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**STUDIES ON BIOACTIVE LIPID MEDIATORS INVOLVED  
IN BRAIN FUNCTION AND NEURODEGENERATIVE  
DISORDERS**

**A. A. A. A. DRBAL**

**PhD**

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Neurodegenerative Disorders**

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sphingomyelin species and endocannabinoids formation; changes in  
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**Abed Alnaser Anter Amer DRBAL**

**Submitted for the Degree of  
Doctor of Philosophy**

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# STUDIES ON BIOACTIVE LIPID MEDIATORS INVOLVED IN BRAIN FUNCTION AND NEURODEGENERATIVE DISORDERS

**Abed Alnaser Anter Amer DRBAL**

**Keywords:** eicosapentaenoic acid, docosapentaenoic acid, sphingomyelin, lithium chloride, lipopolysaccharide, tandem mass spectrometry, oxysterols, Amyotrophic Lateral Sclerosis, gas chromatography, SOD1-mice

## Abstract

Lipids are important for structural and physiological functions of neuronal cell membranes. They exhibit a range of biological effects many are bioactive lipid mediators derived from polyunsaturated fatty acids such as sphingolipids, fatty acid ethanolamides (FA-EA) and endocannabinoids (EC). These lipid mediators and oxysterols elicit potent bioactive functions in many physiological and pathological processes of the brain and neuronal tissues. They have been investigated for biomarker discovery of ageing, neuroinflammation and neurodegenerative disorders. The n-3 fatty acids EPA and DPA are thought to exhibit a range of neuroprotective effects many of which are mediated through production of such lipid mediators.

The aims of this study were to evaluate the effects of n-3 EPA and n-3 DPA supplementation on RBC membranes and in this way assess dietary compliance and to investigate brain sphingomyelin species of adult and aged rats supplemented with n-3 EPA and n-3 DPA to evaluate the effects and benefits on age-related changes in the brain. Furthermore, to study the effects of lithium on the brain FA-EAs and ECs to further understand the neuroprotective effects of lithium neuroprotective action on neuroinflammation as induced by LPS. Finally to examine if circulating oxysterols are linked to the prevalence of ALS and whether RBC fatty acids are markers of this action in relation to age and disease stages. These analytes were extracted from tissue samples and analysed with GC, LC/ESI-MS/MS and GC-MS.

It was found that aged rats exhibited a significant increase in brain AA and decrease in  $\Sigma$ n-3 and  $\Sigma$ n-6 PUFAs when compared to adult animals. The observed increase of brain AA was reversed following n-3 EPA and n-3 DPA supplementation. Sphingomyelin was significantly increased when aged animals were supplemented with n-3 DPA. LPS treatment following lithium supplementation increased LA-EA and ALA-EA, while it decreased DHA-EA. Both oxysterols 24-OH and 27-OH increased in ALS patients and SOD1-mice. Eicosadienoic acid was different in ALS-patients compared to aged SOD1-mice.

These studies demonstrated that dietary intake of n-3 EPA and n-3DPA significantly altered RBC fatty acids and sphingolipids in rat brain. They suggest that n-3 DPA can be a potential storage form for EPA, as shown by retro-conversion of n-3 DPA into EPA in erythrocyte membranes, ensuring supply of n-3 EPA. Also, n-3 EPA and n-3 DPA supplementation can contribute to an increase in brain sphingomyelin species with implications for age effects and regulation of brain development. Effects of lithium highlight novel anti-neuroinflammatory treatment pathways. Both 24-hydroxycholesterol and eicosadienoic acid may be used as biomarkers in ALS thereby possibly helping to manage the progressive stages of disease.

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## Table of Contents

<b>Abstract.....</b>	<b>i</b>
<b>Acknowledgements.....</b>	<b>ii</b>
<b>List of Charts and Figures .....</b>	<b>x</b>
<b>List of Tables .....</b>	<b>xv</b>
<b>List of abbreviations .....</b>	<b>xvii</b>
<b>Chapter 1: Introduction.....</b>	<b>1</b>
1.1 Fatty acids .....	3
1.1.1 Fatty acid biosynthesis .....	4
1.1.2 Eicosapentaenoic acid .....	7
1.1.3 Docosapentaenoic acid .....	7
1.2 Sphingolipids .....	8
1.2.1 Sphingomyelin structure and species .....	9
1.2.2 Sphingomyelin biosynthesis and metabolism.....	11
1.2.3 Sphingomyelin function .....	14
1.3 Endocannabinoids and their congeners.....	15
1.3.1 Biosynthesis of fatty acid ethanolamides and endocannabinoids..	18
1.3.2 Receptors activated by endocannabinoids and related compounds.....	20
1.4 Oxysterols.....	23

1.4.1	Biosynthesis of oxysterols .....	23
1.4.2	Biological activity of oxysterols .....	27
1.5	The role of bioactive lipids in ageing and neurodegenerative disorders.....	30
1.5.1	Omega-3 PUFA in neurodegeneration and brain ageing .....	31
1.5.1.1	Eicosapentaenoic acid.....	33
1.5.1.2	Docosapentaenoic acid .....	34
1.5.2	Sphingomyelin in neurodegeneration and ageing brain .....	35
1.5.3	Fatty acid ethanolamides in brain function and disorders.....	37
1.5.4	Neurodegenerative disorders and oxysterols .....	40
1.5.4.1	Amyotrophic Lateral Sclerosis.....	42
1.6	Lipid analysis .....	44
1.6.1	Gas chromatography in lipid analysis.....	44
1.6.2	Mass Spectrometry in lipid analysis .....	45
1.6.2.1	Ionisation methods used in lipidomics .....	45
1.6.2.2	Mass analysers used in lipidomics .....	48
1.7	Aim and objectives.....	50
<b>Chapter 2:</b>	<b>Materials and Methods .....</b>	<b>53</b>
2.1	Materials .....	54
2.1.1	Chemicals .....	54

2.1.2	Glassware and other consumables .....	55
2.1.3	Equipment.....	55
2.2	Biological and Clinical Samples .....	57
2.2.1	Eicosapentaenoic and docosapentaenoic acid-treated red blood cells and brain tissue .....	57
2.2.2	Microwave-treated rat brain tissue .....	58
2.2.3	Red blood cell and plasma samples from SOD1 mice .....	60
2.2.4	Red blood cell and plasma samples from Amyotrophic Lateral Sclerosis patients.....	61
2.3	Fatty acid analysis .....	62
2.3.1	Preparation of solvents.....	62
2.3.2	Preparation of standards .....	62
2.3.3	Red blood cell lipid extraction.....	63
2.3.4	Preparation of fatty acid methyl esters .....	64
2.3.5	Preparation of internal standard methyl ester.....	65
2.3.6	GC-FID analysis .....	65
2.3.7	Analysis of standards .....	66
2.3.8	Analysis of biological samples.....	67
2.3.9	Calculations.....	67
2.4	Analysis of sphingomyelin.....	68
2.4.1	Preparation of standards .....	68

2.4.2	Brain lipid extraction .....	68
2.4.3	ESI-MS/MS analysis of sphingomyelin species.....	69
2.4.4	LC-ESI-MS/MS analysis of sphingomyelin species.....	70
2.4.5	Limit of quantitation and limit of detection .....	70
2.4.6	Recovery .....	71
2.5	Analysis of fatty acid ethanolamides and endocannabinoids .....	72
2.5.1	Preparation of standards .....	72
2.5.2	Calibration lines.....	72
2.5.3	Limit of quantitation and limit of detection .....	72
2.5.4	Tissue homogenisation and extraction .....	74
2.5.5	Solid phase extraction of fatty acid ethanolamides.....	75
2.5.6	LC/ESI-MS/MS analysis of fatty acid ethanolamides.....	76
2.5.7	Recovery .....	77
2.6	Analysis of oxysterols .....	77
2.6.1	Plasma sterol extraction .....	77
2.6.2	Sterol derivatization.....	78
2.6.3	GC-MS analysis of sterols.....	79
2.7	Statistical analysis .....	79

<b>Chapter 3: The effect of eicosapentaenoic and docosapentaenoic acid supplementation on rat red blood cell fatty acids and brain sphingomyelin.....</b>	<b>80</b>
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3.1	Introduction .....	81
3.2	Materials and methods.....	84
3.3	Results.....	84
3.3.1	The effect of age on rat red blood cell fatty acids .....	84
3.3.2	The effect of EPA and n-3 DPA supplementation on red blood cell fatty acids in adult animals.....	85
3.3.3	The effect of EPA and n-3 DPA supplementation on red blood cell fatty acids in aged animals.....	89
3.3.4	The effect of n-3 DPA long-term supplementation on red blood cell fatty acids of aged animals. ....	90
3.3.5	LC/ESI-MS/MS analysis of brain sphingomyelin species .....	94
3.3.5.1	Method development using bovine brain extract .....	94
3.3.5.2	Analysis of sphingomyelin species in rat brain .....	95
3.3.6	The effect of ageing on rat brain sphingomyelin.....	98
3.3.7	The effect of EPA and n-3 DPA supplementation on adult animal brain sphingomyelin.....	100
3.3.8	The effect of EPA and n-3 DPA supplementation on aged animal brain sphingomyelin.....	101
3.4	Discussion .....	103
<b>Chapter 4: Analysis of brain fatty acid ethanolamides and endocannabinoids following LPS-induced neuroinflammation and lithium treatment .....</b>		<b>113</b>
4.1	Introduction .....	114

4.2	Materials and Method .....	117
4.3	Results.....	117
4.3.1	Optimisation of mass spectrometry conditions and MS/MS assay.....	118
4.3.2	SPE, recovery, linearity, limits of detection and quantification ....	123
4.3.3	Endocannabinoids and fatty acid ethanolamides found in rat brain.....	123
4.3.4	The effect of LPS on rat brain fatty acid ethanolamides and endocannabinoids.....	126
4.3.5	The effect of lithium chloride on rat brain fatty acid ethanolamides and endocannabinoids.....	126
4.4	Discussion .....	128
<b>Chapter 5. Plasma oxysterols and red blood cell fatty acids as potential biomarkers for the diagnosis of Amyotrophic Lateral Sclerosis.....</b>		<b>135</b>
5.1	Introduction .....	136
5.2	Materials and Methods.....	140
5.3	Results.....	141
5.3.1	Plasma oxysterols in ALS patients .....	141
5.3.1.1	The effect of disease on human plasma oxysterols.....	146
5.3.2	Plasma oxysterols in a SOD1-mutant transgenic mice model.....	146
5.3.2.1	The effect of disease on mice plasma oxysterols .....	150

5.3.2.2	The effect of age on mice plasma oxysterols.....	150
5.3.3	Fatty acid levels in ALS human red blood cells .....	155
5.3.3.1	The effect of disease on human red blood cell fatty acids .....	155
5.3.4	Red blood cell fatty acids in SOD1 transgenic mice model .....	155
5.3.4.1	The effect of disease on SOD1 mice red blood cell fatty acids.....	155
5.3.4.2	The effect of age on mice red blood cell fatty acids.....	159
5.4	Discussion .....	161
<b>Chapter 6. General Discussion.....</b>		<b>171</b>
6.1	General discussion .....	173
6.2	Future work.....	188
<b>References... ..</b>		<b>192</b>
<b>APPENDICES.....</b>		<b>247</b>

## List of Charts and Figures

<b>Chart 1.1.</b> The flow of the intended research.....	2
<b>Figure 1.1.</b> Schematic showing the biosynthetic pathways of n-9, n-6 and n-3 PUFA <b>(A)</b> . The converting of EPA to DHA via the Sprecher pathway is shown in part <b>(B)</b> . DPA retro-converted into EPA <b>(C)</b> .....	6
<b>Figure 1.2.</b> The general structure of sphingosine, ceramide and sphingomyelin (1) phosphocholine head group (2) sphingoid base and (3) fatty acid residue .....	10
<b>Figure 1.3.</b> Schematic showing the biochemical pathways of ceramide metabolism: (1) The de novo pathway, (2) sphingomyelin synthase and sphingomyelinase pathways, (3) The exogenous ceramide-recycling pathway, and (4) The salvage pathway. The pathway of sphingomyelin and ceramide synthesis from recycling/salvaging sphingosine .....	13
<b>Figure 1.4.</b> Chemical structures of various fatty acid ethanolamides and endocannabinoids.....	17
<b>Figure 1.5.</b> Fatty acid ethanolamide biosynthetic and hydrolysis pathways <b>(A)</b> . Hydrolysis of two principal endocannabinoids; anandamide (AEA) and 2-arachidonoylglycerol (2-AG) <b>(B)</b> .....	19
<b>Figure 1.6.</b> Summary of the main cholesterol oxygenation reactions <b>A</b> . Enzymatically mediated production species. <b>B</b> . Non-enzymatic pathways taking place in presence of reactive oxygen species (ROS) .....	26

<b>Figure 1.7.</b> A schematic diagram summarizing the major functions, biological roles and the pathologic processes mediated by oxysterols. LXR: liver X receptors; ROS: reactive oxygen species; CNS: central nervous system; CYP450: cytochrome P450.....	28
<b>Figure 1.8.</b> An illustration of how ions are generated from ionised solvent emerging from the capillary during ESI .....	46
<b>Figure 1.9.</b> Diagram showing LC-MS/MS based analyses using an electrospray ion source (ESI) and triple quadruple (3Q) system .....	49
<b>Figure 3.1.</b> ESI-MS/MS spectrum (ESt) of sphingomyelin (SM) species in bovine brain extract.....	95
<b>Figure 3.2.</b> ESI-MS/MS spectrum of rat brain lipid extract (precursors of <i>m/z</i> 184).....	96
<b>Figure 3.3.</b> LC/ESI-MS/MS analysis of sphingomyelin (SM) bovine brain extract.....	97
<b>Figure 3.4.</b> LC/ESI-MS/MS analysis of rat brain extract sphingomyelin (SM) species.....	99
<b>Figure 3.5.</b> SM species of brain in adult animals (3-4 months) following supplementation with EPA (200 mg/kg/day) or n-3 DPA (200 mg/kg/day) for 8 weeks.....	100
<b>Figure 3.6.</b> SM species of brain in aged animals (22-24 months) following supplementation with EPA or n-3 DPA for 8 weeks.....	102

<b>Figure 4.1. A)</b> Proposed mechanism of ionisation of fatty acid ethanolamides. <b>B)</b> Proposed mechanism for the fragmentation of DHA ethanolamide by ESI.....	118
<b>Figure 4.2.</b> ESI-MS/MS spectra of anandamide (A), anandamide-d8 (B), 2-Arachidonoylglycerol (C), 2-Arachidonoylglycerol-d8 (D), N-palmitoylethanolamide (E), N- $\alpha$ -linolenoylethanolamide (F), N-linoleoylethanolamide (G), N-oleoylethanolamide (H), N-stearoylethanolamide (I) and N-docosahexaenoylethanolamide (J).....	121
<b>Figure 4.3.</b> LC/ESI-MS/MS analysis of FA-EAs and 2-AG standards anandamide (AEA), N-palmitoylethanolamide (PA-EA), N-stearoylethanolamide (SA-EA), N-oleoylethanolamide (OA-EA), N-docosahexaenoylethanolamide (DHA-EA), N-linoleoylethanolamide (LA-EA), N-linolenoylethanolamide (ALA-EA), 2-arachidonoylglycerol (2-AG), 1-arachidonoylglycerol (1-AG), and the internal standards (IS) AEA-d8, 2-AG-d8 and 1-AG.....	122
<b>Figure 4.4.</b> Representative calibration lines (1-120 pg analyte in column) of (A) linoleoylethanolamide (LA-EA), (B) arachidonoylethanolamide (AEA), (C) palmitoylethanolamide (PA-EA), (D) docosahexaenoylethanolamide (DHA-EA), (E) stearoylethanolamide (ST-EA), (F) oleoylethanolamide (OA-EA), (G) $\alpha$ -linolenoyl ethanolamide (ALA-EA) and (H) 2-arachidonoyl glycerol (2-AG)....	124
<b>Figure 4.5.</b> LC/ESI-MS/MS analysis of microwaved rat brain FA-EAs and 2-AG.....	125
<b>Figure 5.1.</b> Representative GC-MS traces showing the analysis of one human plasma sample.....	143

**Figure 5.2.** Fragmentation pattern of oxysterol species. The fragmentation process is demonstrated here using 7 $\alpha$ -hydroxycholesterol-TMS as a representative structure for oxysterols.....145

**Figure 5.3:** Chemical structure and molecular weight (M) of oxysterol-TMS derivatives.....148

**Figure 5.4.** Oxysterols identified in human plasma lipid extracts, following GC-MS analysis. **A.** Retention times (Rt min), species and p values. **B.** Plasma oxysterol species those were significantly different when comparing control to ALS patient samples.....149

**Figure 5.5. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in animal aged 35 days SOD1 and wild plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were significantly different in SOD1 animals aged 35 days compared to wild animals aged 35 days.....151

**Figure 5.6. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in animal aged 120 days SOD1 and wild plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were significantly different in SOD1 animals aged 120 days compared to wild animals aged 120 days.....152

**Figure 5.7. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in wild animal aged 35 days and 120 days plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were

significantly different in wild animals aged 35 days compared to wild animals aged 120 days.....153

**Figure 5.8. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in SOD1 animals aged 35 days and 120 days plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were significantly different in SOD1 animals aged 35 days compared to SOD1 animals aged 120 days.....154

**Figure 5.9.** Levels of eicosadienoic acid (20:2n-6) in red blood cell from young (35 days) wild and SOD1 mice and old (120 days) wild and SOD1 mice.....158

**Figure 5.10.** Levels of linoleic acid (C18:2n-6t) and eicosadienoic acid (20:2n-6) in red blood cell from aged (120 days) wild and SOD1 mice.....158

**Figure 5.11.** The effect of age on wild mice red blood cell fatty acid levels when comparing young (35 days) wild animals to old (120 days) ones.....160

**Figure 5.12.** The effect of age on SOD1 mice red blood cell fatty acid levels when comparing young (35 days) SOD1 animals to aged (120 days) ones.....160

**Chart 6.1.** An overview on the findings of the present studies.....172

## List of Tables

<b>Table 1.1.</b> Receptors activated by mammalian endocannabinoids and related fatty acid ethanolamides .....	21
<b>Table 2.1.</b> The MRM transitions, collision energy (CE) and cone voltage (CV) used for the analysis of SM bovine brain extract species, rat brain extract and the internal standard SM d18:1/12:0 (IS).....	71
<b>Table 2.2.</b> Preparation of fatty acid ethanolamide and endocannabinoid composite solution without internal standards.....	73
<b>Table 2.3.</b> Preparation of fatty acid ethanolamides and endocannabinoids composite solution with internal standards (AEA-d8 and 2-AG-d8).....	73
<b>Table 3.1.</b> Fatty acid composition of red blood cells in adult (3-4 months) and aged (22-24 months) animals.....	86
<b>Table 3.2.</b> Fatty acid composition of red blood cells in adult animals (3-4 months) following supplementation with EPA 200 mg/kg/day or n-3 DPA 200 mg/kg/day for 8 weeks.....	87
<b>Table 3.3.</b> Fatty acid composition of red blood cells in aged animals (22-24 months) following supplementation with EPA 200 mg/kg/day or n-3 DPA 200 mg/kg/day for 8 weeks.....	92
<b>Table 3.4.</b> Fatty acid composition of red blood cells in aged animals (17-18 months) supplemented with n-3 DPA 100 mg/rat/day for 3 and 6 months.....	93

<b>Table 3.5.</b> LC/ESI-MS/MS analysis of the rat brain SM. The most abundant SM species found in adult (3-4 months) and aged (22-24 months) rat brain.....	98
<b>Table 4.1.</b> Parameters used for the LC-MS/MS assay of the fatty acid ethanolamides and 2-arachidonoylglycerol standards. MRM: multiple reaction monitoring, CE: collision energy.....	119
<b>Table 4.2.</b> A summary of all fatty acid ethanolamides (FA-EA) and endocannabinoids (ECs) detected in microwaved rat brain subjected to low dose of lithium chloride (LiCl) 1.70 g/kg for 4 weeks, then to high dose of lithium chloride 2.55 g/kg (high LiCl) for 2 weeks follows treatment with LPS at low dose (1 mg/mL at 0.5 ng/h) and high dose (0.5 mg/mL at 250 ng/h) compared to controls.....	127
<b>Table 5.1.</b> Retention times (Rt min) and normalised peak area (peak area/IS area) for all peaks eluted between 19.9 min and 30.4 min during the GC-MS analysis of lipid extract from human plasma.....	144
<b>Table 5.2.</b> Retention times (Rt), molecular weight (MW), oxysterol-TMS m/z and fragment ions (m/z) used to identify oxysterols in human plasma lipid extracts, following GC-MS analysis.....	147
<b>Table 5.3.</b> Red blood cell fatty acids in control subjects and ASL samples.....	156
<b>Table 5.4.</b> Mice red blood cell fatty acids in wild and SOD1 animals.....	157

## List of abbreviations

<b>1-AG</b>	1-Arachidonoylglycerol
<b>20-OH</b>	20-hydroxycholesterol
<b>22-OH</b>	22-hydroxycholesterol
<b>24-OH</b>	24-hydroxycholesterol
<b>25-OH</b>	25-hydroxycholesterol
<b>26-OH</b>	26-hydroxycholesterol
<b>27-OH</b>	27-hydroxycholesterol
<b>2-AG</b>	2-Arachidonoylglycerol
<b>4<math>\beta</math>-OH</b>	4 $\beta$ -hydroxycholesterol
<b>5,6<math>\alpha</math>-epoxy</b>	5,6 $\alpha$ -epoxy-cholesterol
<b>5,6<math>\beta</math>-epoxy</b>	5,6 $\beta$ -epoxy-cholesterol
<b>6-keto</b>	6-ketocholesterol
<b>7-keto</b>	7-ketocholesterol
<b>7<math>\alpha</math>-OH</b>	7 $\alpha$ -hydroxycholesterol
<b>7<math>\beta</math>-OH</b>	7 $\beta$ -hydroxycholesterol
<b>AA</b>	Arachidonic acid

<b>aCSF</b>	artificial Cerebral Spinal Fluid
<b>ADHD</b>	Attention-deficit hyperactivity disorder
<b>AEA</b>	<i>N</i> -arachidonoylethanolamide (anandamide)
<b>ALA</b>	$\alpha$ -Linolenic acid
<b>ALA-EA</b>	$\alpha$ -linolenoylethanolamide
<b>Alk-SMase</b>	Alkaline sphingomyelinase
<b>ALS</b>	Amyotrophic lateral sclerosis
<b>A-SMase</b>	Acid sphingomyelinase
<b>BBB</b>	Blood brain barrier
<b>CB</b>	Cannabinoid receptor
<b>CE</b>	Collision energy
<b>Chol-triol</b>	Cholestane-3 $\beta$ , 5 $\alpha$ , 6 $\alpha$ -triol
<b>CMP</b>	Cytidine monophosphate
<b>CNS</b>	Central nervous system
<b>COX</b>	Cyclooxygenase
<b>CPP-choline</b>	Cytidine diphosphate-choline
<b>CPR</b>	G-protein-coupled receptor

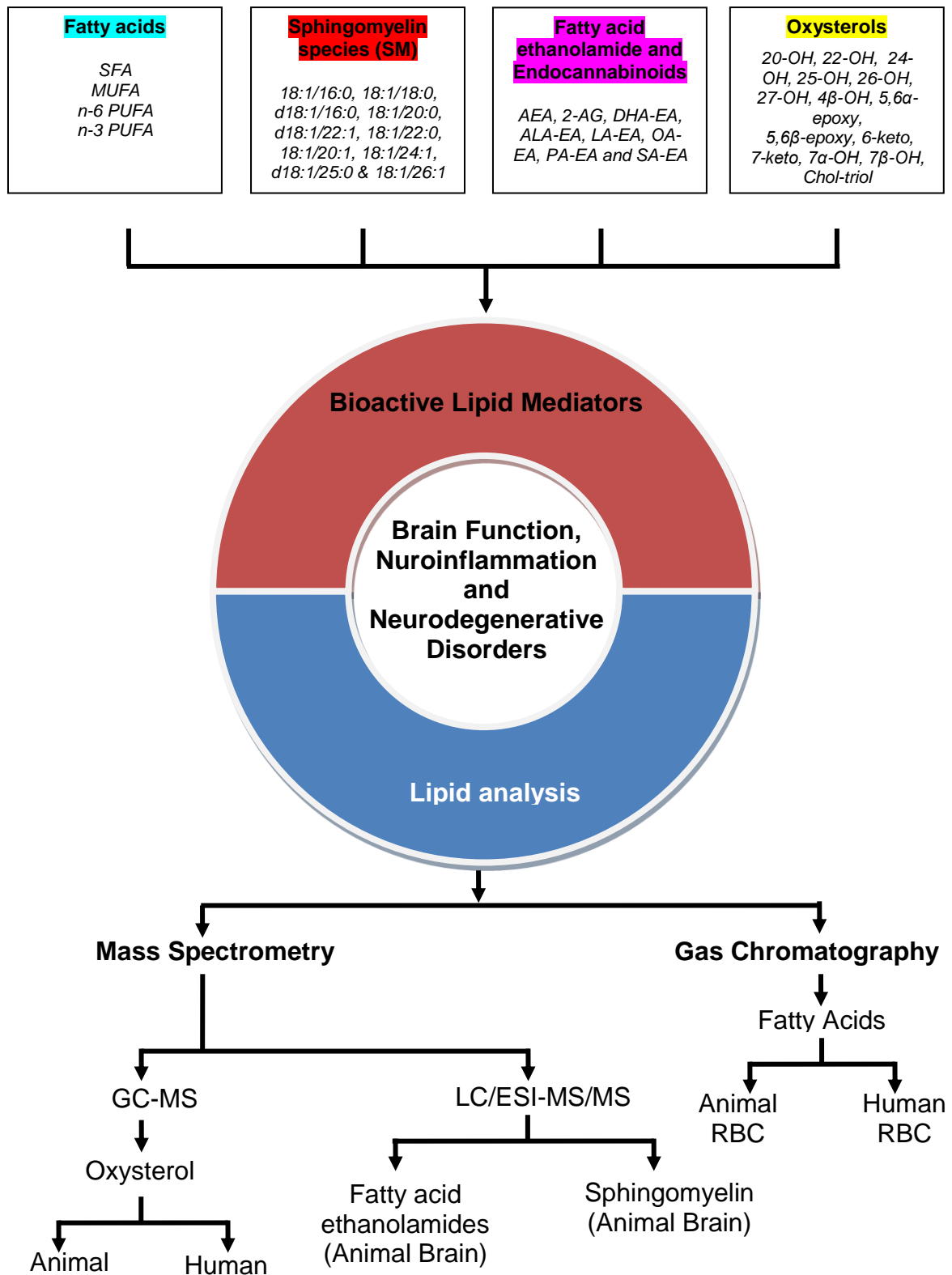
<b>CYP</b>	Cytochrome P450
<b>DCM</b>	Dichloromethane
<b>DGLA</b>	Dihomo- $\gamma$ -linolenic acid
<b>DHA</b>	Docosahexaenoic acid
<b>DHA-EA</b>	<i>N</i> -Docosahexaenylethanolamide
<b>DPA</b>	Docosapentaenoic acid
<b>ECs</b>	Endocannabinoids
<b>EDA</b>	Eicosadienoic acid
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>EI</b>	Electron impact
<b>EPA</b>	Eicosapentaenoic acid
<b>ESI</b>	Electrospray ionization
<b>ESI-MS</b>	Electrospray ionization mass spectrometry
<b>FAAH</b>	Fatty Acid Amide Hydrolase
<b>FA-EA</b>	Fatty acid ethanolamide
<b>FAS</b>	Fatty Acid Synthase
<b>FID</b>	Flame ionization detection

<b>G3P</b>	Glycerol 3-phosphate
<b>GC</b>	Gas chromatography
<b>GC-MS</b>	Gas chromatography-mass spectrometry
<b>GP-NAE</b>	Glycerophospho-NAE
<b>GPR55</b>	G-protein-coupled receptor 55
<b>GSLs</b>	Glycosphingolipids
<b>HDL</b>	High density lipoproteins
<b>HPLC</b>	High-performance liquid chromatography
<b>IS</b>	Internal standard
<b>LA</b>	Linoleic Acid
<b>LA-EA</b>	<i>N</i> -linoleoylethanolamide
<b>LC/ESI-MS/MS</b>	Liquid chromatography-electrospray ionization-tandem mass spectrometry
<b>LC-MS/MS</b>	Liquid chromatography tandem mass spectrometry
<b>LDL</b>	Low-density lipoproteins
<b>LiCl</b>	Lithium chloride
<b>LOD</b>	Limit of detection

<b>LOQ</b>	Limit of quantitation
<b>LOX</b>	Lipoxygenase
<b>LPA</b>	Lysophosphatidic acid
<b>LPS</b>	Lipopolysaccharides
<b>LysoNAPE</b>	N-acyl-lyso-phosphatidylethanolamines
<b>Lyso-PLD</b>	Lysophospholipase
<b>MND</b>	Motor Neuron Disorders
<b>MRM</b>	Multiple reaction monitoring
<b>MS/MS</b>	Tandem mass spectrometry
<b>MUFA</b>	Monounsaturated fatty acid
<b>NAAA</b>	<i>N</i> -acylethanolamine-hydrolyzing acid amidase
<b>NAPE</b>	<i>N</i> -acylphosphatidylethanolamine
<b>NAPE-PLD</b>	<i>N</i> -acylphosphatidylethanolamine Phospholipase D
<b>N-SMase</b>	Neutral sphingomyelinase
<b>OA</b>	Oleic acid
<b>OA-EA</b>	<i>N</i> -oleoylethanolamide
<b>PA</b>	Phosphatidic acid

<b>PA-EA</b>	<i>N</i> -palmitoylethanolamide
<b>PPAR<math>\alpha</math>, <math>\beta</math> and <math>\gamma</math></b>	Peroxisome proliferators-activated receptors $\alpha$ , $\beta$ and $\gamma$
<b>PUFA</b>	Polyunsaturated fatty acid
<b>RBC</b>	Red blood cells
<b>ROS</b>	Reactive oxygen species
<b>S/N</b>	Signal-to-Noise
<b>SA</b>	Stearic acid
<b>SA-EA</b>	<i>N</i> -stearoylethanolamide
<b>SFA</b>	Saturated Fatty Acid
<b>SIM</b>	Selected ion monitoring
<b>SM</b>	Sphingomyelin
<b>SOD1</b>	Superoxide dismutase 1
<b>SPE</b>	Solid phase extraction
<b>TMS</b>	Trimethylsilyl
<b>TRPM8</b>	Transient receptor potential melastatin type 8
<b>TRPV1</b>	Transient receptor potential vanilloid type 1
<b>VLDL</b>	Very low-density lipoproteins

## **Chapter 1: Introduction**



**Chart 1.1.** The flow of the intended research

## 1.1 Fatty acids

Natural fatty acids commonly have chain lengths varying from 4 to 28 carbons, and may be saturated or unsaturated (Davidson and Cantrill, 1985). Unsaturated fatty acids contain one or more double bond termed monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA) respectively. The systematic name of fatty acids is determined by the number of carbons and number of double bonds found in their acyl chain. In a commonly used shorthand notation system, fatty acids are described using the number of carbons in the acyl chain, and number of double bonds. The position of the last double bond in the acyl chain from the carboxyl end is used to define families of PUFA as n-3 PUFA or n-6 PUFA as shown in Figure 1.1 (Markley, 1964, Hames et al., 2005).

Omega (n-3) PUFA such as eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and n-6 PUFA such as arachidonic acid (AA, 20:4n-6) serve a wide range of metabolic functions significant to all forms of life. However, in 1929 and 1930, George and Mildred Burr discovered that certain fatty acids were critical to health and coined the phrase of “essential fatty acids”, with reference to linoleic acid (LA, 18:2n-6) and  $\alpha$ -linolenic acid (ALA, 18:3n-3), mammals cannot biosynthesis them, thus require dietary sources to meet their needs (Burr, 1981, Holman, 1988, , Gurr and Harwood, 2002, Smith, 2012). In addition, PUFA are important nutrients and the ratio of n-3 and/or n-6 PUFA can be correlated with growth, development and cognitive function (Simopoulos, 2002, Yehuda, 2003, Whelan, 2008).

### 1.1.1 Fatty acid biosynthesis

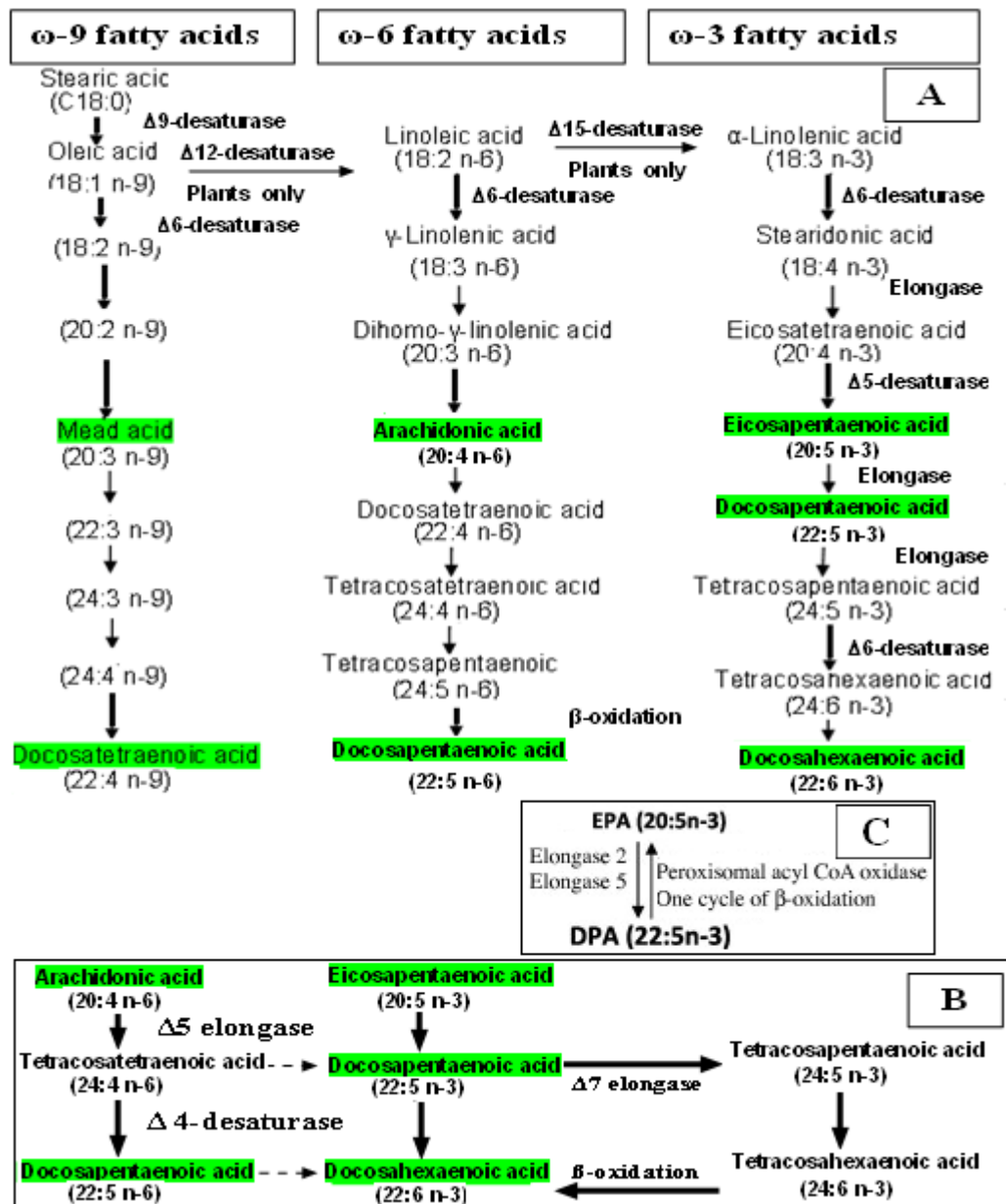
Acetyl-CoA and Malonyl-CoA are the precursors of fatty acids. Malonyl-CoA is the main metabolite need for fatty acid biosynthesis and the enzyme fatty acid synthase carries out a series of C2 elongation steps leading to palmitic acid (C16:0) (Katiyar et al., 1974, Stoops et al., 1975, Wakil et al., 1983, Wakil, 1989). The majority of MUFA are synthesised by introduction of a single double bond between carbon 9 and carbon 10 of a saturated fatty acid (SFA) by action of  $\Delta^9$  desaturases, this reaction takes place in the endoplasmic reticulum. PUFA are synthesised by the insertion of additional double bonds.

Fatty acid chain elongation is a crucial step in the biosynthesis of long chain fatty acids. Elongation beyond the 16-C length of the palmitate product of fatty acid synthase is mainly catalyzed by enzymes (elongases) associated with the endoplasmic reticulum which lengthen fatty acids produced by fatty acyl synthase and the dietary PUFA. Fatty acids esterified to coenzyme A serve as substrates. Malonyl-CoA is the donor of 2-carbon units in a reaction sequence similar to that of fatty acid synthase except that individual steps are catalyzed by separate proteins. A family of enzymes designated fatty acid elongases catalyze the initial condensation step (Kajikawa et al., 2003).

De nova synthesis of fatty acids produces palmitic and stearic,  $\Delta^9$  desaturation of this substrate produces palmitleic (C16:1) and oleic (C18:1). The double bond is at the  $\omega$ -7 or  $\omega$ -9 position further desaturation can only occur between these existing double bonds and the carboxylic end of the chain, because mammals have lost the enzyme for inserting double bound between the existing one and the methyl end of the chain, plants and algae still posses this.

In animals, enzymes such as  $\Delta^4$  desaturase,  $\Delta^5$  desaturase and  $\Delta^6$  desaturase can introduce a new double bond between an existing double bond and the carboxyl group (Figure 1.1). However, linoleic acid (LA, C18:2n-6) and  $\alpha$ -linolenic acid (ALA, C18:3n-3) are not synthesised by animals and classified as essential fatty acids, because animals lack  $\Delta^{12}$  and  $\Delta^{15}$  desaturases which are necessary to introduce double bonds beyond carbon 9 in the acyl chain (Burr, 1981). While, plants metabolise oleic acid (OA, C18:1n-9), because they have  $\Delta^{12}$  and  $\Delta^{15}$  desaturases to synthesis LA and ALA (Gurr and Harwood, 1991, Sprecher et al., 1995, Sprecher, 2000, Calvani and Benatti, 2003).

In mammals, DHA can be synthesized from EPA via the intermediate n-3 DPA, or it can be obtained from diet. Whilst in rat liver DHA synthesis occurs through elongation steps followed by desaturation step which is independent of a  $\Delta^4$ -desaturase (Voss et al., 1991). Other findings, however, have suggested a series of reactions termed the Sprecher pathway (Figure 1.1, B). This pathway describes the biosynthesis of DHA from the EPA via an initial chain elongation yielding 24:5n-3 followed by desaturation yielding 24:6n-3. The chain is then shortened via  $\beta$ -oxidation in peroxisomes or mitochondria to yield DHA (Voss et al., 1991, Sprecher et al., 1995, Sprecher et al., 1999, Sprecher, 2000, De Caterina and Basta, 2001, Meyer et al., 2004, Chen et al., 2009a).



**Figure 1.1.** Schematic showing the biosynthetic pathways of n-9, n-6 and n-3 PUFA (A). The converting of EPA to DHA via the Sprecher pathway is shown in part (B). DPA retro-converted into EPA (C). (Adapted from: (Sprecher et al., 1995, Sprecher, 2000, Meyer et al., 2004, Kaur et al., 2011)).

### **1.1.2 Eicosapentaenoic acid**

EPA is an n-3 PUFA (trivial name timnodonic acid). It is found in oily fish and fish oil, EPA cannot be fully considered an essential fatty acid because the human body can convert the essential fatty acid ALA to EPA. However, the efficiency of this conversion is much lower than the absorption of EPA from the diet, because of the extra metabolic work required to synthesis EPA (Goyens et al., 2006). Moreover, certain health conditions like diabetes or certain allergies may significantly limit the human body's capacity to produce EPA from ALA (Dyerberg et al., 1980, Brenna, 2002, Sinclair et al., 2002Goyens et al., 2006).

### **1.1.3 Docosapentaenoic acid**

There are two isomers of the DPA: (DPA, 22:5n-3) (trivial name clupanodonic acid) that is the product of EPA elongation and (DPA, 22:5n-6) (trivial name osbond acid) that is formed by elongation and desaturation of AA (Figure 1.1). Their physiological behaviour differs extremely despite only differing in the position of two double bonds in the acyl chain (Deng et al., 2009). n-3 DPA is a dietary n-3 PUFA mainly found in fish, fish oil, seal oil and red meat and is substantially higher in fish and fish oils, than the n-6 DPA (Gunstone et al., 1994). The biological properties of DPA have not been thoroughly studied, unlike EPA and DHA, DPA has not been extensively subjected to research due to the limited availability of the pure compound. Generally, in mammals n-3 DPA is found at low levels in most tissues while n-6 DPA content is found at very low levels in most mammalian tissues, except testis tissue (Tam et al., 2000, Tam et al., 2008). N-3 PUFA deficient diet in animals shows a reduction of DHA content. This depletion is accompanied by an increase in n-6 DPA level in most tissues,

especially brain and retina (Guesnet et al., 1988, Homayoun et al., 1988, Lin et al., 1991). Thus, the ratio of n-6 DPA to DHA has been suggested as a biochemical marker of low n-3 PUFA status (Youyou et al., 1986, Moriguchi et al., 2001). Moreover, as shown in (Figure 1.1), the biosynthetic pathways of both DPA isomers are parallel to each other. It has been reported that n-3 DPA is first elongated to tetracosapentaenoic acid (24:5n-3) then desaturated by the activity of  $\Delta 6$ -desaturase to form tetracosahexaenoic acid (24:6n-3) which is then translocated from the endoplasmic reticulum to the peroxisome where it's chain-shortened by activity of  $\beta$ -oxidation to (DHA, 22:6n-3) (Voss et al., 1991, Kaur et al., 2011). Furthermore, it has been found that the retroconversion of n-6 DPA to AA by oxidation occurred in rats when the AA content was decreased by high DHA administration and it did not occur when AA content is maintained at a normal level (Tam et al., 2000, Tam et al., 2008). Also, it has been found that in vitro n-3 DPA is retro-conversion to EPA and in vivo is evident in cells in a number of tissues and animals and is likely to involve the peroxisomal acyl-coA oxidase and one cycle of  $\beta$ -oxidation (Christensen et al., 1993, Reddy and Hashimoto, 2001, Gotoh et al., 2009, Kaur et al., 2010).

## **1.2 Sphingolipids**

Sphingolipids are derivatives of long chain amino alcohol bases such as sphingosine. They comprise a complex range of lipids in which fatty acids are linked via amide bonds to a sphingoid (Figure 1.2). A long-chain base, such as sphingosine, is the simplest possible functional sphingolipid, but ceramides, which contain a fatty acid linked by an amide bond, are the precursors of phospholipids and glycolipids (Igarashi, 1997). Complex sphingolipids are

located mainly in the plasma membrane of mammalian cells where they have a structural function such as in sphingomyelin which has unique and characteristic biological functions, most of which are due to its physical properties and location within membranes. In recent years, it has become apparent that sphingolipids are involved in many different cancers, Alzheimer's disease and other neurological syndromes (Tao et al., 1973, Ramstedt and Slotte, 2006, Zheng et al., 2006, Bryan et al., 2008).

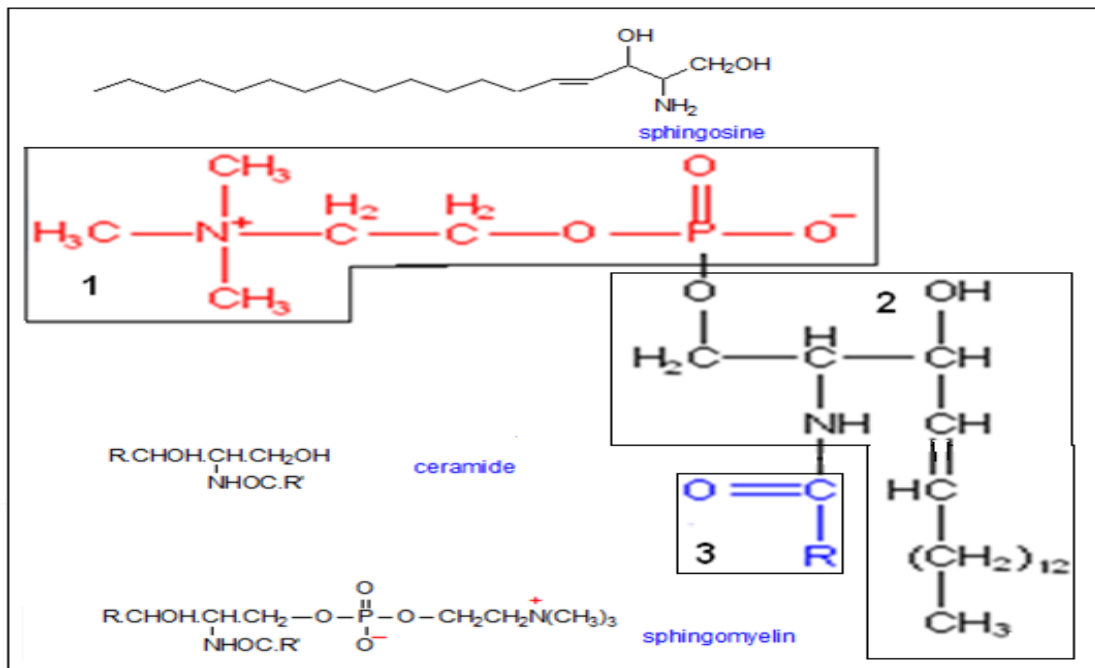
### **1.2.1 Sphingomyelin structure and species**

Sphingomyelin was discovered in the brain and isolated from the neural tissue more than a century ago and is recognised as an important component of the plasma membranes of eukaryotic cells and the most abundant sphingolipid in mammalian cells (Thudichum, 1962, Koval and Pagano, 1991, Ramstedt and Slotte, 2002, Vance and Vance, 2002, van Echten-Deckert and Herget, 2006). SM is particularly important in the nervous system, especially in the myelin sheaths that surround nerve cell axons (Merrill and Jones, 1990), and makes up about 10% of the brain lipids (Mano et al., 1997, Murphy et al., 2001). However, in most mammalian tissues SM content is found to range from 2 to 15% of the total membrane phospholipid depending on the tissue (Koval and Pagano, 1991, Talbott et al., 2000).

Furthermore, different sphingoid bases are used to produce a variety of SM species; the most natural species are C<sub>18</sub> compounds with lower C<sub>20</sub> levels. All the species share a common head group, but can have a variance of acyl chain lengths ranging from 16 to 24 carbons attached to sphingosine amino group, also, varying degree of unsaturation (one chain is saturated (sn-1) and the other

chain (sn-2) could be an unsaturated one giving a range of molecular species (Fenton et al., 2000, Isaac et al., 2003).

Nomenclature of sphingomyelin species is based on the assumption that d18:1(dihydroxy 18:1 sphingosine) is the main base of plasma sphingomyelin species, where the first number refers to the number of carbon atoms in the chain and the second number to the number of double bonds in the chain.



**Figure 1.2.** The general structure of sphingosine, ceramide and sphingomyelin (1) phosphocholine head group, (2) sphingoid base and (3) fatty acid residue (Adapted from: (Horton, 2002)).

For instance, SM (d18:1/18:0) consists of phosphorylcholine, ceramide, OA attached to the C1 position and stearic acid (SA) attached to the C2 position. While, SM(d18:1/16:0) consists of phosphorylcholine, ceramide, OA attached to the C1 position and palmitic acid attached to the C2 position. Also, there is

variability in the fatty acid content of brain SM; about 60% of the fatty acids of SM of the grey matter of brain consist of SA (18:0), while lignoceric (24:0) and nervonic (24:1) acids make up 60% of brain white matter (Isaac et al., 2003). Therefore, there is a variety of SM species that may exhibit various properties in terms of membrane structure and function (Gault et al., 2010). Therefore, powerful analytical methods are required to study these molecular species.

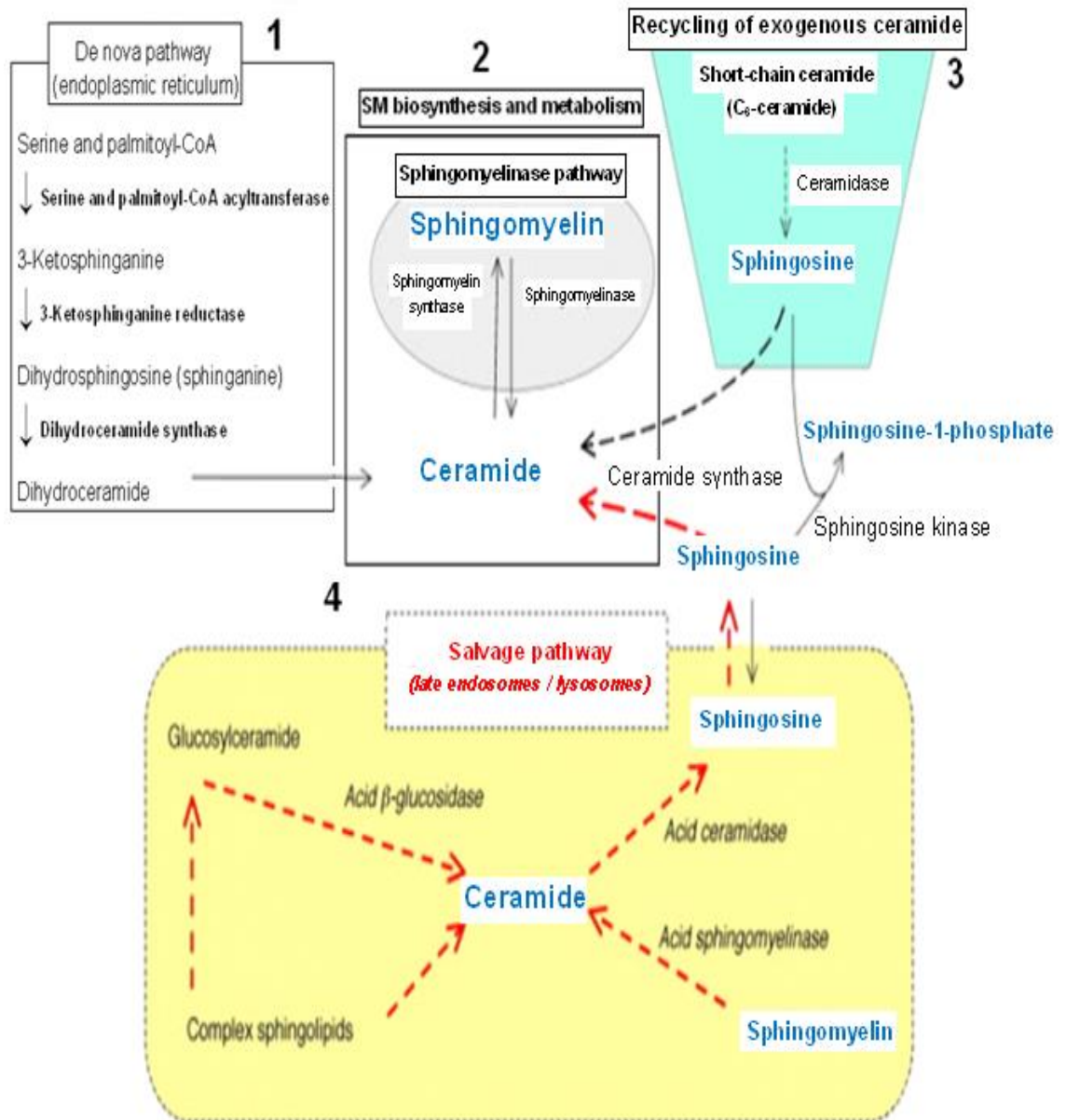
### **1.2.2 Sphingomyelin biosynthesis and metabolism**

The biosynthesis of SM takes place in the Golgi and plasma membrane while its degradation to ceramides by activity of sphingomyelinases takes place in most tissues and cells. Figure 1.3 shows the pathways mediating of SM biosynthesis and metabolism. The transfer of phosphorylcholine from phosphatidylcholine to ceramide, thus generating SM and diacylglycerols is catalysed by a sphingomyelin synthase (Sribney and Kennedy, 1958, Ullman and Radin, 1974, Voelker and Kennedy, 1982, Albi, 2008). However, this reaction is reversible and SM is hydrolysed by acid sphingomyelinase (A-SMase) or neutral sphingomyelinase (N-SMase) to generate ceramide (Voelker and Kennedy, 1982). The reaction of N-acyl-Sphingosine with cytidine diphosphate-choline (CDP-choline) to form SM and cytidine monophosphate (CMP) is catalyzed by ceramide cholinephosphotransferase. This reaction is also reversible (Sribney and Kennedy, 1958, Sribney et al., 1973, Isaac et al., 2003, Dmitrieva et al., 2008).

De novo SM is synthesised via generation of ceramide from serine and palmitoyl CoA in four steps catalysed by acyltransferase, reductase, synthase and desaturase enzymes, respectively (Figure 1.3). Also, SM can be formed

from the recycling ceramide via the formation of sphingosine catalysed by ceramidase and ceramide synthase, thus representing the starting point of the sphingolipid salvage pathway (Kitatani et al., 2008). However, there is a substitute pathway of SM synthesis that has been shown to function in isolated membrane fractions from rat liver and brain. In this pathway SM is synthesised via the transfer of the head group from phosphatidylethanolamine, the ceramide is converted to ethanolamine phosphorylceramide then converted to SM by stepwise methylation of the ethanolamine moiety (Brady et al., 1965, Malgat et al., 1986, Malgat et al., 1987).

Several forms of mammalian sphingomyelinase a lysosomal enzyme present in all tissues, act optimally at low pH (Kanfer et al., 1966, Levade et al., 1986). Its deficiency causes an accumulation of SM and lack of ceramide production in cells and tissues, and is found to be associated with lysosomal lipid storage disorders known as Niemann-Pick disease (Lozano et al., 2001). Moreover A-SMase present in serum and are reported to be secreted by most cells during several pathophysiological processes that include cell growth, stress and inflammatory responses (Spence et al., 1989, Schissel et al., 1996, Arenz, 2010). It is involved in recycling of sphingolipid constituents and may features of cellular signalling as well as in membrane SM turnover (McGovern and Schuchman, 1993, Leventhal et al., 2001). Furthermore, N-SMase is found in the brain, kidney plasma membranes and endoplasmic reticulum (Spence, 1993), and its deficiency affects SM metabolism and biological activity as N-SMase activity is believed to be responsible for stress-induced ceramide generation (Okazaki et al., 1994, Wiegmann et al., 1994, Liu et al., 1997).



