact address:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Forenames:</th>
<th>Contact telephone:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Date of birth:</th>
<th>Gender:</th>
<th>E-mail address:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Have you ever suffered from fits, blackouts, or fainting?</th>
<th>Yes/No</th>
<th>Dates and details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Are you currently on any long-term medication?</th>
<th>Yes/No</th>
<th>Dates and details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Do you suffer from any gastrointestinal disorder (e.g. chrohns disease, irritable bowel syndrome)</th>
<th>Yes/No</th>
<th>Dates and details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Do you smoke? ............... If yes, how many cigarettes per day?

..................

Do you follow a special diet or have any dietary restrictions?

If yes, please give details:

---------------------------------------------------------------------

---------------------------------------------------------------------

Do you take any type of dietary supplement?

If yes, please give details:

---------------------------------------------------------------------

---------------------------------------------------------------------

How many units of alcohol do you consume per week *on average*?

(One unit = ½ pint lager, 1 measure spirits, or 1 glass wine).

---------------------------------------------------------------------
APPENDIX II

1. Density solutions used in the separation of lipoproteins by sequential ultracentrifugation

\[ v_0 d_0 + x = d(v_0 + \bar{v}x) \]
\[ x(1 - d\bar{v}) = v_0(d - d_0) \]
\[ x = \frac{v_0 (d - d_0)}{1 - d\bar{v}} \]

\( x \) = amount of KBr in grams
\( v_0 \) = initial volume in ml
\( d_0 \) = initial density
\( d \) = final density
\( \bar{v} \) = specific volume of KBr at the final density

**Density solution 1.006 g/ml**

11.4 g NaCl + 0.1 g EDTA Na\(_2\) + 500 ml H\(_2\)O + 1 ml 1M NaOH
Dissolve solids and make up to 1000 ml
0.195 M NaCl

**Density solution 1.182 g/ml**

24.98 g NaBr + 100 ml 1.006 g/ml solution
0.195 M NaCl
2.44 M NaBr

**Density solution 1.019 ml**

8 ml 1.182 g/ml + 100 ml 1.006 g/ml

**Density solution 1.063 ml**

1.006 g/ml : 1.182 g/ml

\[ g = \frac{\omega^2 r}{980} \]

\( g \) = relative centrifugal force
\( r \) = the distance between the particle and centre of the rotation in cm (70.1 Ti radius min 40.5 max 82)
\( \omega \) = rotor speed rad/sec
\( \omega = \text{rev/min} \times 2\pi = \text{rev/min} \times (0.10472) \)

\[ \frac{60}{60} \]
APPENDIX III

An individual cholesterol profile of LDL sub fractions

LDL sub fractions, an individual profile of cholesterol content of fractions. Fractions 2 - 17 were used and adjacent fractions were pooled together for vitamin E analysis.
APPENDIX IV

Triacylglycerol concentration in plasma for studies detailed in Chapters 4, 5 and 6

Chapter 4. Plasma triacylglycerol (TAG) concentration following ingestion of 150 mg d6 RRR-α-tocopheryl acetate with four test meals over 6 hours
Values expressed as mean ± SEM (n = 8). There was no significant difference over time

Chapter 5. Plasma triacylglycerol (TAG) concentration following ingestion of 150 mg d6 RRR-α-tocopheryl acetate with 40g fat over 48 hours
Values expressed as mean ± SEM (n = 12). There was a trend towards a significant difference over time (p = 0.06)
Chapter 6. Plasma triacylglycerol (TAG) concentration following ingestion of 150 mg d6 RRR-a-tocopheryl acetate with 40g fat over 48 hours in normolipidemic (NL) and hypercholesterolemic (HC) subjects.

Values expressed as mean ± SEM (n = 8 in each group). There was a significant difference over time in the TAG concentration profile in NL and HC subjects (p < 0.05) and a significant difference between groups (p < 0.05).