**Figure 1.3.** Schematic showing the biochemical pathways of ceramide metabolism: (1) The de novo pathway, (2) sphingomyelin synthase and sphingomyelinase pathways, (3) The exogenous ceramide-recycling pathway, and (4) The salvage pathway. The pathway of sphingomyelin and ceramide synthesis from recycling/salvaging sphingosine is indicated by dotted lines. (Adapted from: (Kitatani et al., 2008)).

In addition, A-SMase and N-SMases are considered as the key enzymes for SM metabolism, catalyzing hydrolysis reactions leading to the generation of ceramide and phosphocholine. However, these enzymes operate in different regions of the cells based on their biochemical roles at both acid and neutral pH (Brady et al., 1966), both of them are activated by a nerve growth factor and most of the cellular stress conditions (Levade et al., 1986, Spence, 1993). Also, dietary SM is hydrolysed in the intestines by alkaline sphingomyelinase (Alk-SMase) to give ceramide, followed by neutral ceramidase to result in free fatty acids and sphingosine (Nilsson, 1968, Vesper et al., 1999).

### **1.2.3 Sphingomyelin function**

Sphingomyelin is displaying various functional and structural properties which are crucial to cell function and plasma membrane structure. SM species are found to be common constituents of plasma membranes of mammalian cells and make up 10-20 % of plasma membrane lipids (Voet et al., 2008).

Biologically, during the building of membranes, SM species serve as a chemically distinct substitute for phosphatidylcholine, and play crucial roles in membrane fluidity regulation. SM contributes to lipid rafts together with cholesterol and controls distribution of cholesterol in cells (Wang and Silvius, 2000, Ramstedt and Slotte, 2002, Bartke and Hannun, 2009, Chalfant and Del Poeta, 2010). It has been reported that membranes containing SM and ceramides have a much lower binding capacity for cholesterol, so SM degradation may play a part in cholesterol homeostasis (Slotte, 1999, Ridgway, 2000). However, the critical functions of SM are not fully understood. The

importance of SM species has been of interest in membrane structure and function (Brouwers et al., 1998, Barenholz and Gatt, 1999).

In addition, there is evidence that breakdown of SM into N-acylsphingosine has been found associated with the induction of apoptotic cell death (Zhang et al., 1997, von Haefen et al., 2002, Tani and Hannun, 2007). SM is also a source of other bioactive compounds; ceramide as a product of SM hydrolysis, which is further hydrolysed to sphingosine and sphingosine-1-phosphate by ceramidase and sphingosine kinase (Hannun, 1994). These metabolites play important roles in cell regulatory pathways: ceramide and sphingosine induce growth arrest, differentiation and apoptosis, while the sphingosine 1 –phosphate stimulates growth and inhibits apoptosis (Hannun and Luberto, 2000, Vance and Vance, 2002). They are also found to take part in cell signalling, brain development and ageing (Hannun and Bell, 1989, , Kolesnick, 1991, Hannun, 1994, Merrill et al., 1997, Huwiler et al., 2000, Hannun et al., 2001) .

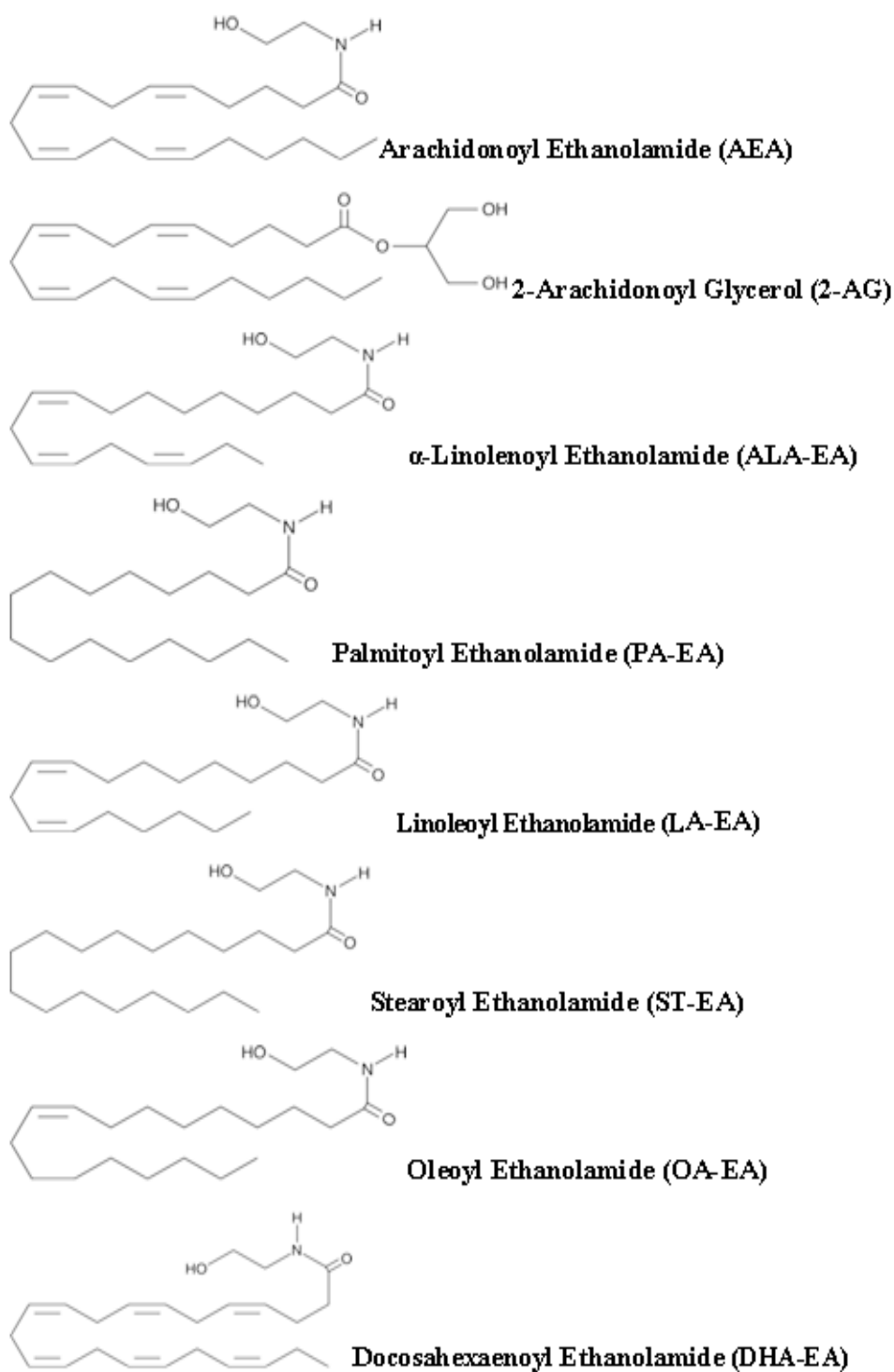
### **1.3 Endocannabinoids and their congeners**

Endocannabinoids (ECs) are lipids that are endogenous agonists of cannabinoid receptors, the best studied ones being the arachidonic acid derivatives (Howlett et al., 2004). N-arachidonylethanolamide or anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al., 1992, Sugiura et al., 1995). AEA is produced by neurones only when need arises acting near its site of biosynthesis (Kingsley and Marnett, 2009). It is transported into the cells and translocates across both directions of cell membranes by binding to a carrier protein (Moore et al., 2005). 2-AG, unlike AEA, is synthesised from AA and

glycerol and is present at relatively high levels in mouse and rat central nervous system, as the most abundant of monoacylglycerol (Stella et al., 1997, Kondo et al., 1998, Sugiura et al., 1999). It has also been found in bovine and human milk (Fride et al., 2005).

Fatty acid ethanolamides (FA-EA), also known as N-acylethanolamides belong to a family of lipids naturally found in both plant and animal tissues, containing several types of acyl groups linked to the nitrogen atom of ethanolamine. AEA is an endogenous FA-EA. It is degraded primarily by the fatty acid amide hydrolase (FAAH) enzyme, which converts AEA into ethanolamine and arachidonic acid.

The FA-EA elicit their functions largely by their binding to the cannabinoid receptors (Thabuis et al., 2011, Lin et al., 2012). Figure 1.4 shows the structure of AEA, 2-AG and the most abundant FA-EA: N-palmitoylethanolamide (PA-EA), N-stearoylethanolamide (SA-EA), N-oleoylethanolamide (OA-EA), N-docosahexaenoylethanolamide (DHA-EA), N-linoleoylethanolamide (LA-EA) and N-linolenoylethanolamide (ALA-EA). PA-EA, SA-EA and OA-EA comprises of  $\geq 25\%$  of total FA-EAs in neuronal cells (Devane et al., 1992, Koga et al., 1997, Mechoulam et al., 1998) while DHA-EA, LA-EA and ALA-EA are less abundant (Mechoulam et al., 1998) and are found in peripheral tissues (Koga et al., 1997). However, their functions are not as well-established as AEA and 2-AG.



**Figure 1.4.** Chemical structures of various fatty acid ethanolamides and endocannabinoids. (Adapted from: (Bradshaw and Walker, 2005, Lambert and Muccioli, 2007)).

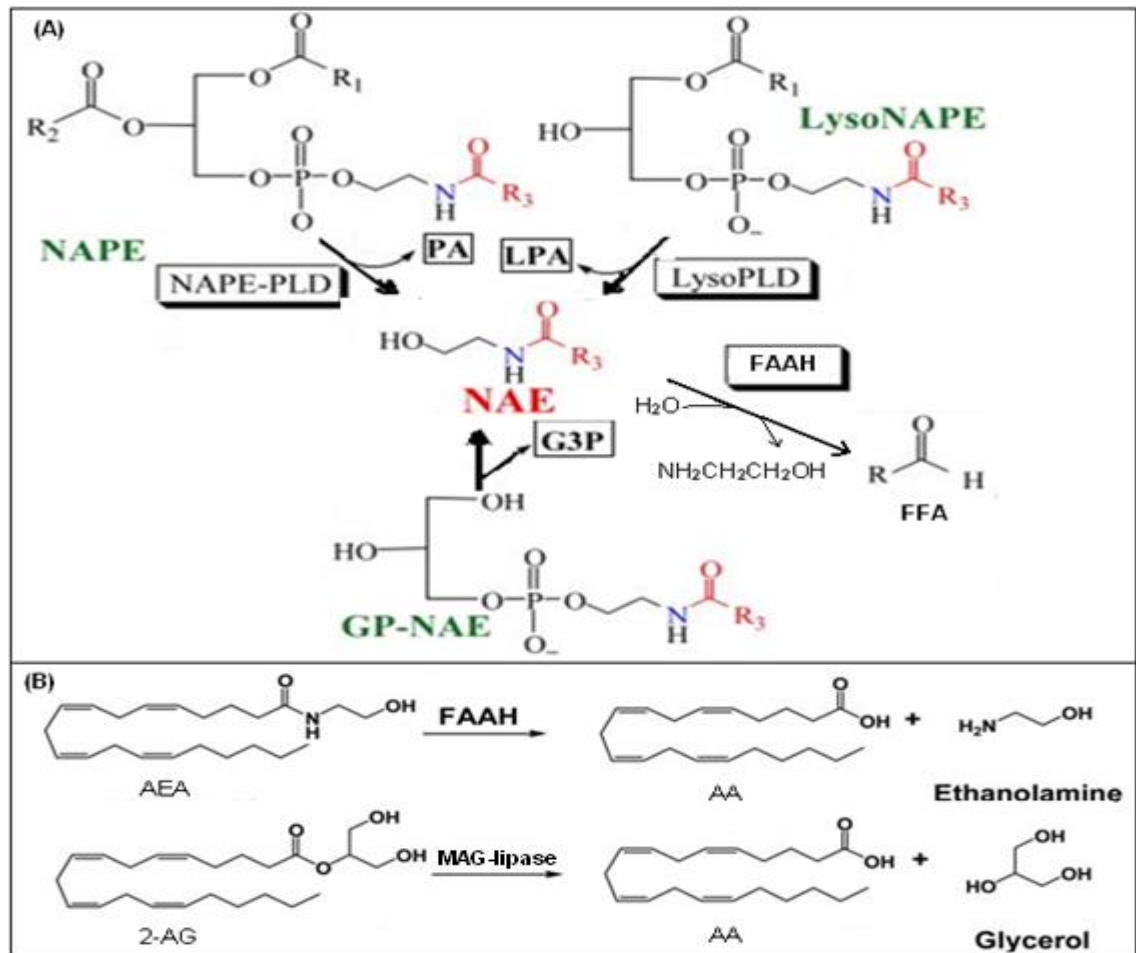
### 1.3.1 Biosynthesis of fatty acid ethanolamides and endocannabinoids

Fatty acid ethanolamides including AEA derive from phosphatidylethanolamine (Di Marzo et al., 1994, Hansen et al., 2000). The enzyme N-acyltransferase transfers fatty acids such as AA from phosphatidylcholine to the ethanolamine head group of phosphatidylethanolamine resulting in N-acylphosphatidylethanolamines (NAPE). Then, the NAPE is hydrolysed via action of NAPE-phospholipase D (NAPE-PLD) to generate FA-EA and phosphatidic acid (PA) (Di Marzo et al., 1994, Sugiura et al., 1996, Schmid and Berdyshev, 2002, Leung et al., 2006) (Figure 1.5). The previous pathway has also been recently discovered in mice, and this route of FA-EA synthesis is known as transacylation phosphodiesterase pathway (Leung et al., 2006).

The biosynthesis pathway involving a lysophospholipase (Lyso-PLD). This enzyme mediates the hydrolysis of N-acyl-lyso-phosphatidylethanolamines (LysoNAPE) to generate FA-EA and lysophosphatidic acid (LPA) (Figure 1.5).

Another pathway involves activity of phosphodiesterase (Figure 1.5) which cleaves the glycerophospho N-acyl ethanolamine (GP-NAE) to yield FA-EA and glycerol 3-phosphate (G3P) (Simon and Cravatt, 2006, Liu et al., 2008, Farrell and Merkler, 2008). These pathways suggest that the body has back-up methods to produce endocannabinoids and their congeners (Di Marzo et al., 2007, Liu et al., 2008). However, all FA-EAs are quickly metabolised by the enzyme fatty acid amide hydrolase (FAAH) to free fatty acids and ethanolamine (Willoughby et al., 1997) as shown in the following reaction.





**Figure 1.5.** Fatty acid ethanolamide biosynthetic and hydrolysis pathways **(A)**. Hydrolysis of two principal endocannabinoids; anandamide (AEA) and 2-arachidonoylglycerol (2-AG) **(B)**. AA: arachidonic acid, FAAH: fatty acid ethanolamide hydrolase, MAG-lipase: monoacylglycerol lipase, FFA: free fatty acids, NEA: N-acylethanolamine, GP-NAE: glycerophospho N-acyl ethanolamine, G3P: glycerol 3-phosphate, NAPE: N-acylphosphatidylethanolamines, NAPE-PLD: NAPE-phospholipase D, PA: phosphatidic acid, Lyso-PLD: lysophospholipase, LysoNAPE: N-acyl-lysophosphatidylethanolamines, LPA lysophosphatidic acid. The fatty acyl group is represented by R. Adopted from: (Simon and Cravatt, 2006, Farrell and Merkler, 2008, Ahn et al., 2009)).

Two isomers of FAAH, FAAH-1 and FAAH-2 exhibit different acyl group specificities, but AEA is the preferred substrate (Kurahashi et al., 1997, Lang et al., 1999, Ueda et al., 2000, Wei et al., 2006).

OA-EA and PA-EA are also hydrolysed by FAAH. The substrate preference has been reported to be in order of AEA > OA-EA > PA-EA (Ueda et al., 2000). FA-EA are also metabolised by N-acyl ethanolamine-hydrolyzing acid amidase (NAAA) an enzyme that has no sequence homology with FAAH. It is also known as a novel lysosomal hydrolase and it has been suggested to play unique role in degradation of FA-EAs. Both AEA and PA-EA are hydrolyzed by NAAA (Tsuboi et al., 2007). 2-AG is hydrolysed by a monoacylglycerol lipase (MAG-lipase) as well as FAAH with MAG-lipase being the principle 2-AG metabolizing enzyme in vivo (Figure 1,5) (Dinh et al., 2002). Finally, AEA and other FA-EAs are substrates for cyclooxygenase (COX) and lipoxygenase (LOX) activities (Kozak et al., 2002, Kozak et al., 2003).

### **1.3.2 Receptors activated by endocannabinoids and related compounds**

The ECs are natural compounds, produced in the brain and peripheral tissues and exert their effects through binding to the cannabinoid receptors: CB1 and CB2 (Matsuda et al., 1990, Munro et al., 1993, Smith et al., 1994, Felder et al., 1996, Ong and Mackie, 1999, Pertwee, 1997, Howlett, 2002). CB1 receptors were cloned in 1990 (Gerard et al., 1991), they are primarily found in neural cells, adipose tissue (Herkenham et al., 1990, Bensaid et al., 2003), vascular endothelium (Liu et al., 2000), myocardium (Bonz et al., 2003), and the terminals of the sympathetic nervous system (Gelfand and Cannon, 2006).

The CB2 receptors were cloned in 1993; they are found principally in peripheral tissues and are predominantly expressed in immune and blood cells (Munro et al., 1993). Recently, CB2 was also found in the central nerve system (Gong et al., 2006, Ashton and Glass, 2007, Fernandez-Ruiz et al., 2007). AEA and 2-AG exert their effects by acting as agonist for both CB1 and CB2 (Di Marzo et al., 2002, Zhao et al., 2005), 2-AG has higher affinity for CB1 (Savinainen et al., 2001) (Table 1.1). Also, DHA-EA activated CB receptors in vitro with significant potency, suggesting that it is an endocannabinoid (Brown et al., 2010).

**Table 1.1.** Receptors activated by mammalian endocannabinoids and related fatty acid ethanolamides

<b>Compound</b>	<b>Receptor</b>
AEA	CB1, CB2, PPAR $\alpha$ , PPAR $\gamma$ , TRPV1, TRPM8, TRPV1
2-AG	CB1, CB2
DHA-EA	CB1, CB2
PA-EA	PPAR $\alpha$ , GPR55
SA-EA	TRPV1
ALA-EA	TRPV1
LA-EA	TRPV1
OA-EA	PPAR $\alpha$ , PPAR $\gamma$ , TRPV1, GPR119

Although AEA, 2-AG and DHA-EA bind and activate CB receptors (Di Marzo et al., 2002, Sugiura and Waku, 2000, Yang et al., 2011), FA-EAs such as PA-EA, SA-EA, LA-EA, ALA-EA and OA-EA do not bind to CB1 nor CB2 receptors

(Capasso et al., 2001, Maccarrone et al., 2002b, Conti et al., 2002, Farquhar-Smith et al., 2002, Fu et al., 2003, Terrazzino et al., 2004). However, they target other receptors. For instance, OA-EA binds to peroxisome proliferators-activated receptors (PPAR $\alpha$  and PPAR $\beta$ ) mediating anorexic effects, inhibiting feeding behaviour and stimulating fat breakdown (Rodriguez de Fonseca et al., 2001, Fu et al., 2003, O'Sullivan, 2007).

OA-EA also binds to G-protein-coupled receptor 119 (GPR119) (Overton et al., 2006) and transient receptor potential vanilloid type 1 receptors (TRPV1) (Movahed et al., 2005). PA-EA is ligand for PPAR $\alpha$  and G-protein-coupled receptor 55 (GPR55) mediating neuroprotective, modulating pain and anti-inflammatory effects (Lo Verme et al., 2005, Ryberg et al., 2007). ST-EA binds to TRPV1 receptors with pro-apoptotic activity, which is regulated by nitric oxide in a way opposite to that reported for AEA (Rodriguez de Fonseca et al., 2001, Maccarrone et al., 2002b, Wouters et al., 2002).

Furthermore, AEA is an agonist for PPAR $\alpha$  and PPAR $\gamma$  receptors (O'Sullivan, 2007), TRPV1 (Starowicz et al., 2007), and transient receptor potential melastatin type 8 receptors (TRPM8) (De Petrocellis et al., 2007). These receptors are involved in cell differentiation and death. However, it is still currently unclear how much of the AEA mediated effects and total in vivo activity are attributed to these receptors (Moreno et al., 2004, Bouaboula et al., 2005, Cimini et al., 2005).

## **1.4 Oxysterols**

Cholesterol is involved in membrane structure and function and is an essential component of the cell membrane regulating its fluidity (Yeagle, 1991, Sadava, 2008). Brain contains approximately 20% of the body's stores of cholesterol. It is important for normal brain function and, also, has been found to be implicated in a number of neurological disorders (Jeitner et al., 2011). Oxysterols are oxygenated derivatives of cholesterol. Oxysterols are more polar compared to cholesterol, because of the presence of an extra oxygen atom in their structure in a hydroxyl or epoxide groups. They are usually present at very low levels in biological fluids and tissues and represent end products of cholesterol metabolism (Lange et al., 1995, Schroepfer, 2000, Bjorkhem and Diczfalusy, 2002, Lordan et al., 2009). Oxysterols are generally believed to be important physiological mediators in cholesterol turnover, homeostasis and many lipid disorders (Lange et al., 1995 Lund and Bjorkhem, 1995, Schroepfer, 2000, Bjorkhem, 2002).

### **1.4.1 Biosynthesis of oxysterols**

The biosynthesis of oxysterols occurs via two different mechanisms: certain oxysterols are produced exclusively by autoxidation of cholesterol through non-enzymatic reactions, whereas others are produced through enzymatic pathways. 25-Hydroxycholesterol (25-OH) and 26-hydroxycholesterol (26-OH) are formed in the mitochondria, and 24-hydroxycholesterol (24-OH) is produced by a specific hydroxylase (Fredrickson and Ono, 1956, Nelson et al., 1981, Esterman et al., 1983, Schroepfer, 2000, Iuliano, 2011,). Most of plasma or tissue oxysterols are synthesized enzymatically via the activity of mitochondrial or

microsomal cytochrome P450 (CYP450), a family of sterol hydroxylases (Russell, 2000, Schroepfer, 2000, Bjorkhem and Diczfalusy, 2002). There are at least 57 CYP enzymes in human tissues (Lewis, 2004) and five of these isoenzymes are found to be involved in oxysterol biosynthesis.

Cytochrome P450 3A4 (CYP3A4), that is also known as cholesterol 4 $\beta$ -hydroxylase, is a major hepatic CYP enzyme that oxidizes cholesterol to 4 $\beta$ -hydroxycholesterol (4 $\beta$ -OH), which is one of the major circulating oxysterols (Breuer, 1995, Bodin et al., 2002).

Cytochrome P450 7A1 (CYP7A1), that is also known as cholesterol 7 $\alpha$ -hydroxylase, is expressed only in liver and metabolizes the conversion of cholesterol to 7 $\alpha$ -OH as an intermediate in the neutral bile acid synthesis pathway. CYP7A1 is the rate limiting enzyme in the bile acid synthesis and plays major roles in hepatic regulation of overall cholesterol balance (Swell et al., 1981, Bjorkhem et al., 1987, Cohen et al., 1992, Beigneux et al., 2002).

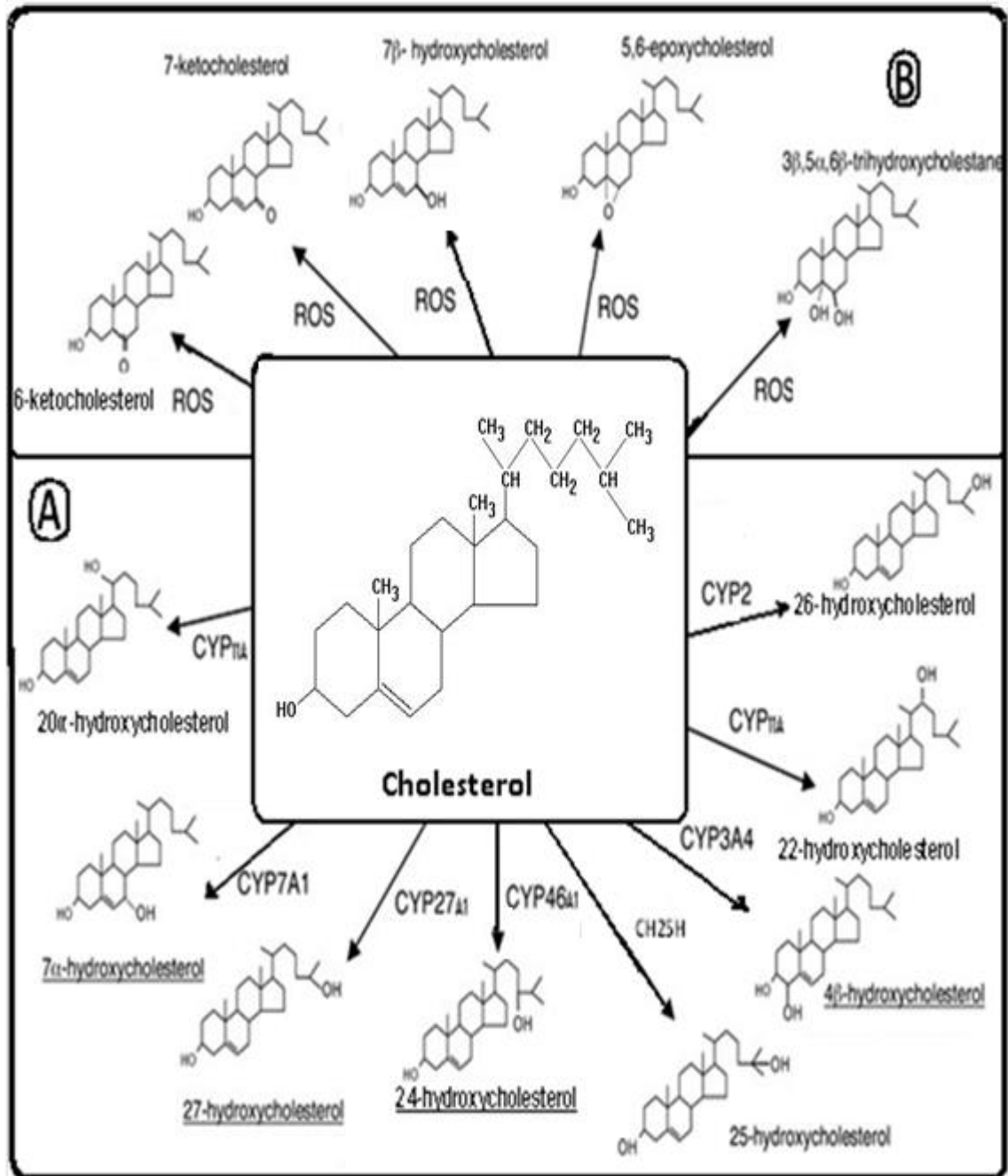
Cytochrome P450 27A1 (CYP27A1), is also known as sterol 27-hydroxylase. It is found in liver mitochondria, generates 27-hydroxycholesterol (27-OH) and 26-OH by introducing a hydroxyl group to the carbon at the 27, 26 positions in cholesterol, respectively. These are intermediates in the alternative acid pathway of bile acid synthesis (Chiang, 1998, Javitt, 2002, Liao et al., 2011).

Cytochrome P450 46A1 (CYP46A1), that is also known as cholesterol 24-hydroxylase and is expressed in the central nervous system. It plays an important role in brain cholesterol metabolism and metabolizes the conversion of cholesterol to 24-OH which can cross the blood brain barrier (BBB) into

systemic circulation. Therefore, this pathway is considered to be a major route of cholesterol turnover in the brain and part of the cholesterol transport process (Lund et al., 1999, Bogdanovic et al., 2001, Liao et al., 2011).

Cytochrome P450 11A (CYP11A), that is also known as cholesterol 20-22 desmolase, is a side-chain cleavage enzyme that generates 20-hydroxycholesterol (20-OH) and 22-hydroxycholesterol (22-OH) from cholesterol during the synthesis of steroid hormones. Its cellular location is still unclear, because it lacks the mitochondrial-targeting transit peptide (Sugano et al., 1996, Tajima et al., 2001, Kim et al., 2008).

In addition, a specific hydroxylase, cholesterol 25-hydroxylase, which belong to a group of enzymes that utilise oxygen and di-iron as cofactors, catalyzes cholesterol hydroxylation reactions. This is not a cytochrome P450 enzyme, but is involved in 25-OH synthesis from cholesterol (Nelson et al., 1981, Lund et al., 1993, Lund et al., 1998). Figure 1.8 shows a summary of these enzymes and their products. The cholesterol non-enzymatic oxidation pathways were discovered more than a century ago upon exposure of cholesterol to air to produce oxidized species. Since the cholesterol structure has a double bond at position 5,6 it makes it more predisposed to non-enzymatic oxidations. Thus, these reactions may occur in arterial wall, smooth muscle cells, endothelial cells and liver or all tissues where oxidation may take place. Moreover, cholesterol is transported around the body bound to lipoproteins; therefore autoxidation can also occur within cell membranes ( Lordan et al., 2009, Iuliano, 2011).



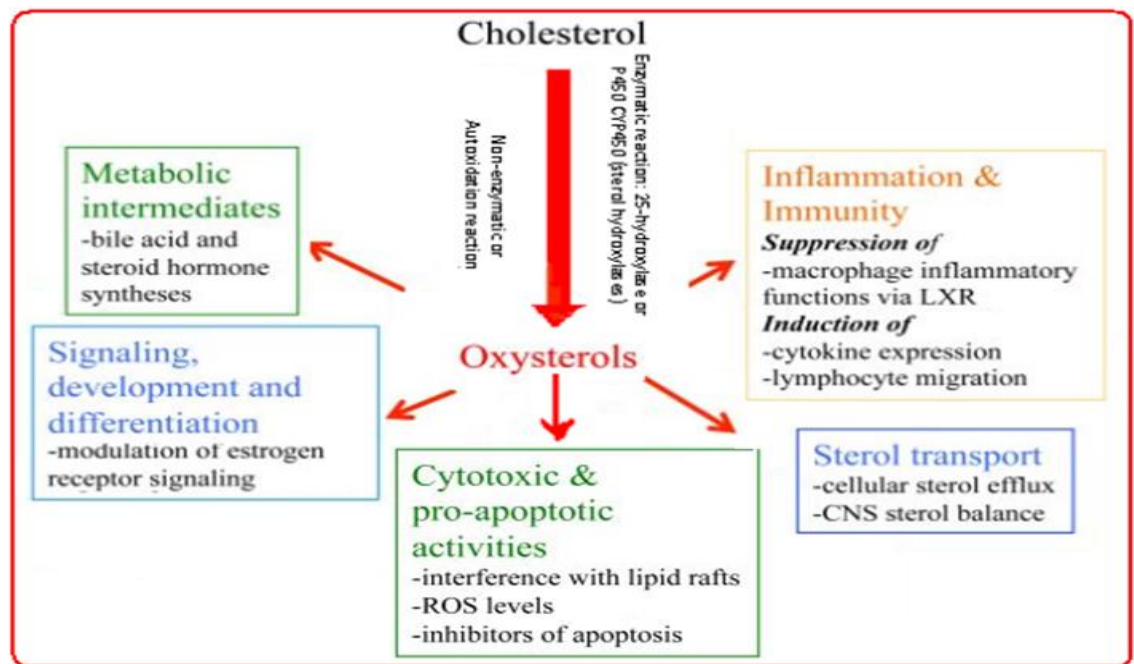
**Figure 1.6.** Summary of the main cholesterol oxygenation reactions **A.** Enzymatically mediated production species (adapted from (Smith, 1987, Maerker, 1987, Bjorkhem, 2002, Lund et al., 1998). **B.** Non-enzymatic pathways taking place in presence of reactive oxygen species (ROS). (Adapted from: (Bjorkhem, 2002, Javitt, 2008, Iuliano, 2011, Olkkonen et al., 2012)).

Furthermore, it has been reported that 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) was first isolated and characterized from bovine liver in 1939 while in 1957, 7-ketocholesterol (7-keto) was discovered in smooth muscle cells. Both oxysterols have also been detected in atherosclerotic tissue and it is believed to be synthesised via cholesterol autoxidation reactions (Haslewood, 1939, Smith, 1987, Hughes et al., 1994, Javitt, 2008, Iuliano, 2011).

Reactive oxygen species (ROS) such as peroxide or hydroperoxide species lead to cholesterol oxidation and generate different species of oxysterols (Smith, 1987, Bjorkhem, 2002, Javitt, 2008, Iuliano, 2011). Autoxidation of cholesterol bound to low density lipoproteins (LDL) was found to be increased in plasma of hypercholesterolemic patients. (Colles et al., 1996). The oxysterol species found were mainly 7 $\beta$ -OH, 7-keto (oxidized at position 7 of cholesterol), and 5,6 $\alpha$ -epoxy-cholesterol (5,6 $\alpha$ -epoxy) and cholestane-3 $\beta$ , 5 $\alpha$ , 6 $\alpha$ -triol (Chol-triol) (Breuer et al., 1996). These species can imitate the effects of oxidized LDL on various cell types such smooth muscle cells (Zhou et al., 1993) and endothelial cells (Boissonneault et al., 1991).

#### **1.4.2 Biological activity of oxysterols**

Oxysterols are present in the circulation and in tissues (Bjorkhem et al., 2002), and contribute to several biologic processes and pathways including bile acid synthesis, intracellular lipid transport (Russell, 2000), cholesterol efflux (Venkateswaran et al., 2000) and lipoprotein metabolism (Vaya et al., 2001) (Figure 1.7).



**Figure 1.7.** A schematic diagram summarizing the major functions, biological roles and the pathologic processes mediated by oxysterols. LXR: liver X receptors; ROS: reactive oxygen species; CNS: central nervous system; CYP450: cytochrome P450. (Adapted from: (Olkkonen et al., 2012)).

Moreover, oxysterols are involved in various pathologic processes and are related to cell differentiation (Hanley et al., 2000) and apoptosis (Panini and Sinensky, 2001). They contribute to cholesterol elimination from cells and regulation of cholesterol secretions into bile acid (Okuda, 1994, Lund et al., 1996, Babiker et al., 1999, Ikonen, 2006).

On the other hand, cholesterol is converted by mitochondrial (CYP27A1) to 27-OH which is present in almost all tissues, except in neuronal cells. However, under normal conditions, 27-OH plasma levels are about twice that of 24-OH (Lutjohann et al., 1996), this ratio was found to be altered with ageing (Galli et al., 1999) or neurodegenerative disorders (Lutjohann et al., 1996, Lund et al.,

1999, Bjorkhem and Meaney, 2004, Bjorkhem et al., 2009). Therefore it has been considered that oxysterols, especially 7 $\alpha$ -OH, 27-OH and 25-OH may play important roles as intermediates in different cholesterol catabolic (hepatic and extra-hepatic) pathways (Diczfalusy et al., 1996, Norlin et al., 2000). For instance, these oxysterols are subjected to several degradation steps to finally generate bile acids (Bjorkhem and Eggertsen, 2001, Bjorkhem, 2002, Javitt, 2002, Porter et al., 2010, Crosignani et al., 2011).

In addition, it has been considered that enzymatic hydroxylation of cholesterol into 20-OH and 22-OH is found to be involved in steroid hormone synthesis in the endocrine system (Hall, 1986, Miller, 1988, Crosignani et al., 2011). It has also been shown that 7 $\beta$ -OH and 7-keto are potent inducers of apoptosis in bovine and human endothelial cells (Lizard et al., 1997, Harada-Shiba et al., 1998). Also, these oxysterols have been identified in hypercholesterolemic subjects at different stages of development of coronary artery disease and may play critical roles in atherosclerosis (Carpenter et al., 1995, Brown et al., 1997,).

Oxysterols also function as endogenous regulators of gene expression, selective receptor modulators, as signalling molecules with key roles in cell differentiation, inflammation processes and neurodegenerative disorders (Umetani et al., 2005, Beyea et al., 2007, Umetani et al., 2007, Wong et al., 2007, DuSell et al., 2008, Kim et al., 2010, Jeitner et al., 2011).

## **1.5 The role of bioactive lipids in ageing and neurodegenerative disorders**

Neurodegeneration is a collective term that describes the progressive loss of structure or function of neurons, including cell death. Many neurodegenerative diseases including: Amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease occur as a result of neurodegenerative processes. Neurodegeneration can be found in many different levels of neuronal circuitry ranging from molecular to systemic (Rubinsztein, 2006, Thompson, 2008).

Moreover, neuroinflammation is defined as a host defence mechanism associated with neutralization of an insult and restoration of normal structure and function of brain; it also refers to the activation of immune cells in CNS in response to a wide variety of stimuli, including neurodegenerative disorders, peripheral nerve damage, or stress (Skaper, 2007). In the 1980s, researchers began to turn their attention to the diseases of neuroinflammation and older age, like many of the gravest diseases of old age, ageing brain disease entails a gradual failure of the brains functions. Even in normal, non-diseased brains, the ageing brain has been observed to present with a gradual decrease in communication among neurons (Craik and Salthouse, 2000). However, the ageing process itself has been defined by a slow deterioration of homeostatic functions throughout the lifespan (Sparkman and Johnson, 2008). Furthermore, neuroinflammation has been found to be associated with the pathogenesis of ageing-related diseases. Also, the ageing brain is found to display an imbalance between brain pro-inflammatory and anti-inflammatory cytokines; a shift towards a pro-inflammatory state increases the risk of neuroinflammation and makes an

aged brain more vulnerable to the pathogenesis factors, infection and stress (Sparkman and Johnson, 2008, Liu et al., 2012). Although it has been found to serve as a neuroprotective mechanism associated with repair and recovery, neuroinflammation also contributes to brain dysfunction (Moore and O'Banion, 2002), it has recently emerged as a key player in many neurodegenerative diseases (Minghetti, 2005, Esposito et al., 2008b, Rao et al., 2009).

Furthermore, multiple aspects of brain structure and function affected by ageing are thought to depend on sufficient brain lipid concentration and interactions among PUFA and their metabolites (Bazan, 1989, Wolfe and Pellerin, 1989, Bourre et al., 1990, Clandinin, 1999, Webster, 2001, Aid et al., 2003, Kitajka et al., 2004, Little et al., 2007, Rapoport, 2008). Consequently, various types of diseases including inflammatory diseases, neurodegenerative disorders, brain inflammation and other age related disorders have been found to be associated with alterations in brain lipid composition ( Adibhatla et al., 2006, Muralikrishna and Hatcher, 2007).

### **1.5.1 Omega-3 PUFA in neurodegeneration and brain ageing**

The n-3 PUFA, especially DHA, work as the basic building blocks of the brain cells and nervous system by protecting the brain health (Youdim et al., 2000, Lauritzen et al., 2001a, Freemantle et al., 2006). These n-3 fatty acids are known to be crucial for brain cell membrane fluidity, brain development, improved of brain cells communication, acquired in nerve growth properties, as a nerve protectant and important for the transmission of brain signals (Edwards et al., 1998, Horrocks and Yeo, 1999, Singh, 2005, Kidd, 2007, van Gelder et al., 2007). However, n-3 PUFA deficiency can lead to mood problems such as

depression and are more likely also associated with many neurodegenerative disorders (Florent et al., 2006, Dyall and Michael-Titus, 2008, Lukiw and Bazan, 2008, McNamara, 2010).

On the other hand, n-3 PUFA supplementation is known to control chronic inflammatory processes involved in brain disorders, reduce neuroinflammation and reduce the activity of the natural killer immune cells (NK cells) (Kelley, 2001, Calder, 2009, Wall et al., 2010). Accordingly, it may also be used as an important key in fighting against neurodegenerative disorders ( Cho et al., 2001, Calon and Cole, 2007, Palacios-Pelaez et al., 2010).

In addition, it has been reported that brain ageing and/or chronic degenerative conditions reduce the brain's ability for conversion of essential fatty acids to EPA and DHA. It is also indicated that lack of these n-3 PUFA in the body can cause a communication breakdown in the brain. Changing the fluidity of cell membranes alters their physical properties, such as permeability and protein activity. However, there evidence suggests that adequate EPA and DHA intake are essential for the adult brain. They impact the brain's structure and signalling systems, and help promote nervous system development and optimal memory function. Also, deficiency of n-3 fatty acids block abnormal brain cell signaling in brain ageing conditions (Russell and Burgin-Maunder, 2012, Levant et al., 2008, Freemantle et al., 2006). Also, other studies have shown that supplemental essential fatty acids, such as ALA and conditionally essential fatty acids including DHA and EPA improve the symptoms of brain ageing, help to protect brain cells and ameliorate the symptoms of mental disorders (Cho et al., 2001, Calder and Yaqoob, 2009).

### **1.5.1.1 Eicosapentaenoic acid**

EPA as one of n-3 PUFA has been shown to have beneficial effects on brain function (Lauritzen et al., 2001b), bipolar disorder (Stoll et al., 1999a), learning improvement (Ikemoto et al., 2001) and other conditions, such as schizophrenia, and are found to be protective against neuronal cell apoptosis and neuronal degeneration after neuronal injury (Peet, 2003). EPA in association with AA has been found to play important roles in neuronal cells growth and development (Lima et al., 2002, King et al., 2006).

Although the content of EPA in the brain is extremely low and unless EPA is administered directly there is no significant accumulation of EPA in the brain (Yehuda et al., 1999). However, recent studies suggest that EPA may affect depression and more importantly suicidal tendencies (Matsudaira, 2007). In these disorders, it has been found that the levels of EPA were significantly lower in suicidal patients compared to healthy controls (Huan et al., 2004). It has been reported that dietary supplementation with EPA increase EPA but not DHA concentrations in plasma while supplementation with both EPA and DHA reduce plasma AA concentrations which resulted in the increase of a specific long-chain n-3 PUFA in plasma or tissues that depended on the supplemented fatty acid of interest (Arterburn et al., 2006).

Other studies have indicated that EPA is involved in changes following neuroinflammation as it acts as a precursor of prostaglandin-3, thromboxane-3 and leukotriene-5, because it acts as a substrate for both COX-2 and 5-LOX, leading to increased production of these different forms of eicosanoids which are believed to be less potent pro-inflammatory than those formed from AA (De

Caterina and Basta, 2001, Guesnet et al., 2004, Calder, 2006). Also, it has been found that EPA is the precursor of inflammation resolving product resolvin E1 ( Serhan et al., 2002, Ariel and Serhan, 2007).

In addition, EPA decreases production of circulating pro-inflammatory cytokines such as tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 1 (IL-1) (Calder, 1997, Calder, 2006a). Thus EPA may exert effects on both the generation of inflammatory mediators and on the resolution of inflammatory processes. Therefore, for these reasons, it was hypothesised that inclusion of EPA in the diet of patients with chronic inflammatory diseases may be of benefit.

#### **1.5.1.2 Docosapentaenoic acid**

The physiological function and pharmacological effects of n-3 DPA have not been fully elucidated. EPA and DPA are primarily metabolized to DHA, and DPA can be retro-converted to EPA (Cutler et al., 2004, Kaur et al., 2010). Also, it has been found that supplementation of DPA or EPA increased both DPA and DHA in both young and aged animals' brain, which reflected that DPA and EPA exerted essentially similar effects in brain tissue and both of them may exert neuroprotective and anti-inflammatory effects (Kaur et al., 2010, Kelly et al., 2011).

The primary mechanism by which DPA and EPA restore neuronal function remains to be determined. However, it has been reported that EPA had an antioxidative effect in marine microorganisms by protecting cells from the effects of the growth inhibition factors such as exogenous H<sub>2</sub>O<sub>2</sub> (Okuyama et al., 2008). Also, has been suggested EPA protected cells from the effects of

oxidation of cellular proteins and breakage of the cell structure (Nishida et al., 2006). A recent study has suggested that it may relate to their antioxidative effects and indicated that age-related changes are inhibited by EPA as neuroprotective effects, also found equally potent effects of n-3 DPA and demonstrate its ability to downregulate age-related changes and oxidative changes in aged animals (Kelly et al., 2011).

### **1.5.2 Sphingomyelin in neurodegeneration and ageing brain**

Phospholipids are the structural components of brain cell membranes, the fatty acyl chain length of these phospholipids, practically PUFA are strongly affect their fluidity, flexibility, selective permeability, and provide unique degree of elasticity (Ollerenshaw, 1989, Nakada et al., 1990, Wallis et al., 2002, Pamplona, 2008).

Although a few membrane lipids including phosphatidylcholine and SM were initially known to have a structural role only, they are found to function significantly in signal transduction via biological membranes (Isaac et al., 2003, Niemela et al., 2004). Moreover, sphingolipids as bioactive lipid mediators are important cell membrane components, may have both brain injury and neurovascular protective (Spiegel and Milstien, 2003, Taha et al., 2006, Testai et al., 2012). Not only the main sphingolipid classes, but also their molecular species are found to be involved in brain function and disorders and impact their biological effects on the neuronal cell membranes; the SM species are part of these major constituents of the lipid bilayer in the cell membrane (Brouwers et al., 1998). However, the brain myelin composition of SM species in all

mammalian species are varied; it has been notable that rat's myelin has far less SM species than those found in human or bovine (Siegel and Agranoff, 1999).

Moreover, SM and its related molecules such as sphingosine phosphate are found to regulate apoptosis. Even though, their novel mediators in the brain have not been distinguished, but their existence can result into brain diseases which are known to be raised from overall sphingolipids biosynthesis defects. Therefore, it has been argued that changes of SM and sphingosine phosphate levels in neurodegenerative brain may be used as early symptoms of brain atrophy (Wiegmann et al., 1994, Albi, 2008, Kolter, 2011).

Sphingomyelin is found to be associated with microdomains in the plasma membrane known as lipid rafts which are characterized by the lipid molecules being in the lipid ordered phase, offering more structure and rigidity compared to the plasma membrane. In these rafts, the acyl chains have low chain motion but the molecules have high lateral mobility due to the higher melting temperature of SM as well as its interactions with cholesterol. Lipid rafts are thought to be involved in many cell processes, such as membrane sorting and trafficking, signal transduction, and cell polarization (Giocondi et al., 2004) and recently they have been speculated to be involved in the cell apoptosis (Zhang et al., 2006).

Sphingomyelin is also found to be accumulating in bone marrow, and brain due to deficiency of the sphingomyelinase, causing irreversible neurological damage characterized by profound brain damage. Also, as a result of multiple sclerosis, patients exhibit up-regulation of certain cytokines in the cerebrospinal

fluid such as tumour necrosis factor alpha. This cytokine activates sphingomyelinase enzymes that catalyze the hydrolysis of sphingomyelin to ceramide, and sphingomyelinase activity has been observed in conjunction with cellular apoptosis (Jana and Pahan, 2010).

On the other hand, it has been recently reported that reduction of sphingomyelin and ceramide accumulation in ageing are associated with the development of a wide variety of brain disorders, also the age-related changes in the ceramide and sphingomyelin content in the tissues are highly determined by the activation of A-SMase in the process of ageing (Har'kavenko et al., 2012). However ceramide, which is generated by hydrolysis of sphingomyelin by sphingomyelinase can stimulate an increase in reactive oxygen species which may cause caspase 3 activation (Pettus et al., 2002, Merrill et al., 2005), also been reported that activation of caspase 3 in neurons is blocked when activity of sphingomyelinase is inhibited and ceramide is known to increase activation of caspase 3 and to causes cell death and induces neuronal cells apoptosis, in cortical neurons ( Movsesyan et al., 2002, Miller et al., 2009).

### **1.5.3 Fatty acid ethanolamides in brain function and disorders**

Non-membrane brain lipids have become evident and have opened the field of research on neuroinflammation and brain disorders. For instance, FA-EAs and ECs serve important functions in neural development, and in the regulation of brain synaptic transmission (Fernandez-Ruiz et al., 1999, Freund et al., 2003, Jung et al., 2005). However, It is still uncertain how AEA, 2-AG and the FA-EAs are released from neuronal cells.

AEA is found to induce hypothermia, analgesia and hypomotility (Crawley et al., 1993, Smith et al., 1994, Calignano et al., 1998); it has also been a concern in neural cell differentiation and death (pro-apoptotic) and in anti-tumorigenic properties (Maccarrone et al., 2000, Pisanti et al., 2007). 2-AG is the most prevalent endogenous CB ligand discovered in brain (Mechoulam et al., 1995, Sugiura et al., 1995). It has been proposed that the 2-AG was involved in brain signalling (Sugiura et al., 2006, Matyas et al., 2008, Tanimura et al., 2010). Further, it has been proposed that central levels of 2-AG are affected by drugs of abuse (De Petrocellis et al., 2000, Gonzalez et al., 2002, Vigano et al., 2003, Caille et al., 2007), however its reinforcing effects have not yet been assessed in an animal model of drug abuse.

The DHA-EA has been found to be produced by the same pathway as AEA (De Petrocellis et al., 2000), It has been detected in brain and binds to brain CB1 receptors (Sugiura et al., 1996, Bisogno et al., 1999). It exhibits activities by regulating the neuron-functions and the immune system, also inhibits shaker-related voltage-gated potassium channels in brain (Devane et al., 1992, Poling et al., 1996, Sheskin et al., 1997, Di Marzo et al., 1998, Kozak and Marnett, 2002, Pavlopoulos et al., 2006).

PA-EA has been identified in the brain as anti-inflammatory and mediates analgesia (Calignano et al., 1998, Jaggar et al., 1998), anti-epilepsy, and neuroprotection which may possess neuro-regenerative properties with most of brain cells and being enzymatically inactive (Skaper et al., 1996, Lambert et al., 2001, Franklin et al., 2003, Sheerin et al., 2004, Kopsky and Hesselink, 2010). Moreover, OA-EA, an ethanolamide of OA, is found to be involved in regulating

hunger, liver fat burning effects (lipolysis) and offering a neuroprotection to the brain cells, through non CB receptors, PPAR and TRPV1 (Thabuis et al., 2008, Galan-Rodriguez et al., 2009, Serrano et al., 2011). Whereas limited pathophysiological roles have been reported on ST-EA , it has been reported that ST-ES is an endocannabinoid-like compound that shows pro-apoptotic activity due to elevation of intracellular calcium, activation of the arachidonate cascade and mitochondrial uncoupling, which is regulated by nitric oxide, in a way opposite to that reported for AEA (Maccarrone et al., 2002c).

On the other hand, FAAH is distributed in several brain regions and is the main enzyme catalyzing FA-EAs hydrolysis (Hillard et al., 1995). Endogenous AEA is present at very low levels and has a very short half-life due to the action of FAAH, as a consequence of lacking FAAH in brain, AEA metabolism is found to be severely impaired and leads to an increase in its endogenous level in brain which may be a protective factor in the brain against acute neuronal damage (Cravatt et al., 2001) and may be used as a structural template to develop neuroprotective agents (van der Stelt et al., 2001). Therefore, FAAH has been considered as a principle regulator of FA-EAs and a key player in the endocannabinoids system. It has also been found that the FAAH inhibitors are currently being developed as potential analgesics (Maccarrone, 2006, Sit et al., 2007, Wallace et al., 2007).

In general, it has been reported that the FA-EAs and ECs have anti-inflammatory effects on most of the neuroinflammation disorders, and affect their pathogenesis. For instance, on brain inflammation occurs in myelin degenerative disorders such as Multiple Sclerosis (Martino et al., 2002), also on

neurodegenerative disorders such as Alzheimer's disease (McGeer and Rogers, 1992) and on traumatic brain injury (Dusart and Schwab, 1994).

#### **1.5.4 Neurodegenerative disorders and oxysterols**

There is a relationship between biosynthesis of oxysterols species, analysis of plasma oxysterols and indication of oxysterols as powerful bioactive lipids that regulate brain lipid metabolism. All of these processes contribute to the investigation of the role of cholesterol metabolism in pathogenesis of neurodegeneration and could afford sufficient explanation to the biological mechanisms of cholesterol homeostasis (Shibata and Glass, 2010, Leoni and Caccia, 2011). Brain is surrounded by BBB which prevents cholesterol being taken from the circulation into the brain or releasing brain cholesterol into the circulation. Almost all cholesterol present in the brain is formed by *de novo* synthesis, (Lutjohann et al., 1996, Bjorkhem and Meaney, 2004, Leoni and Caccia, 2011). However, oxysterols can cross BBB and are immediately distributed to most of biological fluids to regulate cholesterol brain homeostasis (Lange et al., 1995, Corsinovi et al., 2011).

24-Hydroxyoxysterol has been proposed as a marker for brain cholesterol turnover (Bretillon et al., 2000, Bjorkhem, 2006), approximately all the 24-OH present in plasma originates from the brain and its plasma levels may reflect the number of metabolically active neuronal cells in the brain (Lutjohann et al., 1996, Ohyama et al., 2006), also its plasma levels are dependent on the rate of hepatic uptake, clearance and metabolism: its plasma levels are the result of the balance between secretion capacity of the brain and metabolic capacity of the liver (Bretillon et al., 2000).

It has been noticed that flux of 24-OH from brain is critically affected by the number of active neuronal cells that are significantly reduced in neurodegenerative diseases compared to healthy controls (Papassotiropoulos et al., 2000). It has also been suggested that as a consequence of losing neurons by neurodegeneration, the 24-hydroxylase levels might be reduced leading to a decrease in 24-OH formation, subsequently lowering the release of 24-OH into the circulation, suggesting that brain cholesterol oxidation and brain 24-OH elimination are closely related to neurodegenerative diseases (Björkhem, 2006, Shafaati et al., 2011).

Moreover, it has been reported that, associated with increased brain cholesterol turnover, inflammation was found associated with increased brain cholesterol clearance by showing an influence on the brain amount of 24-OH. Increased brain cholesterol synthesis is often, but not always, found to be associated with higher 24-OH production. Also specific inhibition of CYP46A1 was found to be associated with reduced cholesterol synthesis. Therefore, an increased brain cholesterol turnover or modification of cholesterol metabolism was found to be associated with higher amounts of 24-OH production and release in the blood circulation. On the other hand, a reduced cholesterol turnover is found to be associated with reduced production and release of 24-OH (Leoni and Caccia, 2013).

27-Hydroxycholesterol is almost solely generated by non-neuronal cells such as liver and lung, and is found to be significantly increased in neurodegenerative diseases such as in Alzheimer's disease patients compared to healthy controls. This is thought to be due to a defect in the BBB generated by the disease

(Blennow et al., 1990). It has also been reported that Alzheimer's disease reduces brain levels of CYP7b, the critical enzyme in 27-OH metabolism (Yau et al., 2003, Heverin et al., 2005, Meaney et al., 2007) (Figure 1.6).

Conversion of cholesterol into 24-OH in the brain is of critical importance for central cholesterol homeostasis (Lutjohann et al., 1996), also the flux of 27-OH into the brain may be the missing link between hypercholesterolemia and neurodegenerative diseases; it has been recently hypothesised that the balance between 24-OH and 27-OH is of importance in neurodegenerative disorder such as ALS (Bjorkhem et al., 2009, Leoni and Caccia, 2011). Therefore, the brain and plasma levels of oxysterols might be considered as diagnostic markers in neurodegenerative disease (Bjorkhem et al., 2009).

#### **1.5.4.1 Amyotrophic Lateral Sclerosis**

Amyotrophic lateral sclerosis which is also referred to as motor neurone disease (MND) is an adult-onset neurodegenerative disease characterized by gradual death of motor neurones, which then stops muscles from working and leads to weakness and wasting of muscles, causing increased loss of mobility in the limbs, and difficulties with speech, swallowing and breathing. It is also characterized by progressive loss of spinal cord and cortical motoneurons, and it is usually fatal within 2–5 years of diagnosis. The exact cause is still unknown, the biochemical origin and the etiology of the disease are not yet fully elucidated, and there is no definite diagnostic test, no treatment and cure have been reported (Rowland, 1994).

Approximately 10% of ALS cases are inherited, with the remaining 90% of cases originating randomly (Kunst, 2004). The inherited cases are thought to be due to mutations in the gene for the cytosolic copper-zinc superoxide dismutase (SOD1), it is found to be important in the defence against oxidative stress in tissue where it detoxifies superoxide anion radicals to hydrogen peroxide, and further reduces this to water (Noor et al., 2002, Muller et al., 2006). Moreover, expression of a mutant SOD1 protein, with or without residual SOD1 activity, is necessary for the occurrence of ALS phenotype (Clement et al., 2003, Kunst, 2004). Although, there is an evidence that the toxicity of mutant SOD1 in ALS is not due to loss of activity, but to the gain of one or more toxic functions that are independent of SOD1 activity (Nirmalanathan and Greensmith, 2005). It is believed that mutant SOD1 stimulates oxidative stress and induces mitochondrial dysfunction, excitotoxicity, inflammation, and protein aggregation (Tu et al., 1997, Agar and Durham, 2003, Mariani et al., 2005).

It has been reported that in ALS there is increased oxidative DNA damage as indicated by elevated levels of 8-hydroxy-2'-deoxyguanosine in plasma, urine and cerebrospinal fluid (Mariani et al., 2005). Several studies have shown increased lipid peroxidation and DNA damage in transgenic mice expressing mutant SOD1 and in neural tissue or sera from ALS patients (Agar and Durham, 2003, Simpson et al., 2004). Also, studies in animal models containing this mutation has revealed the presence of intracellular aggregates of SOD1 and increased oxidative damage, suggesting the involvement of reactive oxygen species in this pathology (Poon et al., 2005, Smietana et al., 2010).

## **1.6 Lipid analysis**

Lipidomics describe the research field that involves the identification and quantification of multiple lipid species and their molecular species in biological fluids and tissues. This rapidly expanding field has been driven and developed recently by rapid advances in mass spectrometry and it is helped by computational methods. It is useful in recognising the role of lipids in many metabolic diseases, lipid disorders and cells in their physiological or pathological state (Wenk, 2005).

HPLC and GC are extensively used in lipidomic analysis to separate lipids prior to mass spectrometry analysis. Key applications include HPLC/electrospray ionization (ESI) coupled with tandem mass spectrometry (LC/ESI-MS/MS) and GC/chemical or electron ionisation coupled with mass spectrometry (GC-MS).

### **1.6.1 Gas chromatography in lipid analysis**

Gas Chromatography has become most popular and suitable to analyse fatty acids in mammalian cells. Fatty acids are analysed as methyl ester derivatives (FAME) which characterised by low boiling point (Morrison and Smith, 1964, Eder, 1995, Masood et al., 2005, Hallmann et al., 2008), and it is recommended to use short and highly polar fast column to separate them (Destailats and Cruz-Hernandez, 2007). Flam Ionisation Detection (FID) is more convenient and sensitive detector; it is used with mixture of air and hydrogen as a carrier gas for FAME analysis (Eder, 1995, Gutnikov, 1995, McNair and Miller, 1998, McNair and Miller, 2009).

## **1.6.2 Mass Spectrometry in lipid analysis**

Mass spectrometry is an extremely powerful technique in analytical chemistry. Three main units are making up the mass spectrometers: ion source, mass analyzer and detector. In addition, HPLC or GC is coupled with mass spectrometry are used to separate an extensive range of liquids.

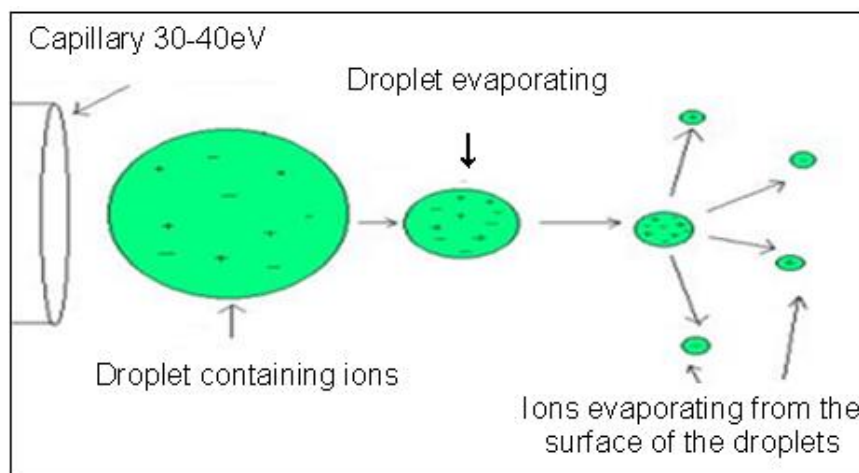
### **1.6.2.1 Ionisation methods used in lipidomics**

The most popular ionisation technique is electrospray ionisation (ESI). As shown in Figure 1.8, LC-MS/MS lipid detection started from HPLC separation, and then passed to mass analysers using an electrospray ion source (ESI). The main function of the ion source is to convert sample molecules to sample ions, mass analyser being the region in which ions are separated via mass techniques based on their mass while the detector is the data storage and recording device of the separated ions (Berger, 1999, Sparkman, 2000).

The LC/ESI-MS/MS is being used in analytical platforms in lipid bioanalysis in some applications including for the biomarker validation purposes in clinical and preclinical pharmaceutical research and development studies.

The ESI-MS is a soft-ionization method that hardly ever disrupts analytes chemical nature prior to mass analysis. It is being developed as a powerful tool used in structural identification and characterization of unknown ionised species, depends on formation of gaseous ions from polar and mostly non-volatile molecules, which makes it suitable for a variety of lipids. The ESI produces mainly singly charged molecular-related ions, usually protonated in positive ionisation mode  $[M+H]^+$ , and deprotonated in negative ionisation mode  $[M+H]^-$ ,

with very little extra energy remaining to cause fragmentation of the sample ions (Fenn et al., 1989) (Figure 1.8).



**Figure 1.8.** An illustration of how ions are generated from ionised solvent emerging from the capillary during ESI. (Adapted from: (Ho et al., 2003)).

The required material is ionised in several ways and then passed through an electromagnetic field and then identified based on their mass to charge ratio ( $m/z$ ). From the source, precursor ions are derived and taken under a fragmentation process yielding product ions, which are further ionised to species of ions, then identified by comparing the fragmentation precursor ions pattern to their product ions. The major advantages of ESI-MS/MS are high accuracy, sensitivity, reproducibility, and the applicability of the technique to complex solutions without prior derivatisation (Gross and Han, 2007).

On the other hand, the most common form of ionization is electron ionization (EI), also known as electron impact. Electrons interact with gas phase atoms or molecules to produce ions:  $(M + e^- \rightarrow M^{+*} + 2 e^-)$  where (M) is the analyte molecule being ionized, ( $e^-$ ) is the electron and ( $M^{+*}$ ) is the resulting ion (Davis

et al., 1987, Macek et al., 1995). Molecules introduced into mass spectrometry are bombarded with free electrons emitted from a filament and then fragmented in a characteristic and reproducible way. Two techniques could be applied, hard ionization technique results in creation of more fragments of low mass to charge ratio ( $m/z$ ), and soft ionization technique charges by molecular collision with an introduced gas. The GC-MS molecular fragmentation pattern is dependent upon the electron energy applied to the system, typically 70 eV (electron Volts) facilitate comparison of the generated spectra with library spectra software (Stein and Scott, 1994, Hoffmann and Stroobant, 2001, Amirav et al., 2008). GC-MS is used as a primary analytical technique for analysis of oxysterols in biological fluids as trimethylsilyl (TMS) derivatives. Their TMS are very easy to prepare and show excellent GC characteristics. It is a mild derivatisation method that displaces the active hydrogen atoms of hydroxyl groups to form silyl ethers that are usually more volatile than the underivatized oxysterols, and further prevents interaction of oxysterols with the stationary phase of GC column (Borjesson et al., 1998Luzon-Toro et al., 2007).

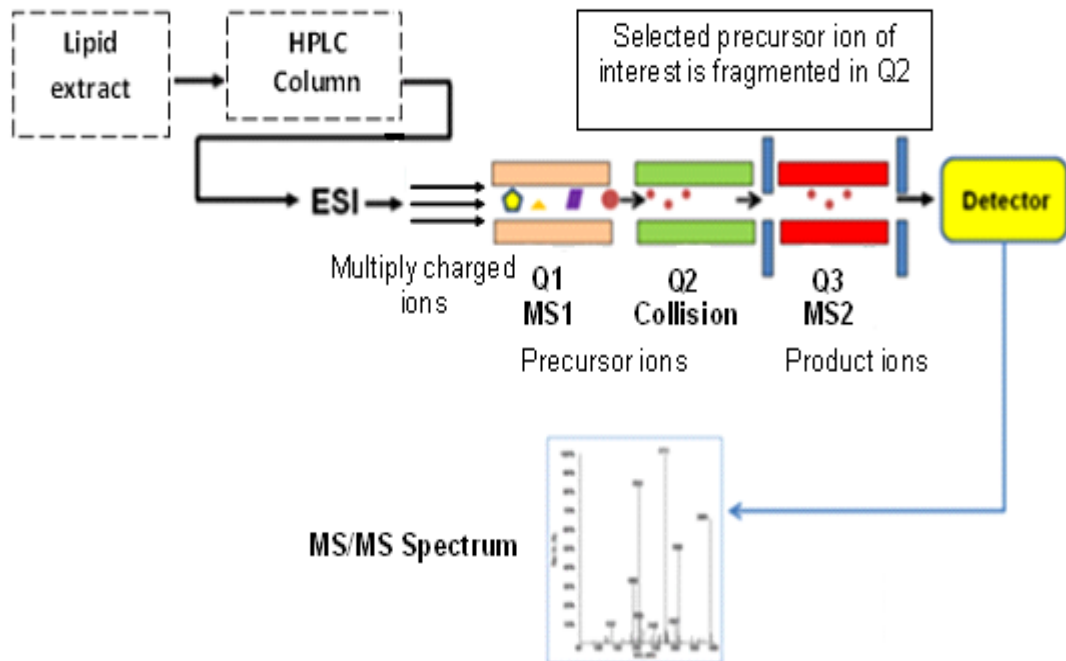
Moreover, TMS groups increase the total ion current and sensitivity of detection of oxysterol species. In general, EI mass spectra of TMS ethers exhibit a significant  $[M-15]^+$  ion formed by loss of a methyl group bonded to silicon, which is very useful in determining the molecular mass, and may be employed for structural deductions. Also, polysilylated compounds often yield ions containing more than one silyl residue, forming different fragment ions. This process may involve the movement of an electron pair from one of the Si-CH<sub>3</sub> bonds (H-O) bond resulting in the formation of another highly stable ion  $e^+$  ( $m/z$ ),

or loss of a methyl radical from one silyl group and subsequent rearrangement through another ion (Blau and Halket, 1993; Segura et al., 1998).

#### **1.6.2.2 Mass analysers used in lipidomics**

The ESI operates through a basic principle in which samples flow to a narrow capillary from the solvent at 0.2 mL/min rate, eluted as droplet ions thrown by ESI while behaving as shown in the Figure 1.8. A range of ESI-MS/MS methods have been developed for analysis of different classes, subclasses, and individual lipid species from biological extracts (Murphy et al., 2001, Murphy et al., 2005).

A typical triple-quadrupole mass spectrometry system contains three quadrupoles; Q1, Q2, and Q3, through which ions are accelerated. A quadrupole consists of four parallel metal rods, by carefully choosing the rod voltage; a quadrupole can serve as an ion filter, only allowing ions of a certain  $m/z$  value to be detected by the mass spectrometry. As shown in Figure 1.9, Q1 and Q3 serve as ions ( $m/z$ ) filters, and Q2 is used as a collision cell to fragment pre-selected ions (parent ions) from Q1. All fragments are then passed on to Q3 being set to filter specific fragments (Hoffmann and Stroobant, 2001). For instance, in the case of AEA, several dissociation fragments (daughter ions) are found, but the most dominant fragment is at  $m/z$  62, which represents the ethanolamine moiety of AEA (Felder et al., 1996, Weber et al., 2004, Yang et al., 2005). While in the case of SM a full scan ( $m/z$  650 -  $m/z$  900) monitored by a parent ion scan of  $m/z$  184 ESI in positive ion mode  $[M+H]^+$  has been reported as the best range to achieve a qualifying separation for SM species (Haroldsen and Gaskell, 1989).



**Figure 1.9.** Diagram showing LC-MS/MS based analyses using an electrospray ion source (ESI) and triple quadrupole (3Q) system. (Adapted from, (Banerjee and Mazumdar, 2012, Hoffmann and Stroobant, 2001).

## 1.7 Aim and objectives

Neuroinflammation present in ageing brain is found to be associated with cell membrane changes and increases in the oxidative stress that can lead to neurodegeneration disorders and impaired brain function. The n-3 PUFA; EPA and DHA, are incorporated in many parts of the body including cell membranes (Lazzarin et al., 2009) and play a role in anti-inflammatory processes and in the viscosity of cell membranes (Conquer et al., 2000, Smith et al., 2011). Also, they are essential for fetal development and healthy ageing (Dunstan et al., 2007). They are also precursors of several metabolites that are potent lipid mediators, considered to be beneficial in the prevention or treatment of several diseases (Serhan et al., 2008). Low intake of dietary EPA and DHA is thought to be associated with increased inflammatory processes, poor fetal development and risk of the development of neurodegenerative diseases such as Alzheimer's disease (Swanson et al., 2012). Although, the exact molecular mechanism of n-3 PUFA action is not well known, they have been shown to exert beneficial effects in inflammatory conditions and neurological disorders.

To date, the influence of EPA and n-3 DPA on profiling of PUFA and on SM species synthesis in the brain and as a precursor to ceramide remain unclear and are still a field of major interest. It is thought that EPA and n-3 DPA may be incorporated into RBC membranes and change RBC fatty acid profiles and brain in situ synthesised SM as a structural phospholipid. In addition, neurodegeneration disorders can result or influence the synthesis of brain oxysterols. It is known that oxysterols have been involved in various neural regulatory processes. However, till today, there is no clarity on how plasma

oxysterols can be affected by ALS and how they can be used as markers in its detection. Moreover, neuroinflammation has recently emerged as a key player in many degenerative diseases. It has been found that neuroinflammation caused by LPS infusion stimulates brain AA metabolism in rats. Lithium treatment reduces this effect indicating a potentially important therapeutic action of lithium. However, no study has been applied to investigate this effect on production of brain FA-EAs and ECs in neuroinflammation.

The main aims of this project have been set:

To evaluate the effects of EPA and/or n-3 DPA supplementation on changes in RBC and brain SM species by investigating RBC membrane fatty acids in adult and aged rats supplemented with EPA and n-3 DPA to assess compliance, and to investigate SM species in brains of adult and aged rats supplemented with EPA and n-3 DPA to evaluate the effects and benefits on brain ageing and age-related changes.

Also, to study the effects of lithium on the brain FA-EAs and ECs of rats subjected to head-focused microwave irradiation to further understand and provide a new possible mechanism on lithium neuroprotective action during neuroinflammation subjected to lithium treatment.

Finally to see if circulating oxysterols were linked to the prevalence of ALS and whether RBC fatty acids were linked to this action in related to age and disease stages.

For the purpose of this thesis, our specific objectives were:

1. Analyse RBC fatty acids in order to investigate the compliance of a diet supplemented with n-3 EPA and DPA.
2. Use LC/ESI-MS/MS to analyse brain SM species in samples treated with EPA and n-3 DPA.
3. Use LC/ESI-MS/MS to analyse brain ECs and their congeners in rats that were subjected to head-focused microwave irradiation, lithium conditioned and LPS-treated, to investigate the effect of lithium treatment on rat brain ECs.
4. Analyse RBC fatty acids in order to investigate the compliance of ALS and ALS transgenic model SOD1 Mice (pre-symptomatic stage and disease end-stage) with disease stages and ageing.
5. Use EI/GCMS to analyse plasma oxysterol species in ALS patients and animal model to investigate the compliance of ALS and ALS transgenic model SOD1 Mice (pre-symptomatic stage and disease end-stage) with disease stages and ageing.

## **Chapter 2: Materials and Methods**

## 2.1 Materials

### 2.1.1 Chemicals

Trimethylpentane (isooctane) and toluene (pesticide residue analysis, PRA grade solvents) were purchased from ACROS Organics (Loughborough, UK). Potassium carbonate (ACS reagent), heneicosaenoic acid C21:0, sodium sulphate (anhydrous), potassium chloride, 2,6-di-tert-butyl-4-methylphenol (BHT), boron trifluoride (BF<sub>3</sub>) in methanol (14% w/v), fatty acid methyl esters (FAME) mixed standard (37 component mix FAME), docosapentaenoic acid methyl ester (C22:5n-3) (98.5%), docosatetraenoic acid methyl ester (C22:4n-6) (98.5%), eicosadienoic acid methyl ester (C20:2) (98.5%), vaccenic acid methyl ester (C18:1n-7) (98.5%), glacial-acetic acid HPLC-grade and tetratriacontane C34 (98%) were purchased from Sigma-Aldrich, (Poole, Dorset, UK). Methanol HPLC-grade, acetonitrile HPLC-grade, chloroform HPLC-grade, dichloromethane (DCM) HPLC-grade, ethanol HPLC-grade, formic acid HPLC-grade were purchased from Fisher Scientific, (Loughborough, UK). Potassium hydroxide was purchased from ReAgent Chemicals (Runcorn, UK). 4 $\beta$ -Hydroxycholesterol (d7) (100%), SM from bovine brain ( $\geq$ 97.0%) and SM (d18:1/12:0) in 1 mL ethanol solution were purchased from Avanti Polar Lipids, INC (Alabaster, Alabama, USA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) purchased Thermo Scientific (Rockford, USA). Oleoylethanolamide (OA-EA), 2-arachidonoylglycerol (2-AG), arachidonylethanolamide (AEA), docosahexaenylethanolamide (DHA-EA), stearoylethanolamide (ST-EA), palmitoylethanolamide (PA-EA), linoleoylethanolamide (LA-EA), arachidonoyl ethanolamide-*d*8 (AEA-*d*8) and 2-arachidonoyl glycerol-*d*8 (2-AG-*d*8) were purchased from Cayman Chemicals

(Ann Arbor, MI, USA). Nitrogen gas was supplied by BOC gasses (UK). Strata<sup>®</sup> Solid phase extraction (SPE) cartridges (1 mL, 100 mg silica-based) were purchased from Phenomenex (Macclesfield, UK).

### **2.1.2 Glassware and other consumables**

Disposable glass Pasteur pipettes (150mm length), disposable pipette tips, glass syringe (5mL), glass vials scintillation (20mL) with caps and Eppendorf tubes Fisher Scientific (Loughborough, UK). Fixed needle gas tight syringes (50  $\mu$ l and 10  $\mu$ l) were purchased from SGE (Milton Keynes, UK). Gastight Hamilton syringe (250  $\mu$ l) was purchased from Sigma-Aldrich, (Dorset, UK). Wide necked glass vials (10 mL), glass vials (1.5 mL), glass flat bottom insert vials (150  $\mu$ l), PTFE/red silicon rubber septa and preassembled screw cap were supplied by LSL Laboratory (Rochdale, UK). Glass insert vials (100  $\mu$ l), amber glass bottles (1.5 and 2 mL) (12 x 32 mm) for autosampler use, round and flat glass tubes (15 mL) were purchased from International LTD (Poole, Dorset, UK). Glass wool used for filtration was purchased from Robinson Healthcare Ltd, (Chesterfield, UK). Narrow range (2.5-4.5) pH indicator paper strips were purchased from Merck (Nottingham, UK). Ultra filtered Milli-Q grade water obtained by filtering with minimum resistance of 18  $\Omega$  was used throughout.

### **2.1.3 Equipment**

GC-FID system (Agilent Technologies, 6850) equipped with an autosampler (6850 network GC) systems and coupled to a hydrogen generator (Claind, HG2000). Data was acquired using software Chemstation Revision (Agilent Technologies, B2.01). GC capillary column, BPX-70 of length 60 m, internal diameter 0.25mm and film 0.25  $\mu$ m. A 12-position SPE vacuum manifold and

drying attachment to the manifold for sample preparation and elution were purchased from Phenomenex (Macclesfield, UK). GC-MS system consisting of GC (Agilent technologies, 7890A), MS (Agilent technologies 5975C) connected by inert XL EI/CI MSD with Triple-Axis Detector and GC Column; 15m x 0.25mm, 0.25mm HP-5MS 5% phenyl methyl siloxane phase fused silica column were purchased from Agilent Technologies UK Ltd (Wokingham, UK). Waters Alliance 2695 HPLC pump with Waters 2690 autosampler coupled to an ESI triple quadrupole (Quattro Ultima) mass spectrometer. Instrument control and data acquisition were performed using the MassLynx™ V4.0 software. All were purchased from Waters (Elstree, UK).

Mini glass (Dounce) homogeniser (2 mL) was obtained from Wheaton (NJ, USA). Mani vortex was purchased from Hook and Tucker Instruments LTD (Croydon, UK). Precision Balance was supplied by Oakleyweigh (Aylesbury, UK). A React-Therm<sup>®</sup> heating module block was supplied by Thermo Scientific (Cramlington, UK). A SANYO Micro Centaur microcentrifuge was supplied by MSE (London, UK). Vortex Whirlmixer and refrigerated centrifuge Sovrall RT6000B were purchased from DuPont (Stevenage Herts, UK). Ultrasonic water bath sonicator was purchased from Ultrawave Limited (Cardiff, UK). Centrifuge; Jouan B3.11 refrigerated centrifuge, purchased from Rhys Scientific Ltd (Chorley, UK)

## **2.2 Biological and Clinical Samples**

### **2.2.1 Eicosapentaenoic and docosapentaenoic acid-treated red blood cells and brain tissue**

All animal work described in this study, was performed by Professor Marina Lynch's research group at Trinity College, Dublin, Ireland. All animal work in this part of the study was performed under a licence granted by the Minister of Health and Children (Ireland) under the Cruelty to Animal Act, 1876 and European Community Directive, 86/609/EC.

Study A: Adult (3-4 months) and aged (22-24 months) male Wistar rats weighting 250-300g were used; the rats were assigned into three groups: The first group (six rats) received 200 mg/kg/day EPA. The second group (seven rats) received 200 mg/kg/day n-3 DPA. The third group (six rats) was the control group and received normal laboratory chow (Red Mills, Ireland) supplemented with monounsaturated fatty acids to ensure isocaloric intake with the control diet. The study lasted 8 weeks.

Study B: This long term feeding study was performed over a period of 6 months using seventeen to eighteen month old male Wistar rats. The rats were allocated into three groups: The first group (four rats) received control diet for 3 months then n-3 DPA 100 mg/rat/day for 3 months. The second group (six rats) received n-3 DPA 50 mg/rat/day for 3 months, and following this a dose of n-3 DPA 100 mg/rat/day for a further 3 months was introduced. The third group (nine rats) was the control group and received normal laboratory chow (Red Mills, Ireland) supplemented with monounsaturated fatty acids to ensure isocaloric intake with the control treatment.

At the end of the dietary supplementation period the animals were sacrificed and blood was collected using EDTA (anticoagulant) tubes. Red blood cells (RBC) were isolated immediately by centrifugation at 3000 rpm for 10 min at 4 °C. At the same time the brains were rapidly removed, collected and placed on ice. The cortical tissue was then sliced and aliquots placed in sterile eppendorf tubes. All samples (RBC and brain) were frozen to -80 °C instantly and then transferred to University of Bradford in dry ice where they were stored at -80 °C awaiting extraction and analysis.

### **2.2.2 Microwave-treated rat brain tissue**

All animal work described in this study, was undertaken by Prof Stanley Rapoport's research group, at NIH Laboratory of Neurosciences, Bethesda, MD, USA. All animal work in this part of study was performed under a protocol (#06-026) approved by the Animal Care Committee of Eunice Kennedy Shriver National Institute of Child Health and Human Development, in accordance with NIH guidelines on the care and use of laboratory animals. Two-month-old male Fischer F344 rats obtained from Taconic Farms (Rockville, Maryland, USA) were housed in a facility with a 12/12 light dark cycle. One group of rats was fed *ad libitum* Purina 5001 chow diet containing 1.70 g LiCl/kg (low LiCl) for 4 weeks, followed by chow containing 2.55 g LiCl/kg (high LiCl) for 2 weeks both diets were supplied by Harlan Teklad (Madison, WI, (USA) as previously mentioned (Basselin et al., 2007). This regimen produces brain lithium concentrations of about 0.7 mM, therapeutically relevant to bipolar disorder (Chang et al., 1999, Chang et al., 1996). Control rats were fed lithium-free Purina 5001 chow diet for 6 weeks. Water and NaCl solution (0.45 M) were

available ad libitum to both groups. The 5001 diet contained (as percent of total fatty acids): 25.2% SFA, 33.3% MUFA, 35.1% LA, 3.1% ALA, 0.39% AA, 1.25% EPA and 1.62% DHA (Basselin et al., 2010).

Rats were anesthetized and an indwelling cerebroventricular cannula was fixed in place as described (Basselin et al., 2010, Basselin et al., 2007, Hauss-Wegrzyniak et al., 1998, Rosenberger et al., 2004). Artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3.0 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 1.2 mM Na<sub>2</sub>PO<sub>4</sub>, pH 7.4) or Escherichia coli LPS serotype 055:B5 were purchased from Sigma (St. Louis, Missouri, USA) at a low dose (1 mg/mL at 0.5 ng/h) or a high dose (0.5 mg/mL at 250 ng/h) was infused into the fourth ventricle through the cannula (Model 3280P, 28 gauge) was purchased from Plastics One Inc (Roanoke, Virginia, USA) via an osmotic pump (Model 2002) was purchased from Alzet® (Cupertino, California, USA). Before surgery, the prefilled pump was placed in sterile 0.9% NaCl at 37°C overnight to start immediate pumping. Post-operative care included triple antibiotic ointment applied to the wound, and 5 mL of sterile 0.9% NaCl (s.c.) to prevent dehydration during recovery. Following 6 days of LPS or aCSF infusion, starting had been on a control or lithium diet for 36 days, rats were anesthetized with Nembutal® (40 mg/kg, i.p.) was purchased from Fritz Logistics Service Co., Ltd (Los Angeles, USA) and subjected to head-focused microwave irradiation (5.5 kW, 3.6 s; Cober Electronics, Stamford CT). Brains were removed and stored at -80°C instantly and then transferred to University of Bradford, UK in dry ice where they were immediately stored in -80 °C awaiting for extraction and analysis.

### **2.2.3 Red blood cell and plasma samples from SOD1 mice**

All animals were bred and maintained by the biological services in the University College London, Institute of Neurology. The samples were provided by Dr Andrea Malaspina, Centre of Neuroscience and Trauma, Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry. The experiments described in this study were carried out according to the Helsinki agreement on animal experimentation, under licence from the UK Home Office and following approval from the UCL Institute of Neurology's Ethical Review Panel. Transgenic mice expressing human SOD1<sup>G93A</sup> mutant protein (TgN[SOD1-G93A]1Gur; Jackson Laboratories, Bar Harbour) were maintained by breeding male heterozygous carriers with female (C57BL/6 x SJL) F1 hybrids. The presence of superoxide dismutase1 (SOD1) G93A mutation was confirmed by PCR reaction from ear biopsies in all mice at the age of 3 weeks.

Depending on genotype, the mice were randomly assigned to one of 2 groups: SOD1<sup>G93A</sup> mice (Group SOD1), and Wild type, age-matched littermates (Group WT). Blood sampling was carried out in mice from each experimental group at 35 and 120 days of age, representing pre-symptomatic and late stage disease. Blood was collected into an EDTA tube by a cardiac puncture, under terminal anaesthesia using pentobarbital. Each tube was then centrifuged at 14,000 rpm for 8 min. The plasma was transferred into a cryotube and protease inhibitor added (Sigma; 1:100, v/v) for the purpose of concurrently inhibiting several classes of proteases during sample preparation. The RBC layer was transferred into a cryotube and snap frozen using liquid nitrogen. In total, plasma and RBCs

samples collected from SOD1 mice (6 animals), WT mice (6 animals) at each time point were used for further analysis as follow: Wild type pre-symptomatic stage, age 35 days; Wild type end-stage, age 120 days; SOD1G93A pre-symptomatic stage, age 35 days; and SOD1G93A end-stage, age 120 days. All samples were stored at -80°C, and transferred to University of Bradford in dry ice where they were stored in -80 °C awaiting analysis.

#### **2.2.4 Red blood cell and plasma samples from Amyotrophic Lateral Sclerosis patients**

All clinical samples used for this work were provided by Dr Andrea Malaspina, Centre of Neuroscience and Trauma, Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry. Written informed consent was obtained from all participants, and the study was approved by the ethics committee of Queen Mary University of London and University College London, Institute of Neurology, UK.

Peripheral blood of ALS patients and healthy controls were collected in EDTA blood tubes, which was processed within one hour after blood was taken with spinning speed at 3500 rpm, acceleration at 5 and deceleration at 4, for 10 minutes at 20°C. The plasma was aliquotted, and the RBC layer was also collected. All the samples were then stored at -80°C, and transferred to University of Bradford in dry ice where they were stored in -80 °C awaiting analysis.

## **2.3 Fatty acid analysis**

### **2.3.1 Preparation of solvents**

Extraction solvent: 0.01% (w/v) BHT in 2:1 chloroform: methanol (0.030 g BHT dissolved in a mixture of 200 mL chloroform and 100 mL methanol). 0.5 M Potassium chloride (KCl) in 50% methanol: (7.45 g KCl dissolved in 100 mL of water, and add 100 mL methanol). 10% (w/v) Potassium carbonate ( $K_2CO_3$ ) solution: (10.0 g of  $K_2CO_3$  dissolved in 100 mL of water). Toluene: Methanol 50/50 (v/v): (10 mL toluene mixed with 10 mL methanol and stored in a glass container). All solvents were keep on the bench at room temperature and used for up to one month.

### **2.3.2 Preparation of standards**

The commercially available mixture of fatty acid methyl ester (FAME) standards (100 mg) contained 37 fatty acid methyl esters, namely: C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1n-9t, C18:1n-9c, C18:2n-6t, C18:2n-6c, C20:0, C18:3n-6, C20:1, C18:3n-6, C20:1, C18:3n-3, C21:0, C22:0, C20:3n-6, C22:1n-9, C20:3n-3, C20:4n-6, C23:0, C22:2, C24:0, C20:5n-3, C24:1, C22:6n-3. This mixture was dissolved in 1 mL DCM to give 100 mg/mL mixed standard solution. This solution was aliquoted to 100  $\mu$ L aliquots, each placed in 1.5 mL amber vial. The vials were sealed and stored at -20 °C. FAME standard aliquots should be stable for one month if they are returned to the -20 freezer post injection.

Individually purchased fatty acid methyl ester standards C22:5n-3 (10 mg), C22:4n-6 (25 mg), C20:2 (25 mg) and C18:1n-7 (100 mg) were prepared separately as follows: each compound was dissolved in 1 mL DCM to give 10

mg/mL, 25 mg/mL, 25 mg/mL and 100 mg/mL solutions respectively. The different solutions were aliquoted into 100  $\mu$ L, each stored in 1.5 mL amber vial. The vials were sealed and stored at -20 °C. These aliquoted solutions should be stable for one month if kept at -20 °C freezer.

The internal standard (IS) C21:0 (10 mg) was dissolved in 10 mL methanol-chloroform (1:2, v/v) containing BHT (0.01 % w/v) to give 1 mg/mL solution of IS as free acid and then stored in -20 °C, stable for up to 1 week.

### **2.3.3 Red blood cell lipid extraction**

Red blood cell samples were taken out of the freezer (-80 °C) and thawed in an ice box. Once the samples had melted, then 0.5 mL of RBC was transferred to a clean glass tube, 1mL of water was added, vortexed for 1 min and left to stand in an ice box for another 1 min. The vortexing and standing step was repeated 3 times. The sample was then transferred to another clean glass tube labelled as extraction tube (T1) containing 4 mL of ice cold extraction solvent (sections 2.3.1). The extraction tube (T1) was vortexed for 5x1 min while it was kept cool on ice for at least 30 s in between each vortex step. It was then centrifuged at 4 °C, 3000 rpm for 5 min. The lower organic layer was transferred into another clean tube labelled as extraction tube (T2) using a glass Pasteur pipette. Further 4 mL of extraction solvent was added to the aqueous layer remaining in (T1) and the extraction was repeated. The organic solvent was removed and combined with extract in tube (T2). 0.5M KCl in methanol (2mL) was then added to the combined organic extract, vortexed, centrifuged and the top aqueous layer was removed and discarded. Another 2mL of 0.5M KCl in methanol were added to the extract tube, vortexed and centrifuged as above.

After that the bottom organic layer was transferred to another clean tube labelled as extraction tube (T3). Approximately 4 spatula-tips of anhydrous sodium sulphate was then added and this solution was shaken gently until a “snow storm” effect was observed. The organic extract was then filtered through a filter made from glass wool tightly-packed in Pasteur pipettes. Prior to using them, the filters had been washed three times with extraction solvent and flushed with air to dry them. After filtering the solution, the filters were washed with 2mL extraction solvent and the eluent was collected, and dried under a fine stream of nitrogen gas. The lipid residue was reconstituted in 1mL of extraction solvent, transferred to a clean glass vial, closed tightly, sealed with parafilm and stored at -80 °C.

#### **2.3.4 Preparation of fatty acid methyl esters**

The lipid extracts were taken out of the freezer (-80 °C) and kept in an ice box for few minutes, 200 µl of the lipid extract was transferred to a clean glass tube labelled as derivatization tube (D1) and then the solvent dried under nitrogen. A 45 µl of IS C21:0 (1 mg/mL) free acid was added (to give a final concentration of 900 µg/µl in the FAME). The solvent was then removed with nitrogen. After that, 250 µl of toluene: methanol (50/50 v/v) and 250 µl of 14% BF<sub>3</sub>-methanol solution (working in the fume cabinet) were added, the vial was flushed with nitrogen, sealed, vortexed and placed into a pre-heated block set at 100 °C and left to heat for 90 min. After this the vial was placed in an ice bath for 10 min to cool down. Then, 1.5 mL of 10% K<sub>2</sub>CO<sub>3</sub> solution (w/v) and 2 mL of trimethylpentane were added, vortexed and the solution centrifuged for 5 min at 3000 rpm. The upper layer of this solution, which contains FAME, was removed

carefully and transferred to a clean glass tube labelled as derivatization tube (D2), using disposable glass Pasteur pipettes. To ensure all the FAME were extracted, another 1.5mL of 10%  $K_2CO_3$  solution (w/v) and 2mL of trimethylpentane were added to residual solution in tube (D1), vortexed and the solution centrifuged for 5 min at 3000 rpm. The upper layer of this solution, which contains any remaining FAME, was removed, transferred and combined with the first extract in tube (D2). The combined extract was evaporated to dryness under nitrogen. The residue was reconstituted in 50 $\mu$ L DCM. To ensure all the extract was taken, the solvent was added then vortexed and centrifuged for 1 min at 3000 rpm, 4 °C, and then transferred to a clean insert vial using a glass syringe. The vials were placed on the autosampler for overnight analysis.

### **2.3.5 Preparation of internal standard methyl ester**

In order to confirm the retention time of the IS, 900 $\mu$ L of IS free acid stock solution (1mg/mL) was transferred into a clean derivatisation tube to be esterified as described above (see section 2.3.4). The final sample of IS methyl ester was re-dissolved in 1mL DCM to give a final concentration 900ng/  $\mu$ L IS methyl ester. A 100  $\mu$ L of this solution was then transferred to a clean 250  $\mu$ L insert vial awaiting to be analysed by GC-FID. The remaining IS methyl ester solution was stored at - 20°C and it should be stable for up to one month.

### **2.3.6 GC-FID analysis**

The FID and injector were maintained at 220 °C and 250 °C respectively. The main carrier gas was helium which was used at a flow rate of 21 cm/s. A mixture of hydrogen and air was used as the detector gas. A GC capillary column, BPX-70 of length 60 m, internal diameter 0.25mm and film 0.25  $\mu$ m

was used. The total run time was set up for 65 min. The starting oven temperature was set at 70 °C for 2 min then increased to 150 °C at a rate of 20 °C per min and held at 150 °C for 5 min. Then raised up from 150 °C to 218 °C at a rate of 2.50 °C per min, and from 218 °C it was increased to 225 °C at a rate of 0.60 °C per min and held at 225 °C temperature for 10 min. Finally, raised up to 230 °C at a rate of 2.50 °C per min and held for 3 min. The injection volume was 1 µL. For the results described in (chapter 3, red blood cells, rats) injections were run at a split mode (30:1). For the results described in (chapter 5, red blood cells, human and rats) injections were run at splitless mode. This allowed the identification of fatty acids found at low concentrations in the biological samples.

### **2.3.7 Analysis of standards**

Initially, individual injections of the IS methyl ester (900 µg / µl) and the FAME mixed standard (100 mg/mL) were injected in order to establish the relevant retention times and there is no overlapping between IS peak and any peaks from the FAME standard mixture. The following solution was prepared: 100 µL of IS methyl ester (900 µg/µL) and 20 µL of FAME (100 mg/mL), then injected and analysed. Also, the four fatty acids C22:5n-3, C18:1n-7, C20:2 and C22:4n-6 which were not included in the 37 component FAME mixed standard were individually prepared, injected and analysed to establish their retention times. Finally, a cocktail of the FAME mixed standard, IS methyl ester and the other four methyl ester standards (C21:0, C22:5n-3, C18:1n-7, C20:2 and C22:4n-6) was injected to make sure there were no overlapping peaks; this was done in duplicate. All the standards were eluted between 18 and 50 min, the first fatty

acid was tetradecanoic acid which eluted at 18.3 min, while the last fatty acid was DHA which eluted at 47.5 min. IS (C21:0) was eluted at 36.4 min. A sample chromatogram of a mixture of all FAME standards and the (IS) is shown in (Appendix - 1, Figure 1).

### **2.3.8 Analysis of biological samples**

Prior to injecting the biological samples, (IS) methyl ester and a mixed standard containing FAME standards and IS methyl ester, were injected each morning to establish the retention times of the day. Biological samples were then analyzed in duplicates. Blank injections of DCM were intervaled between the sample and the standards to avoid any carrying over problems between injections. A total of 26 fatty acids were identified in animal and human RBC, all of them were found to be eluted between 18 and 50 min. List of fatty acids detected in RBC of control and supplemented adult (3-4 months) and aged (22-24 months) animals with 200mg/kg/day EPA or 200mg/kg/day n-3 DPA is presented in (Appendix – 1, Table 1). Also, a sample chromatogram of a typical RBC from animal (adult male Wistar rat) is shown in (Appendix - 1, Figure 2), whereas human is shown in (Appendix - 1, Figure 3).

### **2.3.9 Calculations**

All biological samples were analysed in duplicate injections and all data were expressed as % of weight. Comparison of retention time of FAME in biological samples to mixed FAME standard was used to identify the fatty acids. The FAME peaks were integrated, the average peak area of duplicate injections was taken, normalised by the IS and the weight of IS as methyl ester in the biological sample (939 ng) according to the following equation: (Average of

FAME peak area / Average of IS peak area) x Weight of IS as methyl ester in the biological sample (939 ng). Then the weight % was calculated as following: (FAME weight ng / total FAMES weight ng) x 100). A typical set of calculations is shown in (Appendix –1, Table 1).

## **2.4 Analysis of sphingomyelin**

### **2.4.1 Preparation of standards**

A 1 mg/mL stock solution of SM semi-purified extract from bovine brain  $\geq 97.0\%$  powder was prepared as follows: 1 mg of bovine SM was dissolved in 1 mL chloroform, dried under nitrogen, and then re-dissolved in 1 mL of the mobile phase (70:30:0.1, acetonitrile : methanol : glacial acetic acid). Serial dilutions were then made to make 100  $\mu\text{g/mL}$  SM solution to be used for general scan, and to verify the SM species retention times. SM (d18:1/12:0) 1 mL ethanol solution was used as IS, aliquoted in 20  $\mu\text{L}$ , and stored at  $-20^\circ\text{C}$ .

### **2.4.2 Brain lipid extraction**

SM from rat brain was extracted according to a modified protocol published by Merrill (Merrill et al., 2005). Briefly: The brain samples were removed from  $-80^\circ\text{C}$  and placed on ice, brain slice was taken and weight was recorded (kept between 0.04 to 0.118 g). The brain slice was homogenised using 1mL mini glass homogeniser in 0.75 mL (2:1, v/v) methanol: chloroform and 0.1 mL water. Then 20  $\mu\text{L}$  of SM (d18:1/12:0) (IS) was added to give final concentration of 0.5 nmol/ $\mu\text{L}$ . The homogenate was incubated overnight at  $48^\circ\text{C}$  in a heating block to ensure complete extraction and denaturation of proteins. The tubes were then cooled and alkaline lipid hydrolysis was performed by adding 75  $\mu\text{L}$  of 1M KOH in methanol and the resulting solution was further incubated at  $37^\circ\text{C}$  for 2

hours to complete the extraction; it was then cooled to room temperature. The solution was neutralized with glacial acetic acid. Then, 1 mL chloroform and 2 mL water was added and the solution was vortexed. The mixed solution was centrifuged at (4°C and 5000 rpm for 10 min). The upper layer was carefully discarded and the lower layer was evaporated to dryness under nitrogen.

The lipid residue was re-dissolved in 1mL of mobile phase [70:30:0.1, v:v:v) acetonitrile : methanol : acetic acid] then diluted 1:10 to avoid over loading the column and stored in -20°C for up to one week awaiting analysis.

#### **2.4.3 ESI-MS/MS analysis of sphingomyelin species**

SM species were analysed by ESI-MS/MS according to the protocol published by Merrill (Merrill et al., 2005) with some modifications. Briefly: bovine brain SM and SM d18:1/12:0 at concentrations of (100 µg/mL and 10 ng/mL) were analysed by direct infusion through a syringe pump (flow rate of 10 µL/min) into the mobile phase 50:50:0.1, v:v:v) acetonitrile : methanol : glacial acetic acid (at flow rate 0.2 mL/min); this was repeated for the rat brain extract. The mass spectrometer was operated in the positive ion mode (ES+) and full scan spectra acquired over the mass range of  $m/z$  650-900. The source temperature was set at 120°C and the desolvation temperature was set at 360°C. The sensitivity of the method was optimised by altering the cone voltage (CV) and the collision energy (CE) in the range of 30-40eV and 20-40KV respectively. The cycle time for the MRM assay was 3.34 s, with a 0.1 s interscan delay, dwell time 0.2 s and cone voltage (CV) 35 eV.

#### **2.4.4 LC-ESI-MS/MS analysis of sphingomyelin species**

LC-ESI-MS/MS analysis was performed by normal phase chromatography on a Luna 5u NH<sub>2</sub> 100A column, LC column 30 x 4.6 mm. The elution of SM species was achieved using an isocratic solvent system composed of two mobile phases (A and B) mixed at constant ratio of 70:30 (v/v). Mobile phase A was: acetonitrile with 0.1% glacial acetic acid; Mobile phase B was: methanol with 0.1% glacial acetic acid. The autosampler was set at 8°C. The flow rate was set at 0.2 mL/min throughout the 30 min run time. The injection volume was 10µL and each sample was analysed in duplicate injections.

The analytes were monitored on the positive mode [M+H]<sup>+</sup>. The MRM transitions were set up using the precursor and product ion pairs as shown in Table 2.1. The results were expressed as nmol/g wet weight of tissue at the optimal collision energy.

#### **2.4.5 Limit of quantitation and limit of detection**

The limit of quantitation (LOQ) and the limit of detection (LOD) were verified by using SM (d18:1/12:0). A 0.5 nmol/µL of SM d18:1/12:0 solution was prepared using mobile phase 70:30:0.1 (v:v:v) acetonitrile : methanol : glacial acetic acid. Serial dilutions (1:10, 1:20, 1:50, 1:100, 1:200 and 1:500) were made to make solutions at concentrations of 0.05 nmol/µL, 0.025 nmol/µL, 0.01 nmol/µL, 0.005 nmol/µL, 0.0025 nmol/µL and 0.001 nmol/µL respectively. These solutions were then injected and analysed by LC/ESI-MS/MS as stated in section 2.4.4. The LOQ was determined using a signal-to-noise ratio (S/N) of 10. The LOD was estimated using an S/N ratio of 3. Peak integrations and S/N calculations were performed using the MassLynx™ V.4.0 software.

**Table 2.1.** The MRM transitions, collision energy (CE) and cone voltage (CV) used for the analysis of SM bovine brain extract species, rat brain extract and the internal standard SM d18:1/12:0 (IS).

<b>SM species</b>	<b>MRM <i>m/z</i></b>	<b>CE (KV)</b>	<b>CV (eV)</b>
<b>SM d18:1/12:0 (IS)</b>	647 → 184	30.0	35.0
<b>SM bovine brain extract and Rat brain extract</b>	703 → 184	30.0	35.0
	731 → 184	30.0	35.0
	747 → 184	30.0	35.0
	759 → 184	30.0	35.0
	785 → 184	30.0	35.0
	787 → 184	30.0	35.0
	801 → 184	30.0	35.0
	813 → 184	30.0	35.0
	829 → 184	30.0	35.0
	841 → 184	30.0	35.0

#### 2.4.6 Recovery

The recovery of the extractions was assessed using rat brain. The tissue was homogenized as described section 2.4.2 and spiked with 0.5 nmol/μL of SM (d18:1/12:0). It was extracted as shown in section 2.4.2, and the extract analysed by LC/ESI-MS/MS as described in section 2.4.4. At the same time, 0.5 nmol/μL of IS SM (d18:1/12:0) was analysed by LC/ESI-MS/MS directly. The peak areas of the IS in the spiked brain sample and the directly analysed IS were measured and used to calculate the recovery as follows:

$$\% \text{ Recovery} = \frac{(\text{IS})\text{peak area in the spiked brain sample}}{(\text{IS})\text{peak of the directly analysed}} \times 100$$

## **2.5 Analysis of fatty acid ethanolamides and endocannabinoids**

### **2.5.1 Preparation of standards**

For each of the commercially available AEA, 2-AG, ALA-EA, ST-EA, PA-EA, OA-EA, LA-EA, DHA-EA, and the internal standards AEA-*d8* and 2-AG-*d8*, a 0.01 mg was dissolved in 1 mL ethanol to give 10 ng/μL standard stock solutions. AEA-*d8* and 2-AG-*d8* (10 ng/μL) were then diluted further (1:10) with ethanol to yield final solutions of concentration 1 ng/μL. These solutions were aliquoted to 100 μL aliquots, each placed in a 1.5 mL amber vial. The vials were sealed and stored at -20 °C awaiting to be injected.

### **2.5.2 Calibration lines**

A composite solution of AEA, 2-AG, ALA-EA, ST-EA, PA-EA, OA-EA, LA-EA, DHA-EA was prepared by taking 40 μL of each standard stock solution (10 ng/μL) and adding 860 μL ethanol to the combined solution to yield a composite stock standard solution at a final concentration of 400 pg/μL. Dilutions of the composite solution were made in two ways, one without internal standards as shown in Table 2.2, and the other with internal standards as shown in Table 2.3.

### **2.5.3 Limit of quantitation and limit of detection**

Peak integrations and S/N calculations were performed using the MassLynx™ V.4.0 software. The LOQ was estimated using an S/N of 10. While the LOD was estimated using an S/N ratio of 3.

**Table 2.2.** Preparation of fatty acid ethanolamide and endocannabinoid composite solution without internal standards.

Volume ( $\mu\text{L}$ )			Final concentration of composite standard ( $\text{pg}/\mu\text{L}$ )
Composite stock standard 200 $\text{pg}/\mu\text{L}$	Ethanol	Final volume	
60	40	100	120
40	60	100	80
20	80	100	40
10	90	100	20
5	95	100	10
0.5	99.5	100	1

**Table 2.3.** Preparation of fatty acid ethanolamides and endocannabinoids composite solution with internal standards (AEA-d8 and 2-AG-d8).

Volume ( $\mu\text{L}$ )					Final concentration of composite standard ( $\text{pg}/\mu\text{L}$ )
Composite stock standard 200 $\text{pg}/\mu\text{L}$	AEA-d8 (1 $\text{ng}/\mu\text{L}$ )	2-AG-d8 (1 $\text{ng}/\mu\text{L}$ )	Ethanol	Final volume	
60	10	10	20	100	120
40	10	10	40	100	80
20	10	10	60	100	40
10	10	10	70	100	20
5	10	10	75	100	10
0.5	10	10	79.5	100	1

#### 2.5.4 Tissue homogenisation and extraction

Freshly prepared solution of chloroform: methanol (2:1, v/v) was aliquoted into clean glass vials, 3 mL per vial, and placed on ice, to be used for samples homogenisation to avoid cross contamination. The brain samples were removed from -80°C and placed on ice. Tissue was taken from central cortex of the brain (kept the same for all samples), and immediately the sliced wet weight (kept between 60 to 100 mg) was recorded. The brain tissue was transferred to a glass homogeniser (1 mL capacity), 500 µL (2:1, v/v) chloroform: methanol, was added and the tissue was homogenised (30x up/down strokes and 30x twist strokes). The extract was then transferred (leaving behind any tissue) to a clean flat bottom extraction tube and placed on ice. The homogenisation steps were repeated using a further 5 x 500 µL aliquots of (2:1, v/v) chloroform: methanol. With the last 500 µL, all the remaining tissues were transferred to the extraction tube. The glass homogeniser was rinsed and cleaned with Milli-Q water and ethanol between samples.

40 µL of each (1 ng/µL) internal standard (AEA-*d8* and 2-AG-*d8*) was added to each extraction tube. The extraction tubes were vortexed, then incubated on ice and in the dark for 60 min to ensure complete extraction and denaturation of proteins. 500 µL Milli-Q water was then added to introduce interface between the two layers, the sample was vortexed, and then centrifuged (3000rpm at 4 °C for 5 min) to ensure phase separation. The lower layer was then removed and transferred to a clean round bottom tube using a clean glass Pasteur pipette. The extracts were dried under a fine stream of nitrogen. The dried extract was then reconstituted in 300 µL 1% HPLC grade methanol/chloroform, centrifuged

at (3000rpm at 4 °C for 8 min), and then filtered using a tightly packed glass Pasteur pipette with glass wool, that had been washed with 1% methanol/chloroform. The extract was collected and placed on ice ready for clean up by solid phase extraction.

### **2.5.5 Solid phase extraction of fatty acid ethanolamides**

The solid phase extraction, Strata<sup>®</sup> SPE (1 mL, 100 mg silica-based) cartridges were activated prior to use as follows: The cartridges were attached to a 12 position vacuum manifold and washed with 1 mL chloroform. At the end of the washing phase the sorbent bed had become translucent confirming the silica was ready for sample application. The reconstituted brain extracts (in 300 µL 1% methanol/chloroform) were applied on an activated SPE cartridge. The cartridge was washed with 2x1 mL chloroform to remove any unwanted organic compounds. Fatty acid ethanolamides were eluted from the cartridge using 4x1 mL 10% (v/v) methanol in chloroform. Throughout the washing and elution procedure, vacuum was not applied as a drop wise flow of solvent was achieved by gravity, also care was taken not to allow air to pass through the sorbent. The eluted lipids were collected and placed on ice in a dark place. The solvent was then evaporated under nitrogen. The pressure of nitrogen was maintained such that the flow of gas over the solvent surface caused a gentle ripple. The flow was periodically adjusted as the level of the solvent decreased. Once the solvent had completely evaporated the remaining residue which contained the fatty acid ethanolamides was reconstituted in 50 µL ethanol and transferred to 100 µL insert vials using a glass syringe. The vials were then placed in 2 mL amber glass vials, gently flushed with nitrogen and sealed using

open screw cap with PTFE septa, and stored at -20°C for up to one week ready for LC/ESI-MS/MS analysis.

### **2.5.6 LC/ESI-MS/MS analysis of fatty acid ethanolamides**

In order to optimise the MS/MS conditions required for efficient ionisation of each analyte, individual standards at a concentration of 10 ng/ $\mu$ l were analysed by direct infusion through a syringe pump at flow rate 10  $\mu$ l/min, into the mobile phase at a flow rate of 0.2 ml/min. The sensitivity of method was optimised as the cone voltages and capillary voltages were altered in the range of 30-40eV and 2000-4000V respectively, and ions were formed at the positive ionisation mode  $[M+H]^+$ . Selective fragmentation of  $[M+H]^+$  of each analyte was performed with a collision energy (CE) of 12-20 eV. Argon was used as the collision gas. The source temperature was set at at 120°C and the desolvation temperature was set at at 360°C. The capillary voltage was set at 3500V and the cone voltage at 35 eV. The product ion spectra were recorded with a scan range  $m/z$  50-400. The MRM assay was set up using the most abundant precursor to product ion pairs at the optimal collision energy for each compound.

The FA-EA and 2-AG were separated using an isocratic system composed of two mobile phases (A and B) mixed at constant ratio of 30:70 (v/v). Mobile phase A was acetonitrile: water: formic acid, 2:98:0.1 (v/v/v) and Mobile phase B was acetonitrile: water: formic acid, 98:2:0.1 (v/v/v). All the analytes were eluted by 52 min, the run time was set at 69 min at flow rate at 0.2 mL/min to wash the column prior to the next sample injection. The separation was performed on a C18(2)(Luna 5  $\mu$ l, 150 x 2.0mm) column. The injection volume was 10  $\mu$ l. The autosampler chamber temperature was maintained at 8°C.

## 2.5.7 Recovery

To assess the recovery of the extraction methodology, brain tissue was homogenized as described in section 2.5.4 and spiked with 40  $\mu\text{L}$  of AEA-d8 (1  $\text{ng}/\mu\text{L}$ ) and 25  $\mu\text{L}$  of composite stock standard (200  $\text{pg}/\mu\text{L}$ ). The extract was semi purified by SPE as described in section 2.5.5, and analysed by LC/ESI-MS/MS as described in section 2.5.6. At the same time, and in parallel to the spiked brain sample, a set of un-spiked brain samples were homogenized, extracted and analysed. Moreover, 25  $\mu\text{L}$  of composite stock standard (200  $\text{pg}/\mu\text{L}$ ) was directly analysed by LC/ESI-MS/MS. The peak area of the analytes were integrated and used to evaluate the recovery of the spiked standards by using the following equation:

$$\% \text{ recovery} = \frac{\text{Peak area of spiked sample} - \text{Peak area of unspiked sample}}{\text{Peak area of directly analysed standard}} \times 100$$

## 2.6 Analysis of oxysterols

### 2.6.1 Plasma sterol extraction

The extraction of plasma sterols, including cholesterol and oxysterol species, was performed using a protocol based on the method of Evershed et al (Evershed et al., 1999, Edwards et al., 2009). In detail:

To avoid any possibility of contamination all glassware was rinsed 3 times in DCM. Gloves and glass syringes were used at all times. 11 Plasma samples and 1 method blank were processed in one batch. Both vials and lids were labelled by a unique sample identifier for example T1 to T12. 1mL Plasma of each biological sample was transferred to the corresponding vials. Then, lipid was extracted by adding 1mL of DCM: methanol (2:1 v/v) to each sample. All

vials were kept in an ice box, and then left to stand at room temperature for 15 min. All contents of vials T1 to T12 were pipetted off to other clean vials labelled as extraction vials E1 to E12. Then 2  $\mu\text{L}$  of (1.55 $\mu\text{g}/\mu\text{L}$ ) 4 $\beta$ -hydroxycholesterol (*d7*) (IS) were added to each vial (E1 to E12). The sample vials T1 to T12 were then washed off using another 1mL of DCM: methanol (2:1 v/v) and pipetted off to the corresponding E1 to E12 vials. Vials E1 to E12 were sonicated for 5 min in an ultrasonic water bath, and centrifuged at 4 °C, 2000 rpm for 5 min. The lower layer containing the lipid extract was pipetted off into clean vials labelled as derivatisation vials D1 to D12. To ensure complete extraction of lipids, 1mL of DCM: methanol (2:1 v/v) were added to the residue left in vials E1 to E12, sonicated for 5 min in an ultrasonic water bath, and centrifuged at 4 °C, 2000 rpm for 5 min. Then, the lower layer containing the lipid extract was pipetted off and combined with the lipid extract in the corresponding vials (D1 to D12). This step was repeated twice. The combined lipid extract in D1 to D12 vials was reduced under a stream of nitrogen with gentle heat (heating block surface 40 °C) to about 2 mL, and then transferred to clean small vials labelled D1 to D12. The extract was then gently evaporated under a stream of nitrogen to dryness. Then the samples were ready for derivatisation.

### **2.6.2 Sterol derivatization**

All samples were derivatized to trimethylsilyl ethers as follows: 5-10 drops of BSTFA with 1% TMCS were added to each dry residue using a Pasteur pipette. Then 1 $\mu\text{L}$  C34 (1.55 $\mu\text{g}/\mu\text{L}$ ) was added to each vial of dry residue (reference standard). Then, the samples were left to stand overnight at room temperature for the derivatization reaction to complete. The excess BSTFA was evaporated

off to dryness under nitrogen with gentle heat (heating block surface 40°C). The dry lipid residue containing sterol TMS-ethers were reconstituted in 50µL DCM and analysed by GC-MS which were stable at room temperature for two days.

### **2.6.3 GC-MS analysis of sterols**

The sterol TMS-ethers analysis was carried out by GC-MS. The splitless injector and interface were maintained at 300 °C and 340 °C respectively. Helium was the carrier gas at constant flow at a flow rate of 21 cm/s. The temperature of the oven was programmed to run from 50 °C (2 min) to 350 °C (10 min) at 10 °C/min. The total run time was 40 min. The GC column (15m x 0.25mm, 0.25mm HP-5MS 5% phenyl methyl siloxane phase fused silica) was directly inserted into the ion source where electron impact (EI) spectra were obtained at 70 eV with full scan from *m/z* 50 to 800. All peaks in the total ion chromatograms (TIC) were integrated and normalised based on the peak area of the IS. Data were expressed as mean ± standard deviation (SD).

### **2.7 Statistical analysis**

Statistical analysis was undertaken using the statistical package SPSS version 16.0. Comparison of any two groups was based on the two-tailed independent samples Student's *t* test. To compare more than two groups data was analysed using one way ANOVA with a Bonferroni's post hoc correction for multiple comparisons. Data for the FA-EA and EC study was non-parametric and was analysed using Kruskal-Wallis test with adjustment for multiple comparisons. A value of  $p < 0.05$  was considered to be statistically significant.

**Chapter 3: The effect of eicosapentaenoic and docosapentaenoic acid supplementation on rat red blood cell fatty acids and brain sphingomyelin**

### 3.1 Introduction

Several studies have shown that n-3 PUFA are important for brain function ( Bourre et al., 1988, Wainwright, 1992, Bourre et al., 1993, Innis, 2007, Petursdottir et al., 2008), particularly DHA is associated with brain development and memory (Das, 2003). Deficiencies in n-3 PUFA are implicated in diseases such as cardiovascular disease (Wijendran and Hayes, 2004), Huntington's (Muralikrishna and Hatcher, 2007), bipolar disorder (Stoll et al., 1999a), attention-deficit hyperactivity disorder (ADHD) (Richardson and Puri, 2000a), learning impairments (Ikemoto et al., 2001), schizophrenia and depression (Peet, 2003). It has been indicated that increased n-3 PUFA consumption can reduce many risk factors associated with several diseases (Seo et al., 2005). Moreover, their roles in decreasing effects of neurological deterioration associated with ageing in aged animals have been reported (McGahon et al., 1999b). In addition, oxidative stress a major risk factor has also been described to increase with age and found to be associated with a deficit in neurological functions (O'Donnell et al., 2000) and brain ageing (Floyd and Hensley, 2002).

EPA is considered to be a potent bioactive n-3 PUFA. It has been reported that synthesis of EPA from its precursor ALA (18:3n-3) is relatively inefficient, as a result of this transport of n-3 PUFA from plasma to brain may play important roles because of the limited ability of brain to synthesise EPA, in case of high demand for it (Edmond, 2001, Qi et al., 2002). Therefore, efficient tissue accretion of n-3 fatty acids has been indicated to be significantly dependant on the delivery of EPA directly from dietary sources, rather than from its precursor (Deckelbaum et al., 2006). EPA can be increased by feeding ALA, particularly

when restricting LA intake. In addition, n-3 DPA is also found increased by dietary ALA, thus DPA may have comparable biological effects to EPA (Smink et al., 2010, Smink et al., 2012,). Furthermore, it has been demonstrated that EPA supplementation may improve recovery from several diseases associated with deficiency of n-3 PUFA (Jho et al., 2004). EPA is a precursor of DHA (Qiu, 2003, Leonard et al., 2004) and so can play a major role in the supply of DHA. It has been shown that an intermediate step in the metabolism of DHA from EPA involves the production of n-3 DPA (Figure 1.1) which is an elongated metabolite of EPA (Sprecher, 2000). It has been found evident in a number of tissues that n-3 DPA can also be retro converted to EPA (Gotoh et al., 2009, Kaur et al., 2010), but does not appear to be readily metabolised to DHA, with limited conversion of n-3 DPA to DHA occurring mainly in liver (Voss et al., 1991). However, the literature on n-3 DPA is limited, and its dietary metabolic fate in mammals is currently unknown. Also its effect on brain ageing and bioactive lipid mediators and related pathways have not been extensively studied because of its limited availability as a pure compound (Kaur et al., 2011).

Recent studies have reported beneficial effects of increasing dietary intake of n-3 PUFA. In vitro studies (Kim and Chung, 2007) and in vivo studies (Lonergan et al., 2002, Martin et al., 2002b) have shown that both EPA and DHA possess anti-inflammatory and anti-oxidative properties. Also, similar effects have been reported in aged rats (McGahon et al., 1999a, Little et al., 2007). Furthermore, a recent study reported that aged rats fed on either EPA or n-3 DPA showed neuroprotective effects, and that both n-3 DPA and EPA might reduce the age-related oxidative changes (Kelly et al., 2011).

On the other hand, it has been reported that n-3 PUFA are involved in many pathways such as inhibition or modulation of eicosanoid pathways, which leads to alteration of inflammatory responses. Also, incorporation of n-3 fatty acids into membrane phospholipids effects the composition of membranes. Thus, because these pathways are highly interactive, the biological potential of n-3 PUFA on health and disease must be due to their multiple coordinated mechanisms (Seo et al., 2005). Ceramide, which is generated by hydrolysis of sphingomyelin by activity of sphingomyelinase can stimulate an increase in reactive oxygen species (Pettus et al., 2002), it is also metabolized by sphingomyelin synthase to produce SM (Kitatani et al., 2008) (Figure 1.3). It has been recently reported that EPA and n-3 DPA altered ceramide levels in rat brain (Kelly et al., 2011). Therefore, it is expected that SM and its species may be also altered in aged rat brain by EPA and n-3 DPA supplementation. Moreover, it has been found that n-3 PUFA increase the hydrolysis of plasma membrane SM to ceramide and phosphocholine, and interact with different steps of sphingolipid metabolism. Thus, hydrolysis of SM could affect other metabolic pathways and be involved in regulation of lipid homeostasis such as cellular cholesterol homeostasis (Worgall et al., 2002, Eberle et al., 2004, Deckelbaum et al., 2006). Also ceramide itself has effects on sphingolipid synthesis (Worgall et al., 2004). Therefore, it has been hypothesised that the long-chain n-3 PUFA; EPA and DPA could impact positively on numerous roles and functions of SM in nervous systems such as retardation of ageing.

To date there are no studies investigating the influence of EPA and n-3 DPA supplementation on brain SM. To investigate the manipulation of EPA and n-3

DPA supplementation, RBC fatty acids will be measured to assess compliance and brain SM species in samples prepared from adult and aged rats in each of the treatment groups were analysed by GC and LC-ESI-MS/MS respectively. This study aims to evaluate the effects of EPA and/or n-3 DPA supplementation on changes in RBC fatty acids and brain SM species.

## **3.2 Materials and methods**

All animal work and sample collection was performed by Professor MA Lynch's group, Trinity College, Dublin, Ireland as described in section 2.2.1. All materials used for this study are described in detail in section 2.1.1. Fatty acid analysis method details including solvent preparation, standard preparation and RBC lipid extraction are described in sections 2.3.1, 2.3.2, 2.3.3 & 2.3.4. Fatty acid analysis is performed using GC as described in sections 2.3.6, 2.3.7 & 2.3.8. SM analysis method details including standard preparation and brain lipid extraction are described in sections 2.4.1 & 2.4.2. SM species analysis was performed using LC/ESI-MS/MS as described in section 2.4.3.

## **3.3 Results**

### **3.3.1 The effect of age on rat red blood cell fatty acids**

All fatty acids detected in RBC of both adult (3-4 months) and aged (22-24 months) animals are tabulated in Appendix 1, Table 2. The profile of RBC fatty acids in adult and aged animals is represented in Table 3.1. In detail: The highest weight % of SFA in the RBC of both groups was C16:0 followed by C18:0; the highest weight % of MUFA was C18:1n-9c; the highest weight % of

n-6 PUFA was C20:4n-6 followed by C18:2n-6c, and the highest weight % of n-3 PUFA detected was C22:6n-3.

Statistically significant differences were observed in the concentration of fatty acids in RBC when comparing adult and aged animals. The concentration of the following fatty acids C14:0 ( $p = 0.01$ ), C20:0 ( $p = 0.01$ ), C24:0 ( $p = 0.004$ ) C16:1 ( $p = 0.009$ ), C18:1n-9c ( $p = 0.039$ ), C22:1n-9 ( $p = 0.003$ ) and C22:6n-3 ( $p = 0.001$ ) was significantly higher in aged animals compared to adult ones. Conversely, the concentration of C24:1 ( $p = 0.01$ ), C20:4n-6 ( $p = 0.019$ ) and C18:3n-3 ( $p = 0.022$ ) was significantly lower in aged animals compared to adult ones. Interestingly, the concentration of total n-3 PUFA was not found to be different.

### **3.3.2 The effect of EPA and n-3 DPA supplementation on red blood cell fatty acids in adult animals**

All fatty acids detected in the RBC of adult animals (3-4 months) supplemented with 200 mg/kg/day EPA or 200 mg/kg/day n-3 DPA are tabulated in Appendix 1, Table 1. The profile of RBC fatty acids in adult animals (3-4 months) following supplementation with 200 mg/kg/day EPA or 200 mg/kg/day n-3 DPA for 8 weeks is shown in Table 3.2. In detail: The highest weight % of SFA in RBC of both groups was C16:0 followed by C18:0, C22:0, C17:0 and C24:0; the highest weight % of MUFA was C18:1n-9c followed by C18:1n-7, C17:1, C22:1n-9 and C24:1; the highest weight % of n-3 PUFA was C22:5n-3 followed by C22:6n-3, C18:3n-3, and C20:5n-3, and the highest weight % of n-6 PUFA was C20:4n-6 followed by C18:2n-6c, C20:3n-6 and C20:2.

**Table 3.1.** Fatty acid composition of red blood cells in adult (3-4 months) and aged (22-24 months) animals. Results expressed as weight % of total fatty acids (mean  $\pm$  SD); (n = 6 animals per group), \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001. MFUA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids, UFA: unsaturated fatty acids, ND: not detected.

FATTY ACIDS	Weight % of total fatty acids	
	Adult	Aged
C14:0	0.3 $\pm$ 0.1	0.5 $\pm$ 0.2*
C15:0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1
C16:0	26.9 $\pm$ 1.0	25.5 $\pm$ 0.5
C17:0	1.4 $\pm$ 0.4	0.6 $\pm$ 0.2**
C18:0	13.6 $\pm$ 1.4	12.9 $\pm$ 1.5
C20:0	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1*
C22:0	1.7 $\pm$ 0.3	1.3 $\pm$ 0.3
C23:0	0.2 $\pm$ 0.0	0.2 $\pm$ 0.1
C24:0	0.7 $\pm$ 0.0	2.1 $\pm$ 0.2**
<b><math>\Sigma</math> SFA</b>	<b>45.2 <math>\pm</math> 1.9</b>	<b>43.9 <math>\pm</math> 1.0</b>
C14:1	ND	ND
C15:1	ND	ND
C16:1	0.2 $\pm$ 0.0	1.0 $\pm$ 0.4**
C17:1	1.4 $\pm$ 0.1	1.1 $\pm$ 0.4
C18:1n-9t	0.3 $\pm$ 0.0	0.2 $\pm$ 0.1
C18:1n-9c	6.2 $\pm$ 1.0	7.8 $\pm$ 3.3*
C18:1n-7	2.5 $\pm$ 0.1	2.6 $\pm$ 0.9
C20:1n-9	0.1 $\pm$ 0.0	0.2 $\pm$ 0.1
C22:1n-9	0.2 $\pm$ 0.0	0.9 $\pm$ 0.4**
C24:1	0.8 $\pm$ 0.1	0.4 $\pm$ 0.2*
<b><math>\Sigma</math> MUFA</b>	<b>11.8 <math>\pm</math> 1.0</b>	<b>14.1 <math>\pm</math> 4.3*</b>
C18:2n-6t	0.1 $\pm$ 0.0	0.2 $\pm$ 0.1
C18:2n-6c	12.9 $\pm$ 1.7	12.2 $\pm$ 0.1
C18:3n-6	ND	ND
C20:2	0.4 $\pm$ 0.0	0.2 $\pm$ 0.1
C20:3n-6	0.4 $\pm$ 0.0	0.4 $\pm$ 0.1
C20:4n-6	22.8 $\pm$ 1.5	18.4 $\pm$ 3.5*
C22:2	ND	ND
<b><math>\Sigma</math> n-6 PUFA</b>	<b>36.5 <math>\pm</math> 1.5</b>	<b>31.4 <math>\pm</math> 3.1</b>
C18:3n-3	2.1 $\pm$ 0.7	1.2 $\pm$ 0.3*
C20:3n-3	ND	ND
C20:5n-3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0
C22:5n-3	1.8 $\pm$ 0.2	1.7 $\pm$ 0.2
C22:6n-3	3.0 $\pm$ 0.3	3.5 $\pm$ 0.9**
<b><math>\Sigma</math> n-3 PUFA</b>	<b>8.2 <math>\pm</math> 0.6</b>	<b>6.1 <math>\pm</math> 1.9</b>

Statistically significant differences were observed in the concentration of fatty acids in RBC when comparing adult animals supplemented with either EPA or n-3 DPA to controls. The concentration of the following fatty acids C22:0 ( $p = 0.008$ ) and C23:0 ( $p = 0.046$ ) were significantly lower in adult animals supplemented with n-3 DPA compared to control ones. Whereas the concentration of C24:0 ( $p = 0.01$ ) and C17:1 ( $p = 0.026$ ) was significantly lower in adult animals supplemented with EPA compared to control ones.

Conversely, the concentration of C17:0 ( $p = 0.0001$ ) and C24:1 ( $p = 0.002$ ) was significantly lower in adult animals supplemented with either EPA or n-3 DPA compared to control ones. While, the concentration of C22:1n-9 ( $p = 0.015$ ), C18:3n-3 ( $p = 0.046$ ), C20:5n-3 ( $p = 0.003$ ) and C22:5n-3 ( $p = 0.0001$ ) was significantly higher in adult animals supplemented with either EPA or n-3 DPA compared to control ones. Moreover, the concentration of C22:1n-9 ( $p = 0.015$ ) and C24:0 ( $p = 0.0001$ ) was significantly lower in adult animals supplemented with EPA compared to adult animals supplemented with n-3 DPA.

Finally, the concentration of C22:0 ( $p = 0.008$ ) and C24:0 ( $p = 0.001$ ) and C20:5n-3 ( $p = 0.0001$ ) was significantly higher in adult animals supplemented with EPA compared to adult animals supplemented with n-3 DPA. Interestingly, no difference was found in the concentration of C20:4n-6 between both groups compared to control ones.

**Table 3.2.** Fatty acid composition of red blood cells in adult animals (3-4 months) following supplementation with EPA 200 mg/kg/day or n-3 DPA 200 mg/kg/day for 8 weeks. Results expressed as total fatty acids weight % (mean  $\pm$  SD); (n = 6 per group). \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001. ND: not detected, MFUA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids.

FATTY ACIDS	Weight % of total fatty acids		
	Control	n-3 DPA	EPA
C14:0	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1
C15:0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0
C16:0	26.9 $\pm$ 1.0	25.5 $\pm$ 0.6	26.7 $\pm$ 1.3
C17:0	1.4 $\pm$ 0.4	0.6 $\pm$ 0.1***	0.6 $\pm$ 0.0***
C18:0	13.6 $\pm$ 1.4	13.3 $\pm$ 0.9	13.8 $\pm$ 1.0
C20:0	0.3 $\pm$ 0.1	0.4 $\pm$ 0.0	0.3 $\pm$ 0.1
C22:0	1.7 $\pm$ 0.3	1.3 $\pm$ 0.2**	1.5 $\pm$ 0.2**
C23:0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0*	0.2 $\pm$ 0.0
C24:0	0.7 $\pm$ 0.0	0.7 $\pm$ 0.1	0.5 $\pm$ 0.1**
<b><math>\Sigma</math> SFA</b>	<b>45.2 <math>\pm</math> 1.9</b>	<b>42.2 <math>\pm</math> 1.2</b>	<b>44.3 <math>\pm</math> 2.1</b>
C14:1	ND	ND	ND
C15:1	ND	ND	ND
C16:1	0.2 $\pm$ 0.0	0.2 $\pm$ 0.0	0.3 $\pm$ 0.1
C17:1	1.4 $\pm$ 0.1	1.2 $\pm$ 0.1*	1.2 $\pm$ 0.1*
C18:1n-9t	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0
C18:1n-9c	6.2 $\pm$ 1.0	6.4 $\pm$ 1.0	6.3 $\pm$ 1.5
C18:1n-7	2.5 $\pm$ 0.1	2.4 $\pm$ 0.1	2.3 $\pm$ 0.1
C20:1n-9	0.1 $\pm$ 0.0	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0
C22:1n-9	0.2 $\pm$ 0.0	1.1 $\pm$ 0.2**	0.7 $\pm$ 0.0**
C24:1	0.8 $\pm$ 0.1	0.5 $\pm$ 0.1**	0.6 $\pm$ 0.1**
<b><math>\Sigma</math> MUFA</b>	<b>11.8 <math>\pm</math> 1.0</b>	<b>12.7 <math>\pm</math> 1.0</b>	<b>11.4 <math>\pm</math> 1.5</b>
C18:2n-6t	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
C18:2n-6c	12.9 $\pm$ 1.7	14.2 $\pm$ 1.7	13.9 $\pm$ 2.4
C18:3n-6	ND	ND	ND
C20:2	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1
C20:3n-6	0.4 $\pm$ 0.0	0.5 $\pm$ 0.0	0.5 $\pm$ 0.1
C20:4n-6	22.8 $\pm$ 1.5	22.0 $\pm$ 1.4	20.6 $\pm$ 2.3
C22:2	ND	ND	ND
<b><math>\Sigma</math> n-6 PUFA</b>	<b>36.5 <math>\pm</math> 1.5</b>	<b>37.1 <math>\pm</math> 1.0</b>	<b>35.4 <math>\pm</math> 1.2</b>
C18:3n-3	2.1 $\pm$ 0.7	2.6 $\pm$ 0.7*	2.7 $\pm$ 0.3*
C20:3n-3	ND	ND	ND
C20:5n-3	0.2 $\pm$ 0.1	0.6 $\pm$ 0.1**	1.1 $\pm$ 0.2***
C22:5n-3	1.8 $\pm$ 0.2	3.6 $\pm$ 0.4***	3.3 $\pm$ 0.4***
C22:6n-3	3.0 $\pm$ 0.3	2.8 $\pm$ 0.1	2.8 $\pm$ 0.3
<b><math>\Sigma</math> n-3 PUFA</b>	<b>8.2 <math>\pm</math> 0.6</b>	<b>9.6 <math>\pm</math> 0.8</b>	<b>10.5 <math>\pm</math> 0.8</b>

### **3.3.3 The effect of EPA and n-3 DPA supplementation on red blood cell fatty acids in aged animals**

All fatty acids detected in the RBC of aged animals (22-24 months) supplemented with 200 mg/kg/day EPA or 200 mg/kg/day n-3 DPA are tabulated in Appendix 1, Table 1.

The profile of RBC fatty acids in aged animals (22-24 months) following supplementation with 200 mg/kg/day EPA or 200 mg/kg/day n-3 DPA for 8 weeks is presented in detail in Table 3.3.

The highest weight % of SFA in RBC of both groups was C16:0 followed by C18:0; the highest weight % of MUFA was C18:1n-9c followed by C18:1n-7, C17:1; the highest weight % of n-3 PUFA was C22:6n-3 followed by C22:5n-3, C20:5n-3 and C18:3n-3, and the highest weight % of n-6 PUFA was C20:4n-6 followed by C18:2n-6c, C20:3n-6. Statistically significant differences were observed in the concentration of fatty acids in RBC within the aged subjects supplemented with either EPA or n-3 DPA. The concentration of the following fatty acids C15:0 ( $p = 0.001$ ), C16:0 ( $p = 0.035$ ), C17:1 ( $p = 0.028$ ) and C22:5n-3 ( $p = 0.0001$ ) was significantly higher in adult animals supplemented with n-3 DPA compared to control ones.

While the concentration of C17:1 ( $p = 0.028$ ), C24:1 ( $p = 0.0001$ ), C20:5n-3 ( $p = 0.0001$ ) and C22:5n-3 ( $p = 0.0001$ ) was significantly higher in aged animals supplemented with EPA compared to control ones. Moreover, the concentration of C24:0 ( $p = 0.001$ ), C16:1 ( $p = 0.0001$ ) and C18:3n-3 ( $p = 0.008$ ) was significantly lower in aged animals supplemented with n-3 DPA compared to control ones, and the concentration of C24:0 ( $p = 0.0001$ ) and C22:1n-9 was

significantly lower in aged animals supplemented with EPA were significantly lower compared to control ones.

On the other hand, the concentration of C15:0 ( $p = 0.001$ ), C16:0 ( $p = 0.008$ ), C24:0 ( $p = 0.0001$ ), C24:1 ( $p = 0.0001$ ) and C20:5n-3 ( $p = 0.0001$ ) was significantly higher, while the concentrations of C16:1 ( $p = 0.045$ ) and C18:3n-3 ( $p = 0.012$ ) were significantly lower in aged animals supplemented n-3 DPA compared to those animals being supplemented with EPA. Conversely, the concentration of C20:4n-6 was found to have no significant changes in aged animals supplemented with either EPA or n-3 DPA compared to control ones.

#### **3.3.4 The effect of n-3 DPA long-term supplementation on red blood cell fatty acids of aged animals.**

All the fatty acid detected in the RBC of aged animals (17-18 months) supplemented with 100 mg/rat/day n-3 DPA for 3 months or 50 mg/kg/day n-3 DPA for 3 months, followed by 100 mg/kg/day for a further 3 months as presented in (appendix 1, Table 1).

The RBC fatty acid profile in aged animals (17-18 months) following supplementation with 100 mg/rat/day n-3 DPA for 3 months or 50 mg/kg/day n-3 DPA for 3 months, followed by 100 mg/kg/day for a further 3 months is presented in detail in Table 3.4. The highest weight % of SFA in RBC of both groups was C16:0 followed by C18:0, C24:0 and C22:0; the highest weight % of MUFA was C18:1n-9c followed by C18:1n-7, C17:1 and C24:1; the highest weight % of n-3 PUFA was C22:6n-3 followed by C22:5n-3 and C18:3n-3, and the highest weight % of n-6 PUFA was C20:4n-6 followed by C18:2n-6c, C20:3n-6 and C20:2.

Statistically significant differences were observed when the concentration of RBC fatty acids of aged animals was compared to the ones supplemented with n-3 DPA for either 3 or 6 months. The concentration of C14:0 ( $p = 0.019$ ), C17:0 ( $p = 0.036$ ) and C22:5n-3 ( $p = 0.0001$ ) was significantly higher, while the concentration of C24:0 ( $p = 0.0001$ ) and C18:3n-3 ( $p = 0.0001$ ) was significantly lower in aged animals supplemented with n-3 DPA for 6 months compared to the control group. Conversely, the concentration of C17:0 ( $p = 0.036$ ), C20:0 ( $p = 0.001$ ), C22:5n-3 ( $p = 0.000$ ) and C22:6n-3 ( $p = 0.015$ ) was significantly higher. The concentration of C18:3n-3 ( $p = 0.043$ ) was significantly lower in aged animals supplemented with n-3 DPA for 3 months compared to the control group. Moreover, the concentration of C20:0 ( $p = 0.005$ ) and C24:0 ( $p = 0.029$ ) was significantly higher in aged animals supplemented with n-3 DPA for 3 months compared to aged animals supplemented with n-3 DPA for 6 months. Interestingly, the concentration of C20:4n-6 was not found to be different in aged animals supplemented with n-3 DPA for either 3 or 6 months when compared to the control group.

**Table 3.3.** Fatty acid composition of red blood cells in aged animals (22-24 months) following supplementation with EPA 200 mg/kg/day or n-3 DPA 200 mg/kg/day for 8 weeks. Results expressed as total fatty acids weight % (mean  $\pm$  SD); (n = 6 animals per group). \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001. MFUA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids, UFA: unsaturated fatty acids, ND: not detected.

FATTY ACIDS	Weight % of total fatty acids		
	Control	n-3 DPA	EPA
C14:0	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1	0.4 $\pm$ 0.2
C15:0	0.3 $\pm$ 0.1	0.6 $\pm$ 0.2**	0.3 $\pm$ 0.0**
C16:0	25.5 $\pm$ 0.5	26.7 $\pm$ 1.1*	25.3 $\pm$ 0.3**
C17:0	0.6 $\pm$ 0.2	0.6 $\pm$ 0.0	0.5 $\pm$ 0.0
C18:0	12.9 $\pm$ 1.5	12.6 $\pm$ 1.3	12.5 $\pm$ 2.4
C20:0	0.5 $\pm$ 0.1	0.3 $\pm$ 0.2	0.4 $\pm$ 0.1
C22:0	1.3 $\pm$ 0.3	1.3 $\pm$ 0.4	1.1 $\pm$ 0.2
C23:0	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0
C24:0	2.1 $\pm$ 0.2	1.5 $\pm$ 0.3***	1.3 $\pm$ 0.4***
<b><math>\Sigma</math> SFA</b>	<b>43.9 <math>\pm</math> 1.0</b>	<b>44.0 <math>\pm</math> 0.9</b>	<b>41.7 <math>\pm</math> 2.4</b>
C14:1	ND	ND	ND
C15:1	ND	ND	ND
C16:1	1.0 $\pm$ 0.4	0.3 $\pm$ 0.0***	0.7 $\pm$ 0.2*
C17:1	1.1 $\pm$ 0.4	1.5 $\pm$ 0.1**	1.4 $\pm$ 0.2**
C18:1n-9t	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.0
C18:1n-9c	7.8 $\pm$ 3.3	8.3 $\pm$ 1.7	9.1 $\pm$ 2.6
C18:1n-7	2.6 $\pm$ 0.9	2.9 $\pm$ 0.3	3.0 $\pm$ 0.5
C20:1n-9	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0
C22:1n-9	0.9 $\pm$ 0.4	0.6 $\pm$ 0.1	0.2 $\pm$ 0.1***
C24:1	0.4 $\pm$ 0.2	0.8 $\pm$ 0.1***	0.6 $\pm$ 0.1***
<b><math>\Sigma</math> MUFA</b>	<b>14.1 <math>\pm</math> 4.3</b>	<b>14.8 <math>\pm</math> 1.8</b>	<b>15.4 <math>\pm</math> 3.0</b>
C18:2n-6t	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.2 $\pm$ 0.1
C18:2n-6c	12.2 $\pm$ 0.1	12.8 $\pm$ 1.9	14.0 $\pm$ 2.9
C18:3n-6	ND	ND	ND
C20:2	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0	0.2 $\pm$ 0.1
C20:3n-6	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.6 $\pm$ 0.2
C20:4n-6	18.4 $\pm$ 3.5	21.0 $\pm$ 2.1	18.4 $\pm$ 2.4
C22:2	ND	ND	ND
<b><math>\Sigma</math> n-6 PUFA</b>	<b>31.4 <math>\pm</math> 3.1</b>	<b>33.5 <math>\pm</math> 0.8</b>	<b>33.5 <math>\pm</math> 1.2</b>
C18:3n-3	1.2 $\pm$ 0.3	0.5 $\pm$ 0.2**	1.1 $\pm$ 0.4*
C20:3n-3	ND	ND	ND
C20:5n-3	0.2 $\pm$ 0.0	0.6 $\pm$ 0.1***	2.0 $\pm$ 0.5***
C22:5n-3	1.7 $\pm$ 0.2	3.2 $\pm$ 0.3***	3.1 $\pm$ 0.5***
C22:6n-3	3.5 $\pm$ 0.9	3.8 $\pm$ 0.5	3.4 $\pm$ 0.6
<b><math>\Sigma</math> n-3 PUFA</b>	<b>6.1 <math>\pm</math> 1.9</b>	<b>8.2 <math>\pm</math> 1.0</b>	<b>9.8 <math>\pm</math> 1.5</b>

**Table 3.4.** Fatty acid composition of red blood cells in aged animals (17-18 months) supplemented with n-3 DPA 100 mg/rat/day for 3 and 6 months. Results expressed as total fatty acids weight % (mean  $\pm$  SD); (n = 6 per group). \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001. PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids, UFA: unsaturated fatty acids, MFUA: monounsaturated fatty acids, ND: not detected.

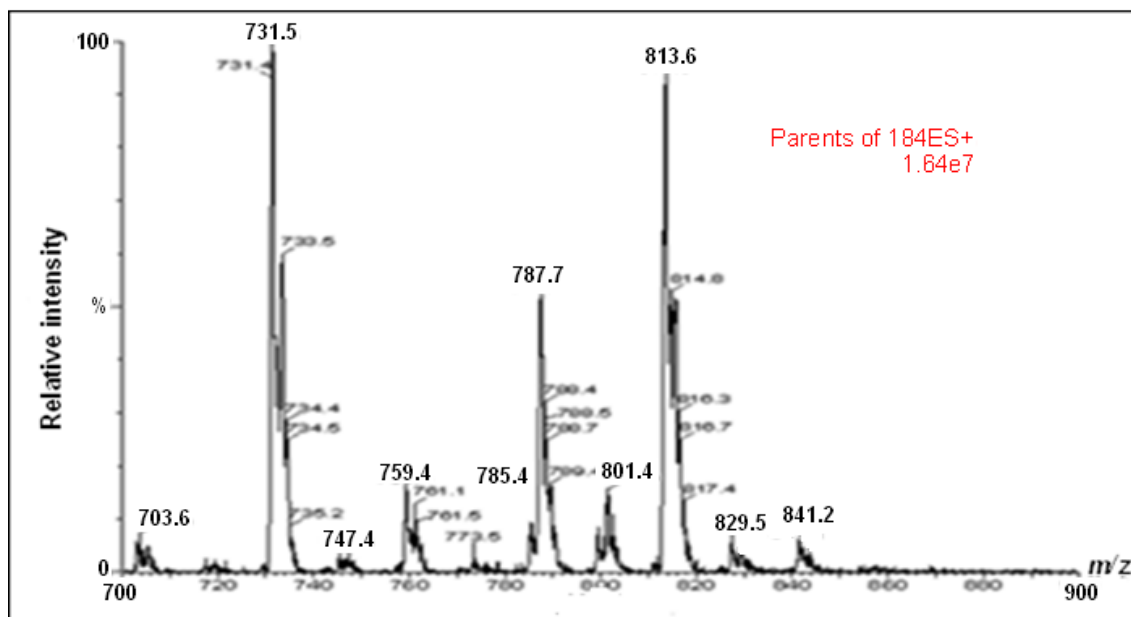
FATTY ACIDS	Weight % of total fatty acids		
	Aged animals	Post n-3 DPA	Post EPA
C14:0	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0	0.3 $\pm$ 0.1*
C15:0	0.3 $\pm$ 0.1	0.2 $\pm$ 0.0	0.3 $\pm$ 0.0
C16:0	25.1 $\pm$ 1.6	26.1 $\pm$ 1.9	26.7 $\pm$ 0.8
C17:0	0.4 $\pm$ 0.1	0.6 $\pm$ 0.1*	0.6 $\pm$ 0.0*
C18:0	13.1 $\pm$ 1.3	14.5 $\pm$ 1.3	13.8 $\pm$ 1.4
C20:0	0.4 $\pm$ 0.1	0.9 $\pm$ 0.3**	0.5 $\pm$ 0.1**
C22:0	1.1 $\pm$ 0.2	1.3 $\pm$ 0.2	1.1 $\pm$ 0.2
C23:0	0.1 $\pm$ 0.0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0
C24:0	2.7 $\pm$ 0.2	2.5 $\pm$ 0.1*	2.1 $\pm$ 0.2***
<b><math>\Sigma</math> SFA</b>	<b>43.6 <math>\pm</math> 1.9</b>	<b>46.5 <math>\pm</math> 0.9</b>	<b>45.4 <math>\pm</math> 1.4</b>
C14:1	ND	ND	ND
C15:1	ND	ND	ND
C16:1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0
C17:1	1.5 $\pm$ 0.2	1.4 $\pm$ 0.3	1.5 $\pm$ 0.1
C18:1n-9t	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0
C18:1n-9c	7.6 $\pm$ 1.3	6.6 $\pm$ 0.7	6.9 $\pm$ 0.6
C18:1n-7	2.4 $\pm$ 0.2	2.4 $\pm$ 0.1	2.4 $\pm$ 0.3
C20:1n-9	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
C22:1n-9	0.3 $\pm$ 0.1	0.2 $\pm$ 0.0	0.3 $\pm$ 0.1
C24:1	0.6 $\pm$ 0.1	0.7 $\pm$ 0.1	0.6 $\pm$ 0.2
<b><math>\Sigma</math> MUFA</b>	<b>13.2 <math>\pm</math> 1.2</b>	<b>12.0 <math>\pm</math> 0.7</b>	<b>12.4 <math>\pm</math> 0.5</b>
C18:2n-6t	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
C18:2n-6c	13.3 $\pm$ 2.1	12.5 $\pm$ 0.7	14.1 $\pm$ 1.4
C18:3n-6	ND	ND	ND
C20:2	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1
C20:3n-6	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.4 $\pm$ 0.0
C20:4n-6	22.7 $\pm$ 2.1	21.0 $\pm$ 2.1	20.9 $\pm$ 1.8
C22:2	ND	ND	ND
<b><math>\Sigma</math> n-6 PUFA</b>	<b>39.4 <math>\pm</math> 2.2</b>	<b>34.4 <math>\pm</math> 2.2</b>	<b>35.9 <math>\pm</math> 1.7</b>
C18:3n-3	1.9 $\pm$ 0.6	1.1 $\pm$ 1.2*	0.3 $\pm$ 0.1***
C20:3n-3	ND	ND	ND
C20:5n-3	0.2 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1
C22:5n-3	1.3 $\pm$ 0.3	2.3 $\pm$ 0.4***	2.4 $\pm$ 0.2***
C22:6n-3	3.1 $\pm$ 0.4	3.9 $\pm$ 0.3*	3.4 $\pm$ 0.4
<b><math>\Sigma</math> n-3 PUFA</b>	<b>6.5 <math>\pm</math> 1.0</b>	<b>7.6 <math>\pm</math> 0.8</b>	<b>6.4 <math>\pm</math> 0.4</b>

### 3.3.5 LC/ESI-MS/MS analysis of brain sphingomyelin species

#### 3.3.5.1 Method development using bovine brain extract

Semi-purified SM extract from bovine brain and SM d18:1/12:0 were prepared in mobile phase (70:30:0.1, acetonitrile: methanol: glacial acetic acid). Solutions of 100 µg/mL SM semi-purified extract and 10 ng/mL SM d18:1/12:0 were used for: a) MS/MS general scans and b) to establish retention times and MRM transition. General scans were acquired by direct infusion of these solutions as described in section 2.4.3 and determined the SM fragmentation patterns. The ESI-MS/MS spectrum of SM molecular species found in the SM bovine brain extract is shown in Figure 3.1. Optimal ionisation was observed when the collision energy was set at 30 KV and the cone voltage at 35 eV as shown in Table 2.1. The full scan was acquired between  $m/z$  650-900. Individual SM were recorded following a precursor ion scan of  $m/z$  184 ESI in positive ion mode  $[M+H]^+$ . This experiment provided information on the number of SM species and was used to set up the MRM transitions shown in Table 2.1.

LC/ESI-MS/MS analysis was performed in normal phase; the best separation and peak resolution of the bovine brain extract SM species was achieved using an isocratic elution system composed of acetonitrile with 0.1% acetic acid and methanol with 0.1% acetic acid mixed ratio of 70:30 (v/v) at flow rate 0.2 mL/min. Figure 3.3 shows the 10 SM species found in the SM bovine brain extract. Although, all SM species were eluted by 5 min, the run time was set up to 30 min to wash the column prior to the next injection. The LOQ and LOD were found to be 0.5 and 0.001 nmol respectively. The recovery was 82%.



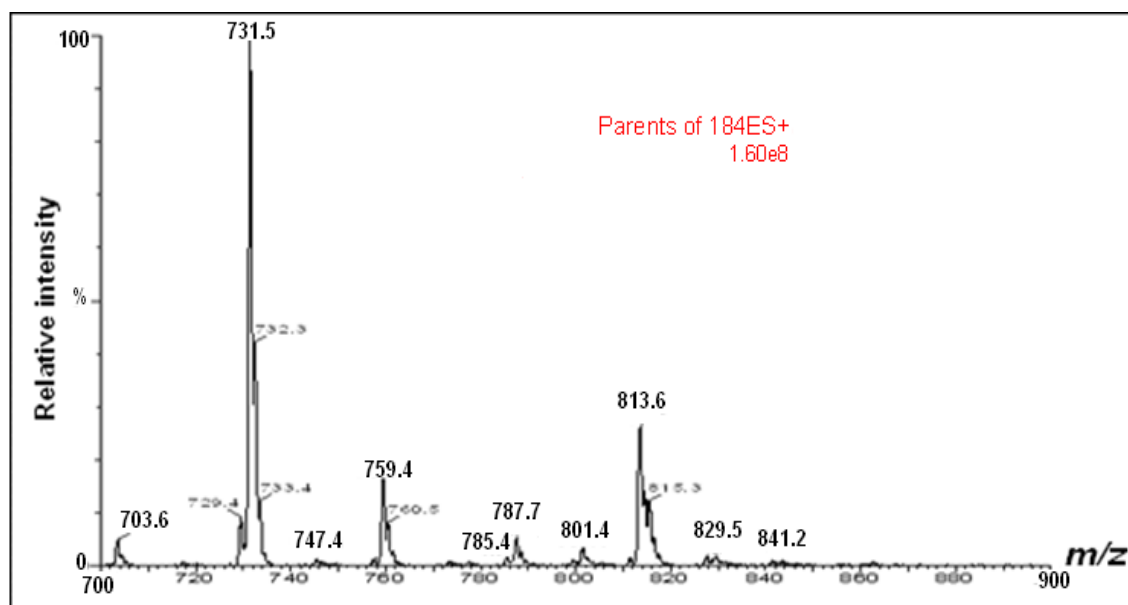
**Figure 3.1.** ESI-MS/MS spectrum (ES<sup>t</sup>) of sphingomyelin (SM) species in bovine brain extract. 100 µg/mL SM extract solution was introduced directly into the mass spectrometer at a flow rate of 10 µL/min.

### 3.3.5.2 Analysis of sphingomyelin species in rat brain

Tissue homogenate of rat brain was left overnight at 48°C methanol: chloroform to complete lipid extraction and facilitate protein denaturation as described previously (Merrill et al., 2005). An alkaline lipid hydrolysis step then was performed using 1M KOH in methanol to remove most of the interfering glycerolipids, in particular phosphatidylcholines that can mask sphingomyelins in a simple MS scan

An MS/MS general scan was performed to identify rat brain SM species. The ESI- MS/MS spectrum of SM molecular species detected in rat brain extract is shown in Figure 3.2.

LC/ESI-MS/MS analysis was performed as described in section 2.4.4 and the most abundant SM species found in rat brain extract are shown in Figure 3.4. Although all species eluted within 5 min, the run time was set up to 30 min to wash the column prior to the next injection. SM (d18:1/12:0) was added prior to extraction at concentration of 0.5 nmol/ $\mu$ L as internal standard. The ratio between the analyte species and SM (d18:1/12:0) was used to quantify rat brain SM species. The brain slice wet weight (g) was also recorded and the results were expressed as nmol/g wet tissue.



**Figure 3.2.** ESI-MS/MS spectrum of rat brain lipid extract (precursors of  $m/z$  184). The extract was introduced directly into the mass spectrometer at a flow rate of 10  $\mu$ L/min.

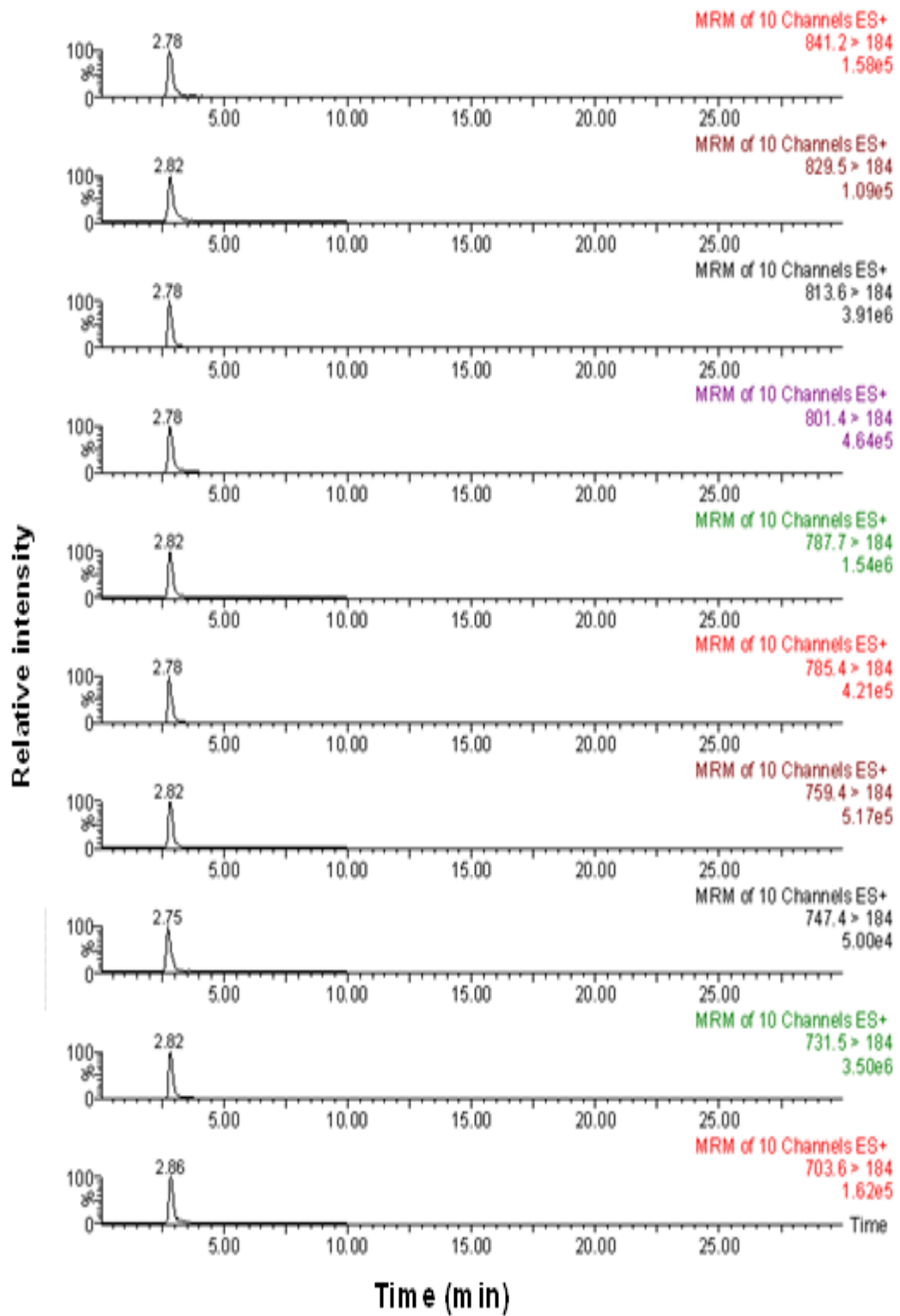


Figure 3.3. LC/ESI-MS/MS analysis of sphingomyelin (SM) bovine brain extract.

### 3.3.6 The effect of ageing on rat brain sphingomyelin

Two groups of animals were used in this study: adult (3-4 months) and aged (22-24 months) animals. A total of 10 SM species were detected in brain extract of both groups (Table 3.5). The most abundant rat brain SM species was *m/z* 731(SM18:1/18:0) followed by *m/z* 813(SM18:1/24:1) and *m/z* 759 (SM18:1/20:0); low abundant rat brain SM species were *m/z* 747(SMd18:1/16:0) followed by *m/z* 841(SM18:1/26:1) and *m/z* 829(SMd18:1/25:0). No statistically significant differences were observed in the levels of SM species when comparing adult to aged animals. However, some trends were observed: the levels of *m/z* 703(SM18:1/16:0), *m/z* 731(SM18:1/18:0), *m/z* 759 (SM18:1/20:0), *m/z* 785(SMd18:1/22:1), *m/z* 787(SM18:1/22:0), and *m/z* 813(SM18:1/24:1) were slightly lower, whereas, the levels of *m/z* 801(SM18:1/20:1), *m/z* 829(SMd18:1/25:0) and *m/z* 841(SM18:1/26:1) were higher in brain aged animals compared to adult ones.

**Table 3.5.** LC/ESI-MS/MS analysis of the rat brain SM. The most abundant SM species found in adult (3-4 months) and aged (22-24 months) rat brain. Data expressed as nmol/g wet tissue (mean  $\pm$  SD); (n = 6 animals per group).

<i>m/z</i>	SM species	Adult rats (3-4 months) (nmol/g)	Aged rats (22-24 months) (nmol/g)
<b>703</b>	18:1/16:0	13.1 $\pm$ 3.6	11.1 $\pm$ 3.7
<b>731</b>	18:1/18:0	187.2 $\pm$ 56.6	133.5 $\pm$ 39.6
<b>747</b>	d18:1/16:0	0.3 $\pm$ 0.2	0.3 $\pm$ 0.04
<b>759</b>	18:1/20:0	25.1 $\pm$ 7.3	20.4 $\pm$ 4.2
<b>785</b>	d18:1/22:1	5.8 $\pm$ 1.3	4.9 $\pm$ 2.4
<b>787</b>	18:1/22:0	7.0 $\pm$ 1.5	4.5 $\pm$ 1.6
<b>801</b>	18:1/20:1	2.6 $\pm$ 0.6	3.1 $\pm$ 1.1
<b>813</b>	18:1/24:1	74.4 $\pm$ 11.8	57.1 $\pm$ 23.0
<b>829</b>	d18:1/25:0	1.1 $\pm$ 1.2	1.3 $\pm$ 0.4
<b>841</b>	18:1/26:1	0.6 $\pm$ 0.2	0.7 $\pm$ 0.3

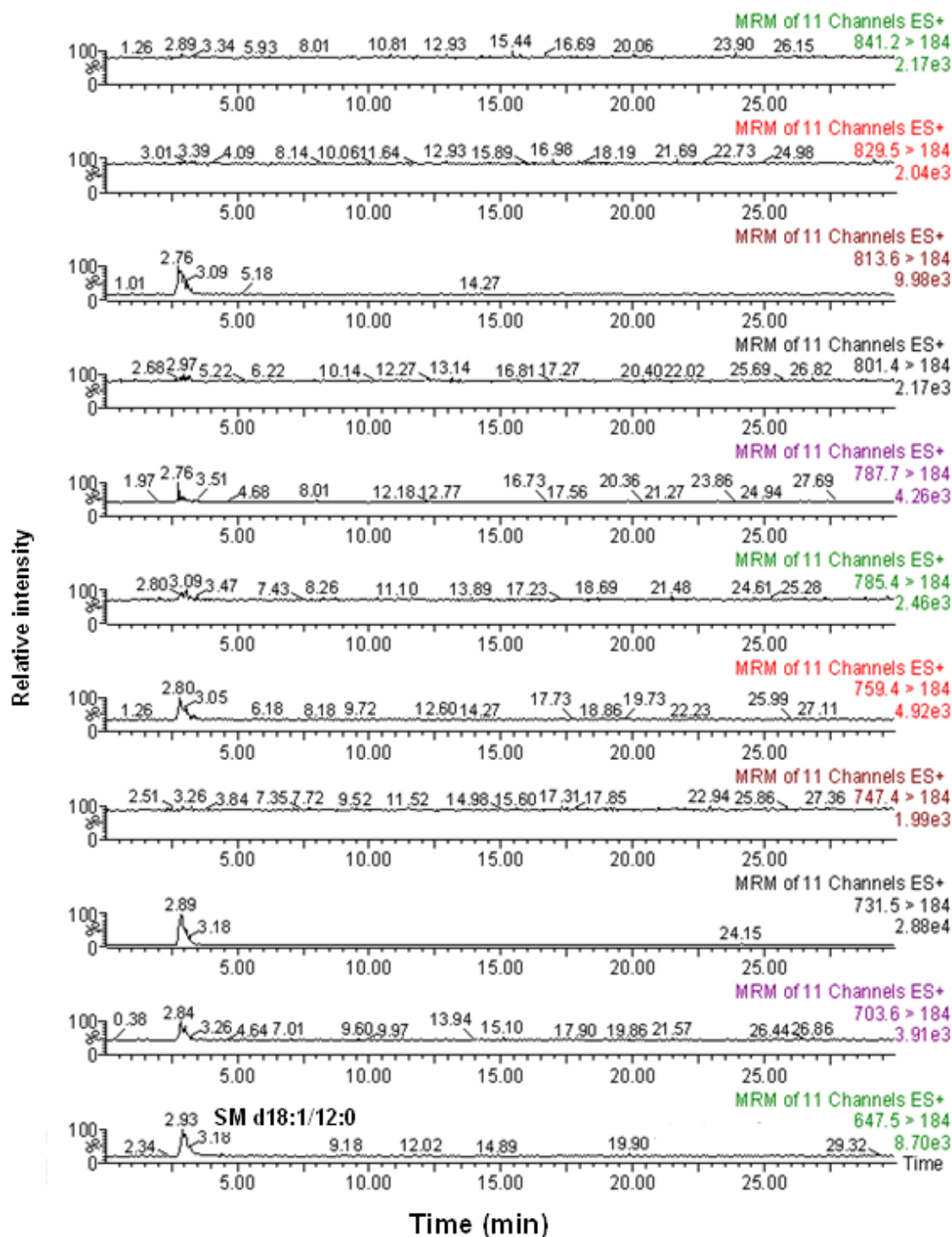
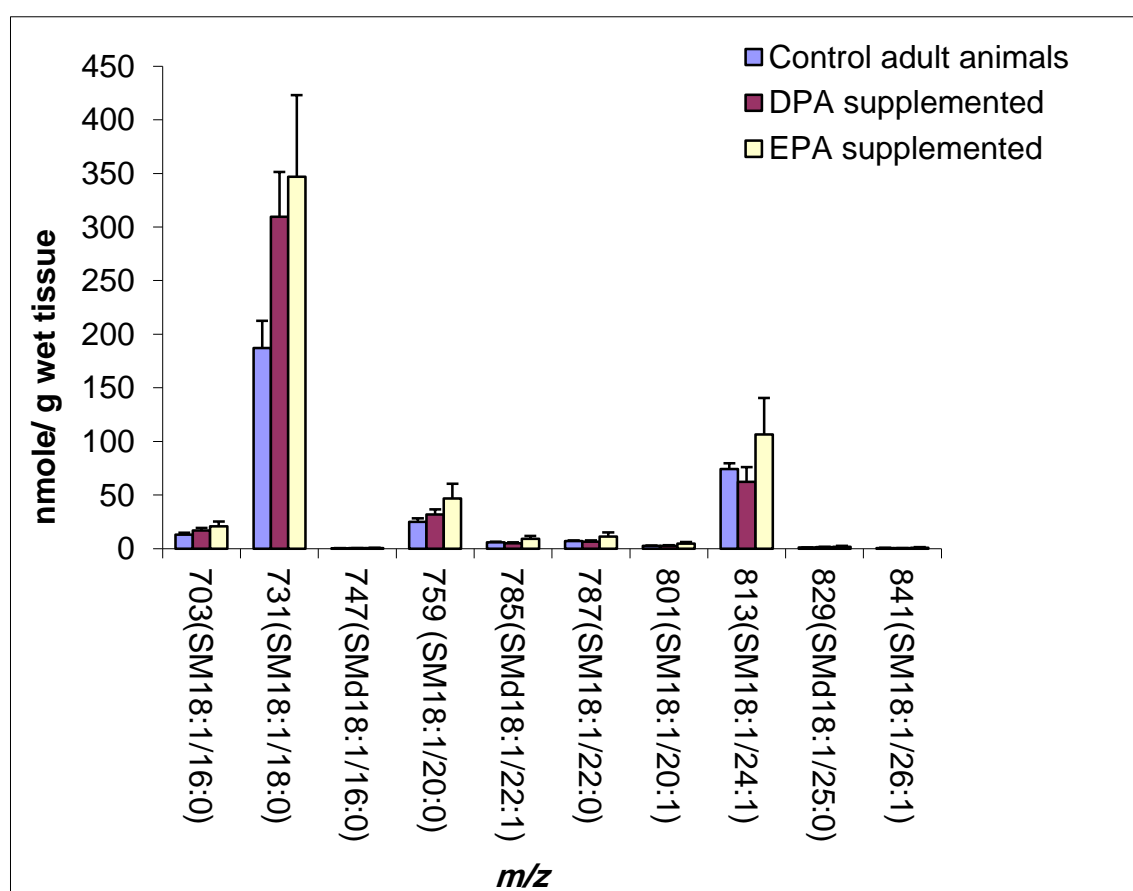


Figure 3.4. LC/ESI-MS/MS analysis of rat brain extract sphingomyelin (SM) species.

### 3.3.7 The effect of EPA and n-3 DPA supplementation on adult animal brain sphingomyelin

The effect of 200 mg/kg/day EPA or 200 mg/kg/day n-3 DPA supplementation for 8 weeks on brain SM species of adult animals (3-4 months) results are shown in detail in Figure 3.5. Even though there are trends of slight increase in the levels of  $m/z$  703(SM18:1/16:0),  $m/z$  731(SM18:1/18:0) and  $m/z$  759 (SM18:1/20:0) in adult animals supplemented with either EPA or n-3 DPA compared to control ones, these changes were not statistically significant.

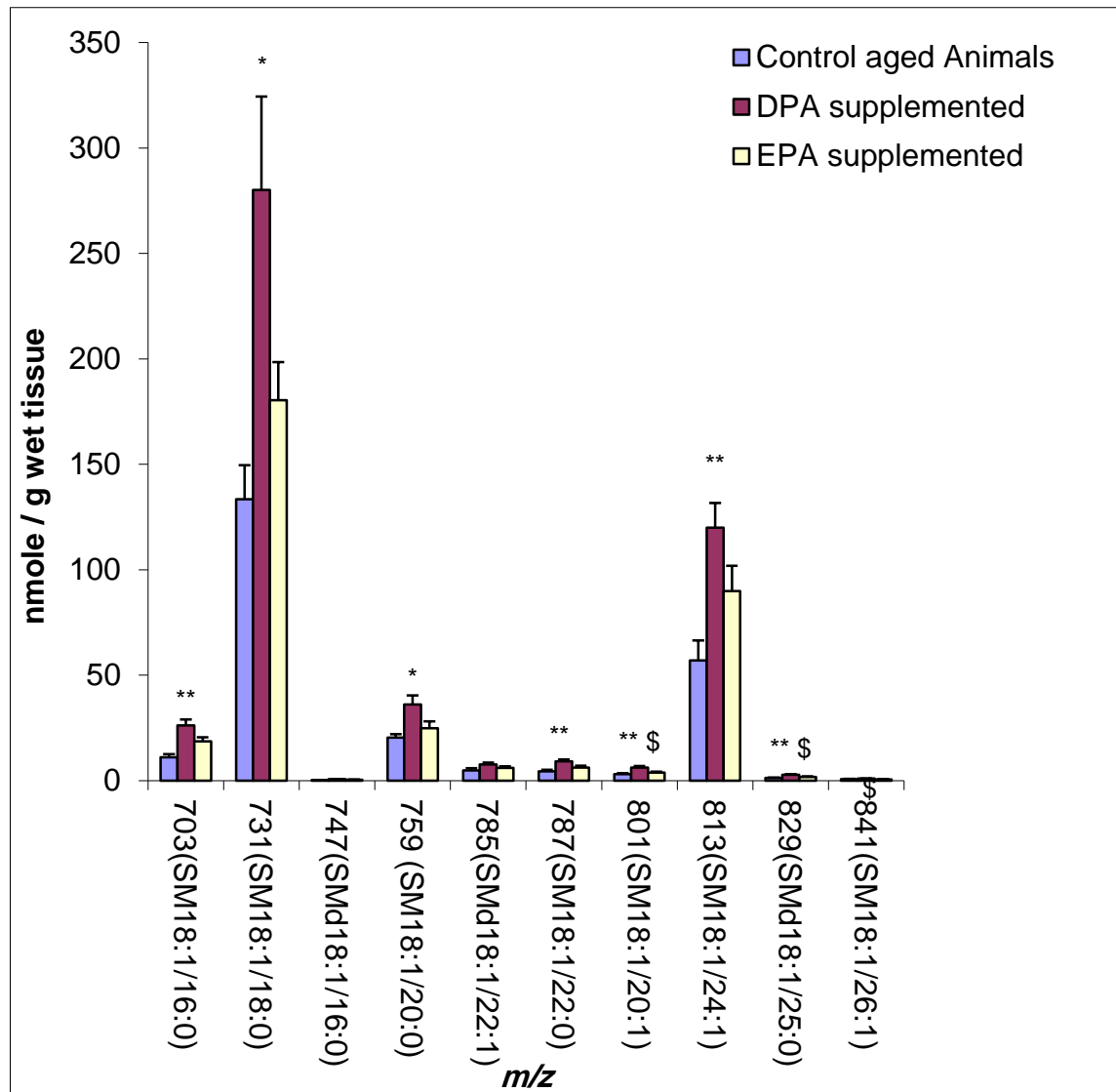


**Figure 3.5.** SM species of brain in adult animals (3-4 months) following supplementation with EPA (200 mg/kg/day) or n-3 DPA (200 mg/kg/day) for 8 weeks. Results expressed as nmol/g wet weight tissue (mean  $\pm$  SD);  $n = 6$  animals per group).

### 3.3.8 The effect of EPA and n-3 DPA supplementation on aged animal brain sphingomyelin

The effect of 200 mg/kg/day EPA or 200 mg/kg/day n-3 DPA supplementation for 8 weeks on brain SM species of aged animals (22-24 months) is shown in Figure 3.6. Statistically, significant differences were observed on most of brain SM species levels, when the aged animals the supplemented with n-3 DPA. The levels of the following SM species  $m/z$  703(SM18:1/16:0) ( $p=0.001$ ),  $m/z$  731(SM18:1/18:0) ( $p=0.011$ ),  $m/z$  759 (SM18:1/20:0) ( $p=0.016$ ),  $m/z$  787(SM18:1/22:0) ( $p=0.004$ ),  $m/z$  801(SM18:1/20:1) ( $p=0.004$ ), 813(SM18:1/24:1) ( $p=0.004$ ) and  $m/z$  829(SMd18:1/25:0) ( $p=0.002$ ) were significantly higher in aged animals supplemented with n-3 DPA compared to animals in the control group. However, no significant changes were observed in the levels of brain SM species of the aged animals that were supplemented with EPA, even though there was a trend towards increased levels of  $m/z$  703(SM18:1/16:0),  $m/z$  731(SM18:1/18:0),  $m/z$  759 (SM18:1/20:0) and  $m/z$  813(SM18:1/24:1).

Furthermore, the levels of  $m/z$  801(SM18:1/20:1) ( $p=0.018$ ),  $m/z$  829(SMd18:1/25:0) ( $p=0.038$ ) and  $m/z$  841(SM18:1/26:1) ( $p=0.045$ ) were significantly higher in aged animals supplemented with n-3 DPA compared to aged animals being supplemented with EPA. Generally, all the brain SM species were higher in aged animals' supplemented n-3 DPA compared to those animals being supplemented with EPA, although not all changes were significantly different.



**Figure 3.6.** SM species of brain in aged animals (22-24 months) following supplementation with EPA or n-3 DPA for 8 weeks. Results expressed as nmol/g wet weight tissue (mean  $\pm$  SD; n = 6 animals per group). Aged animals supplemented with n-3 DPA compared to control \*p<0.05 and \*\*p<0.01; aged animals supplemented with EPA compared with aged animals supplemented with n-3 DPA \$p<0.05.

### 3.4 Discussion

Nearly one third of brain fatty acids belong to the n-3 PUFA family (Bourre and Dumont, 1991). They are considered to play a crucial role in brain function, appear to be important for brain memory, brain performance and behavioural function as well as in normal growth and development, they are also found to help with reducing inflammation (Belluzzi et al., 2000, Seddon et al., 2001, Yashodhara et al., 2009).

SM is the most abundant sphingolipid (Tepper et al., 2000, Andrieu-Abadie and Levade, 2002). It can comprise as much as 50% of the lipids in certain tissues, and makes up about 10% of the lipids of the brain (LeBaron et al., 1981), it tends to be in greatest concentration in the plasma membranes and especially in the outer leaflet. In general, the fatty acids found esterified in SM are very-long-chain saturated and monounsaturated, including odd-numbered ones. The absolute proportions of each fatty acid and sphingoid base (sphingosine or dihydrosphingosine) can vary markedly between SM species (Ramstedt and Slotte, 2002, Merrill et al., 2009).

This study focused on studying the effect of EPA and n-3 DPA supplementation on brain SM species in the rat. The first objective investigated RBC membrane fatty acids in adult and aged rats following supplementation with EPA and n-3 DPA to assess compliance. Incorporation indicates that the treatment was successful and that brain tissue can be used for such studies. There are studies showing that RBC accretion correlated to DHA accretion in brain tissue (Huang et al., 2007), but not much is known about EPA and DPA. The profile of RBC

fatty acids of adult control rats was compared to that of aged control rats in order to investigate if there were any alterations in the normal fatty acid profile following EPA and n-3 DPA supplementation.

EPA is found only at low levels in the cell membranes and it is not stored in significant quantity in the brain, where it seems to have a functional, rather than a structural role. One of the major factors in regulating the activity of fatty acids in the brain is the blood-brain-barrier (BBB). It has been shown that EPA does not cross the BBB (Yehuda et al., 1999). Also, it has been recently hypothesized that EPA is more rapidly  $\beta$ -oxidised upon its entry into the brain and that might be a reason for the low level of EPA in brain phospholipids (Chen et al., 2009b). However, other studies have considered the significant pharmacological effect of EPA on brain cell function (McNamara and Carlson, 2006, Gorjão et al., 2009). In this respect it can improve brain function at the very simplest level by improving blood flow (Richardson and Puri, 2000b, Puri et al., 2000). Thus, it is very important to find out how EPA is transported into and functions in the brain. One such way could be by transport via circulation. EPA could be incorporated into RBC and once RBC reach the brain, EPA could be released from the membrane as free fatty acid in the brain. EPA could then act in the brain or could be converted into DHA or other fatty acid such as n-3 DPA.

The n-3 DPA is an elongation product of EPA and it may be the bioactive metabolite of EPA that is responsible for its beneficial effects. To date, the literature on n-3 DPA is limited and there is not much information about the action of n-3 DPA. However, the available data suggests it has beneficial health effects (Kaur et al., 2011), but there is very little evidence supporting these

beneficial effects and its pharmacological effects. One of the reasons for this may be that pure n-3 DPA not been readily available and its properties have not been systematically examined. Another reason may be related to the fact that n-3 DPA is found in combination with EPA and DHA in natural products. Therefore, supplementation studies with n-3 DPA alone are needed to further investigate its biological and pharmacological effect (Burdge et al., 2007, Tam et al., 2008, Kaur et al., 2011, Miller et al., 2012, Linderborg et al., 2013).

This study has also shown that supplementation of adult rats with EPA and n-3 DPA for eight weeks or n-3 DPA for three and six months lead to no significant changes in AA levels of RBC. This could be due to the fact that n-6 and n-3 PUFA may compete for the same metabolizing enzymes. Thus, an increase in n-3 PUFA substrates will result in the metabolism of n-3 at the expense of n-6 PUFA. Also, when there is a high ALA level it could inhibit the metabolism of DHA and AA, thus limiting the availability of the precursor to form DHA (Portolesi et al., 2007). This study also shows that the supplementation also significantly increased both EPA and n-3 DPA, respectively in RBC; this indicates that the rats are able to use n-3 DPA to provide more EPA in the body possibly by retro-conversion and/or visa versa (Figure 1.1). Also, the conversion of EPA to n-3 DPA or vice versa indicates the possibility that n-3 DPA can be a potential storage form for EPA in erythrocyte membranes. These findings are in agreement with two recent studies *in vivo* that also provide evidence for retro-conversion of n-3 DPA into EPA (Gotoh et al., 2009, Kaur et al., 2010).

Furthermore, the results of this study also suggested that n-3 DPA may act as a reservoir of the major long-chain n-3 PUFA in rats. These present findings are

in agreement with a recent clinical study that found; n-3 DPA supplementation significantly increased the proportions of EPA in plasma, also EPA supplementation significantly increased the proportion of EPA in plasma and RBC, while EPA supplementation did not alter the proportions of n-3 DPA or DHA, and also showed that DPA and EPA demonstrated different and specific incorporation patterns. This, suggested that DPA may act as a reservoir of the major long-chain n-3 PUFA in humans (Miller et al., 2012)

Although the level of EPA only is increased, increased levels of n-3 DPA in erythrocyte membranes may act to sustain a constant supply of EPA. The overall findings indicate that supplementation with both EPA and n-3 DPA has similar effects on the fatty acid composition of RBC membrane of aged animals. This finding agreed with previously reported studies which indicated that the neuroprotective effects of DPA in aged rats are similar to the EPA effects (Martin et al., 2002a, Kelly et al., 2011).

DHA is one of the major brain n-3 PUFA (Lauritzen et al., 2001b) It is critical for optimal brain health and function at all ages and it was recently found that it provides brain-boosting benefits in infants and ageing adults (Lukiw and Bazan, 2008). In this study it was found that DHA increased in aged animals supplemented with n-3 DPA compared to adult rats supplemented with n-3 DPA. This could be due to more n-3 DPA being converted to DHA in the aged animals than in adults. Also, in adult rats, higher levels of DHA are used for growth and development by different body tissues such as brain and neuronal cells, than in older rats and this could lead to most of DHA being used by these tissues and thereby decreasing its level in RBC.

Moreover, ALA and n-3 DPA were found to be increased in the RBC of adult rats supplemented with EPA when compared to aged rats supplemented with EPA. Since ALA is the precursor of n-3 PUFA, it may be possible that its metabolism by  $\Delta 6$ -desaturase is inhibited by EPA supplementation. Also a recent study suggested that it is possible to identify positive correlations between EPA and its precursor ALA (Destailats et al., 2010). At the same time, the EPA supplement in adult rats is utilized to produce n-3 DPA without affecting the DHA concentration.

In our study, the SFA; tetradecanoic acid (C14:0), eicosanoic acid (C20:0) and tetracosanoic acid (C24:0) and the MUFA palmitoleic acid (C16:1 cis-9), cis-OA (C18:1) and erucic acid (C 22:1 n-9) were found to be higher in the aged rats RBC compared to adult rats. In the brain, it has been shown that the concentration of SFA is increased in aged rats (Bourre, 2004). Moreover, it has been reported that the fatty acid profile in the brain membranes may be a reflection of the fatty acid profiles of RBC (Ballabriga, 1994).

Furthermore, the results obtained from this present study confirmed that there were differences in the fatty acid relative % of adult rats compared to aged rats. Significantly, there was a decrease in AA, ALA and nervonic acid concentration in RBC of aged compared with adult rats which is in agreement with (Suzuki et al., 1989) who signify that the concentration of AA tended to decrease with increasing age, while in contrast DHA tended to significantly increase. This indicates that the PUFA metabolism is affected by age. However, AA was further significantly unchanged in the RBC of animals supplemented with EPA and/or n-3 DPA.

The second aim of this study was to investigate SM in brain of adult and aged rats following supplementation with EPA and n-3 DPA. SM is found in cell membranes together with other phospholipids and sphingolipids. Qualitative and quantitative analysis of SM requires a sensitive and specific extraction protocol to separate them from these lipids. In this study lipid extraction was performed using KOH in methanol hydrolysis. Therefore, most of the interfering glycerolipids, in particular phosphatidylcholines that can be found associated with SM were removed prior to the analysis and SM was clearly left in the extract ready for the chromatographic analysis. LC/ESI-MS/MS) was applied for analysis of brain SM, because it is currently the only technology with the requisite structural specificity, sensitivity, quantitative precision and relatively high-throughput capabilities for such analyses of SM species found in small samples and quantity.

This method is quick, inexpensive and afforded a good intensity for the major abundant SM species and used a normal-phase column (Luna 5u NH<sub>2</sub> 100A column, LC column 30 x 4.6 mm) to separate and identify all the SM species being eluted with the same retention time. The separation of SM species on a normal-phase column depends on the polar head group and saturation and length of the fatty acid chain (Taguchi et al., 2000). Moreover, it has been found that the level of collision energy is very important for the sensitive identification of the SM species (Larsen et al., 2001, Ekroos et al., 2002, Hsu and Turk, 2003, Wenk et al., 2003).

All the individual SM species detected in this present study were found to be fragmented using the same CE (30 KV) and showed only a common fragment

ion at  $m/z$  184 due to the loss of the phosphocholine head group. The formation of this ion was in agreement with the literature (Hsu and Turk, 2003). The formation of  $m/z$  184 is typical of all phosphocholine - containing lipids (Haroldsen and Gaskell, 1989) and is the diagnostic fragment ion for this specific class of phospholipids (Kim and Lee, 1994, Brugger et al., 1997, Hsu et al., 1998, Murphy et al., 2001).

The method was then applied to compare the most abundant SM species found in brain of adult and aged rats: a total of 10 protonated species of SM were detected. The brain of ageing rats showed some decrease but there were no significant changes when compared with brain of adult rats. These findings are not in agreement with previous reports showing that the SM contents in the rat brain increased with age (Giusto et al., 1992). There were no differences in animals and age groups between the two studies. Therefore, these different findings could be related to using a less sensitive analytical technique; to using gas liquid chromatography (GLC) and different columns, or it could be due to using different regions of the brain.

The effect of n-3 DPA (20:5n-3) supplementation on the SM species of rat brain in association with ageing effects has not been clarified yet. Therefore, this study focused on exploring any alteration of rat brain SM species in adult and aged rats supplemented with n-3 DPA and compared these effects to EPA. In this present study adult animals show no statistically significant changes in brain SM species when supplemented with either EPA or n-3 DPA. However, in this study it was found that there are statistically significant changes observed in

most of the brain SM species levels, when the aged animals were supplemented with n-3 DPA, but not with EPA.

It has been suggested that PUFA can interact with different steps of sphingolipid metabolism (Worgall et al., 2002). It has also been reported that N-SMase is activated by n-3 PUFA such as EPA and DHA and results in the generation of ceramide (Robinson et al., 1997). A recent study has reported that increases in the activities of sphingomyelinase were observed in cortical tissue of aged rats and these increases were attenuated in tissue of aged rats supplemented by either n-3 DPA or EPA (Kelly et al., 2011).

Furthermore, other studies have shown that sphingolipids, including ceramide and sphingosine, accumulate in the brain during ageing and any alterations in sphingolipid metabolism increases the risk and progression of age-related disease (Giusto et al., 1992, Lightle et al., 2000, Cutler and Mattson, 2001, Kavok et al., 2003, Mattson et al., 2008). However, changes in sphingolipid metabolising enzyme activities during development and ageing have not been fully studied. Also, it has been reported that the key enzymes in sphingolipid metabolism, the plasma membrane N-SMase activity, was significantly increased in developing rat brain tissues, also the A-SMase increased in the brain during ageing. Although, it has been noted that the activity of A-SMase was relatively lower than that of N-SMase. The A-SMase activity was suggested to be a good candidate for maintenance of ageing brain (Spence and Burgess, 1978, Buccoliero and Futerman, 2003).

In addition, it has been found that a decreased conversion of ceramide to SM might also contribute to age-related increases in ceramide levels (Sacket et al., 2009). It is also an indication that ageing can slow the synthesis of SM and/or a slower synthesis of SM resulted in ageing (Lightle et al., 2000). Moreover, it has been reported that activity of sphingomyelin synthase and ceramide synthase is lower than sphingomyelinase and ceramidase in developed and aged mice brain (Jensen et al., 2005). Thus, N-SMase and ceramidase might produce ceramide and sphingosine in the brain during ageing, thereby leading to an accumulation of ceramide and/or sphingosine and lower the SM content (Lahiri and Futerman, 2007).

However, in our study there are significant changes in brain SM species of aged animals supplemented with n-3 DPA. Although we did not identify any significant changes with EPA supplementation, these changes are the reverse of those observed with sphingosine, as no significant changes were observed (Kelly et al., 2011), and indicate that these fatty acids may modulate the sphingomyelinase pathway. Also, these present findings could support the previous finding on n-3 DPA and EPA that showed a markedly increased S1P: ceramide ratio, which is important because S1P has been shown to endow resistance to apoptotic cell death (Bartke and Hannun, 2009), while ceramide is generally damaging to cells and is generated by hydrolysis of SM by sphingomyelinase. Thus, it appears that the modulatory effect of EPA and n-3 DPA on SM may lead to a shift in the balance of S1P: ceramide ratio towards an antiapoptotic phenotype and is indicative of deterioration in cell activity, cases, and an indicator of cell death.

In summary, ageing has been reported to be associated with a decrease in the brain content of n-3 PUFA, such as EPA and DHA, and with decreased neuroplasticity (Dyall et al., 2007). Thus, the main findings of this study show that EPA and n-3 DPA supplementation was not only able to compensate the loss of n-3 PUFA due to ageing, but also contributed to an increase in brain SM species and could probably contribute to the attenuation of the age effect and to regulate brain development. Also, results show that increases of SM species occurs with ageing rats supplemented by both EPA and n-3 DPA. This indicates that both EPA and n-3 DPA could play important roles in activation and regulation of brain SM biosynthesis. Also, they could consistently affect many of the body physiological processes, including cell proliferation, cell differentiation, and cell apoptosis. This increase in the level of sphingomyelin synthesis could result from the relatively higher activity of sphingomyelin synthase, the decreased generation of ceramide from SM via N-SMase, A-SMase and ceramidase. The data suggest the possibility that dietary manipulations of SM metabolism with EPA and n-3 DPA might prove effective in treating various age-related diseases, particularly brain diseases. However, additional studies with animal models are needed to determine whether the observed changes in brain SM species indeed do or do not play a crucial role in the neuroprotective effect of n-3 fatty acids on brain ageing. Such studies could also help to understand the functionally important interplay between the SM species and n-3 PUFA supplementation in the brain.

**Chapter 4: Analysis of brain fatty acid ethanolamides and endocannabinoids following LPS-induced neuroinflammation and lithium treatment**

## 4.1 Introduction

Endocannabinoids are endogenous lipid mediators, found in many animal and human cells, tissues and body fluids, with important pharmacological properties. Somewhat higher concentrations are reported in the brain and peripheral tissues (Fontana et al., 1995, Howlett and Mukhopadhyay, 2000, Howlett et al., 2004). The endocannabinoid system consists of endogenous ligands (AEA and 2-AG) and congeners, target receptors (CB1, CB2), synthesis (NAPE-PLD; DAG lipase) and degradation enzymes (FAAH, MAG-Lipase) (Figure 1.5) (Maccarrone et al., 2010). Both AEA and 2-AG are endogenous cannabinoids, identified both in the periphery and in the brain (Piomelli et al., 1999, Panikashvili et al., 2001, Maccarrone et al., 2002a). Many other endogenous mediators that have been identified in mammalian brain and peripheral tissues including: PA-EA, ST-EA and OA-EA (Bisogno et al., 1997, Lambert and Di Marzo, 1999, Balvers et al., 2009, Bisogno et al., 2009).

The most abundant FA-EAs in brain comprise about 25% of total brain FA-EAs they are AEA, OA-EA and PA-EA (Koga et al., 1997, Arafat et al., 1989, Okamoto et al., 2004, Walker et al., 2005, Thabuis et al., 2008, Merriam et al., 2011) and LA-EA, ALA-EA and DHA-EA are found at lower levels (Mechoulam et al., 1998). These compounds have become the focus of many studies, investigating their properties as modulators of neuroinflammatory and apoptotic responses.

It has been reported that, both AEA and 2-AG have anti-inflammatory and neuroprotective properties (Panikashvili et al., 2001, van der Stelt et al., 2001,

Eljaschewitsch et al., 2006, Panikashvili et al., 2006). Furthermore, it has been suggested that increased levels of FA-EAs and ECs may be neuroprotective and limit brain damage (Eljaschewitsch et al., 2006, Centonze et al., 2007), and when decreased may be contributing to the pathology of neuroinflammation (Cabranes et al., 2005).

Lipopolysaccharides are known inflammatory stimuli. They stimulate brain AA release from phospholipids and metabolism, but lithium pre-treatment reduces this effect and down-regulates brain AA metabolism (Basselin et al., 2010). Also, lithium has been used to treat neuroinflammation and neurodegenerative disorders, especially bipolar disorder for almost 50 years, and remains the most commonly used treatment for its manic phase (Cade, 1949, Harwood and Agam, 2003).

It has been suggested that lithium affects the AA cascade by decreasing AA turnover within brain phospholipids and decreasing levels of its brain derivative such as anandamide (AEA), the ethanolamide of AA (Chang et al., 1996). Lithium is known to reduce oxidative stress (Machado-Vieira et al., 2007) and was also found to protect irradiated hippocampal neurons in mice from apoptosis, resulting in better performance in learning and memory function. It was also found to prevent neurocognitive deficit in patients with cancer (Yazlovitskaya et al., 2006, Khasraw et al., 2012).

Recently emerging evidence suggests that lithium can have a neuroprotective effect (Foland et al., 2008). Also, it has been recently reported that lithium treatment is partly mediated by inhibiting inflammation and by promoting

proliferation and survival of neural stem and progenitor cells (Lai et al., 2011). However, there are only limited prospective clinical data on the use of lithium as a neuroprotectant of neuroinflammation. Also, its roles and actions on brain lipid mediators such as AEA, 2-AG and their congeners are not fully defined.

Bioactive lipid mediators such as eicosanoids have been found to rapidly change in the brain during global ischemia and following death (Bazan, 1970, Cenedella et al., 1975, Galli and Racagni, 1982, Petroni et al., 1987, Petroni et al., 1989, Murphy, 2010). Also, it has been found that rat decapitation and increased duration of ischemia leads to an increase in brain AA levels (Cenedella et al., 1975, Bosisio et al., 1976, Murphy, 2010). However, head-focused microwave irradiation has been found to significantly attenuate this increase in the AA release (Cenedella et al., 1975, Galli and Racagni, 1982, Murphy, 2010). Thus, brain rapid fixation using a head-focused microwave irradiation technique has been considered to be critical in assessing true basal levels of brain bioactive lipid mediators derived from PUFA (Bazan, 1970, Murphy et al., 1994, Murphy, 2010).

To date there is no study investigating the effect of lithium treatment on brain FA-EAs and ECs. Lithium may stimulate brain FA-EAs and EC metabolism, but the use of head-focused microwave treatment can stop AA production and help to quantify brain FA-EAs and EC levels correctly.

Previous study has identified AEA in rat brain subjected to head-focused microwave treatment (Bazinet et al., 2005). Other studies have used tissue collected under ordinary ischemic and decapitation procedures (Richardson et

al., 2007, Artmann et al., 2008, Zhang et al., 2010) or in rat plasma (Giuffrida et al., 2000) or in human serum (Schreiber et al., 2007) to measure endocannabinoids (AEA and 2-AG) in various tissues (Hardison et al., 2006).

The main aim of this present study was to determine the effects of lithium on brain FA-EAs and ECs following induction of neuroinflammation caused by LPS infusion and subjected to lithium pre-treatment. Brain tissue was collected following head-focused microwave irradiation. The results of this study could provide new information on lithium induced neuroprotective action.

## **4.2 Materials and Method**

All animal work and sample collection were undertaken by Prof Rapoport's research group at NIH Laboratory of Neurosciences, Bethesda, MD, USA as described in section 2.2.2.

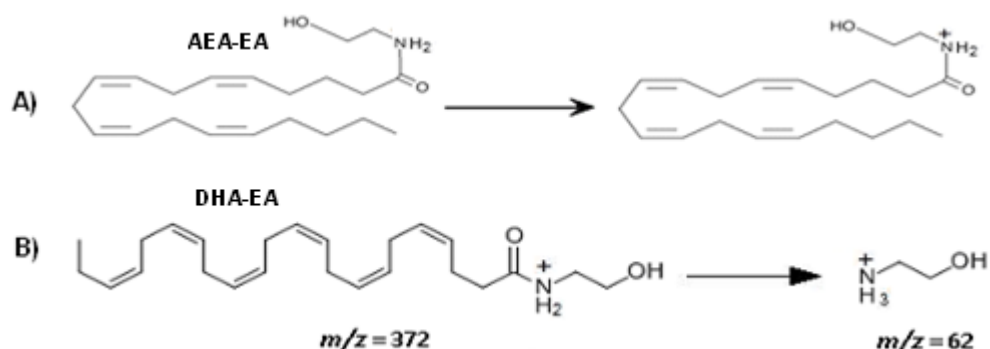
All materials that have been used in this study are mentioned in detail in section 2.1. Standard preparation and lipid extractions are described in sections 2.5.1, 2.5.4, 2.5.5 & 2.5.6. FA-EAs and ECs analysis was performed using LC/ESI-MS/MS as described in section 2.5.6.

## **4.3 Results**

Method development included: a) optimising MS/MS conditions, b) optimising LC/ESI-MS/MS assay, c) optimising solid phase extraction of FA-EAs and ECs from brain tissue, and d) assessing recovery, linearity, LOD and LOQ of the method.

### 4.3.1 Optimisation of mass spectrometry conditions and MS/MS assay

The amide group of FA-EAs easily become positively charged making these compounds highly suitable for ESI operated in positive ion mode as shown in Figure 4.1A. Individual FA-EAs and 2-AG standards were directly infused into the mobile phase flow; the cone voltage capillary voltage and individual CE for all standards were optimised for sensitivity as shown in Figure 4.2.

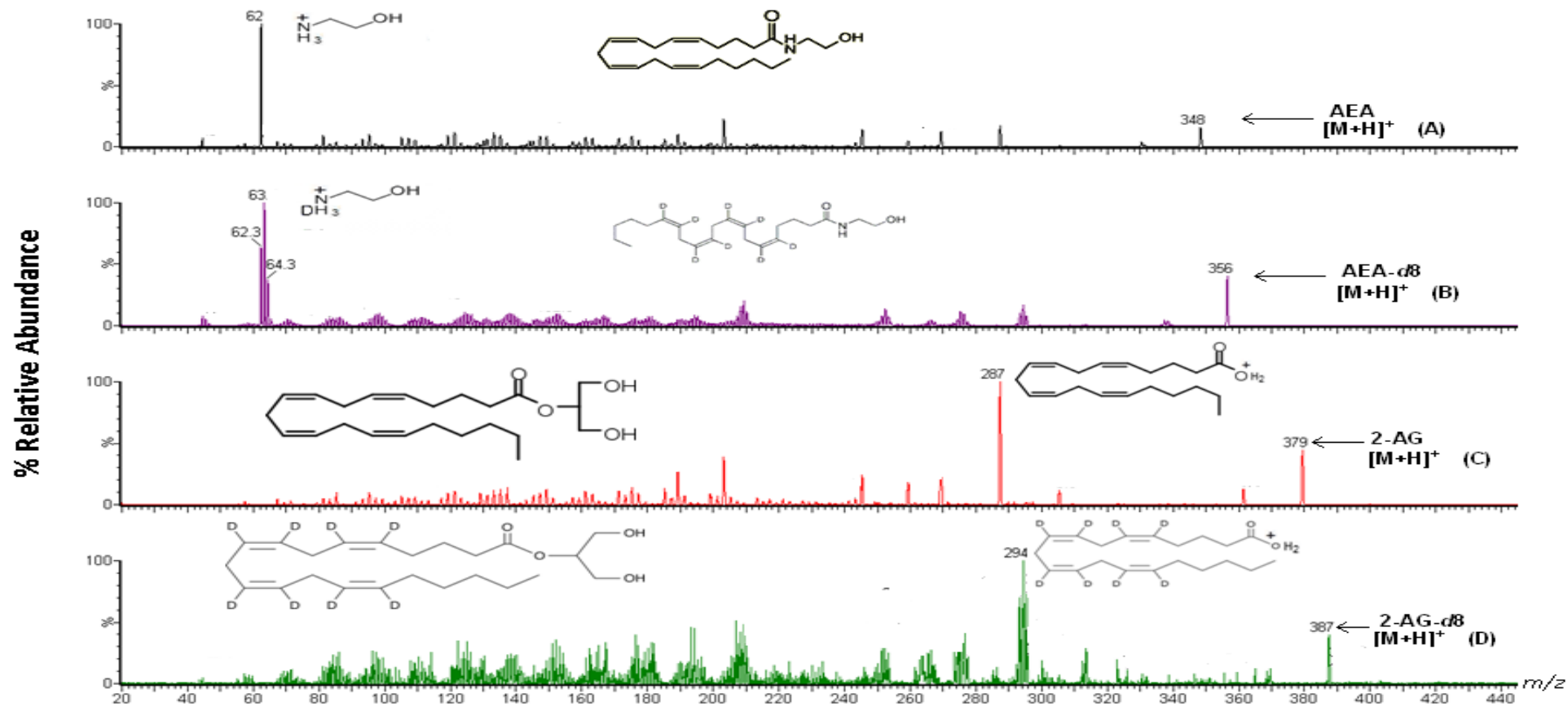


**Figure 4.1. A)** Proposed mechanism of ionisation of fatty acid ethanolamides. **B)** Proposed mechanism for the fragmentation of DHA ethanolamide by ESI.

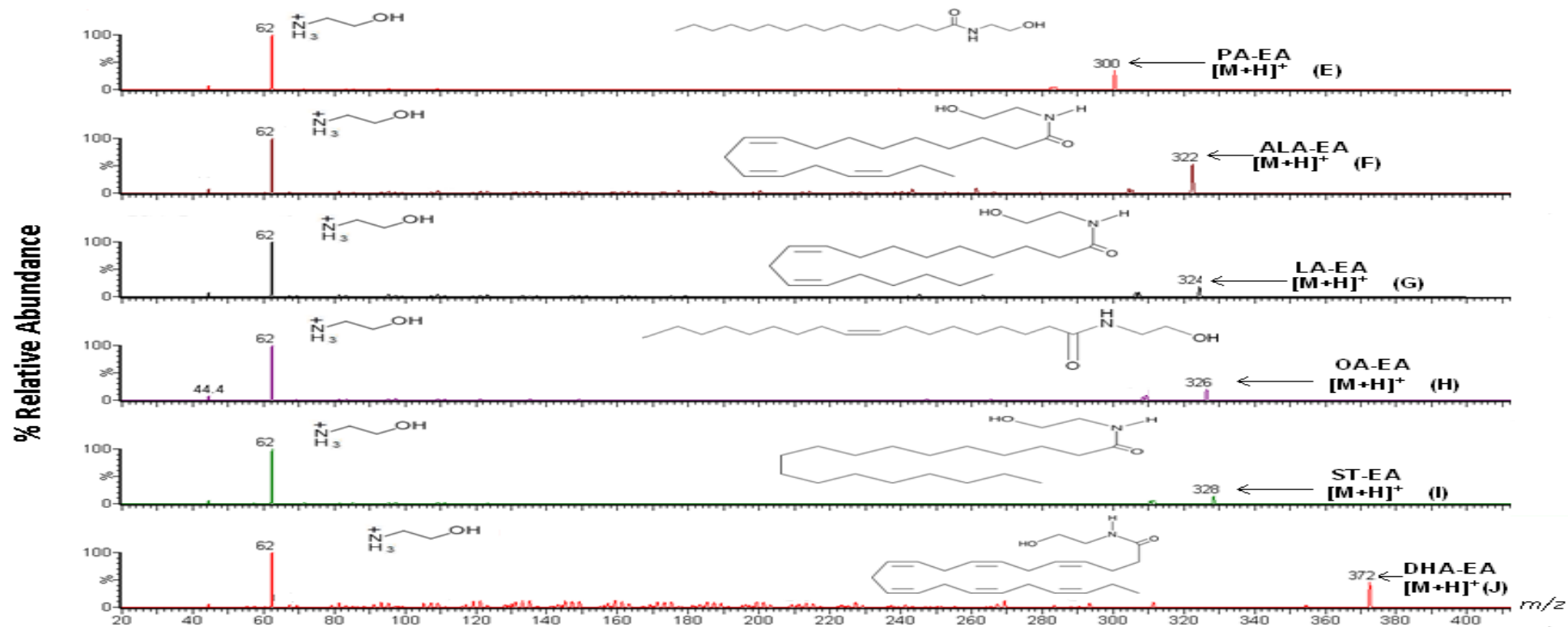
A general mechanism for the proposed fragmentation of FA-EAs is shown in Figure 4.1B. The MRM assay was set as shown in Table 4.1 using ethanolamine-derived ion *m/z* 62 whilst the deuterated IS AEA-*d*8 gives a product ion at *m/z* 63. The deuterated IS 2-AG-*d*8 gives a product ion at *m/z* 294. The principle product ion for 2-AG was *m/z* 287 which corresponds to 2-arachidonoyl protonated ion after losing glycerol.

**Table 4.1.** Parameters used for the LC-MS/MS assay of the fatty acid ethanolamides and 2-arachidonoylglycerol standards. MRM: multiple reaction monitoring, CE: collision energy.

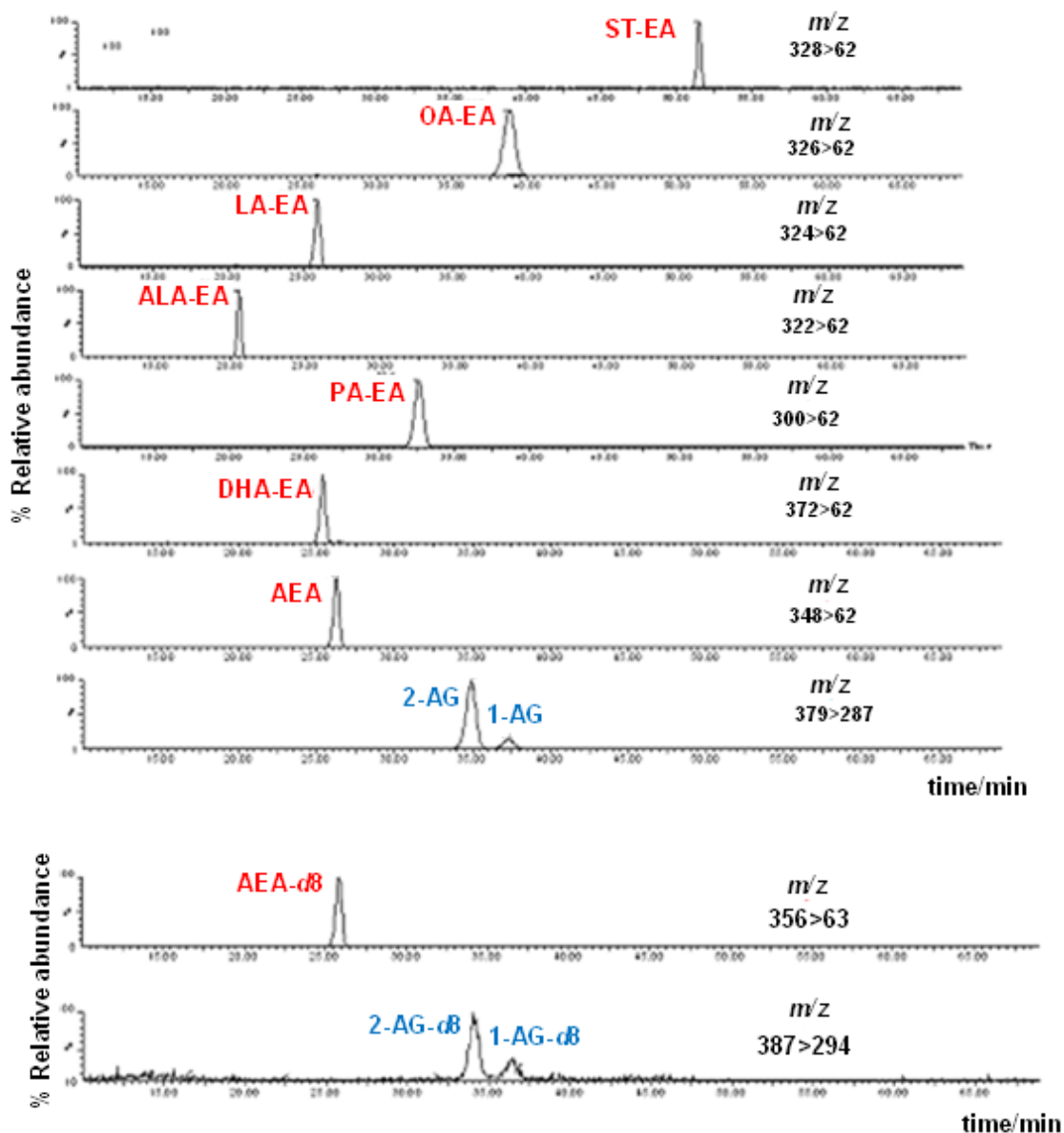
<b>Compounds</b>	<b>MW</b>	<b>Parent ion (m/z)</b>	<b>Product ion (m/z)</b>	<b>MRM (m/z)</b>	<b>CE (eV)</b>
PA-EA	299	300	62	300>62	14
ALA-EA	321	322	62	322>62	12
LA-EA	323	324	62	324>62	17
OA-EA	325	326	62	326>62	16
ST-EA	327	328	62	328>62	16
AEA	347	348	62	348>62	16
DHA-EA	371	372	62	372>62	14
2-AG	378	379	287	379>287	20
<b>Internal standards</b>					
AEA- <i>d</i> 8	355	356	63	356>63	16
2-AG- <i>d</i> 8	386	387	294	387>294	20



(Figure 4.2 continues overleaf...)



**Figure 4.2.** ESI-MS/MS spectra of anandamide (A), anandamide-d8 (B), 2-Arachidonoylglycerol (C), 2-Arachidonoylglycerol-d8 (D), N-palmitoylethanolamide (E), N- $\alpha$ -linolenylethanolamide (F), N-linoleoylethanolamide (G), N-oleoylethanolamide (H), N-stearoylethanolamide (I) and N-docosahexaenylethanolamide (J).



**Figure 4.3.** LC/ESI-MS/MS analysis of FA-EAs and 2-AG standards anandamide (AEA), N-palmitoylethanolamide (PA-EA), N-stearoylethanolamide (SA-EA), N-oleoylethanolamide (OA-EA), N-docosahexaenylethanolamide (DHA-EA), N-linoleoylethanolamide (LA-EA), N-linolenylethanolamide (ALA-EA), 2-arachidonoylglycerol (2-AG), 1-arachidonoylglycerol (1-AG), and the internal standards (IS) AEA-d8, 2-AG-d8 and 1-AG, using 70% acetonitrile (isocratic system) on a C18(2)(Luna 5  $\mu$ l, 150 x 2.0mm) column.

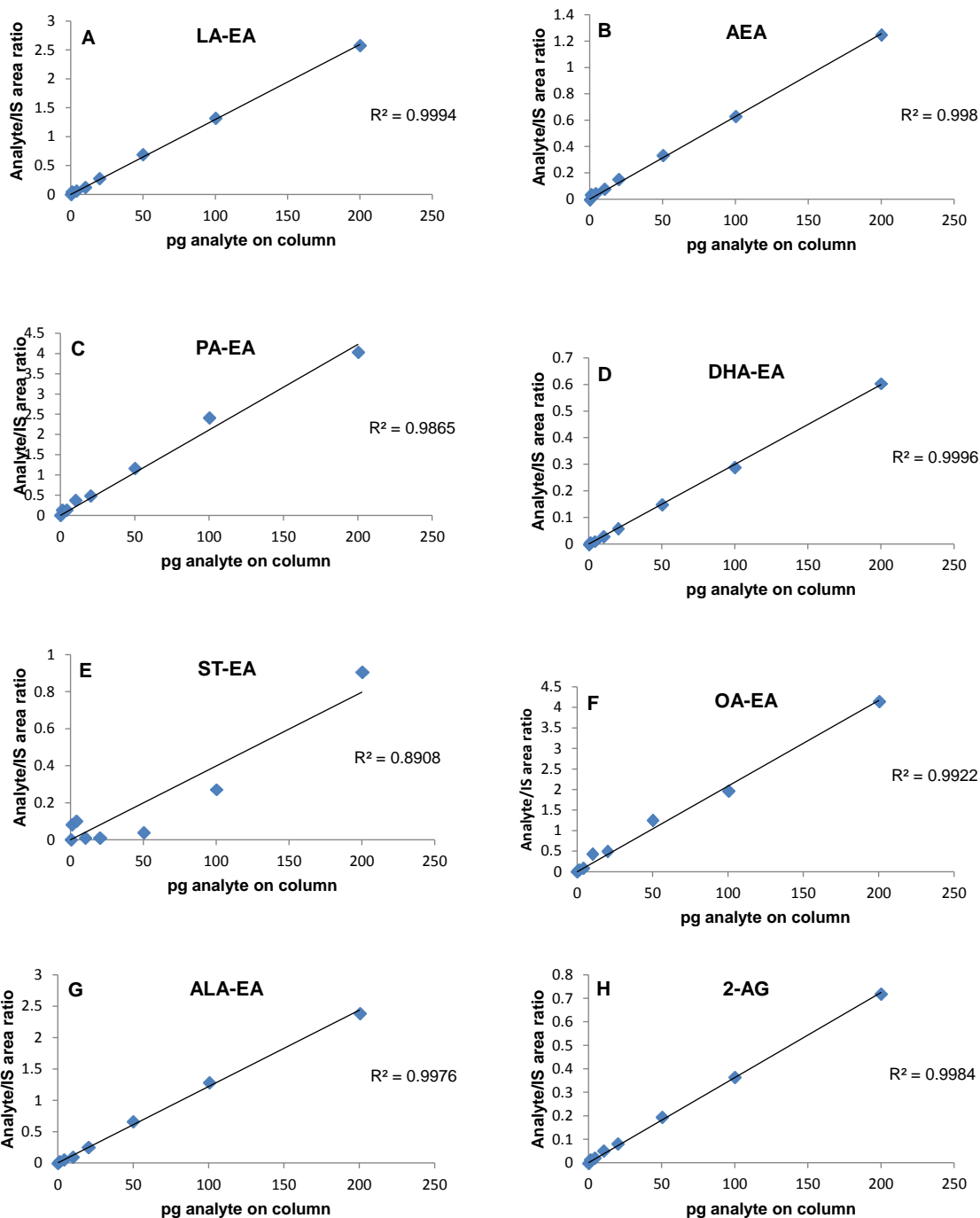
### **4.3.2 SPE, recovery, linearity, limits of detection and quantification**

FA-EAs and ECs were extracted from rat brain and the reconstituted extract was analysed without any further purification. However, the sensitivity was low. Therefore, a solid phase extraction (SPE) step was applied to clean up the extract. Following this step the sensitivity was found improved.

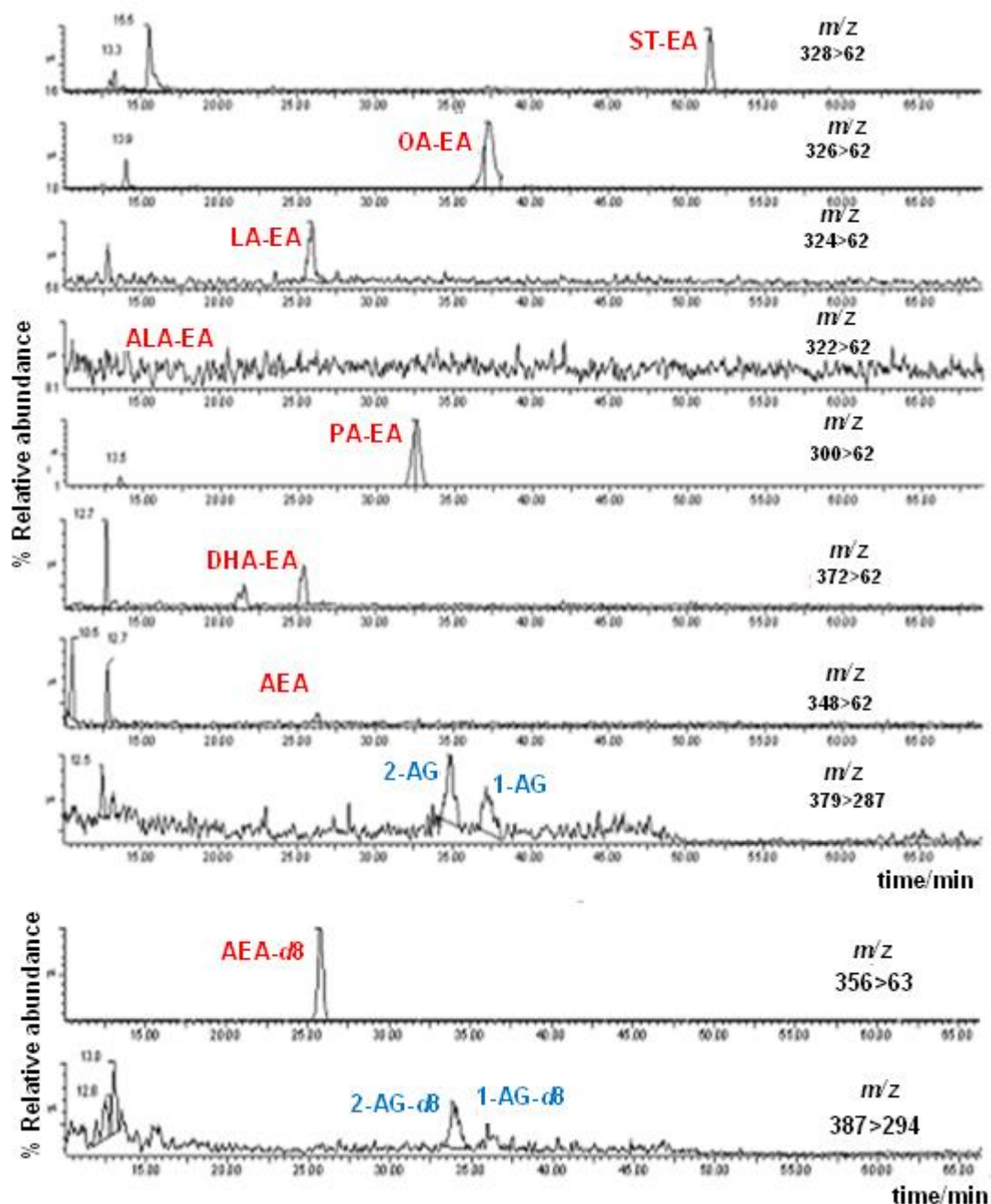
The recovery in the assay of all the metabolites ranged from 88% to 122%. Standard calibration curves were created for each compound (Table 2.2) and the least-squares linear regression method was used to calculate the calibration lines. Linear correlations ( $r^2 = 0.89-0.99$ ) were observed for the concentrations over range of 1 pg to 120 pg on the column (Figure 4.4), and used to quantitative the analytes. The limits of detection were found to be in the range of 0.25-1 pg on the column, and the limits of quantitation were 1-5 pg on the column.

### **4.3.3 Endocannabinoids and fatty acid ethanolamides found in rat brain**

Seven FA-EAs and 2-AG were identified in rat brain and eluted between 25 and 52 min (Figure 4.5), ALA-EA eluted at 20.5 min, DHA-EA at 25.3 min, LA-EA at 25.9 min, AEA at 26.2 min, PA-EA at 32.5 min, OA-EA at 38.7 min, ST-EA at 51.5 min and 2-AG at 34.9 min, 1-AG (isomer of 2-AG) at 37.3 min. IS AEA-*d8* at 25.7 min, IS 2-AG-*d8* at 33.8 min, 1-AG-*d8* (isomer of 2-AG-*d8*) at 36.3 min. All of them were eluted with 70% of acetonitrile (isocratic system). Normalized peak areas were used as following: Analytes peak area / internal standard peak area of the same sample. By comparing their ratios of peak areas to the standard curves (Figure 4.4) and using tissue wet weight, their concentrations were calculated and expressed as pg/mg wet weight.



**Figure 4.4.** Representative calibration lines (1-120 pg analyte in column) of (A) linoleoylethanolamide (LA-EA), (B) arachidonylethanolamide (AEA), (C) palmitoylethanolamide (PA-EA), (D) docosahexaenylethanolamide (DHA-EA), (E) stearoylethanolamide (ST-EA), (F) oleoylethanolamide (OA-EA), (G)  $\alpha$ -linolenoyl ethanolamide (ALA-EA) and (H) 2-arachidonoyl glycerol (2-AG).



**Figure 4.5.** LC/ESI-MS/MS analysis of microwaved rat brain FA-EAs and 2-AG; anandamide (AEA), N-palmitoylethanolamide (PA-EA), N-stearoylethanolamide (SA-EA), N-oleoylethanolamide (OA-EA), N-docosahexaenoylethanolamide (DHA-EA), N-linoleoylethanolamide (LA-EA), N-linolenoylethanolamide (ALA-EA), 2-arachidonoylglycerol (2-AG), 1-arachidonoylglycerol (1-AG), deuterated IS (AEA-d8) and the deuterated IS (2-AG-d8) and its isomer 1-AG.

#### **4.3.4 The effect of LPS on rat brain fatty acid ethanolamides and endocannabinoids**

The effect of low dose LPS (1 mg/mL at 0.5 ng/h) and/or high dose LPS (0.5 mg/mL at 250 ng/h) on microwaved rat brain fatty acid ethanolamides and endocannabinoids is shown in Table 4.2. However, the treatment did not result in statistically significant differences.

#### **4.3.5 The effect of lithium chloride on rat brain fatty acid ethanolamides and endocannabinoids**

The effect of four weeks LiCL treatment (1.70 g/kg) followed by 2 weeks LiCL (2.55 g/kg) on microwaved rat brain FA-EA and ECs of neuroinflammation induced by LPS infusion at low dose (1 mg/mL at 0.5 ng/h) and/or high dose (0.5 mg/mL at 250 ng/h) are shown in Table 4.2.

The effect of LiCL treatment compared to untreated subjects shows that only LA-EA was significantly increased ( $p=0.044$ ). Moreover, ALA-EA ( $p=0.035$ ) and LA-EA ( $p= 0.033$ ) were significantly increased in LiCL treated low LPS compared to untreated low LPS, while DHA-EA was significantly decreased ( $p= 0.006$ ), while no significant changes have been noticed on the other FA-EA and 2-AG. However, LiCL treated high LPS compared to untreated high LPS shows that only LA-EA was significantly increased ( $p=0.031$ ), whereas no significant changes have been noticed on the other FA-EA and 2-AG.

**Table 4.2.** A summary of all fatty acid ethanolamides (FA-EA) and endocannabinoids (ECs) detected in microwaved rat brain subjected to low dose of lithium chloride (LiCL) 1.70 g/kg for 4 weeks, then to high dose of lithium chloride 2.55 g/kg (high LiCl) for 2 weeks following treatment with LPS at low dose (1 mg/mL at 0.5 ng/h) and high dose (0.5 mg/mL at 250 ng/h) compared to controls. \$ p<0.05 (High LPS vs LiCL high LPS); \* p<0.05, \*\* p<0.01 (Low LPS vs LiCL low LPS); # p<0.05 (Control vs LiCL treated). LPS: Lipopolysaccharide. Results are presented as pg of each analyte per mg of tissue (mean  $\pm$ SD), n = 8 animals per group.

pg/mg tissue						
FA-EA and ECs	Control	Low LPS	High LPS	LiCL treated	LiCL low LPS	LiCL high LPS
<b>PA-EA</b>	684.3 $\pm$ 410.3	531.2 $\pm$ 335.6	601.6 $\pm$ 551.6	481.5 $\pm$ 386.1	255.4 $\pm$ 197.3	692.9 $\pm$ 472.1
<b>OA-EA</b>	61.3 $\pm$ 42.6	43.8 $\pm$ 26.5	49.1 $\pm$ 25.5	44.6 $\pm$ 43.1	22.4 $\pm$ 20.7	59.8 $\pm$ 45.9
<b>ST-EA</b>	64.1 $\pm$ 17.4	57.6 $\pm$ 36.4	37.7 $\pm$ 38.7	25.0 $\pm$ 11.5	23.6 $\pm$ 14.6	56.7 $\pm$ 28.5
<b>2-AG</b>	14.3 $\pm$ 8.4	23.7 $\pm$ 19.9	17.3 $\pm$ 9.8	36.3 $\pm$ 25.5	18.6 $\pm$ 9.6	19.9 $\pm$ 12.9
<b>1-AG</b>	249.7 $\pm$ 217.5	240.7 $\pm$ 194.9	199.9 $\pm$ 190.8	230.6 $\pm$ 173.3	147.6 $\pm$ 142.1	213.3 $\pm$ 151.8
<b>T-AG</b>	264.0 $\pm$ 223.0	264.4 $\pm$ 2.9.2	217.2 $\pm$ 198.1	266.9 $\pm$ 218.6	166.3 $\pm$ 151.5	233.1 $\pm$ 163.6
<b>ALA-EA</b>	0.1 $\pm$ 0.1	0.01 $\pm$ 0.01*	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.01*	0.1 $\pm$ 0.1
<b>LA-EA</b>	1.0 $\pm$ 0.5 <sup>#</sup>	1.0 $\pm$ 1.0*	0.9 $\pm$ 0.3 <sup>\$</sup>	1.2 $\pm$ 1.1 <sup>#</sup>	1.2 $\pm$ 0.4*	1.6 $\pm$ 1.0 <sup>\$</sup>
<b>AEA</b>	0.8 $\pm$ 0.4	0.6 $\pm$ 0.4	0.5 $\pm$ 0.3	0.8 $\pm$ 0.1	0.6 $\pm$ .04	0.3 $\pm$ .01
<b>DHA-EA</b>	5.4 $\pm$ 3.4	4.4 $\pm$ 1.8**	4.0 $\pm$ 1.5	3.9 $\pm$ 3.3	3.2 $\pm$ 0.7**	3.7 $\pm$ 2.3

#### 4.4 Discussion

Neuroinflammation contributes to degenerative diseases including: neuronal degenerative diseases, Alzheimer's disease, oxidative stress, and cell apoptotic processes. It has been reported that neuroinflammation caused by LPS infusion stimulates brain AA release and metabolism in rats, but lithium pre-treatment reduces this effect, indicating a potentially important therapeutic action of lithium (Basselin et al., 2007). However, no study has been applied to investigate lithium treatment effects on production of brain FA-EAs and ECs in LPS induced neuroinflammation. Therefore, the aim of this present study was to study the effects of lithium on the brain FA-EAs and ECs of rats subjected to head-focused microwave irradiation. Also, to further understand and provide a new possible mechanism of lithium neuroprotective action during neuroinflammation caused by LPS infusion subjected to lithium treatment.

In order to measure brain FA-EA and EC, an LC/ESI-MS/MS protocol was developed and applied to measure PA-EA, ST-EA, OA-EA, ALA-EA, LA-EA, AEA, DHA-EA, and 2-AG from microwaved rat brain tissue. The extraction protocol included a SPE clean up step. Good recovery that ranged from 88% to 122% was achieved.

The LC/ESI-MS/MS assay applied in this study allowed us to detect FA-EAs and ECs of rat microwaved brain tissue. The method was sensitive enough to detect low levels of these mediators. Quantification of AEA, PA-EA, DHA-EA, ST-EA, L-EA, ALA-EA and OA-EA was based on MRM assays using the fragment  $m/z$  62 representing the ethanolamine moiety. This was in agreement

with other published protocols (Felder et al., 1996, Weber et al., 2004, Yang et al., 2005).

The assay of 2-AG was based on the transition  $m/z$  287 to  $m/z$  92, that corresponds to the formation of the glycerol fragment (Bradshaw et al., 2006). The detection of 2-AG and 2-AG-d8 was complicated by the presences of isomers of 1-AG and 1-AG-d8 respectively. These could have been formed during the extraction. The detection of more than one peak (Figurer 4.3) confirmed their presences in the extract. In the case of 2-AG, the combined peak of the two isomerises (total AG, T-AG) was used, as described in other studies (Carrier et al., 2004, Richardson et al., 2007).

Microwaved brain tissue was used in this study. It has been reported that brain AEA is regulated enzymatically and its brain levels are increased in response to ischemia and post-mortem delay (Deutsch et al., 1997, Bazinet et al., 2005). Also, it was hypothesised that ordinary decapitation may have similar effects on brain levels of unesterified AA and its brain mediators. Consequently, brain FA-EAs and ECs may be affected by ordinary decapitation. Thus, an immediate head-focused microwave irradiation was used to rapidly inactivate FA-EAs and ECs metabolising enzymes prior to decapitation, thereby enabling their accuracy and precise measurements in rat brain tissue to be determined. However, in the present study OA-EA, ST-EA, ALA-EA, AEA, LA-EA and 2-AG were only found at low levels. It has been reported recently on the essentiality of head-focused microwave irradiation to fix brain lipids prior to analysis (Farias et al., 2008). Therefore, these findings may simply reflect that heat-induced microwave irradiation may breakdown brain FA-EA and EC or their trapping in

heat-denatured proteins (Farias et al., 2008, Golovko and Murphy, 2008). Therefore, our study is recommended to further perform these findings with a new study to be reported on brain FA-EA heat stability and recovery.

The present finding of AEA occurrence at low levels was in agreement with previous findings which reported that AEA levels in head-focused microwaved rat brain were lower than generally reported in non head-focused microwave treated brains (Bazinet et al., 2005). This could be due to lithium reducing brain AA metabolism to AEA. Furthermore, in our study LA-EA and ALA-EA were found to be affected by LiCL treatment and increases statistically significant. LA-EA and ALA-EA are metabolic products of LA and ALA respectively; these findings may indicate that lithium activates LA and ALA metabolism pathways in the brain, particularly their conversion to their ethanolamide derivatives.

DHA-EA was found at high levels and was found to be affected by the lithium and low LPS treatment which decreased the level statistically significantly. This finding suggests that LiCL works to reduce brain DHA being metabolised to DHA-EA. A recent study has reported that DHA metabolism to DHA-EA is a significant biochemical mechanism for neurite growth and offers a new molecular insight into hippocampal neurodevelopment and function (Kim et al., 2011).

Our finding of 2-AG being present at high levels was in agreement with other studies reported on 2-AG as the most abundant monoacylglycerol found in mouse and rat brain (Kondo et al., 1998, Sugiura et al., 1999). This was not affected by lithium/LPS treatments. Although the analysis was complicated by

its isomer 1-AG, both isomers were reported as total AG because it is still unknown to what extent 2-AG is converted to 1-AG during the extraction process. Also, it is not known if 1-AG was already present in rat brain.

PA-EA was present at high levels in control microwaved rat brain; it was found to be reduced by lithium and attenuated by lithium/low LPS treatment, but increased with lithium/high LPS, although these changes are not statistically significant. The present findings are still in agreement with previous reports on PA-EA anti-inflammatory activity in neuronal cells (Lambert et al., 2002, Lo Verme et al., 2005). It attenuates pain sensation (Calignano et al., 1998) and has a variety of biological functions related to ameliorating chronic pain and inflammation (O'Sullivan, 2007) and recently it has been reported that it has a neuroprotective effect (Koch et al., 2011). However, none of the previous studies have linked PA-EA activities to lithium effects. In addition, the presence of PA-EA at high levels indicates an effect of lithium in reducing PA-EA hydrolysis by FAAH with a corresponding increase in PA-EA levels in brain.

Also, it has been reported that PA-EA could enhance or replace AEA effects on brain activity and is regarded as a shorter and fully saturated analogue of anandamide (Jonsson et al., 2001, Ho et al., 2008). This may explain the present findings on AEA which was found at low levels and was not affected by lithium/LPS treatment. Also, a study has recently demonstrated that an imbalance and alteration in brain PA-EA and AEA levels (increased PA-EA/AEA ratio) exhibit analgesia in acute and inflammatory pain (Calignano et al., 1998, De Filippis et al., 2010). Brain OA-EA and ST-EA were also found at high levels

in this study, and their concentration was not affected by lithium LPS treatment suggests that lithium does not affect FAAH.

There is increasing evidence that inflammatory changes may be a contributing factor in a number of brain or neurodegenerative disorders. Systemic administration of LPS has been used as a model of acute inflammation in numerous studies (Sadeghi et al., 1999, Lonergan et al., 2004, Kavanagh et al., 2004). Also, LPS has been used to induce neuroinflammation in rats and, depending on the dose, LPS may contribute to initiating degenerative and ischemic brain diseases (Haus-Wegrzyniak et al., 1998, Hunot and Hirsch, 2003, Rosenberger et al., 2004).

During the neuroinflammatory response, the brain enzymes (phospholipases) that produce AA, DHA, PA, OA, ST, ALA and LA are activated, resulting in release of these mediators from neuronal cells (Phillis et al., 2006). Also, LPS infusion activates the AA cascade, because of that, all the AA metabolites can interfere with brain function, structure, and energy metabolism (Rosenberger et al., 2004).

In addition, lithium is known to ameliorate neurodegeneration, suppress neuroinflammation and improve behavioural performance in a mouse model of traumatic experience (Yu et al., 2012). It has been recently reported that lithium modifies and decreases brain AA and DHA release and metabolism in a rat LPS-induced model of neuroinflammation (Basselin et al., 2007, Ramadan et al., 2012). Therefore, it has been hypothesised that these effects may also impact on FA-EA brain synthesis. Also, lithium treatment is found to significantly

improve learning ability and memory in association with increased brain preservation (Yu et al., 2012). Furthermore, it has been reported to be the primary drug of choice for the treatment of bipolar disorder (Harwood and Agam, 2003).

The neuroprotective effects of FA-EAs and ECs have been described by several authors (Monory et al., 2006, Zhang et al., 2008, Noonan et al., 2010). Their ability to decrease inflammation in the animal spinal cord has been reported over two decades ago (Lyman et al., 1989), and several studies have supported this with recent evidence indicating that symptoms and inflammatory changes were more profound in CB2 receptor knockout mice (Palazuelos et al., 2008). The neuroprotective and anti-inflammatory effect of 2-AG has also been reported (Panikashvili et al., 2005). However, the mechanisms by which FA-EAs and ECs mediate their neuroimmunomodulatory effects are not well elucidated.

Moreover, it has been thought that brain FA-EAs, if not controlled, may cause a number of health effects and impact brain function and performance. Their brain levels are regulated and controlled by the hypothalamus to avoid creating their negative health effects. Furthermore, it has been reported that these bioactive lipids play important roles in energy homeostasis, cell proliferation, metabolic homeostasis, and regulation of inflammatory processes. For instance, AEA activates the cannabinoid receptors (CB1/CB2) (Matsuda et al., 1990, Devane et al., 1992) and exhibits anti-inflammatory properties. Also PA-EA is inactive at both cannabinoid receptor types, but was shown to bind to PPAR- $\alpha$ . This receptor was suggested to mediate some of the anti-inflammatory effects of PA-

EA (Lo Verme et al., 2005), but is unable to exert lipolysis effects (Matias et al., 2007).

Whereas, OA-EA is also inactive on both cannabinoid receptors, it activates (PPAR- $\alpha$ ) with more potency than PA-EA; OA-EA, is also able to exert lipolysis effects, but shows opposing effects as compared to the other FA-EAs (Fu et al., 2003) and exerts anorexic actions (Rodriguez de Fonseca et al., 2001, Fu et al., 2003). Moreover, the FA-EAs and ECs, their metabolic enzymatic machinery and their receptors have been detected in all central and peripheral tissues involved in the control of energy intake, processing and storage, including the hypothalamus (Berrendero et al., 1998, van der Stelt et al., 2001, Osei-Hyiaman et al., 2005, Engeli et al., 2005) and they also stimulate lipogenesis and fat accumulation (Cota et al., 2003).

In conclusion, all these reports taken together with the present results may indicate that lithium could exert positive effects in developing potent, novel molecules with anti-inflammatory and protective properties against neuroinflammation and traumatic brain injury that involves activation of inflammatory cells and production of local pro-inflammatory mediators that can amplify brain damage. Thus, medicinal use of lithium and the potential of fatty acid amide-targeted therapy of neuroinflammation may represent an exciting opportunity for the development of new drugs for treatment of brain diseases, disorders and general inflammatory diseases.

**Chapter 5. Plasma oxysterols and red blood cell fatty acids as potential biomarkers for the diagnosis of Amyotrophic Lateral Sclerosis**

## 5.1 Introduction

About 25% of the total body cholesterol is located in the brain. Cholesterol is involved in the maturation of the CNS and is necessary for vital brain functions (Bjorkhem and Meaney, 2004, Dietschy and Turley, 2004, Leoni and Caccia, 2011). Cholesterol also serves as a precursor for the synthesis of oxysterols (Ioannou, 2001) as shown in Figure 1.6 and is converted to oxysterols by either enzymatic pathways (Russell, 2000) or autoxidation via non-enzymatic pathways (Iuliano, 2011).

The sensitivity of cholesterol to enzymatic and non-enzymatic oxidation has generated significant interest in oxysterols as potential markers for the *in vivo* study of oxidative stress (Russell, 2000, Javitt, 2008). The biosynthetic pathways and biological activities of oxysterols have been studied to clarify the causes of many diseases, understanding their role in several conditions such as neurological diseases and ageing (Luoma, 2007, Javitt, 2008, Bjorkhem et al., 2009, Leoni and Caccia, 2011). Furthermore, lipid composition of red blood cells and plasma in neurodegenerative diseases such as Amyotrophic lateral sclerosis (ALS) has attracted the attention of many researchers in the recent years (Acar et al., 2012).

ALS is one of the most common motor neuron diseases. It causes muscle weakness, muscle atrophy and lose the ability to initiate and control all voluntary movement, but not always, spared until the terminal stages of the disease and over time, patients experience dysphagia, dysarthria, spasticity and hyperreflexia (Chio et al., 2011). A defect on chromosome 21 codes

for superoxide dismutase causing mutations in the gene that produces the powerful antioxidant Cu/Zn superoxide\_dismutase (SOD1) enzyme which found to be associated with approximately 20% of cases of inherited ALS (Reaume et al., 1996, Al-Chalabi and Leigh, 2000).

It has recently been reported that there are significantly higher plasma and brain cholesterol levels in ALS patients when compared to healthy controls. Also there is a probability of a high risk of cholesterol autoxidation that may result in elevation of oxysterols levels in ALS patients (Zhao et al., 2012). Moreover, hypercholesterolemia has been found to be an important risk factor associated with neurodegenerative disorders such as Alzheimer's disease (Bodovitz and Klein, 1996, Simons et al., 1998, Koudinov and Koudinova, 2001, Puglielli et al., 2003). Management and adjustment of plasma and brain cholesterol levels is regulated by mechanisms that control brain and central nervous system cholesterol levels. The exchange of cholesterol between the brain and plasma is prevented by the BBB. However, conversion of cholesterol to oxysterols that can cross the BBB is the most important mechanism for eliminating excess cholesterol from the brain (Bjorkhem and Diczfalusy, 2002, Meaney et al., 2007, Vaya et al., 2011).

Oxysterol species are present in the human circulation, and species that have been identified in plasma include: 7 $\alpha$ -OH, 24-OH, 25-OH, 4 $\alpha$ -OH and 4 $\beta$ -OH (Russell, 2000, Luoma, 2007, Bjorkhem et al., 2011, Farez et al., 2011). The levels of these species in the circulation can be altered because of neuron diseases and ageing (Javitt and Javitt, 2009, Jeitner et al., 2011), and some species have been considered as possible neurodegenerative disease

biomarkers (Lordan et al., 2009, Leoni and Caccia, 2011). Although, oxysterols are detected in healthy human or animal plasma at very low levels compared to cholesterol, they have been found at higher levels associated with neurodegenerative disorders such as Multiple Sclerosis, Alzheimer's disease and Huntington's disease (Leoni and Caccia, 2011).

Recently it has been reported that oxysterols also have the capacity to induce inflammatory responses and play roles in cell differentiation processes, and are implicated in a number of neurological disorders (Olkkonen et al., 2012). Conversely, oxysterols have been considered as a major cause of tissue and cellular dysfunction that plays a major role in ageing and most age-related and oxidative stress-related diseases (Negre-Salvayre et al., 2010). It has been hypothesised that the flux of oxysterols to and from the brain may be used as a key to prevent or slow down neurodegeneration or for diagnosis of many brain disorders such as ALS (Anchisi et al., 2012).

Analysis of oxysterols present challenging analytical problems, because of their presence in trace amounts in biological systems (Bjorkhem et al., 2011). To identify the oxysterols in human or animal plasma, there is need for careful and effective sample extraction methods as well as the use of highly sensitive analytical instruments (Kumar et al., 2011). GC-MS is the analytical technique of choice for oxysterols which requires a critical derivatisation step to convert oxysterols to non-polar compounds. Silylation is the major derivatization procedure for GC/MS oxysterol analyses which has typically been performed after forming their TMS derivatives (Birkofer et al., 1982, Blau and Halket, 1993, Segura et al., 1998). These derivatives are less polar and thermally-stable than

free oxysterols; these properties enable them to exhibit a lower limit of detection, enhanced peak intensity and give high resolution results on GC-MS analysis (Blau and Halket, 1993, Dutta and Appelqvist, 1997, Ubhayasekera et al., 2004).

On the other hand, n-3 PUFA have been found to have a wide range of beneficial effects in several neuronal health and disease conditions. It has been indicated that n-3 PUFA concentration affects blood lipid profile and cell membrane lipid composition (Wainwright, 2002, SanGiovanni and Chew, 2005). Also, it has been proposed that a diet rich in n-3 PUFA could reduce by 50–60% the risk of developing the ALS disease (Veldink et al., 2007). Despite the proposed dietary n-3 PUFA supplementation benefits as a potential means to delay the onset of the MNDs and/or their rate of progression, there is inadequate evidence to support this. Furthermore, there is a lack of studies to examine ALS that could induce any changes in human or animal RBC fatty acid profiles. RBC fatty acid composition is considered to be commonly used to assess fatty acids in human and animal studies, and there is evidence that the PUFA profile of blood cells might reflect neural tissue levels (Benatti et al., 2004, Acar et al., 2012). Moreover, RBC membrane lipids are considered as an index of tissue lipid status, and they represent the longer-term fatty acid status that is found to be less sensitive to dietary fluctuations than plasma lipids (Farquhar, 1962, Carlson et al., 1986, Makrides et al., 1994, Acar et al., 2012).

When oxidation of cholesterol and fatty acids mostly occurs within cell membranes it causes alterations in their fluidity, permeability, and structure and damages other cellular constituents (Blache et al., 1998, Iuliano, 2011). Therefore, it has been hypothesised that these alterations could be significant in

the pathophysiological role of cholesterol and oxidized fatty acids in neurological diseases.

Transgenic mice deficient in SOD1, an intrinsic redox enzyme, display elevated oxidative stress, decreased body mass, decreased musculoskeletal mass, and decreased whole muscle strength compared to wild-type mice. All these effects are observably associated with ageing. Therefore, this model is used to study ALS-like diseases and give information about human ALS (Huang et al., 1999, Vasilaki et al., 2006).

In this study we used tissues from SOD1 mutant-deficient mice. We used adult and aged rats as a model to study circulating oxysterols and compare levels to the wild-type mice. This could help in understanding the role of plasma oxysterols in neurodegenerative diseases, and explore the relevance of SOD1 mutant-deficient mouse models to ALS patients. Therefore, this study was set to examine the hypothesis whether oxysterols and RBC fatty acids are altered in ALS *in vivo* and whether such abnormalities are correlated with each other in relation to age and disease states. The first objective was to check whether plasma oxysterol species are affected by ALS in human and in SOD1 mutant-deficient mice. The second objective was to analyse RBC fatty acid profiles and find changes in ALS and in SOD1 mutant-deficient mice.

## **5.2 Materials and Methods**

All animals and clinical samples used for this work were provided by Dr Andrea Malaspina, Centre of Neuroscience and Trauma, Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry as

described in sections 2.2.3 and 2.2.4 respectively. All materials which have been used for this study are described in detail in section 2.1.1, Fatty acid analysis method details including: solvents preparation, standards preparation and RBC lipid extraction are described in sections 2.3.1, 2.3.2, 2.3.3 & 2.3.4 respectively. Fatty acid analysis is performed using GC as described in sections 2.3.6, 2.3.7 & 2.3.8 respectively. Oxysterol analysis method details and plasma lipid extraction details are described in section 2.6.1, Oxysterol species analysis is performed using GC-MS as described in section 2.6.3.

## **5.3 Results**

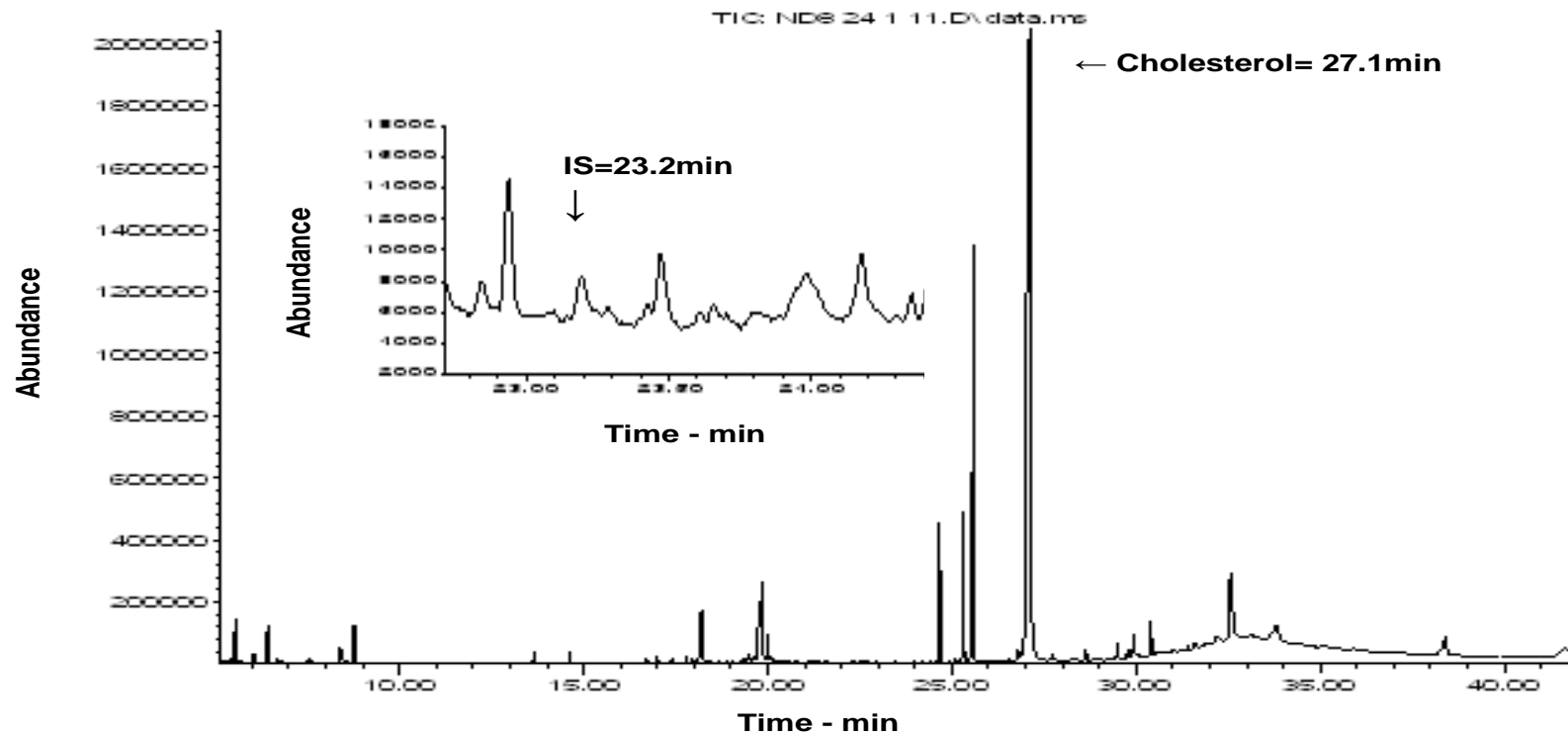
### **5.3.1 Plasma oxysterols in ALS patients**

GC-MS analysis of human plasma lipid extracts was applied to identify the most abundant oxysterol species. Under the conditions employed in this experiment and according to the literature (Evershed et al., 1999) and previous work of the group (Dr Stern, personal communication), oxysterol species are expected to elute between 19.9 and 30.4 min, as shown in Figure 5.1. Overall, 42 peaks were recorded in this area including the internal standard (IS) (23.2/min) and cholesterol (27.1/min). Firstly, the peak area of all peaks eluted between 19.9 and 30.4 min were integrated, the normalisation was based on using IS peak area as following: peak area/IS area. Data was expressed as mean  $\pm$  standard deviation (SD). Table 5.1 shows the normalised peak areas of all relevant peaks.

A total of 14 out of 42 peaks (Table 5.1) were found to be statistically different. Those peaks that eluted at 21.2, 23.5, 26.6, 28.2 and 30.0 min were found to be significantly decreased in plasma of ALS patients when compared to controls.

While those peaks eluted at 24.7, 25.1, 25.3, 25.6, 26.2, 26.8, 27.9, 29.8 and 29.9 min were found significantly increased in plasma of ALS patients when compared to controls. Finally, the electronic impact (EI) spectra for each peak were used to identify the compounds that eluted at these retention times. The identification process was based on the  $m/z$  value for the molecular ion (M), the fragmentation patterns and information from the literature. Figure 6.2 shows the fragmentation patterns for oxysterols as reported in the literature (Breuer and Bjorkhem, 1990, Dzeletovic et al., 1995, Zhang et al., 2001, Jiang et al., 2007, Rossmann et al., 2007).

Table 5.2 summarizes the retention time, molecular weight of oxysterols and  $m/z$  of oxysterol-TMS ethers, as well as the main fragment ions used to identify oxysterol species following GC-MS analysis of the lipid extract from human plasma samples. A total of 14 peaks were identified as oxysterols according to their retention time, molecular ions and  $m/z$  oxysterol-TMS fragment ions. For example: 24-OH (24.7 min) was identified using the fragment ions  $m/z$  129, 145, 412 and 456; and 27-OH (25.6 min) was identified using  $m/z$  147, 257, 411 and 457. The oxysterol with shortest retention time was 7 $\alpha$ -OH (22.1 min) and the oxysterol with the longest retention time was 7-keto (30.0). More details are presented in Appendix 2 and Appendix 3. Figure 5.3 shows the chemical structures and molecular weight of TMS derivatives of all oxysterols that were identified in human plasma.



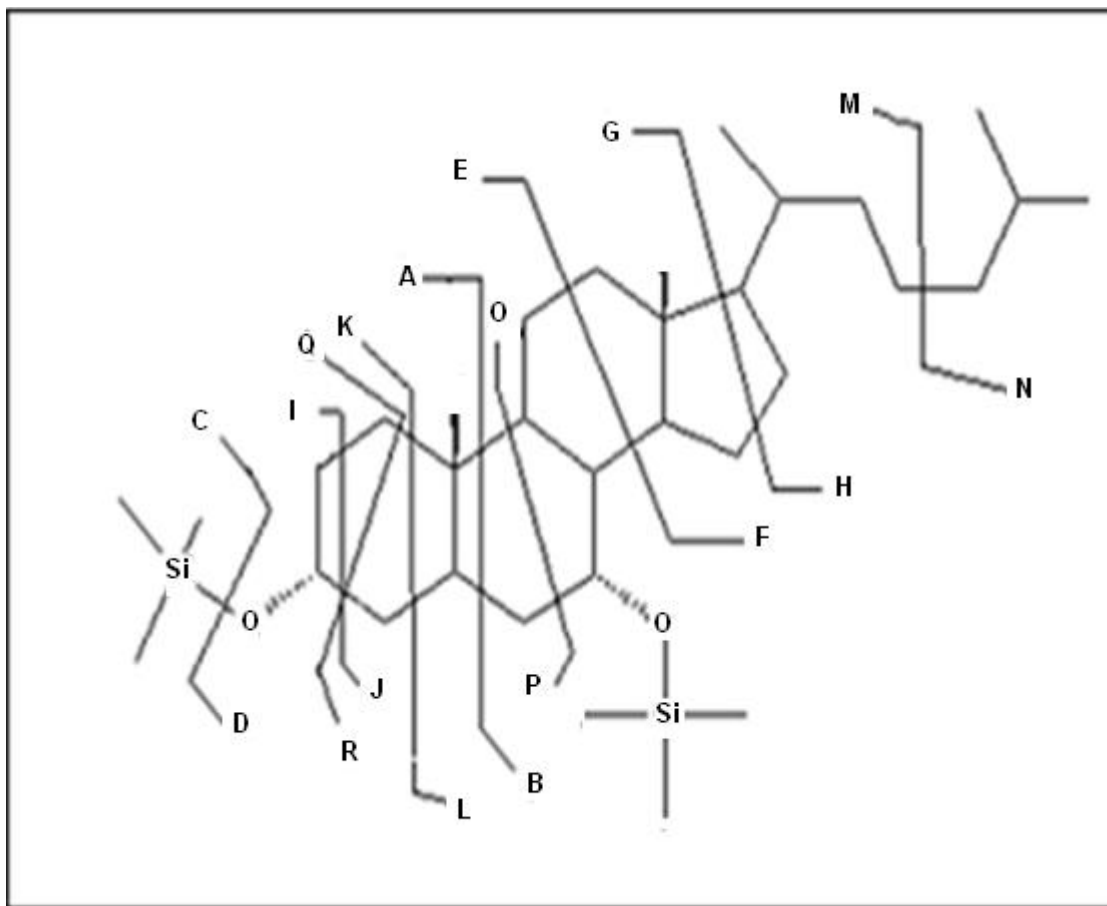
**Figure 5.1.** Representative GC-MS traces showing the analysis of one human plasma sample. The internal standard (IS) (4 $\beta$ -OH (d7)) eluted at 23.2 min and cholesterol eluted at 27.1 min.

**Table 5.1.** Retention times (Rt min) and normalised peak area (peak area/IS area) for all peaks eluted between 19.9 min and 30.4 min during the GC-MS analysis of lipid extract from human plasma. The results in bold were significantly different when comparing controls to ALS Data shown as mean  $\pm$  SD, IS = internal standard.

Rt (min)	Control (n=9) Peak Area	ALS (n=10) Peak Area	P
19.9	82.7 $\pm$ 37.7	168.8 $\pm$ 72.5	
20.3	13 $\pm$ 17.1	7.8 $\pm$ 7.9	
20.6	1 $\pm$ 0.7	1 $\pm$ 1.2	
20.8	1.8 $\pm$ 1	2.4 $\pm$ 2.9	
21.0	1.4 $\pm$ 0.9	0.9 $\pm$ .8	
<b>21.2</b>	<b>2.8 <math>\pm</math> 1.8</b>	<b>3.6 <math>\pm</math> 3.2</b>	<b>0.003</b>
21.9	3.2 $\pm$ 4.6	0.8 $\pm$ 0.8	
22.1	2.2 $\pm$ 1.6	0.8 $\pm$ 0.6	
22.4	2.6 $\pm$ 2.4	1.5 $\pm$ 1.7	
22.5	0.9 $\pm$ 0.6	1.1 $\pm$ 1	
22.6	1.7 $\pm$ 0.8	1.4 $\pm$ 1.1	
22.9	1.7 $\pm$ 2.8	0.8 $\pm$ 0.5	
(IS) 23.2	1 $\pm$ 0	1 $\pm$ 0	
<b>23.5</b>	<b>3.9 <math>\pm</math> 6.9</b>	<b>0.8 <math>\pm</math> 0.7</b>	<b>0.008</b>

Rt (min)	Control (n=9) Peak Area	ALS (n=10) Peak Area	P
23.7	0.8 $\pm$ 0.5	0.7 $\pm$ 0.4	
23.9	1.1 $\pm$ 0.6	2.1 $\pm$ 0.6	
24.2	1 $\pm$ 0.6	1.9 $\pm$ 0.7	
24.4	0.8 $\pm$ 0.4	1.2 $\pm$ 0.3	
<b>24.7</b>	<b>16.9 <math>\pm</math> 11.2</b>	<b>98.4 <math>\pm</math> 31.9</b>	<b>0.0001</b>
24.9	1.4 $\pm$ 1	1.4 $\pm$ 1.4	
<b>25.1</b>	<b>1.1 <math>\pm</math> 0.5</b>	<b>3.5 <math>\pm</math> 1.8</b>	<b>0.007</b>
<b>25.3</b>	<b>16 <math>\pm</math> 10.7</b>	<b>24.7 <math>\pm</math> 5.3</b>	<b>0.002</b>
<b>25.6</b>	<b>38.7 <math>\pm</math> 17.9</b>	<b>302.3 <math>\pm</math> 64.0</b>	<b>0.002</b>
25.8	0.7 $\pm$ 0.5	0.9 $\pm$ 0.8	
26.0	2.8 $\pm$ 3.8	4.2 $\pm$ 5.8	
<b>26.2</b>	<b>0.9 <math>\pm</math> 0.4</b>	<b>1.7 <math>\pm</math> 1.3</b>	<b>0.033</b>
<b>26.6</b>	<b>6.4 <math>\pm</math> 10.3</b>	<b>2.7 <math>\pm</math> 1.9</b>	<b>0.008</b>
<b>26.8</b>	<b>1.9 <math>\pm</math> 1.1</b>	<b>6.1 <math>\pm</math> 6.9</b>	<b>0.0001</b>

Rt (min)	Control (n=9) Peak Area	ALS (n=10) Peak Area	P
(Cholesterol) 27.1	600.9 $\pm$ 156.4	1098. $\pm$ 168.1	
27.8	2 $\pm$ 0.7	2.5 $\pm$ 1	
<b>27.9</b>	<b>0.8 <math>\pm</math> 0.2</b>	<b>1 <math>\pm</math> 0.6</b>	<b>0.046</b>
<b>28.2</b>	<b>1.4 <math>\pm</math> 0.9</b>	<b>0.2 <math>\pm</math> 0</b>	<b>0.010</b>
28.4	1.6 $\pm$ 1.7	2.3 $\pm$ 1.8	
28.8	1.3 $\pm$ 0.7	2 $\pm$ 1.4	
28.9	0.9 $\pm$ 0.3	1.1 $\pm$ 0.4	
29.0	0.9 $\pm$ 0.3	1.4 $\pm$ 0.3	
29.3	1 $\pm$ 0.2	1.8 $\pm$ 0.5	
29.5	4.6 $\pm$ 3	8.1 $\pm$ 4.6	
<b>29.8</b>	<b>2.9 <math>\pm</math> 1.1</b>	<b>3.5 <math>\pm</math> 1.8</b>	<b>0.030</b>
<b>29.9</b>	<b>4.8 <math>\pm</math> 2</b>	<b>11.6 <math>\pm</math> 6.5</b>	<b>0.0001</b>
<b>30.0</b>	<b>6.6 <math>\pm</math> 4.0</b>	<b>0.9 <math>\pm</math> 0.9</b>	<b>0.035</b>
30.4	5.9 $\pm$ 2.7	11.1 $\pm$ 5.8	



**Figure 5.2.** Fragmentation pattern of oxysterol species. The fragmentation process is demonstrated here using 7 $\alpha$ -hydroxycholesterol-TMS as a representative structure for oxysterols. The letters A to R representative all possible fragment ions and the  $m/z$  values of all the fragments are shown in Appendix-3, Tables 1 & 2. Adapted from (Breuer and Bjorkhem, 1990, Dzeletovic et al., 1995, Zhang et al., 2001, Jiang et al., 2007, Rossmann et al., 2007).

### **5.3.1.1 The effect of disease on human plasma oxysterols**

Analysis of plasma oxysterol from ALS subjects indicated that the levels of 24-OH, 5,6 $\beta$ -epoxy, 5,6 $\alpha$ -epoxy, 27-OH, 26-OH, 20 $\alpha$ -OH, 22-OH, 25-OH hydroxycholesterol and the 6-ketocolesterol were significantly higher in plasma of ALS samples compared to controls. The levels of 7 $\alpha$ -OH, 7 $\beta$ -OH, 4 $\beta$ -OH, chol-triol hydroxycholesterol and 7-ketocolesterol were found to be significantly lower in plasma of ALS samples compared to control subjects. All data is presented in Figure 5.4 (A and B).

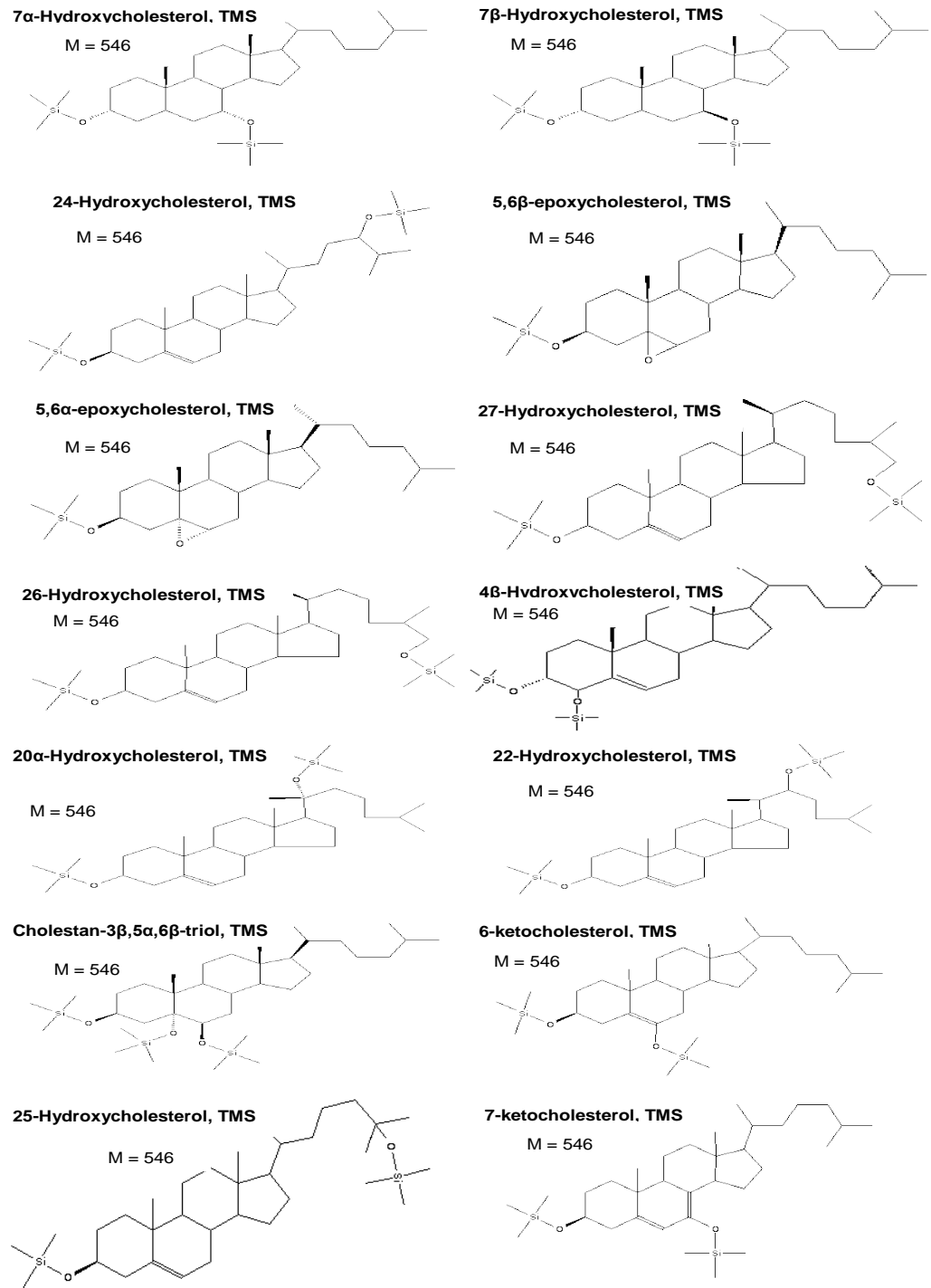
### **5.3.2 Plasma oxysterols in a SOD1-mutant transgenic mice model**

Plasma oxysterol analysis in the SOD1-ve animal model was performed following the same experimental protocol as described for the human plasma samples. All peaks eluted between 19.9 and 30.4 min were recorded. To identify the compounds that eluted at these retention times, the MS spectrum for each peak was used. Following the same process as in human plasma, the identification process was based on retention times, m/z values for the molecular ion (M) and the fragmentation patterns using information from the literature. A total of 14 peaks out of 42 peaks were identified as oxysterols according to their retention time, molecular ions and m/z oxysterols-TMS fragment ions. The oxysterol with shortest retention time was 7 $\alpha$ -OH (22.1 min) and the oxysterol with the longest retention time was 7-keto (30.0 min). A one-way ANOVA with Bonferroni's multiple comparison post-hoc test was conducted to ascertain any statistical significant changes.

**Table 5.2.** Retention times (Rt), molecular weight (MW), oxysterol-TMS m/z and fragment ions (m/z) used to identify oxysterols in human plasma lipid extracts, following GC-MS analysis.

	Rt min <sup>a</sup>	Oxysterols	MW	TMS (m/z)	Fragmentation pattern (m/z) <sup>b</sup>	References	Figure <sup>c</sup>
1	22.1	7 $\alpha$ -hydroxycholesterol (7 $\alpha$ -OH)	402	546	57, <b>73</b> , <b>105</b> , 129, 147, 159, <b>255</b> , <b>325</b> , 351, <b>368</b> , <b>411</b> , <b>456</b> , 503	(Nielsen et al., 1995) P108, Fig. 3, (Bortolomeazzi et al., 1999) P3072, Fig. 1	Fig 1
2	23.5	7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH)	402	546	57, 73, 129, 147, 159, <b>221</b> , 351, <b>386</b> , <b>411</b> , <b>456</b> , 503	(Bortolomeazzi et al., 1999) P3072, Fig. 1 (Lee et al., 2006) P4874 Tab. 1 & P4876 Fig.1	Fig 2
3	24.7	24-hydroxycholesterol (24-OH)	402	546	<b>129</b> , <b>145</b> , 213, 247, 255, 326, 352, 368, <b>413</b> , <b>456</b> , 503	(Kumar et al., 2011) P244, Tab. 1, (Griffiths and Wang, 2009) P2786	Fig 3
4	25.1	5,6 $\beta$ -epoxy-cholesterol (5,6 $\beta$ -epoxy)	402	474	57, 105, <b>159</b> , 213, 255, 353, <b>368</b> , <b>385</b> , 417, <b>459</b>	(Rossmann et al., 2007) P438, Tab. 1 & Fig.2, (Lee et al., 2006) P4874 Tab. 1 & P4876 Fig.1	Fig 4
5	25.3	5,6 $\alpha$ -epoxy-cholesterol (5,6 $\alpha$ -epoxy)	402	474	57, 105, <b>158</b> , 213, 255, 353, <b>368</b> , <b>385</b> , 417, <b>459</b>	(Rossmann et al., 2007) P438, Tab. 1 & Fig.2, (Lee et al., 2006) P4874 Tab. 1 & P4876 Fig.1	Fig 5
6	25.6	27-hydroxycholesterol (27-OH)	402	547	55, 105, <b>147</b> , 213, 247, <b>257</b> , 326, 352, 368, <b>411</b> , <b>457</b>	(Griffiths and Wang, 2009) P2786	Fig 6
7	26.2	26-hydroxycholesterol (26-OH)	402	546	105, 145, 213, 255, 301, <b>341</b> , 355, 368, <b>382</b> , <b>417</b> , <b>456</b>	(Li et al., 2000) P177, Fig.1	Fig 7
8	26.6	4 $\beta$ -hydroxycholesterol (4 $\beta$ -OH)	402	546	55, 105, 129, <b>147</b> , <b>253</b> , 327, <b>368</b> , <b>417</b> , 441, <b>456</b>	(Breuer, 1995) P2278, Fig. 1 & 2, (Bodin et al., 2002) P31538, Fig.2	Fig 8
9	26.8	20 $\alpha$ -hydroxycholesterol (20 $\alpha$ -OH)	402	547	55, 105, 145, <b>201</b> , 213, 255, 353, 368, 386, <b>461</b> , 502	(Lin et al., 2003) P58, Fig. 1	Fig 9
10	27.9	22-hydroxycholesterol (22-OH)	402	547	55, 105, 145, 213, 247, 255, <b>282</b> , <b>355</b> , 368, <b>382</b> , <b>441</b> , 456, <b>504</b>	(Kumar et al., 2011) P244, Tab. 1,	Fig 10
11	28.2	cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (Chol-triol)	420	637	<b>147</b> , 247, 255, <b>329</b> , 355, <b>367</b> , <b>417</b> , 457, 502	(Rossmann et al., 2007) P438, Fig.1 & Tab. 1, (Lee et al., 2006) P4874 Tab. 1 & P4876 Fig.1	Fig 11
12	29.8	6-ketocholesterol 6-Keto-Chol	418	547	55, <b>131</b> , 247, <b>334</b> , <b>369</b> , 418, <b>431</b> , <b>458</b> , <b>501</b>	(Kemmo et al., 2007) P1441, Fig. 1 & P1442, Tab. 1A	Fig 12
13	29.9	25-hydroxycholesterol (25-OH)	402	546	55, <b>131</b> , 147, 213, 247, <b>271</b> , <b>327</b> , <b>368</b> , <b>415</b> , <b>456</b> , 501	(Nielsen et al., 1995) P108, Fig. 3, (Johnsson and Dutta, 2003) P770, Fig. 2	Fig 13
14	30.0	7-ketocholesterol (7-Keto)	400	545	55, <b>131</b> , 147, 213, 246, <b>332</b> , 367, 416, 457, <b>472</b> , <b>501</b>	(Lee et al., 2006) P4874 Tab. 1 & P4876 Fig.1	Fig 14

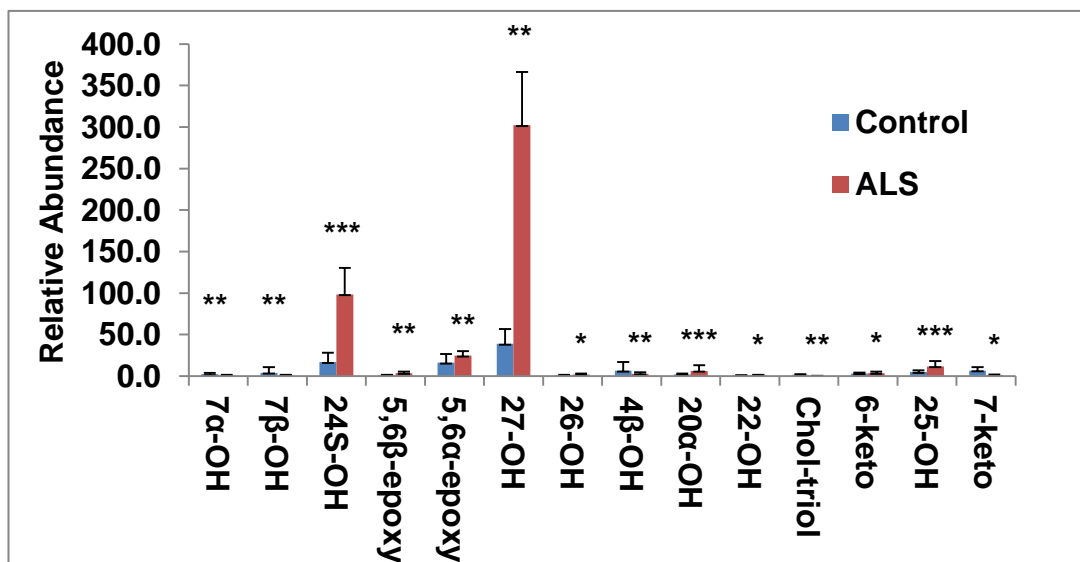
<sup>a</sup> Retention times with reference to **Table 6.1**. <sup>b</sup> Values in bold represent the ions used to identify oxysterols in the present study. <sup>c</sup> All these figures are found in **Appendix 2**.



**Figure 5.3:** Chemical structure and molecular weight (M) of oxysterol-TMS derivatives. (Adapted from: (Bjorkhem and Diczfalusy, 2002, Jiang et al., 2007)).

**A**

Rt (min)	Oxysterols	P
21.2	7 $\alpha$ -hydroxycholesterol (7 $\alpha$ -OH)	0.003
23.5	7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH)	0.008
24.7	24-hydroxycholesterol (24-OH)	0.0001
25.1	5,6 $\beta$ -epoxy-cholesterol (5,6 $\beta$ -epoxy)	0.007
25.3	5,6 $\alpha$ -epoxy-cholesterol (5,6 $\alpha$ -epoxy)	0.002
25.6	27-hydroxycholesterol (27-OH)	0.002
26.2	26-hydroxycholesterol (26-OH)	0.033
26.6	4 $\beta$ -hydroxycholesterol (4 $\beta$ -OH)	0.008
26.8	20 $\alpha$ -hydroxycholesterol (20 $\alpha$ -OH)	0.0001
27.9	22-hydroxycholesterol (22-OH)	0.046
28.2	cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (Chol-triol)	0.010
29.8	6-ketocholesterol (6-Keto-Chol)	0.030
29.9	25-hydroxycholesterol (25-OH)	0.0001
30.0	7-ketocholesterol (7-Keto)	0.035

**B**

**Figure 5.4.** Oxysterols identified in human plasma lipid extracts, following GC-MS analysis. **A.** Retention times (Rt min), species and p values. **B.** Plasma oxysterol species that were significantly different when comparing control to ALS patient samples. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Data shown as mean  $\pm$  SD,  $n=9$  of control,  $n=10$  of ALS patients.

### **5.3.2.1 The effect of disease on mice plasma oxysterols**

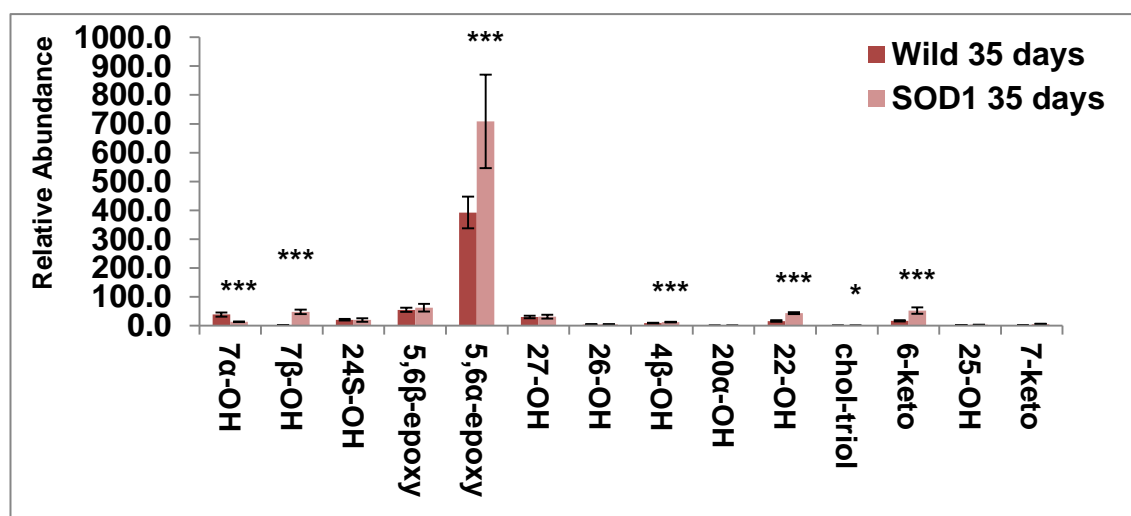
Plasma of SOD1-deficient mice was compared to plasma of wild type mice; both groups were studied at two different stages: pre-symptomatic stage at 35 days and end-stage at 120 days. The plasma levels of  $7\beta$ -OH,  $5,6\alpha$ -epoxy,  $4\beta$ -OH, 22-OH and 6-keto were significantly higher in SOD1-ve animals aged 35 days compared to wild type animals aged 35 days (Figure 5.5). While  $7\alpha$ -OH and chol-triol were significantly lower in SOD1 animals aged 35 days compared to wild animals aged 35 days. However, 24-OH,  $5,6\beta$ -epoxy, 27-OH, 26-OH,  $20\alpha$ -OH, 25-OH and 7-keto did not change. The plasma levels of 24-OH,  $5,6\beta$ -epoxy,  $5,6\alpha$ -epoxy,  $20\alpha$ -OH, 22-OH, 25-OH and 7-keto were significantly higher in SOD1-mutant animals aged 120 days compared to wild type animals aged 120 days, while only  $7\alpha$ -OH was significantly lower in SOD1-ve animals aged 120 days compared to wild animals aged 120 days (Figure 5.6).

### **5.3.2.2 The effect of age on mice plasma oxysterols**

Plasma levels of 24-OH,  $4\beta$ -OH and 22-OH were significantly lower in wild type animals aged 35 days compared to wild type animals aged 120 days, while  $7\alpha$ -OH, 27-OH,  $20\alpha$ -OH and 7-keto were significantly higher as shown in Figure 5.7. The remaining oxysterols did not change. Mice plasma levels of  $5,6\beta$ -epoxy, 27-OH,  $20\alpha$ -OH, 25-OH and 7-keto were significantly higher in SOD1 animals aged 120 days in comparison with SOD1 animals aged 35 days; conversely, the  $7\beta$ -OH,  $4\beta$ -OH, 22-OH and 6-keto were significantly lower, while the  $7\alpha$ -OH, 24-OH,  $5,6\alpha$ -epoxy, 26-OH, Chol-triol, and 7-keto showed no significant changes. All data is presented in Figure 5.8.

**A**

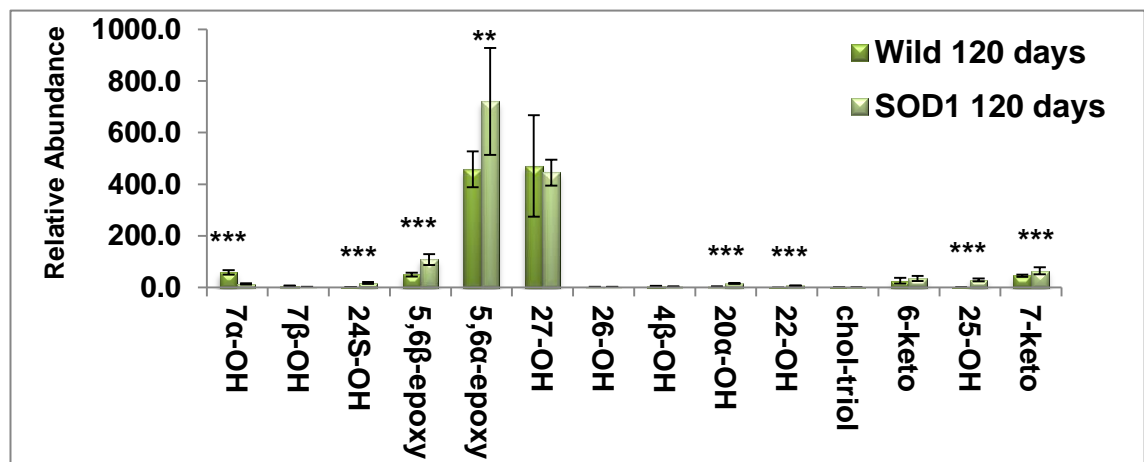
	Wild 35 days	SOD1 35 days		
Rt	Normalised Peak Area	Normalised Peak Area	P	Oxysterol
22.1	38.4 ± 7.4	13.0 ± 1.4	0.0001	7 $\alpha$ -hydroxycholesterol
23.5	1.7 ± 0.3	47.2 ± 7.7	0.0001	7 $\beta$ -hydroxycholesterol
24.7	20.2 ± 2.6	19.6 ± 5.9		24-hydroxycholesterol
25.1	54.3 ± 7.2	62.1 ± 13		5,6 $\beta$ -epoxy-cholesterol
25.3	392.4 ± 54.8	708.0 ± 162.3	0.0001	5,6 $\alpha$ -epoxy-cholesterol
25.6	30.1 ± 4.7	31.0 ± 6.7		27-hydroxycholesterol
26.2	2.8 ± 2.1	3.9 ± 0.6		26-hydroxycholesterol
26.6	8.7 ± 1.3	12.0 ± 1.4	0.0001	4 $\beta$ -hydroxycholesterol
26.8	0.6 ± 0.1	0.9 ± 0.2		20 $\alpha$ -hydroxycholesterol
27.9	15.7 ± 2.9	43.1 ± 3.8	0.0001	22-hydroxycholesterol
28.2	1.0 ± 0.3	0.7 ± 0.2	0.05	cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol
29.8	16.5 ± 2.5	52.4 ± 10.9	0.0001	6-ketocholesterol
29.9	1.4 ± 0.2	2.7 ± 0.4		25-hydroxycholesterol
30.0	2.2 ± 0.4	5.9 ± 0.7		7-ketocholesterol

**B**

**Figure 5.5. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in animal aged 35 days SOD1 and wild plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were significantly different in SOD1 animals aged 35 days compared to wild type animals aged 35 days. \*  $p < 0.05$  and \*\*\*  $p < 0.001$ . Data shown as mean  $\pm$  SD,  $n = 5$  animals SOD1 aged 35 days,  $n = 6$  animals wild aged 35 days.

**A**

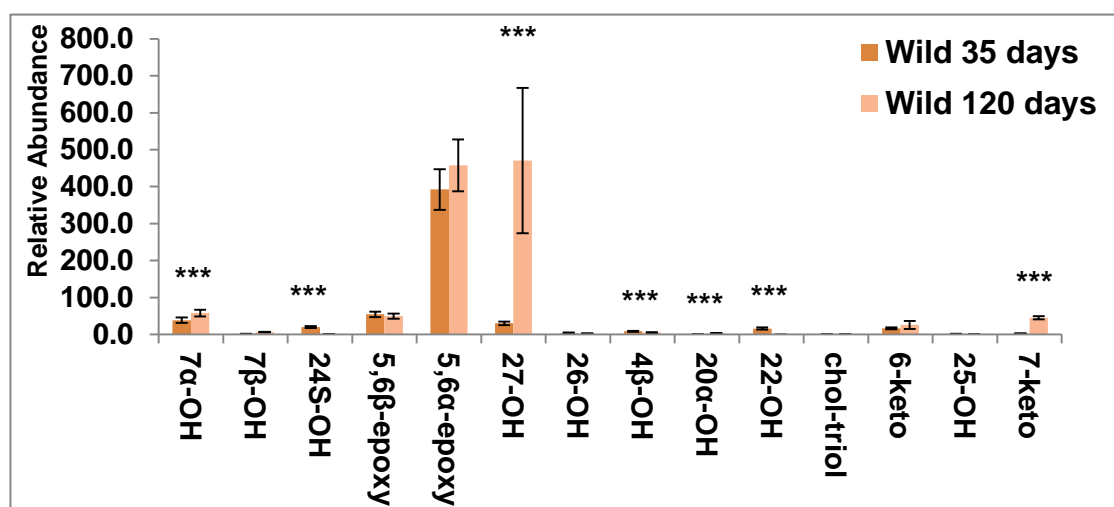
	Wild 120 days	SOD1 120 days		
Rt	Normalised Peak Area	Normalised Peak Area	P	Oxysterols
22.1	57.9 ± 9.0	13.6 ± 2.4	0.0001	7α-hydroxycholesterol
23.5	6.1 ± 0.8	2.0 ± 0.2		7β-hydroxycholesterol
24.7	0.6 ± 0.2	17.0 ± 3.9	0.0001	24-hydroxycholesterol
25.1	49.5 ± 6.9	107.9 ± 21.5	0.0001	5,6β-epoxy-cholesterol
25.3	457.5 ± 69.8	720.7 ± 207.6	0.01	5,6α-epoxy-cholesterol
25.6	470.7 ± 196.8	444.8 ± 50.6		27-hydroxycholesterol
26.2	2.0 ± 0.3	1.8 ± 0.4		26-hydroxycholesterol
26.6	5.0 ± 1.4	3.2 ± 0.8		4β-hydroxycholesterol
26.8	2.6 ± 0.5	15.5 ± 2.2	0.0001	20α-hydroxycholesterol
27.9	0.3 ± 0.1	6.9 ± 0.9	0.0001	22-hydroxycholesterol
28.2	1.0 ± 0.1	0.8 ± 0.2		cholestan-3β,5α,6β-triol
29.8	25.8 ± 10.7	34.2 ± 10.1		6-ketocholesterol
29.9	0.8 ± 0.1	29.3 ± 5.8	0.0001	25-hydroxycholesterol
30.0	45.1 ± 4.3	64.5 ± 13.4	0.0001	7-ketocholesterol

**B**

**Figure 5.6. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in animal aged 120 days SOD1 and wild plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were significantly different in SOD1 animals aged 120 days compared to wild animals aged 120 days. \*\* p<0.01 and \*\*\* p<0.001. Data shown as mean ± SD, n=4 animals SOD1 aged 120 days, n=6 animals wild aged 120 days.

**A**

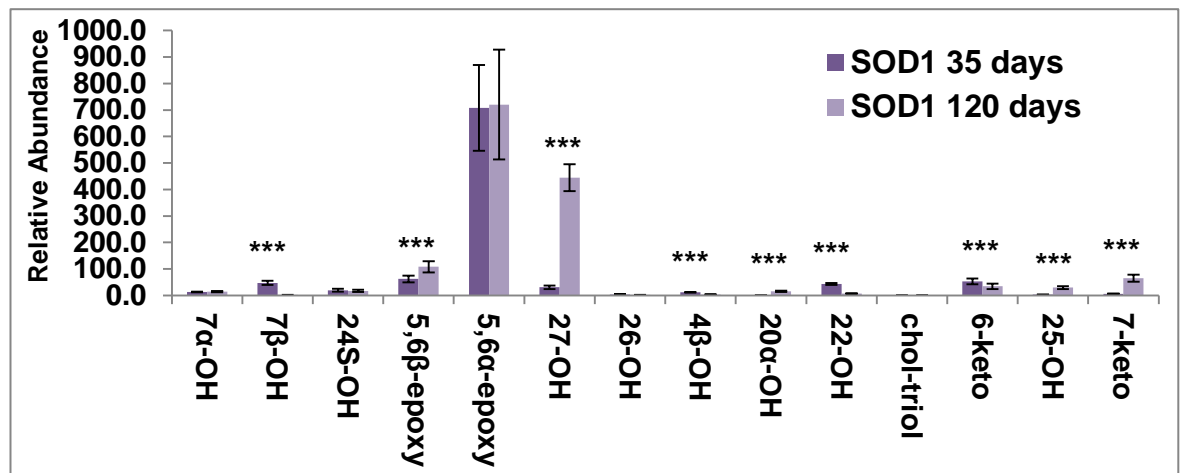
	Wild 35 days	Wild 120 days		
Rt	Normalised Peak Area	Normalised Peak Area	P	Oxysterols
22.1	38.4 ± 7.4	57.9 ± 9.0	0.0001	7 $\alpha$ -hydroxycholesterol
23.5	1.7 ± 0.3	6.1 ± 0.8		7 $\beta$ -hydroxycholesterol
24.7	20.2 ± 2.6	0.6 ± 0.2	0.0001	24-hydroxycholesterol
25.1	54.3 ± 7.2	49.5 ± 6.9		5,6 $\beta$ -epoxy-cholesterol
25.3	392.4 ± 54.8	457.5 ± 69.8		5,6 $\alpha$ -epoxy-cholesterol
25.6	30.1 ± 4.7	470.7 ± 196.8	0.0001	27-hydroxycholesterol
26.2	2.8 ± 2.1	2.0 ± 0.3		26-hydroxycholesterol
26.6	8.7 ± 1.3	5.0 ± 1.4	0.0001	4 $\beta$ -hydroxycholesterol
26.8	0.6 ± 0.1	2.6 ± 0.5	0.0001	20 $\alpha$ -hydroxycholesterol
27.9	15.7 ± 2.9	0.3 ± 0.1	0.0001	22-hydroxycholesterol
28.2	1.0 ± 0.3	1.0 ± 0.1		cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol
29.8	16.5 2.5	25.8		6-ketocholesterol
29.9	1.4 0.2	0.8		25-hydroxycholesterol
30.0	2.2 0.4	45.1	0.0001	7-ketocholesterol

**B**

**Figure 5.7. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in wild animal aged 35 days and 120 days plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were significantly different in wild animals aged 35 days compared to wild animals aged 120 days. \*\*\* p<0.001. Data shown as mean  $\pm$  SD, n=6 animals per group.

**A**

	SOD1 35 days n=5	SOD1 120 days n=4		
Rt	Normalised Peak Area	Normalised Peak Area	P	Oxysterols
22.1	13.0 ± 1.4	13.6 ± 2.4	0.0001	7α-hydroxycholesterol
23.5	47.2 ± 7.7	2.0 ± 0.2	0.0001	7β-hydroxycholesterol
24.7	19.6 ± 5.9	17.0 ± 3.9		24-hydroxycholesterol
25.1	62.1 ± 13.0	107.9 ± 21.5	0.0001	5,6β-epoxy-cholesterol
25.3	708.0 ± 162.3	720.7 ± 207.6		5,6α-epoxy-cholesterol
25.6	31.0 ± 6.7	444.8 ± 50.6	0.0001	27-hydroxycholesterol
26.2	3.9 ± 0.6	1.8 ± 0.4		26-hydroxycholesterol
26.6	12.0 ± 1.4	3.2 ± 0.8	0.0001	4β-hydroxycholesterol
26.8	0.9 ± 0.2	15.5 ± 2.2	0.0001	20α-hydroxycholesterol
27.9	43.1 ± 3.8	6.9 ± 0.9	0.0001	22-hydroxycholesterol
28.2	0.7 ± 0.2	0.8 ± 0.2		cholestan-3β,5α,6β-triol
29.8	52.4 ± 10.9	34.2 ± 10.1	0.01	6-ketocholesterol
29.9	2.7 ± 0.4	29.3 ± 5.8	0.0001	25-hydroxycholesterol
30.0	5.9 ± 0.7	64.5 ± 13.4	0.0001	7-ketocholesterol

**B**

**Figure 5.8. A.** Retention times (Rt min), normalised peak area and p values of oxysterols species identified in SOD1 animals aged 35 days and 120 days plasma lipid extracts, following GC-MS analysis. **B.** Plasma oxysterol species that were significantly different in SOD1 animals aged 35 days compared to SOD1 animals aged 120 days. \*\*\* p<0.001. Data shown as mean ± SD, n=4 animals SOD1 aged 120 days, n=5 animals SOD1 aged 35 days.

### **5.3.3 Fatty acid levels in ALS human red blood cells**

Fatty acid analysis of RBC from ALS subjects was performed in order to investigate the possible effect of ALS on human RBC fatty acids. Overall, 29 fatty acids were identified, all data is summarised in Table 5.3.

#### **5.3.3.1 The effect of disease on human red blood cell fatty acids**

Out of the 29 fatty acids reported in Table 5.3, only one fatty acid, eicosadienoic acid (20:2n-6), was found to be significantly decreased in the ALS samples compared to the control group.

### **5.3.4 Red blood cell fatty acids in SOD1 transgenic mice model**

Following fatty acid analysis of RBC from the SOD1 animal model, a total of 27 fatty acids were identified and quantified in wild type and SOD1 mice, young (35 days) and old (120 days). The results were statistically significantly different.

#### **5.3.4.1 The effect of disease on SOD1 mice red blood cell fatty acids**

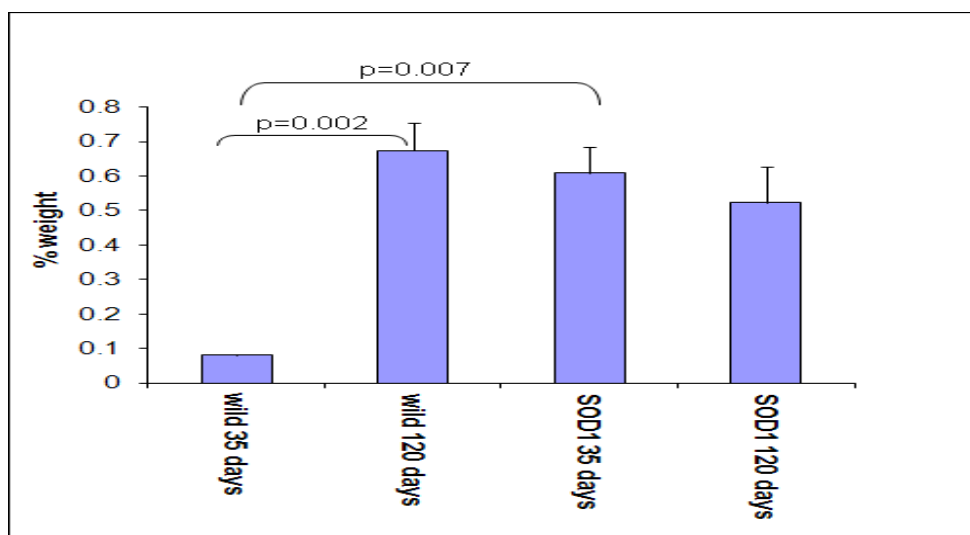
Out of the 27 fatty acids reported in Table 5.4, only one namely eicosadienoic acid (20:2n-6) was found to be significantly increased in young SOD1 mice (35 days) compared to young wild mice, (35 days) as shown in Figure 5.9. Furthermore, only one fatty acid namely linoleic acid (C18:2n-6t), was found to be significantly increased ( $p < 0.05$ ) in old (120 days) SOD1 mice compared to control group (Figure 5.10). However, there was a trend showing reduced levels of 20:2n-6 in old (120 days) SOD1 mice compared to wild animals of the same age (120 days), but this was not statistically significant.

**Table 5.3.** Red blood cell fatty acids in control subjects and ALS samples. Data shown as mean  $\pm$  SD, n=6 animals per group. ND: not detected, SFA: saturated fatty acids, MFUA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, ALS = Amyotrophic lateral sclerosis.

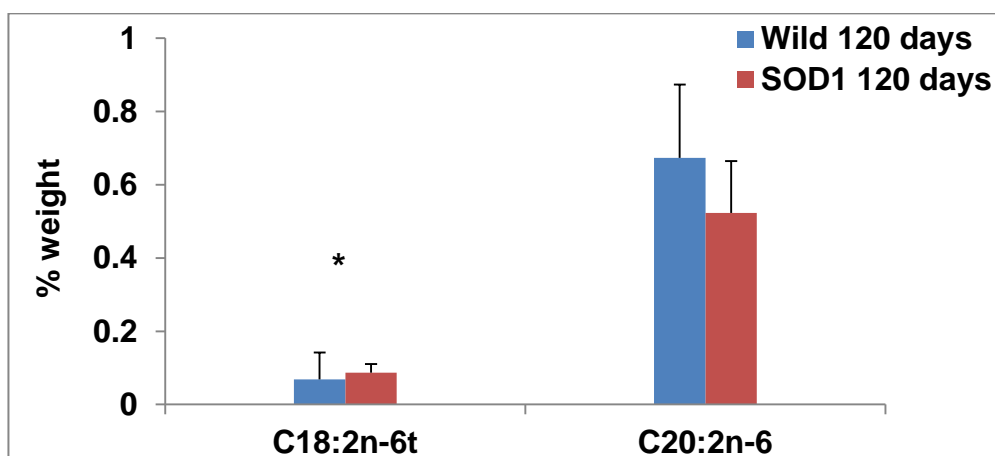
<b>Fatty acids</b>	<b>Controls</b>	<b>ALS</b>
C12	0.08 $\pm$ 0.04	0.06 $\pm$ 0.03
C14:0	0.75 $\pm$ 0.19	0.70 $\pm$ 0.13
C15:0	0.17 $\pm$ 0.03	0.19 $\pm$ 0.04
C16:0	26.70 $\pm$ 1.13	28.83 $\pm$ 3.78
C17:0	0.19 $\pm$ 0.10	0.23 $\pm$ 0.14
C18:0	11.81 $\pm$ 2.33	11.07 $\pm$ 2.20
C20:0	0.36 $\pm$ 0.06	0.34 $\pm$ 0.16
C22:0	0.56 $\pm$ 0.25	0.65 $\pm$ 0.21
C23:0	0.09 $\pm$ 0.04	0.08 $\pm$ 0.03
C24:0	1.01 $\pm$ 0.61	0.93 $\pm$ 0.41
<b><math>\Sigma</math> SFA</b>	41.67 $\pm$ 4.42	43.03 $\pm$ 6.42
C14:1	ND	ND
C16:1	1.09 $\pm$ 0.36	1.34 $\pm$ 0.41
C17:1	0.85 $\pm$ 0.65	1.54 $\pm$ 1.26
C18:1n-9t	ND	ND
C18:1n-9c	19.43 $\pm$ 1.78	21.26 $\pm$ 5.35
C18:1n-7c	0.44 $\pm$ 0.34	0.44 $\pm$ 0.38
C20:1n-9	0.18 $\pm$ 0.04	0.16 $\pm$ 0.08
C22:1n-9	0.08 $\pm$ 0.02	0.09 $\pm$ 0.05
C24:1	1.48 $\pm$ 0.89	1.49 $\pm$ 0.51
<b><math>\Sigma</math> MUFA</b>	23.56 $\pm$ 2.43	26.35 $\pm$ 3.90
C18:2n-6t	ND	ND
C18:2n-6c	20.28 $\pm$ 5.67	17.00 $\pm$ 4.05
<b>C20:2n-6</b>	<b>0.13 <math>\pm</math> 0.05</b>	<b>0.06 <math>\pm</math> 0.01</b> <sup>p=0.024</sup>
C20:3n-6	1.30 $\pm$ 0.17	1.39 $\pm$ 0.35
C20:4n-6	8.32 $\pm$ 2.02	7.88 $\pm$ 2.55
C22:2	0.06 $\pm$ 0.03	0.07 $\pm$ 0.03
<b><math>\Sigma</math> n-6 PUFA</b>	30.10 $\pm$ 5.88	26.39 $\pm$ 3.81
C18:3n-3	0.46 $\pm$ 0.13	0.40 $\pm$ 0.18
C20:5n-3	1.11 $\pm$ 0.41	1.00 $\pm$ 0.77
C22:5n-3	0.90 $\pm$ 0.30	0.91 $\pm$ 0.60
C22:6n-3	2.12 $\pm$ 0.48	1.87 $\pm$ 1.42
<b><math>\Sigma</math> n-3 PUFA</b>	4.59 $\pm$ 0.92	4.18 $\pm$ 2.66

**Table 5.4.** Mice red blood cell fatty acids in wild and SOD1 animals. \$ p<0.05, \$\$ p<0.01 (wild animals aged 35 days vs wild animals aged 120 days); \* p<0.05, \*\* p<0.01, \*\*\* p<0.001 (SOD1 animals aged 35 days vs SOD1 animals aged 120 days); # p<0.05 (wild vs SOD1 animals aged 120 days). FA = Fatty Acids, ND: not detected, MFUA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids. Data shown as mean  $\pm$  SD, n = 6 animals of SOD1 aged 35 days, n = 11 animals of SOD1 aged 120 days, n=6 animals of wild aged 35 days, n=6 animals of wild aged 120 days.

FA	wild 35 days	wild 120	SOD1 35	SOD1 120
C14:0	0.38 $\pm$ 0.03	0.48 $\pm$ 0.13	0.41 $\pm$ 0.09	0.65 $\pm$ 0.26
C15:0	0.18 <sup>\$</sup> $\pm$ 0.01	0.40 <sup>\$</sup> $\pm$ 0.11	0.19 <sup>**</sup> $\pm$ 0.03	0.45 <sup>**</sup> $\pm$ 0.17
C16:0	33.07 $\pm$ 0.78	30.63 $\pm$ 1.49	32.88 $\pm$ 0.70	27.41 $\pm$ 4.98
C17:0	0.41 $\pm$ 0.03	0.55 $\pm$ 0.07	0.38 <sup>**</sup> $\pm$ 0.02	0.67 <sup>**</sup> $\pm$ 0.13
C18:0	13.96 $\pm$ 0.40	12.91 $\pm$ 1.88	12.49 $\pm$ 1.32	15.26 $\pm$ 1.46
C20:0	0.38 $\pm$ 0.03	0.41 $\pm$ 0.08	0.35 $\pm$ 0.03	0.42 $\pm$ 0.06
C22:0	0.80 $\pm$ 0.07	0.86 $\pm$ 0.09	0.86 $\pm$ 0.09	0.75 $\pm$ 0.10
C23:0	0.07 $\pm$ 0.02	0.14 $\pm$ 0.06	0.09 $\pm$ 0.02	0.10 $\pm$ 0.05
C24:0	2.02 <sup>\$\$</sup> $\pm$ 0.04	1.60 <sup>\$\$</sup> $\pm$ 0.19	1.93 <sup>**</sup> $\pm$ 0.38	1.50 <sup>**</sup> $\pm$ 0.17
<b><math>\Sigma</math>SFA</b>	51.26 $\pm$ 0.95	47.97 $\pm$ 1.33	49.58 $\pm$ 1.43	47.20 $\pm$ 4.90
C16:1	0.24 $\pm$ 0.05	0.38 $\pm$ 0.13	0.26 $\pm$ 0.03	1.60 $\pm$ 4.16
C17:1	0.36 $\pm$ 0.09	0.37 $\pm$ 0.03	0.48 $\pm$ 0.05	0.35 $\pm$ 0.14
C18:1n-9t	0.09 $\pm$ 0.03	0.13 $\pm$ 0.03	0.13 $\pm$ 0.02	0.14 $\pm$ 0.05
C18:1n-9c	9.18 $\pm$ 0.99	9.48 $\pm$ 1.15	9.50 $\pm$ 0.51	9.23 $\pm$ 0.68
C18:1n-7	1.21 $\pm$ 0.15	1.44 $\pm$ 0.25	1.27 $\pm$ 0.15	1.06 $\pm$ 0.18
C20:1n-9	0.38 $\pm$ 0.03	0.39 $\pm$ 0.08	0.37 $\pm$ 0.03	0.46 $\pm$ 0.11
C22:1n-9	0.52 $\pm$ 0.07	0.64 $\pm$ 0.12	0.65 $\pm$ 0.11	0.78 $\pm$ 0.15
C24:1	0.79 $\pm$ 0.09	0.85 $\pm$ 0.11	0.87 $\pm$ 0.09	0.74 $\pm$ 0.13
<b><math>\Sigma</math>MUFA</b>	12.75 $\pm$ 1.14	13.68 $\pm$ 0.92	13.51 $\pm$ 0.82	14.35 $\pm$ 4.35
C18:2n-6t	ND	0.07 <sup>#</sup> $\pm$ 0.03	ND	0.09 <sup>#</sup> $\pm$ 0.02
C18:2n-6c	13.09 $\pm$ 0.88	14.46 $\pm$ 1.71	12.63 <sup>*</sup> $\pm$ 0.43	14.84 <sup>*</sup> $\pm$ 1.13
C20:2n-6	0.08 <sup>\$</sup> $\pm$ 0.01	0.67 <sup>\$</sup> $\pm$ 0.20	0.61 $\pm$ 0.18	0.52 $\pm$ 0.34
C20:3n-6	1.38 $\pm$ 0.08	1.30 $\pm$ 0.21	1.43 <sup>***</sup> $\pm$ 0.08	1.03 <sup>***</sup> $\pm$ 0.17
C20:4n-6	14.61 $\pm$ 0.75	15.23 $\pm$ 1.34	15.49 $\pm$ 0.66	15.18 $\pm$ 1.41
C22:2	0.08 $\pm$ 0.01	0.08 $\pm$ 0.034	0.11 $\pm$ 0.02	0.11 $\pm$ 0.05
<b><math>\Sigma</math>n-6 PUFA</b>	29.24 <sup>\$</sup> $\pm$ 0.44	31.82 <sup>\$</sup> $\pm$ 1.88	30.26 $\pm$ 0.64	31.77 $\pm$ 1.64
C18:3n-3	0.17 $\pm$ 0.03	0.15 $\pm$ 0.03	0.156 $\pm$ 0.021	0.155 $\pm$ 0.030
C20:5n-3	0.19 <sup>\$</sup> $\pm$ 0.01	0.25 <sup>\$</sup> $\pm$ 0.04	0.205 $\pm$ 0.024	0.212 $\pm$ 0.038
C22:5n-3	0.94 $\pm$ 0.05	0.79 $\pm$ 0.14	0.896 <sup>**</sup> $\pm$ 0.106	0.641 <sup>**</sup> $\pm$ 0.093
C22:6n-3	4.96 $\pm$ 0.57	5.17 $\pm$ 0.41	5.217 $\pm$ 0.301	5.399 $\pm$ 0.643
<b><math>\Sigma</math>n-3 PUFA</b>	6.26 $\pm$ 0.58	6.37 $\pm$ 0.52	6.474 $\pm$ 0.294	6.407 $\pm$ 0.697



**Figure 5.9.** Levels of eicosadienoic acid (20:2n-6) in red blood cell from young (35 days) wild and SOD1 mice and old (120 days) wild and SOD1 mice. Data shown as mean  $\pm$  SD, n = 6 animals of SOD1 aged 35 days, n = 11 animals of SOD1 aged 120 days, n=6 animals of wild aged 35 days, n=6 animals of wild aged 120 days.



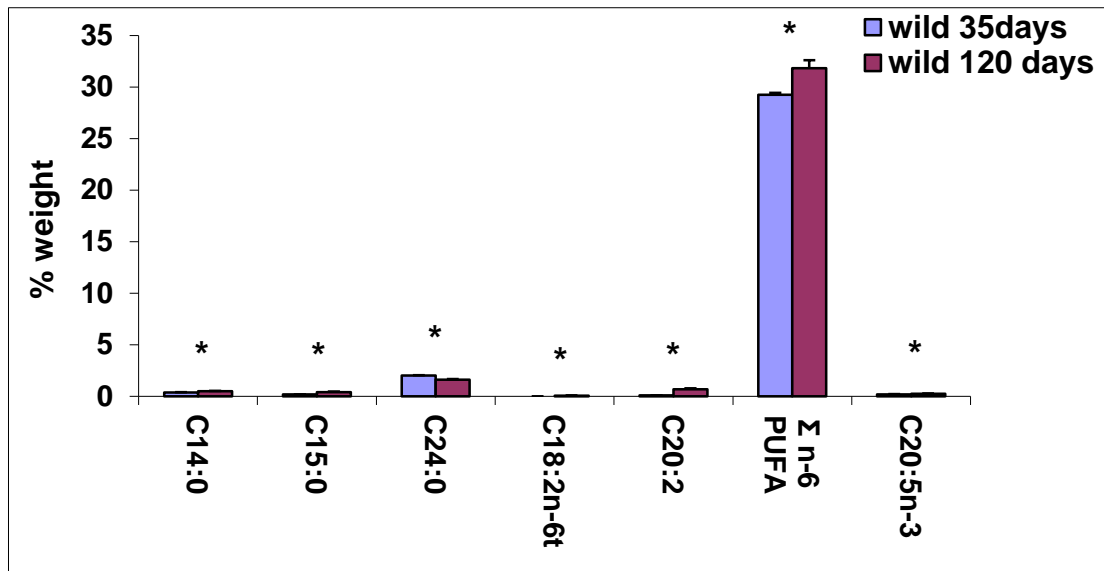
**Figure 5.10.** Levels of linoleic acid (C18:2n-6t) and eicosadienoic acid (20:2n-6) in red blood cell from aged (120 days) wild and SOD1 mice. \* p < 0.05. Data shown as mean  $\pm$  SD, n = 6 animals of old (120 days) wild mice, n = 11 animals of old (120 days) SOD1 mice.

#### 5.3.4.2 The effect of age on mice red blood cell fatty acids

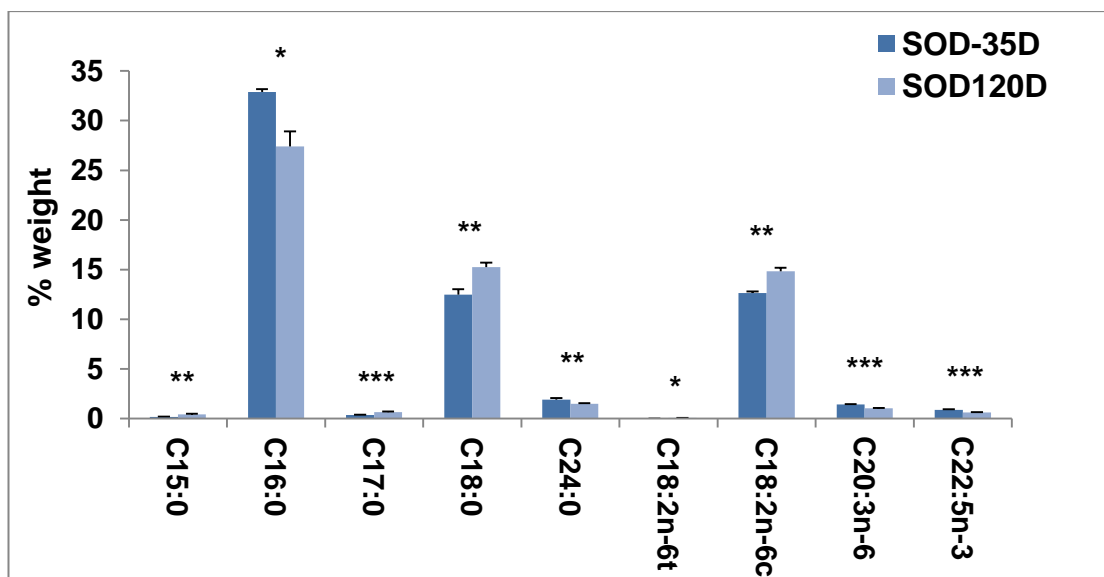
Figure 5.11 shows the effect of age on wild type mice RBC fatty acids. myristic acid (C14:0)  $p=0.002$ , pentadecylic acid (C15:0)  $p=0.022$ , LA (C18:2n-6t)  $p<0.05$ , EPA (C20:5n-3)  $p=0.021$  and  $\Sigma$ n-6PUFA  $p=0.02$  were found significantly increased in old (120 days) wild mice compared to young (35 days) wild mice. Only lignoceric acid (C24:0)  $p=0.014$  was found to be significantly decreased.

Figure 5.12 shows the effect of age on SOD1 mice RBC fatty acids. Pentadecylic acid (C15:0)  $p=0.002$ , margaric acid (C17:0)  $p<0.001$ , SA (C18:0)  $p=0.003$ , LA (C18:2n-6t)  $p<0.05$  and its isomer linoleic acid (C18:2n-6c)  $p=0.005$  were found to be significantly increased in old (120 days) SOD1 mice compared to young (35 days) SOD1. Palmitic acid (C16:0)  $p=0.017$ , lignoceric acid (C24:0)  $p=0.004$ , DGLA (C20:3n-6)  $p<0.0001$  and DPA (C22:5n-3)  $p<0.0001$  were found to be significantly decreased.

Moreover, as shown in Table 5.4, EPA (C20:5n-3) and  $\Sigma$ n-6PUFA were increased in SOD1 mice (120 days) compared to SOD1 (35 days), at the same time, C17:0 and C18:2n-6c were increased in wild mice (120 days) compared to wild (35 days). Also C16:0, C18:0, C20:3n-6 and 22:5n-3 were found decreased in the wild mice (120 days) compared to wild (35 days). However, none of these changes reached statistical significance. Furthermore, as shown in Figure 5.9, EDA C20:2n-6 was found to be significantly increased  $p=0.002$  in wild mice (120 days) compared to wild mice (35 days). Conversely, there was a trend showing reduced levels of EDA C20:2n-6 (Figure 5.10) in SOD1 mice (120 days) compared to SOD1 mice (35 days), but this was not statistically significant.



**Figure 5.11.** The effect of age on wild mice red blood cell fatty acid levels when comparing young (35 days) wild type animals to old (120 days) ones. \*  $p < 0.05$  and \*\*  $p < 0.01$ . Data shown as mean  $\pm$  SD,  $n = 6$  animals per group.



**Figure 5.12.** The effect of age on SOD1 mice red blood cell fatty acid levels when comparing young (35 days) SOD1 animals to aged (120 days) ones. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Data shown as mean  $\pm$  SD,  $n=6$  animals SOD1 35 days,  $n=11$  animals SOD1 120 days.

## 5.4 Discussion

A GC-MS method was used to identify and evaluate oxysterols in human and mice plasma. The oxysterols were analysed as TMS ether derivatives which are thermally stable during GC analysis (Zhang et al., 2001). Also, TMS derivatives produce more appropriate diagnostic fragments useful for structural elucidation (Dzeletovic et al., 1995, Babiker and Diczfalusy, 1998, Guardiola et al., 2004, Armentrout et al., 2005, Griffiths and Wang, 2009).

In the present study, plasma oxysterols were monitored in full-scan mode as TMS derivatives. Identification was based on comparison of retention times and fragmentation patterns on published information (Girao et al., 1998, Echarte et al., 2001, Tomoyori et al., 2002, van de Merbel et al., 2011).

In this study, fourteen oxysterol species were identified in human plasma (Table 5.1). The main ones were: 27-OH, 24-OH, 5,6-epoxy, 7-keto, 4 $\beta$ -OH, 7 $\beta$ -OH, 6-keto and 7 $\alpha$ -OH. These findings are in agreement with previous studies (Schroepfer, 2000, Griffiths et al., 2008, Wang et al., 2009) who have reported that 7 $\alpha$ -OH, 7 $\beta$ -OH, 24-OH, 25-OH, 27-OH, 5,6 $\alpha$ -epoxy and chol-triol are found in human plasma at levels between 3 ng/mL and 150 ng/mL. In agreement with other research findings was that 24-OH, 26-OH, 27-OH, 4 $\beta$ -OH and 7 $\alpha$ -OH were the most abundant in plasma of healthy volunteers, while the other oxysterols were found present at low levels (Dzeletovic et al., 1995, Babiker and Diczfalusy, 1998). Furthermore, other studies reported that human plasma 7 $\beta$ -OH was much lower or sometimes hardly found (Kudo et al., 1989).

Findings of oxysterols in mice plasma in this study (Figures 5.5 and 5.6) are also in agreement with other publications. Even though the identification and levels of oxysterols in rodent circulation is less well studied, it has been reported that their levels are of the same order of magnitude as in human (Rosen et al., 1998, Li-Hawkins et al., 2000, Wang et al., 2009). However, oxysterols have been identified in cortex and spinal cord of mouse central nervous system, these include 7 $\alpha$ -OH, 7 $\beta$ -OH, 22-OH, 24-OH, 25-OH and 27-OH. Of these, 24-OH is found to be entirely biosynthesised in brain (Wang et al., 2009).

Any alteration of lipid metabolism during brain and/or neuronal cell disorders may critically impact on the causes of neuronal death in neurodegenerative diseases (Medina-Meza et al., 2011). ALS is characterized by cholesterol oxidation that is associated with an abnormal lipid metabolism. It has been demonstrated that oxysterols are considered as important diagnostic markers in neurodegenerative disease (Bjorkhem et al., 2009). Analysis of plasma oxysterols may elucidate the role of cholesterol metabolism in the pathogenesis of neurodegenerative disorders (Iuliano et al., 2009, Leoni and Caccia, 2011). It has been found that oxidation of cholesterol to 24-OH in brain (Lund et al., 1999) and 27-OH in spinal cord (Heverin et al., 2005) is essential to preserve proper brain and CNS functions (Lund et al., 1999, Pfrieger, 2003). The findings also show that 24-OH, 27-OH and 4 $\beta$ -OH are the most important and are in higher concentration in human circulation (Jeitner et al., 2011). Another study reported that 24-OH is almost exclusively formed in brain which indicates that circulating 24-OH may be originating from brain (Lund et al., 1999).

It has been suggested that the flux of 24-OH from brain into the circulation through the BBB is considered to be the major pathway of brain cholesterol turnover and homeostasis (Lund and Bjorkhem, 1995, Lutjohann et al., 1996, Bjorkhem et al., 1998, Bjorkhem, 2002). The present findings are in agreement with a recent study which found that, plasma 24-OH is increased in the first stages of Alzheimer's disease (Zuliani et al., 2011), and this is also in agreement with another study in a German population of patients with Alzheimer's disease that were reported to have slightly higher plasma 24-OH than the control population (Lutjohann et al., 2000). However, this is in disagreement with Bjorkhem and colleagues (2006), who have reported that patients with advanced Alzheimer's disease and cerebral inflammatory diseases had slightly lower plasma 24-OH and the most severe cases had the lowest levels (Bjorkhem et al., 2006). Results are also in disagreement with Bretillon and colleagues (2000), who found that patients suffering from Multiple Sclerosis at a later stage of the disease and over a long period had significantly reduced levels of plasma 24-OH (Bretillon et al., 2000). Also it is in disagreement with Leoni and Caccia (2011), who have indicated that plasma 24-OH was found to be reduced in Multiple Sclerosis, Alzheimer's disease and Huntington's disease according to the degree of brain atrophy (Leoni and Caccia, 2011). These variations are probably related to the consequence of losing neurons containing CYP46 which is responsible for converting cholesterol to 24-OH (Norlin et al., 2000). Therefore, the present finding suggests that as the disease progress; brain no longer exports 24-OH or producing 24-OH.

Moreover, another study found that plasma 24-OH is significantly reduced in Huntington's disease patients at all disease stages (Leoni et al., 2008). Whereas, other studies found that plasma 24-OH is insignificant in young Huntington's disease patients within the early stages of the disease, and in some cases, even increased (Bjorkhem et al., 2006, Dujmovic, 2011, Leoni and Caccia, 2011). These published observations are in contradiction to our findings as shown in Figure 5.4 which found that plasma 24-OH is significantly increased in ALS patients compared to controls. Conversely, another study has indicated that in normal adult human, brain cholesterol turnover gives rise to a daily flux of 24-OH into the circulation (Lutjohann et al., 1996), while our finding has reported high plasma 24-OH is associated with ALS. This indicates that ALS might increase brain 24-OH flux into circulation and this is related to extend of disease manifestations, stages of disease and age.

Another oxysterol, 27-OH, mainly formed in peripheral tissues (extrahepatic and non-cerebral sources, is the most abundant oxysterol in the circulation of normal humans and is capable of passing into the brain (Duane and Javitt, 1999, Meaney et al., 2002). Brain and plasma levels of 27-OH are found to be correlated with the corresponding levels of cholesterol in the circulation indicating that there is an equilibrium between them in healthy states and in diseases which is based on the severity of the disease (Vaya et al., 2001, Heverin et al., 2005). In addition, excessive levels of 27-OH have been associated with Alzheimer's disease (Bjorkhem et al., 2009). Therefore, plasma 27-OH might be an important diagnostic marker in neurodegeneration disorders, exploring the correlation between hypercholesterolemia and ALS.

In our study, plasma 27-OH was found to be significantly increased (Figure 5.4) in ALS subjects compared to normal ones. This finding is in agreement with Bjorkhem and colleagues (2001). They found that plasma 27-OH in Smith-Lemli-Opitz syndrome was found to be increased (Bjorkhem et al., 2001). Also it is in agreement with other studies reporting on hypercholesterolemic patients which found plasma 27-OH levels were higher in hypercholesterolemic patients compared to normal individuals (Bjorkhem et al., 2009). Also our finding is in agreement with recent studies that have reported that 27-OH is an important pathogenetic factor and is significantly increased in patients with a rare hereditary spastic paresis disease that is characterized as massive neurodegeneration due to mutation at CYP7B1, and as a result of damage and dysfunction of the nerves. Authors also reported that reduction of plasma 27-OH levels may reduce or prevent its neurological symptoms (Schule et al., 2010, Leoni and Caccia, 2011). Even with normal levels of total plasma cholesterol; plasma 27-OH is still found in higher levels in patients suffering from ALS (Bjorkhem, 2002). Therefore, increasing plasma 27-OH can be proposed as biomarker for cholesterol homeostasis and neurodegenerative diseases (Leoni and Caccia, 2011).

Our results show increased 25-OH. This is in agreement with a recent study which reported that plasma 25-OH was increased in patients with a rare hereditary spastic paresis disease (Schule et al., 2010). 25-OH is a relatively minor oxysterol in mammalian plasma (Chen et al., 2002), but has been suggested to have important tissue specific functions and is biologically an important metabolite. It has a regulatory effect on SM biosynthesis and SM is

required together with cholesterol, for formation of lipid rafts in cell membranes (Lund et al., 1998, Bjorkhem and Diczfalusy, 2002, Chen et al., 2002).

Our findings on the elevated 5,6 $\beta$ -epoxy, 5,6 $\alpha$ -epoxy, 7 $\alpha$ -OH, 7 $\beta$ -OH and 7-Keto levels are in part in agreement with Alkazemi and colleagues (2008). Their study reported that all these species are increased in plasma of patients are associated with oxidative stress diseases (Alkazemi et al., 2008). Moreover, our finding on 4 $\beta$ -OH agrees with Bodin and colleagues (2002), who have found that 4 $\beta$ -OH is present at high levels in plasma of subjects suffering from chronic neurological disorders (Bodin et al., 2002). Different findings might be attributed to advanced stages of disease, different ALS pathogenetic mechanisms or to systemic neuroinflammation associated with ALS. Also these species may be involved in the development of ALS and may not be good markers for ALS.

In order to understand these findings, we worked with a transgenic mouse model. In this study we compared the later stages (120 days) to pre-symptomatic stages (35 days) of SOD1 mice. No significant changes were noted in plasma 24-OH in pre-symptomatic stages of SOD1 mice as shown in Figure 5.5 in comparison wild mice. While significant increases in 24-OH were found in SOD1 mice (120days) compared to wild mice (120days) (Figure 5.6). These findings are in agreement with Lutjohann and colleagues (2002). They found no differences in plasma 24-OH between wild type and transgenic mice carrying the Swedish mutation (APP23) at pre-stages of Alzheimer's disease (Lutjohann et al., 2002). But they have recently found that plasma 24-OH in a mouse model of Huntington's disease was markedly reduced compared to controls (Zuccato et al., 2010, Karasinska and Hayden, 2011).

Plasma 24-OH was affected by age (Figure 5.7) and was significantly ( $p=0.0001$ ) decreased in wild type mice (120 days) compared to wild type mice (35 days). However, no significant changes (Figure 5.8) were noticed in plasma 24-OH levels when examining the old SOD1 transgenic mice. Therefore, these findings suggested that in the prolonged SOD1 defacing, a decreased flux of 24-OH through the BBB into the circulation may be related to the defacing. Also, 24-OH may be related to the severity of the disease and not with the age.

Furthermore, this study has also shown the significant increase of 5,6 $\beta$ -epoxy ( $p=0.0001$ ), 5,6 $\alpha$ -epoxy ( $p=0.01$ ), 20 $\alpha$ -OH ( $p=0.0001$ ), 22-OH ( $p=0.0001$ ), 25-OH ( $p=0.0001$ ) and 7-keto ( $p=0.0001$ ) in plasma of later stage (120 days) SOD1 mice. Whereas, 7 $\beta$ -OH ( $p=0.0001$ ), 5,6 $\alpha$ -epoxy ( $p=0.01$ ), 4 $\beta$ -OH ( $p=0.0001$ ), 22-OH ( $p=0.0001$ ) and 6-keto ( $p=0.0001$ ) is found significantly increased in plasma of pre-symptomatic stages (35 days) SOD1 mice. Plasma 7 $\alpha$ -OH ( $p=0.0001$ ) was significantly decreased in plasma of both stages SOD1 mice.

Moreover, 27-OH ( $p=0.0001$ ), 20 $\alpha$ -OH ( $p=0.0001$ ) and 7-keto ( $p=0.0001$ ) were significantly decreased in plasma of both wild and SOD1 mice (Figures 5.7 and 5.8). 5,6 $\beta$ -epoxy and 25-OH were significantly increased in SOD1 mice. Whereas, 4 $\beta$ -OH ( $p=0.0001$ ) and 22-OH ( $p=0.0001$ ) were significantly decreased in plasma of both wild and SOD1 mice. 7 $\beta$ -OH ( $p=0.0001$ ) and 6-keto ( $p=0.0001$ ) was significantly decreased in SOD1 mice. While, plasma 7 $\alpha$ -OH was significantly decreased ( $p=0.0001$ ) in wild mice. The present findings of 27-OH and 7 $\alpha$ -OH are in agreement with recent study reports where 27-OH was significantly increased, while 7 $\alpha$ -OH was significantly decreased in mice suffering motor neuron disease on developing and ageing (Wang et al., 2009).

In general, the present findings show that plasma oxysterols are detected in healthy humans and wild type mice. Levels of all the fourteen identified oxysterol species in ALS patients were shown to be different (Figure 5.4) from healthy controls. Significant changes in the SOD1 mice model varied according to disease stage (Figure 5.5) and age of mice (Figure 5.6). These differences may be related to specific actions of the mutant SOD1 gene on the biosynthesis of oxysterols, or due to different stages of the disease, or possibly as a consequence of varied metabolism (Schule et al., 2010), and may be linked to the differences in the environmental, nutritional or dietary causes for these observations. High 24-OH in plasma in ALS may open new possibilities to study changes of brain cholesterol homeostasis in different pathological conditions. Also, 27-OH may be the missing link between hypercholesterolemia and ALS disease. Furthermore, the balance of plasma 24-OH and 27-OH might be important for the initiation/progression of neurodegenerative diseases and this could be used as diagnostic markers to assess' therapeutic strategies.

RBC fatty acid profiles of ALS patients and the SOD1 animal transgenic model were also studied following age and disease stage changes. We found a significant increase in eicosadienoic acid (Table 5.3) in ALS patients compared to control ones. This finding is in contrast to findings of Woods and colleagues (2004) whose report showed that eicosadienoic acid does not accumulate to a significant extent in cellular membranes. This fatty acid is an elongation product of LA and is a common minor component of animal tissues, typically representing about 1-2% of the total phospholipid fatty acids (Woods et al., 2004). However, it is difficult to attribute biological significance to this increase,

given the rarity of the fatty acid in biological systems. Therefore, this finding should be further investigated and the analysis repeated using different group of samples. Eicosadienoic acid was also significantly increased in SOD1 mice (35 days) as shown in Figure 5.10 compared to control and its precursor LA (C18:2n-6t) was also found to be significantly increased in SOD1-deficient mice (120 days) compared to wild mice (120 days).

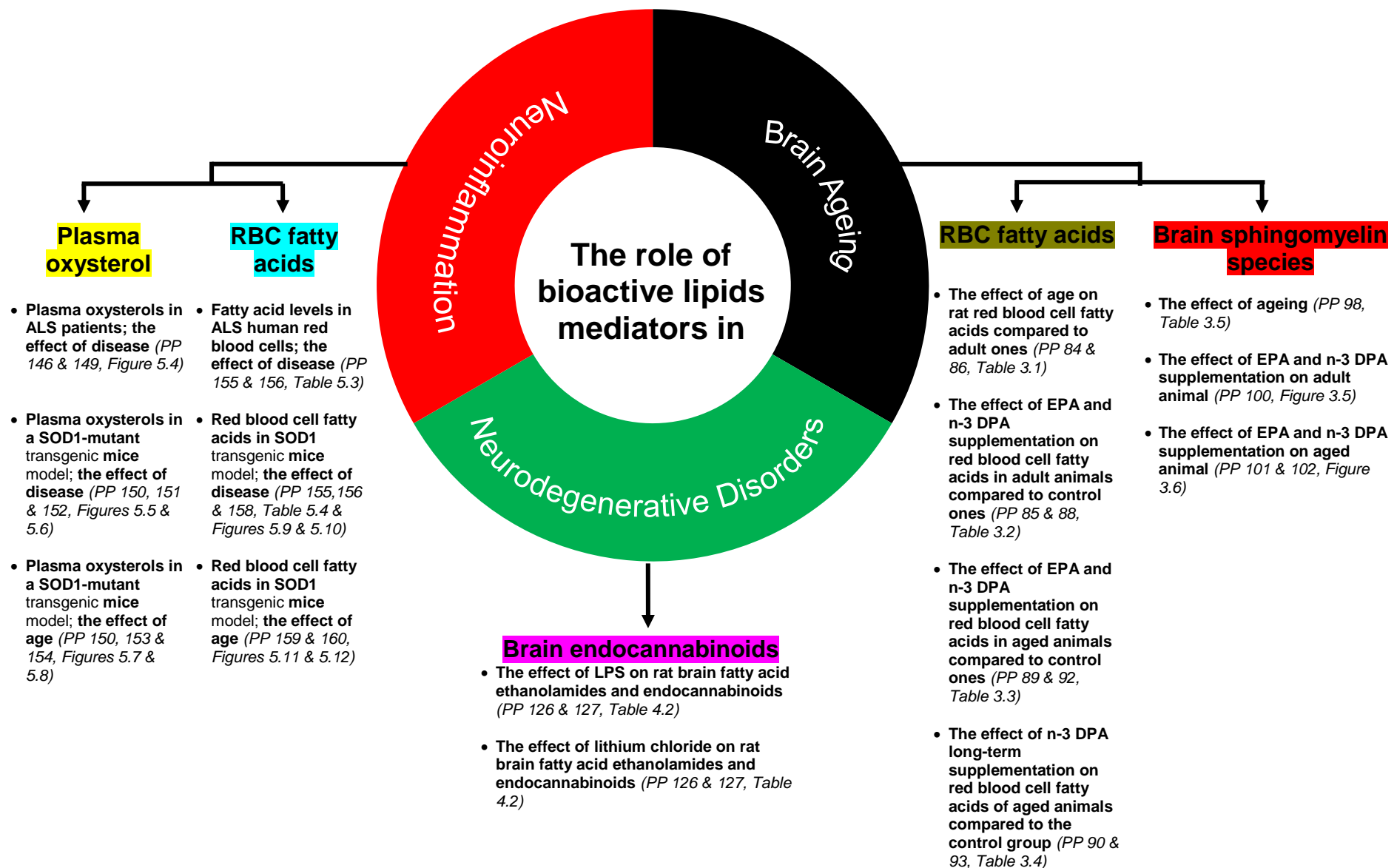
Significant age-related changes in variations in RBC fatty acid profiles of wild type and SOD1 mice have been noticed in the present study; SFA; myristic acid C14:0 ( $p=0.05$ ), pentadecylic acid C15:0 ( $p=0.022$ ), and PUFA; LA C18:2n-6t ( $p=0.05$ ), EPA C20:5n-3 ( $p=0.021$ ) and  $\Sigma$ n-6PUFA ( $p=0.02$ ) (Figures 5.12) were significantly increased in wild type mice (120 days) compared to wild type mice (35 days). While lignoceric acid C24:0 ( $p=0.014$ ) was significantly decreased. Also, SFA; pentadecylic acid C15:0 ( $p=0.002$ ), margaric acid C17:0 ( $p=0.0001$ ) and SA C18:0 ( $p=0.003$ ) and PUFA; LA C18:2n-6t ( $p=0.05$ ) and its isomer LA C18:2n-6c ( $p=0.005$ ) (Figure 5.13) were significantly increased in SOD1 mice (120 days) compared to SOD1 mice (35 days). SFA; palmitic acid C16:0 ( $p=0.017$ ), lignoceric acid C24:0 ( $p=0.004$ ) and the PUFAs; dihomo-gamma-linolenic acid C20:3n-6 ( $p=0.0001$ ) and DPA C22:5n-3 ( $p=0.0001$ ) were found significantly decreased.

However, eicosadienoic acid was significantly ( $p=0.002$ ) increased in wild mice (120days) and ( $p=0.007$ ) in SOD1 mice (120days) compared to wild mice (35 days), it is considered to be exclusively formed as a direct elongation of endogenous LA (C18:2n-6c) (Kanoh and Lindsay, 1972). Also, neither DHA nor AA were found at significant levels in the present study, either in relation to age

or disease stages in RBC of ALS patients and the mice model compared to controls.

Overall, the present findings suggest an age-related effect on lipid profiles and SOD1 appears to have an added effect on these changes. Eicosadienoic acid levels were found significantly different in both, ALS patients and SOD1 mice, suggesting that this fatty acid may be an indication or marker for the biochemical changes underlying ALS disease. The findings are consistent with what has been reported on oxysterols as bioactive molecules, and support the hypothesis that oxysterols such as 24-OH and 27-OH might be markers of brain-induced damage. Oxysterols tend to exert biological activities relevant to neurodegeneration mechanisms; these activities can include the regulation of enzymes involved in the neuroinflammation and fatty acid metabolism (Lemaire-Ewing et al., 2005, Micheletta and Iuliano, 2006, Arca et al., 2007). Therefore, the variations we observed in plasma oxysterols and RBC fatty acids might be used as indicators of brain damage during ALS, reflecting the BBB dysfunction and alteration of brain lipid metabolism.

## **Chapter 6. General Discussion**



**Chart 6.1.** An overview on the findings of the present studies

## 6.1 General discussion

Lipids are vital to the health of CNS in animals and humans. The assessment of blood lipids is very frequent in clinical research as it is assumed to reflect the lipid composition of peripheral tissues and can be linked to health and disease states. Recently, it has been suggested that dysregulation of brain lipids and bioactive lipid mediators may contribute to the pathology of neuroinflammation, neurodegenerative diseases and brain ageing (Phillips and de Oliveira, 2008, Iuliano et al., 2009).

Fatty acids are important lipids and are considered as major biological regulators. Evidence in both humans and animals indicates that PUFA, and especially the n-3 PUFA, have potent bioactive properties in comparison with other types of lipid fatty acids (Seo et al., 2005). Fatty acids may influence enzyme activity, cell differentiation and proliferation (Clandinin et al., 1991).

The conditionally essential PUFA, AA and DHA, make up approximately 20% of fatty acids in the mammalian brain (Contreras et al., 2000). Normal brain DHA and AA levels can be maintained by elongation and desaturation of their precursors (ALA and LA) respectively, within the liver (Igarashi et al., 2007). Multiple aspects of brain metabolism, function, and structure are thought to depend on having adequate brain concentrations of AA and DHA as well as on interactions among these PUFA and their metabolites (Contreras and Rapoport, 2002).

Moreover, brain diseases, such as Alzheimer disease ALS, appear to involve disturbed PUFA metabolism (Kim et al., 2005, Esposito et al., 2008a). Thus,

understanding the dynamics of brain PUFA metabolism could help to interpret brain function in health and disease. Furthermore, brain cells need a certain degree of flexibility to function properly. This is accomplished by maintaining a balance of different types of fatty acids in the cell membrane. Therefore, certain levels of brain PUFA regulate lipid metabolism. An n-3 PUFA intake like EPA and DPA reduce DHA and increase AA suggest being the main up-regulate formation of both enzymatic- and free radical-derived inflammatory mediators (Farias et al., 2008).

In addition, studies on animals and humans have revealed that cholesterol metabolism is disrupted in neurodegenerative diseases. It suggests that ALS may be characterized by cholesterol oxidation that could be associated with an abnormal lipid metabolism and an abnormal fatty acid profile. It is thought there is a relationship between the biosynthesis of oxysterol species and levels of oxysterols in plasma.

Also, there are indications that oxysterols are powerful bioactive lipids that regulate lipid metabolism (Shibata and Glass, 2010). Moreover, it has been suggested that the n-3 fatty acid, ALA, might protect neurones, while DHA found in brain membranes makes it easier for them to change shape and transmit electrical signals (Freund-Levi et al., 2006, Calon and Cole, 2007). Therefore, supplements of n-3 fatty acids could lead to increased DHA in blood and tissues and lower the risk of developing neurodegenerative diseases (Calon and Cole, 2007).

Effects of dietary n-3 PUFA on brain biochemistry, membrane physical chemistry, enzyme activities and their carrier function, particularly during brain development and ageing, in conjunction with their benefit on reducing risks of brain ageing diseases and the neurodegenerative disorders have been reported. Thus, it has been found that animal model studies support such clinical studies, indicating that n-3 PUFA dietary deficiency results in altered brain composition and function (Bourre, 2005).

Previously reported studies showed that EPA possessed neuroprotective properties (Shahidi and Wanasundara, 1998, Okamoto et al., 2007), and could modulate inflammatory processes. In addition, the discovery of FA-EA offered other ways through which n-3 PUFA might influence brain biochemistry. Also, EPA and n-3 DPA supplementation may contribute to the treatment or delay many of the aspects of brain disorders (Neuringer et al., 1986, Makrides et al., 1994).

n-3 DPA is an elongation product of EPA and can be derived from EPA metabolism in mammalian cells (Kanayasu-Toyoda et al., 1996) which exhibit similar actions of EPA (Kaur et al., 2010). Recently, it has been reported that n-3 DPA possesses neurorestorative effects; it also decreases the coupled activation of sphingomyelinase which may be related to its ability to decrease age-related oxidative changes (Kelly et al., 2011). However, to date there have been no studies that examine the relationship between EPA and n-3 DPA intake, SM species in brain and PUFA metabolism.

Analytical techniques such as GC, LC/ESI-MS/MS and GC/MS have enabled the profiling of several lipid mediators simultaneously from biological body fluids or tissues. Therefore, these techniques foster understanding of how a wide range of lipid mediators express their effects in health and disease states.

The focus of Chapter 3 was to examine the effect of EPA and n-3 DPA supplementation on brain SM species as a means of understanding their effects on brain ceramide. I investigated the manipulation of EPA and n-3 DPA supplementation on profiling of RBC lipids as a marker of compliance. Since the effect was age related, I had to assess old and young animals.

In this study, tetradecanoic acid, eicosanoic acid, tetracosanoic acid, palmitoleic acid, cis-OA and erucic acid were found to be increased with ageing, also DHA was significantly increased. In contrast, AA, ALA and nervonic acid were significantly decreased with ageing. These findings indicate that fatty acid metabolism is affected by age. However, AA in aged animals was not affected by either EPA or n-3 DPA supplementation, while DHA was increased in aged animals supplemented with n-3 DPA but not with EPA. This may be due to aged animals being more able to use n-3 DPA to form DHA than younger adult animals. Also, DHA is used by brain and neuronal cells in rats for development and growth. Reduced DHA in RBC of adult animals may be indicative that DHA is a large contributor to brain growth in the developing nervous system.

ALA and n-3 DPA in RBC of adult rats supplemented with EPA were found to be increased compared to aged rats, which indicated that EPA may stimulate  $\Delta 6$ -desaturase in aged animals in order to facilitate ALA metabolism as a

precursor of n-3 PUFA. At the same time, EPA supplementation in adult rats is utilized to produce more n-3 DPA without affecting the DHA concentration. Thus, n-3 DPA may be the bioactive metabolite of EPA in brain that is responsible for its beneficial effects. A long period of n-3 DPA intake showed no significant changes in AA levels of RBC, while it increased levels of EPA and n-3 DPA. This could mean that n-6 and n-3 PUFA may compete for the same metabolizing enzymes. Thus, an increase in n-3 PUFA substrates will result in the metabolism of n-3 at the expense of n-6 PUFA. Also, an imbalance between n-3 and n-6 PUFA may play a role in brain development, ageing related diseases and neurodegenerative diseases (Simopoulos, 1989, Soderberg et al., 1991, Martinez, 1995). Overall these findings indicate that EPA and n-3 DPA supplementation has similar effects on the fatty acid composition of RBC membrane of aged animals.

Significant increases were found in brain SM species of aged animals supplemented with n-3 DPA. Although, no significant changes with EPA supplementation have been reported. These changes are the reverse of those recently observed in sphingosine levels as no significant changes were observed (Kelly et al., 2011). Thus, n-3 was DPA found to have a greater effect on brain SM levels than EPA and indicates that n-3 DPA may modulate the sphingomyelinase pathway. The lack of effect of EPA on brain SM species in ageing animals during this study may be due to the absence of appropriate stimuli.

However, ageing has been shown to alter brain phospholipid composition and consequently the PUFA profile, such as decreased brain levels of EPA and

DHA (Dyall et al., 2007). The regulation of brain SM levels as a precursor of ceramide may have profound effects on ageing-related diseases. Ceramide is synthesized from SM and accumulates in the cell (Figure 1.3) and is further metabolized to free fatty acids and sphingosine, the precursor of sphingosine-1-phosphate (S1P) (Pettus et al., 2002, Milhas et al., 2010). Also, these changes would support recent findings that n-3 DPA and EPA supplementation increased the S1P: ceramide ratio (Kelly et al., 2011) which is important, because ceramide and sphingosine promote apoptosis and generally damage cells, whereas sphingosine-1-phosphate is anti-apoptotic and elicits resistance to apoptotic cell death (Bartke and Hannun, 2009). The balance between these pro- and anti-apoptotic sphingolipids determines the survival of various cells.

Thus, it appears that the modulatory effect of EPA and n-3 DPA on brain SM may lead to a shift in the balance of S1P: ceramide ratio towards an anti-apoptotic phenotype and indicative of deterioration in cell activity, cases, an indicator of cell death. Furthermore, EPA or n-3 DPA intake may play important roles in activating and regulating brain SM biosynthesis.

Many fatty acid ethanolamides (FA-EAs) and endocannabinoids (ECs) such as; AEA, PA-EA, ST-EA and 2-AG have been found to display potent anti-inflammatory and neuroprotective effects. The effects of lithium treatment on neuroinflammation and brain levels of FA-EAs and ECs, in order to provide new information on lithium induced neuroprotective action, are not being realised. Therefore, in chapter 4 an LC/ESI-MS/MS assay was used to profile FA-EAs and 2-AG in head-focused microwave irradiation of brain tissue subjected to neuroinflammation induced by LPS infusion in lithium pre-treated rats. This

method gave good peak resolutions with a recovery range from 88 % to 122 % depending on the analyte; this was achieved by cleaning up the extract with an SPE procedure. Therefore, this modified assay can be applied to profiling of a wide range of FA-EAs and ECs from brain tissue extracts without including any derivatisation steps.

The work presented here is intended to be an update on the involvement of FA-EAs and ECs in the process of inflammation because it has been suggested that a possible mechanism of their actions involves increased production of eicosanoids that promote the resolution of inflammation (Burstein and Zurier, 2009). It is also suggested that FA-EA and EC formation can be stimulated through neuroinflammation induced by LPS. Thus, these mediators can be detected at sites of inflammation in the brain where they are thought to mediate their neuroprotective and anti-inflammatory actions to aid recovery.

It was recently reported that lithium can modify brain AA and DHA metabolism in a rat LPS model of neuroinflammation. Lithium may affect brain levels of FA-EAs and is proposed as a factor for reduced risk of neuroinflammation. During this study, lithium treatment *in vivo* was used to investigate changes in FA-EA and EC profiles within the microwaved brain and to determine their relationship to the effect of different doses of LPS infusion. This should highlight new approaches to understanding lithium neuroprotective action in neuroinflammation.

A wide range of FA-EAs and ECs have been identified and quantified in our study including: AEA, PA-EA, DHA-EA, ST-EA, LA-EA, ALA-EA, OA-EA and 2-

AG. Some of them like PA-EA, T-AG, ST-EA and OA-EA were present at high levels compared to the others and were not affected significantly by lithium treatment. Also, neuroinflammation induced by LPS did not show any changes in brain levels of FA-EAs; AEA, DHA-EA or PA-EA which could exert their effects as neuroprotective and anti-inflammatory mediators. However, lithium treatment showed significant increases in LA-EA and ALA-EA and significant decreases in DHA-EA concentrations.

LA-EA and ALA-EA are prominent among the AEA and DHA-EA analogs respectively, found in neural tissue. Their significant increases may indicate that they have anti-inflammatory effects in response to the neuroinflammation induced by LPS. They were also activated and potentiated their anti-inflammatory action as a response to lithium treatment. LA-EA has recently been reported to exert anti-inflammatory effects and reduce LPS-induced inflammation in animals (Ishida et al., 2013).

PA-EA is found to be elevated with lithium treatment which may be an attempt to enhance or replace the low levels of AEA even though these levels were not affected by lithium treatment. The endogenous PA-EA is a congener of AEA (Petrosino et al., 2010). It has been reported that PA-EA acts by enhancing the anti-inflammatory and anti-nociceptive effects exerted by AEA which is often produced together with PA-EA, and activates cannabinoid CB1 and CB2 receptors or TRPV1 receptors (Di Marzo et al., 2001, De Petrocellis et al., 2001, Smart et al., 2002, Ho et al., 2008). Also, receptors for which AEA shows more potency than CB2, such as CB1, TRPV1 (Zygmunt et al., 1999) and PPAR $\gamma$  (Bouaboula et al., 2005) can be involved in PA-EA-induced effects. Thus, these

actions suggest that the most likely mechanism of action of PA-EA might be the so-called 'entourage' effects due to the PA-EA-induced inhibition of the enzyme catalyzing the AEA degradation (FAAH) that leads to an enhancement of its tissue levels (Costa et al., 2008), resulting in its increasing analgesic and anti-inflammatory actions. The 'entourage' effect may represent a novel route for molecular regulation of endogenous FA-EAs and ECs activity in neuroinflammation.

It has been reported that lithium treatment reduce brain AA turnover and availability (Chang et al., 1996), the present finding with DHA-EA could indicate that lithium treatment may also reduce brain DHA turnover and availability. Consequently, brain DHA-EA may also be reduced as it is derived from DHA. This is an unexpected finding, as it has been reported that animals treated with LPS show an exaggerated neuroinflammatory response (Abraham and Johnson, 2009) which could lead to more DHA-EA being produced as an anti-inflammatory and organ-protective agent in the brain.

Furthermore, The FA-EAs are amides of PUFA, for instance, AEA is the amide of AA (20:4  $\omega$ -6) and ethanolamine that attenuates pain sensation (Calignano et al., 1998), PA-EA is the amide of PA (16:0) and ethanolamine and has anti-inflammatory activity that also attenuates pain sensation (Calignano et al., 1998, Lambert et al., 2002), OA-EA is the amide of OA (18:1) and ethanolamine and has anorexic effects and reported by stimulates fat breakdown. ST-EA is the amide of stearic acid (18:0) and ethanolamine that has pro-apoptotic activity (Rodriguez de Fonseca et al., 2001). In addition n-3 N-acylethanolamines are endogenously synthesised from n-3 fatty acids (Brown et al., 2011). It has also

been reported that dietary supplementation with fatty acids like n-3 PUFA is found to be therapeutically effective (Stoll et al., 1999b, Innis, 2008). Many studies suggest that introducing lithium into the supplement may have beneficial effects in reducing risk of diseases such as bipolar disorder (Rapoport and Bosetti, 2002) reduce inflammation in the brain and may also be important for preventing oxidative stress (Jenkinson et al., 1999a). Therefore, the current findings could open a new field of investigating if n-3 PUFA supplementation like DHA, under conditions of an imbalance between n-3 and n-6 PUFAs, would improve synthesis of brain n-3-EAs and inhibit neuroinflammation. Also, it has been suggested that a combination of lithium treatment and n-3 PUFA supplementation may be useful for attenuating neuroinflammation. However, increasing PUFA intakes should only be recommended when an adequate antioxidant intake is ensured (Jenkinson et al., 1999b).

In general, neuroinflammation is present in the majority of acute and chronic neurological disorders. N-3 PUFA are well known as anti-inflammatory agents in many non-neural tissues (Orr et al., 2013). However, their role in neuroinflammation is less well studied. Moreover, such rigorous studies that test the direct effects of n-3 PUFA in neuroinflammation *in vivo* are lacking. Also, which n-3 PUFA directly target brain inflammatory pathways still is not well elucidated. In addition, their relationship with the brain n-3 PUFA bioactive metabolites may provide novel therapeutic targets for neurological disorders with a neuroinflammatory component. Also, a preclinical study with all classes of FA-EAs and ECs is still needed to validate our findings in relation to the benefit of lithium on treatment of the neuroinflammation. Such a study may be

include the endogenous examples of FA-EAs and ECs, point to a variety of their pro-inflammatory activity and therapeutic targets and the important roles for these bioactive lipid mediators in the physiology of inflammation and the neurodegenerative disorders.

The maintenance of cholesterol homeostasis is of great importance to supply tissues with the appropriate amount of cholesterol and prevent its accumulation that may affect health. Cholesterol has high sensitivity to the enzymatic and non-enzymatic oxidation processes that lead to formation of oxysterols. Plasma cholesterol has been found to be associated with motor neuron loss in ALS Disease (Dorst et al., 2011, Zhao et al., 2012). Neurodegenerative diseases such as Alzheimer's disease are reported to be associated with a disturbed cholesterol metabolism (Vega and Weiner, 2007). Also, it has been argued that plasma oxysterols could serve as markers of oxidative stress (Micheletta and Iuliano, 2006). Since the BBB efficiently prevents cholesterol uptake from the circulation into the brain, *de novo* synthesis is therefore responsible for almost all cholesterol present in brain (Lutjohann et al., 1996, Dietschy and Turley, 2004); any surplus cholesterol in the brain is oxidized and rapidly removed as oxysterols (Di Paolo and Kim, 2011). Therefore, brain cholesterol level and turnover and oxysterol levels have numerous activities in the brain and are suspected of playing major roles in neurological disorders such as Alzheimer disease, Multiple Sclerosis, and Huntington disease (Jeitner et al., 2011) and (Leoni and Caccia, 2011). Also, they may play essential roles in the regulation of various cellular biological processes and functions including

induction of cell death (apoptosis) (Morin et al., 1991, Lordan et al., 2009, Vejux and Lizard, 2009).

It was thought that a balance in the ratio of plasma to brain oxysterol levels may be used as a marker of many neurodegenerative diseases. Brain and plasma oxysterol exchange is controlled by BBB (Bjorkhem, 2006); many species of oxysterol have been used as biomarkers of neurodegenerative disorders (Bjorkhem et al., 2009, Leoni and Caccia, 2011).

In contrast, n-3 PUFA have a wide range of beneficial effects in several neuronal health and disease conditions especially by affecting membrane lipid composition of the n-3 PUFA that are supposed to be highly oxidisable. Generally, oxidation usually occurs within the cell membranes and leads to damage and changes their physic-chemical properties (Block et al., 2010). Many researchers are still trying to explore the effects of oxidation and abnormalities of fatty acid metabolism and cholesterol oxidation on neurodegeneration.

Therefore, the interest in Chapter 5 was to investigate whether plasma oxysterol species and RBC fatty acid are affected by ALS in human and SOD1 knock-out mice in related age and disease states. Recently, there have been many contributions to the field of oxysterol metabolism, but there are a number of specific problems that have been associated with this field of research. For instance, the enormous complexity of the biological effects of oxysterols, most oxysterols are not commercially available for standardisation, a lack of uniformity in the analytical chemistry procedures, with particular reference to

mass spectrometry methods, the level of sterol compounds present in the diet. It is also important to highlight the complicated problems of cholesterol autoxidation which results in numerous artefacts during sample processing (Iuliano and Lizard, 2013).

Several major oxysterols are formed as intermediates in several pathways of converting cholesterol to bile acids or steroid hormones. Also their roles as readily transportable forms of sterol are well established (Schroepfer, 2000, Bjorkhem and Diczfalusy, 2002, Oikkonen and Lehto, 2004). Of these species, the most abundant oxysterols that have been reported in human serum, generated enzymatically, are 27-OH, 24-OH, 7 $\alpha$ -OH, and 4 $\beta$ -OH (Oikkonen et al., 2012). While the most abundant oxysterols generated through autoxidation include 7-keto and 7 $\beta$ -OH which have prominent cytotoxic and pro-apoptotic properties (Lordan et al., 2009).

However, in our study, 24-OH was found to be increased in ALS and it is exclusively formed in brain (Prasanthi et al., 2009). This probably indicated that ALS states may stimulate activity of CYP46 as the enzyme responsible for converting cholesterol to 24-OH in brain. Also, ALS may lead to dysfunction of BBB and an increased flux of 24-OH into the circulation providing a possible major pathway of brain cholesterol turnover and homeostasis.

27-OH was found to be significantly increased in ALS. It is mainly formed in peripheral tissues (Duane and Javitt, 1999), and its brain and plasma levels are found to be correlated with the corresponding levels of cholesterol circulation in healthy people/animals and in diseases based on the severity of diseases.

Therefore, its concentration could be considered as an important diagnostic marker in ALS.

25-OH was also found to be significantly increased in relation to age and disease stages. Conversion of cholesterol to 25-OH *in vitro* was found to be catalysed by different cytochrome P450 enzymes such as CYP27A1 and CYP3A4, but the importance of these reactions *in vivo* remains unclear. Presence of 25-OH at high levels in plasma indicates that it is an important regulator of cholesterol metabolism (Diczfalusy, 2013).

The biochemical and biological properties of 4 $\beta$ -OH, 7 $\beta$ -OH, 7 $\alpha$ -OH and 7-keto are not well known. The activities levels of these oxysterols were characterised in myelin synthesizing cells of ALS and SOD1 knock-out mice. They were found to be significantly decreased in relation to the advanced stages of disease and in ageing. It has been reported that at high concentrations, 4 $\beta$ -OH is not only able to inhibit cell growth, but also to induce cell death associated with a loss of membrane integrity. These effects are lower than the effects of 7-keto and 25-OH (Nury et al., 2013). Therefore, myelin cell synthesis of these species may be attenuated by ageing and prolonged period of disease and therefore their levels in plasma are reduced.

Other plasma oxysterols were also found to be significantly increased; 5,6 $\beta$ -epoxy, 5,6 $\alpha$ -epoxy, 26-OH, 20 $\alpha$ -OH, 22-OH and 6-keto in relation to the later stage of disease and ageing. These changes could indicate that raised cholesterol levels may be a consequence of the late stage of the disease and/or ageing. Oxysterols are present in mammalian tissues at very low concentrations,

as mixtures accompanied by a high excess of cholesterol (Olkonen et al., 2012). Thus, as a result of the generally increased content of cholesterol, plasma levels of oxysterols would also be expected to be increased. The above findings and the potent regulatory activity that several oxysterols have on cellular cholesterol homeostatic mechanisms (Gill et al., 2008) prompted an intensive research effort into oxysterols in the context of lipid homeostasis and neurodegeneration.

Novel physiologic activities of oxysterols have emerged from this research. Oxysterols are believed to act as endogenous regulators of gene expression in lipid metabolism and as signaling molecules with key roles in developmental, differentiation, and inflammation processes. Recently, novel oxysterol receptors responsible for these activities have been identified. All of these are opening new perspectives on oxysterol function and inducing novel interest in these compounds in the field of biomedical research.

Moreover, the above mentioned variable findings may be related to the advanced stage of disease, to different ALS pathogenetic mechanisms or to the systemic neuroinflammation associated with ALS. These conditions may affect their net flux from circulation into the brain or from brain into the circulation. Also, they are suggested to play major roles on BBB functions and performance and in the development of ALS disease.

In addition, these findings reported differences in the levels of some oxysterols between mutant SOD1 knock-out and ALS patient; these differences may be related to specific actions of the mutant SOD1 gene on the biosynthesis of

oxysterols. Furthermore, maintaining the balance of plasma 24-OH and 27-OH might be important for attenuating the neurodegenerative diseases, and consequently could be used as diagnostic markers and as basis for their use in any therapeutic strategy.

Overall, the present findings can be used as key factors in relation to the ageing effect in the brain. The significant increase of eicosadienoic acid and LA with age and disease state needs to be validated before they may be used as markers for underlying ASL. Furthermore, 24-OH and 27-OH are consistent as markers for oxidative stress and lipid peroxidation. Also, they may reflect the BBB dysfunction and alteration of brain lipid metabolism. Therefore, further investigations of oxysterols and their impact on neuroinflammation, such as regulation of their biosynthetic enzymes that may be involved in the neuroinflammation and fatty acid metabolism, are required for developing improved strategies for the prevention and treatment of various types of brain diseases and neuronal disorders.

## **6.2 Future work**

The precise causes of ageing-associated alterations, neuroinflammation and neurodegenerative disorders are still not clearly identified, but there is evidence that bioactive lipids derived from enzymatic and reactive oxygen species mediated reactions that may play important roles in these processes. Even though increased inflammation is found to be accompanied by normal brain ageing, and ageing may lead to the accumulation of disabilities and diseases that limit normal body functions, it remains uncertain to what extent the

inflammatory changes associated with ageing explain why ageing is an important risk factor for all major neurodegenerative diseases.

Both EPA and n-3 DPA effects may not be related to their direct action, but may be related to the action of their metabolites. Although EPA supplementation has been well studied, n-3 DPA supplementation and its relationship with EPA and other PUFA supplementations are still not well elucidated. Mechanisms underlying EPA and n-3 DPA specific responses need to be better understood to compare n-3 DPA with EPA in preventing and treating brain ageing and age related diseases. Also, more arguments could be raised as to which of these n-3 PUFA may perform best on treatment of neurodegenerative disorders. Therefore, further investigations to fully resolve how the balance of different n-3 fatty acids affects brain function, cell membranes composition, membrane function and several biochemical pathways within cells, tissues, and organs is needed.

However, more extensive research is also required in order to investigate the biological effects of pure n-3 DPA *in vitro* and *in vivo* as there are still questions that remain unanswered. For example is n-3 DPA an effective precursor of DHA in brain? is it a significant reservoir of EPA in the body?, is n-3 DPA conserved from  $\beta$ -oxidation relative to other n-3 PUFA?, does n-3 DPA have any unique/specific biological properties.

Furthermore, it has been reported that n-3 DPA and EPA markedly increased the S1P: ceramide ratio; S1P has been shown to provide resistance to apoptotic cell death while ceramide is damaging to cells. Thus based on the fact that

sphingomyelin is a precursor of ceramide, additional studies with animal models are needed to determine whether the observed changes in brain SM species may or may not play a causal role in the neuroprotective effect of n-3 fatty acids on brain ageing. Such studies could also help to understand the brain's important functional interplay between the SM species and n-3 PUFA supplementation.

On the other hand, although several hypotheses have been suggested to explain the mechanism of lithium action as a molecular target in the treatment of neuroinflammation the actual mechanisms remain unclear to date. Therefore, further studies using a large sample size and involving lithium treatment of animals and focusing on the investigation of changes in brain FA-EAs and ECs levels in relation to functional end points in brain diseases in which inflammation occurs might clarify the underlying cellular mechanisms.

Disturbances in cholesterol metabolism and its conversion to oxysterols may also play a role in neuropathological conditions. Cholesterol and fatty acid oxidation cause alterations in cell membrane fluidity, permeability, and structure. These alterations could alter membrane functions and signify the pathophysiological roles of cholesterol and fatty acid oxidation in neurological diseases.

Despite normal or insignificant changes in human RBC fatty acid profiles, particularly of eicosadienoic acid, the present study indicates that ALS presents with increased levels of the most abundant plasma oxysterols (i.e. 24-OH and 27-OH) which are closely related to the altered eicosadienoic acid profiles,

based on the disease stages. This was also closely related to alterations of fatty acid profiles observed with ageing. These emerging associations highlight the usefulness of combined measurements of oxysterols and fatty acids, particularly the eicosadienoic acid, as a means of understanding the lipid bases of the disease mechanism in ALS. Also, these combined determinations may be seen as important prognostic factors for managing risks or progressive stages of disease, and of monitoring its therapeutic strategies, including those based on antioxidants, and dietary manipulation.

Therefore, comprehensive studies using more factors that influencing oxysterol metabolism such as the roles of the oxysterol receptors and their metabolic enzymes in the regulation of cholesterol and sterol metabolism, as well as effects on n-3 PUFA metabolism and the benefits of n-3 PUFA supplementation are needed to confirm and validate our present findings. To increase accuracy, a large sample size should be used. Also, future work should include the importance of studying the nutritional intervention strategies in controlling oxysterol levels and disease progression.

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## **APPENDICES**

## Appendix - 1: Fatty acid analysis

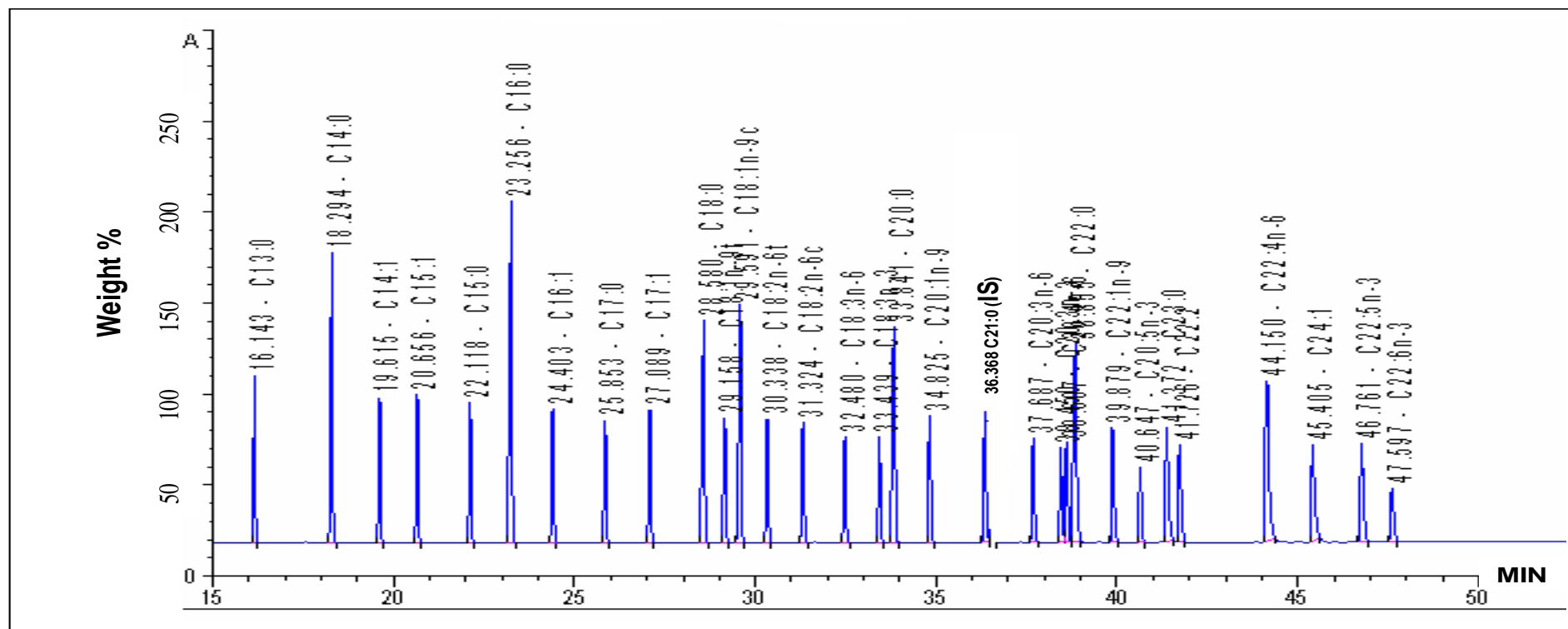


Figure 1. Representative GC-FID chromatogram of cocktail FAME standards (SIGMA).

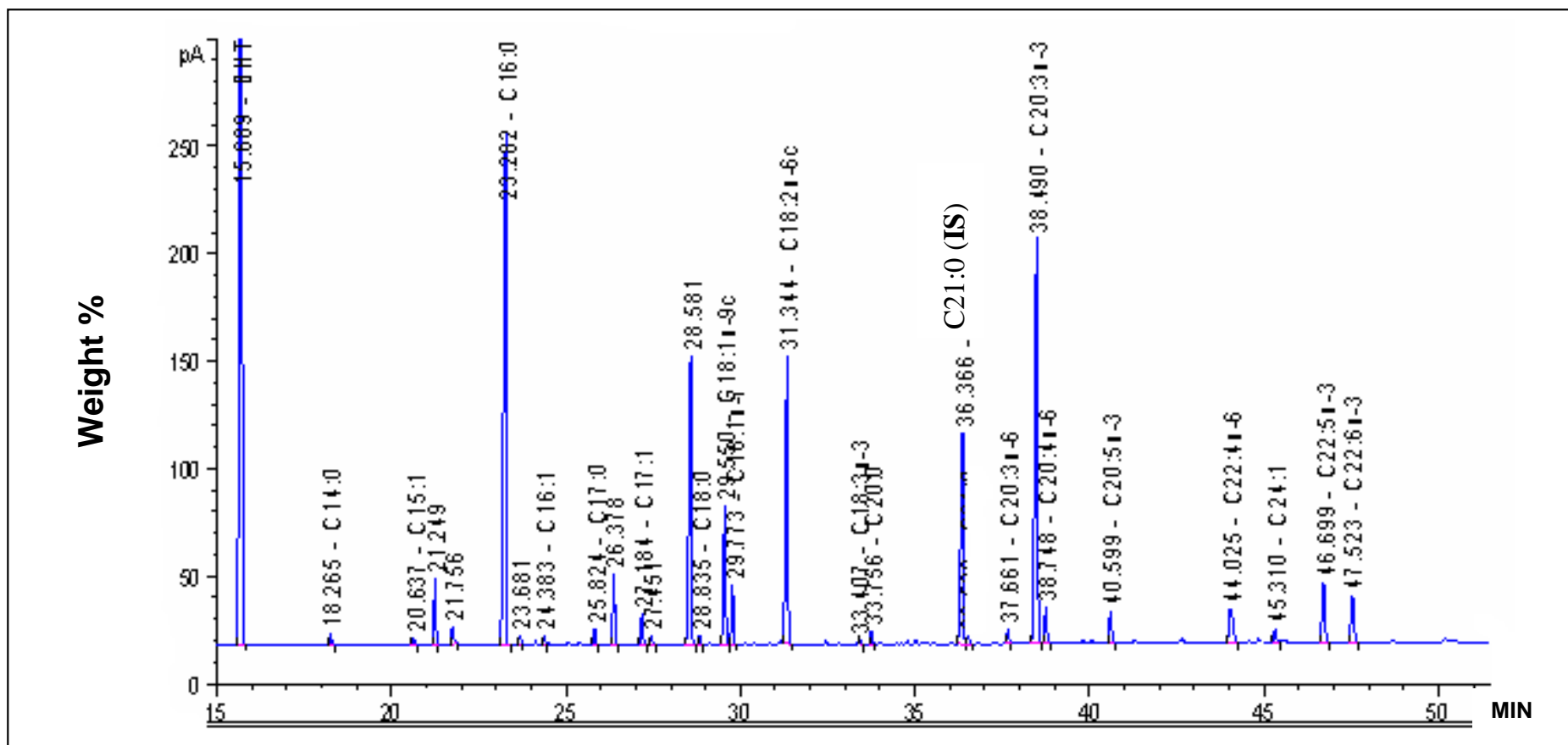


Figure 2. Representative GC-FID chromatogram showing rat RBC fatty acids (adult male Wistar rat).

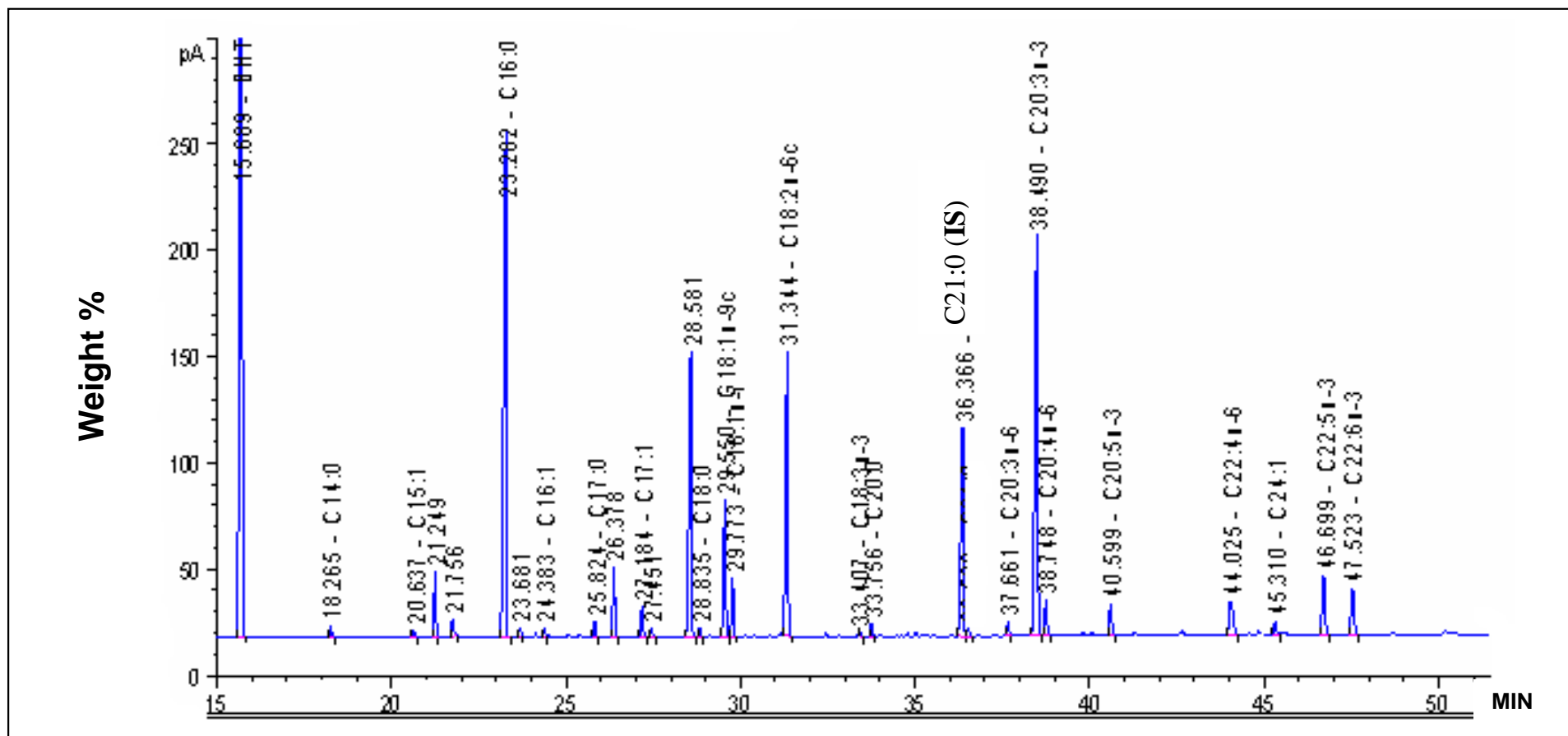


Figure 3. Representative GC-FID chromatogram showing human fatty acids.

Table 1. Typical set of calculations for FAME analysis.

FATTY ACID	AREA 1	AREA 2	AVERAGE	MW FAME	weight of FAME (ng)	% weight
C4	0.0	0.0	0.0	89	0.00	0.00
C6	0.0	0.0	0.0	118	0.00	0.00
C8	0.0	0.0	0.0	158	0.00	0.00
C10	0.0	0.0	0.0	186	0.00	0.00
C11	0.0	0.0	0.0	200	0.00	0.00
C12	0.0	0.0	0.0	214	0.00	0.00
C13	0.0	0.0	0.0	228	0.00	0.00
C14	18.7	33.5	26.1	242	29.28	0.29
C14:1	0.0	0.0	0.0	240	0.00	0.00
C15	18.8	33.5	26.2	256	29.34	0.29
C15:1	0.0	0.0	0.0	254	0.00	0.00
C16	1812.3	3175.8	2494.1	270	2798.15	27.37
C16:1	14.9	25.8	20.4	268	22.83	0.22
C17	192.1	74.5	133.3	284	149.56	1.46
C17:1	98.3	173.3	135.8	282	152.36	1.49
C18	937.3	1646.6	1292.0	298	1449.48	14.18
C18:1n-9t	24.0	44.5	34.3	296	38.43	0.38
C18:1n-9c	377.8	666.1	522.0	296	585.59	5.73
C18:1n-7c	160.1	281.3	220.7	282	247.61	2.42
C18:2n-6t	8.7	3.8	6.2	294	7.00	0.07
C18:2n-6c	835.9	1496.3	1166.1	294	1308.28	12.80
C18:3n-6	21.8	15.8	18.8	292	0.00	0.00
C18:3n-3	35.4	70.6	53.0	292	59.42	0.58
C20	0.0	0.0	0.0	326	0.00	0.00
C20:1n-9	0.0	16.6	8.3	324	9.30	0.09
C20:2	22.7	36.8	29.8	322	33.38	0.33
C20:3n-6	27.9	49.3	38.6	320	43.31	0.42
C20:4n-6	1522.0	2672.9	2097.5	318	2353.19	23.02
C20:3n-3	0.0	0.0	0.0	320	0.00	0.00
C22	112.8	197.8	155.3	354	174.24	1.70
C22:1n-9	9.7	20.2	15.0	352	16.80	0.16
C20:5n-3	17.3	32.4	24.9	316	27.88	0.27
C23	10.9	21.0	16.0	368	17.89	0.18
C22:2	0.0	0.0	0.0	350	0.00	0.00
C24:0	23.0	43.0	33.0	382	37.02	0.36
C24:1	49.7	99.0	74.4	380	83.42	0.82
C22:5n-3	130.6	229.9	180.3	344	202.23	1.98
C22:6n-3	224.3	396.3	310.3	342	348.14	3.41
IS 21:0	604.9	1069.0	<b>837.0</b>	340	<b>939</b>	
TOTAL					<b>10224.11</b>	

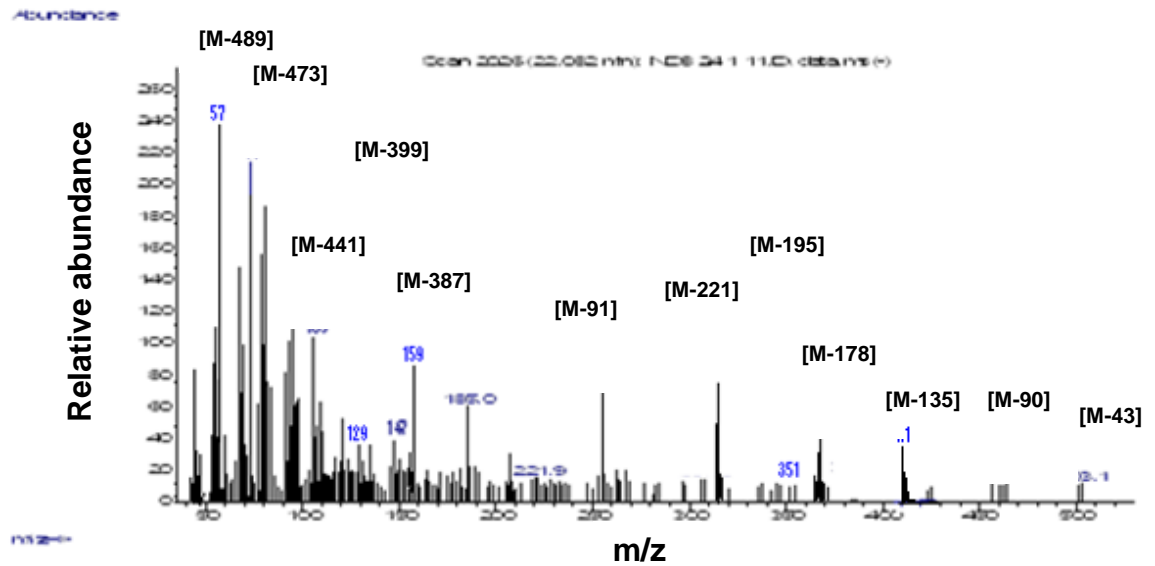
Table 2. List of fatty acids detected in rat red blood cell samples. SFA: saturated fatty acids, MFUA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

<b>SFA</b>	
C14:0	Tetradecanoic acid (Myristic acid)
C15:0	Pentadecanoic acid (Pentadecylic acid)
C16:0	Hexadecanoic acid (Palmitic acid)
C17:0	Heptadecanoic acid (Margaric acid)
C18:0	Octadecanoic acid (Stearic acid)
C20:0	Eicosanoic acid (Arachidic acid)
C22:0	Docosanoic acid (Behenic acid)
C23:0	Tricosanoic acid (Tricosylic acid)
C24:0	Tetracosanoic acid (Lignoceric acid)
<b>MUFA</b>	
C16:1	Palmitoleic acid
C17:1	Heptadecenoic acid
C18:1n-9t	Oleic acid
C18:1n-9c	cis-Oleic acid
C18:1n-7	Vaccenic acid
C20:1n-9	Eisosenoic acid
C22:1n-9	Erucic acid
C24:1	Nervonic acid
<b>n-6 PUFA</b>	
C18:2n-6t	trans-Linoleic acid
C18:2n-6c	cis-Linoleic acid
C20:2	eicosadienoic acid
C20:3n-6	Dihomo-gamma-linolenic acid (DGLA)
C20:4n-6	Arachidonic acid (AA)
<b>n-3 PUFA</b>	
C18:3n-3	$\alpha$ -Linolenic acid (ALA)
C20:3n-3	Eisosatrienoic acid (ETE)
C20:5n-3	Eicosapentaenoic acid (EPA)
C22:5n-3	Docosapentaenoic acid (DPA)
C22:6n-3	Docosahexaenoic acid (DHA)

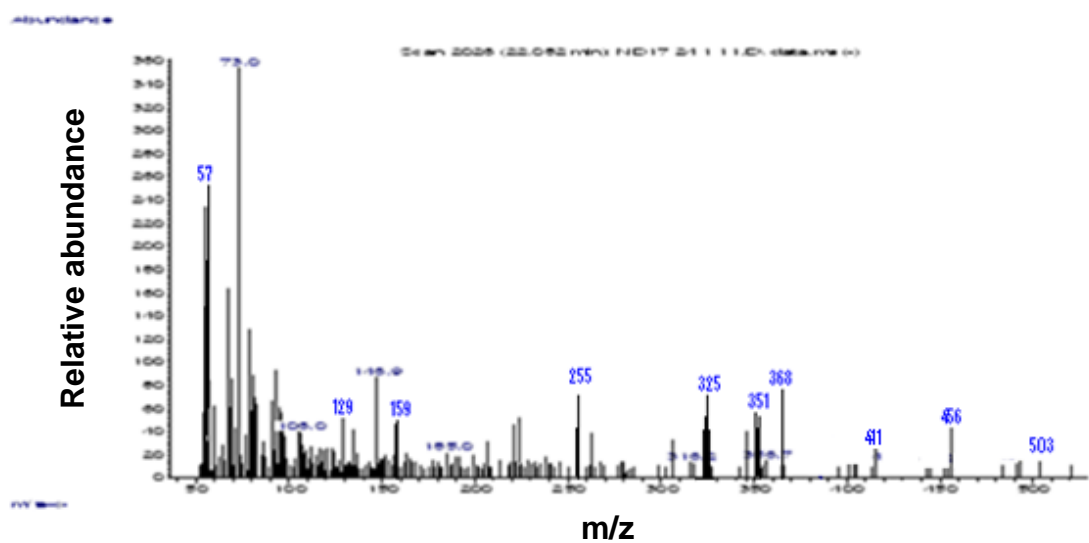
## Appendix - 2: Identification of oxysterols

**Figure 1.** EI spectrum of the compound eluting at 22.1 min (see table 6.3) identified as **7 $\alpha$ -hydroxycholesterol (7 $\alpha$ -OH)**.

### Healthy control

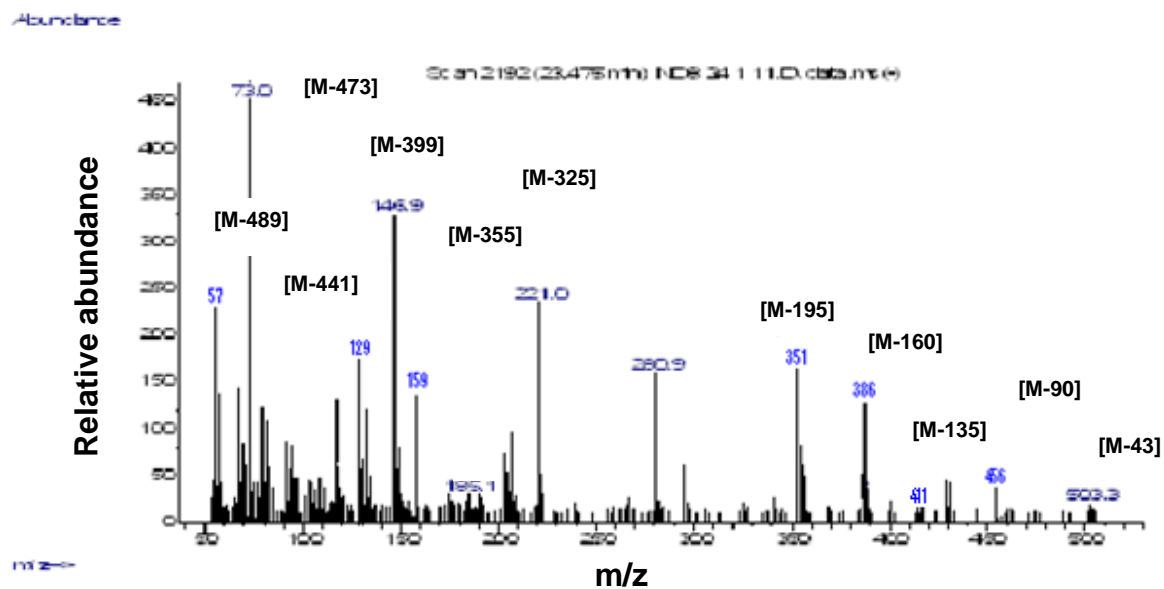


### ALS patients

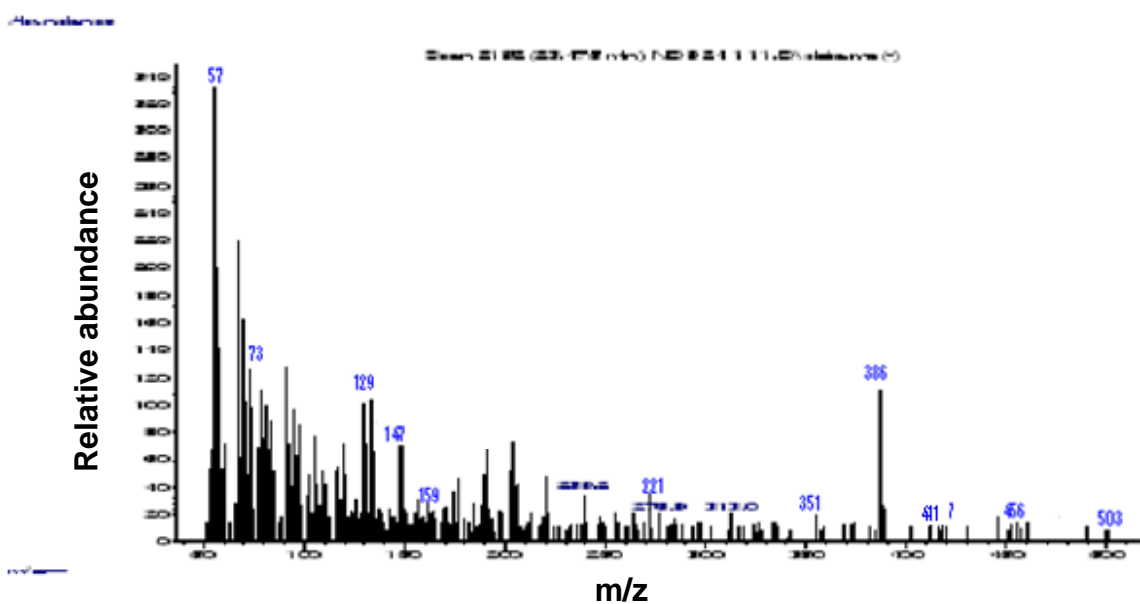


**Figure 2.** EI spectrum of the compound eluting at 23.5 min (see table 6.3) identified as **7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH)**.

### Healthy control

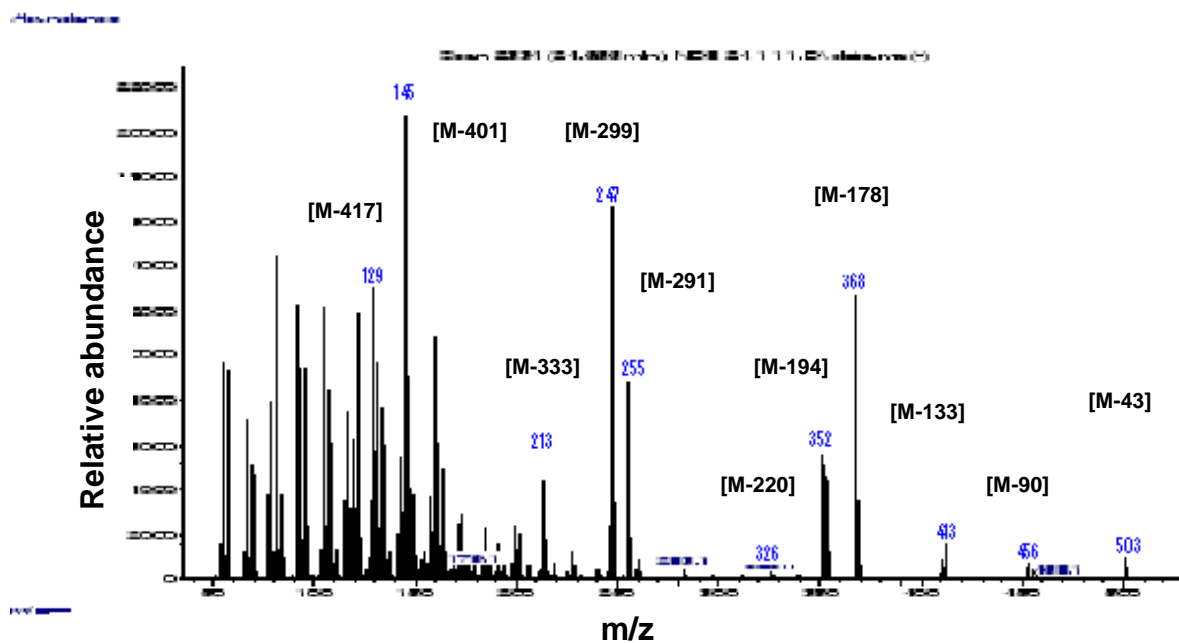


### ALS patients

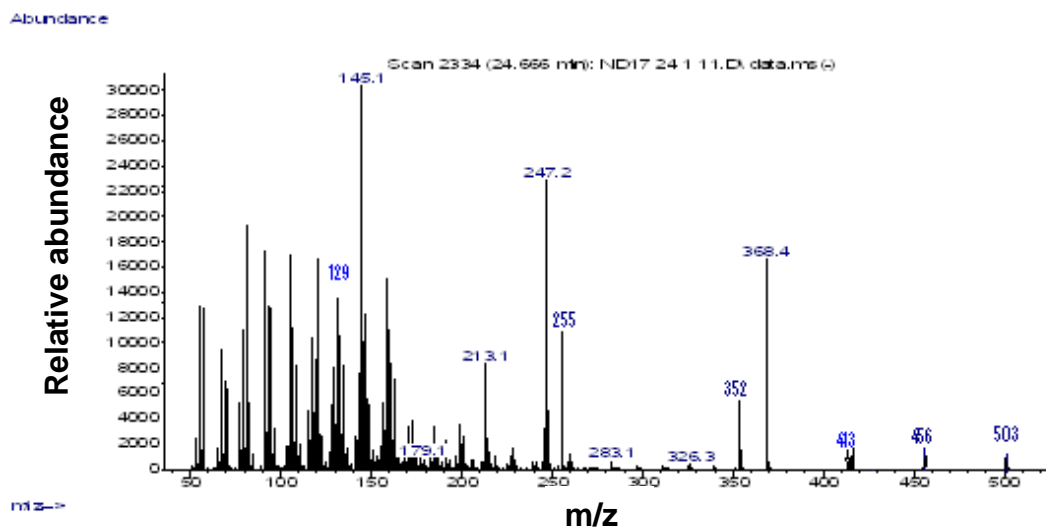


**Figure 3.** EI spectrum of the compound eluting at 24.7 min (see table 6.3) identified as **24-hydroxycholesterol** (24-hOH).

### Healthy control

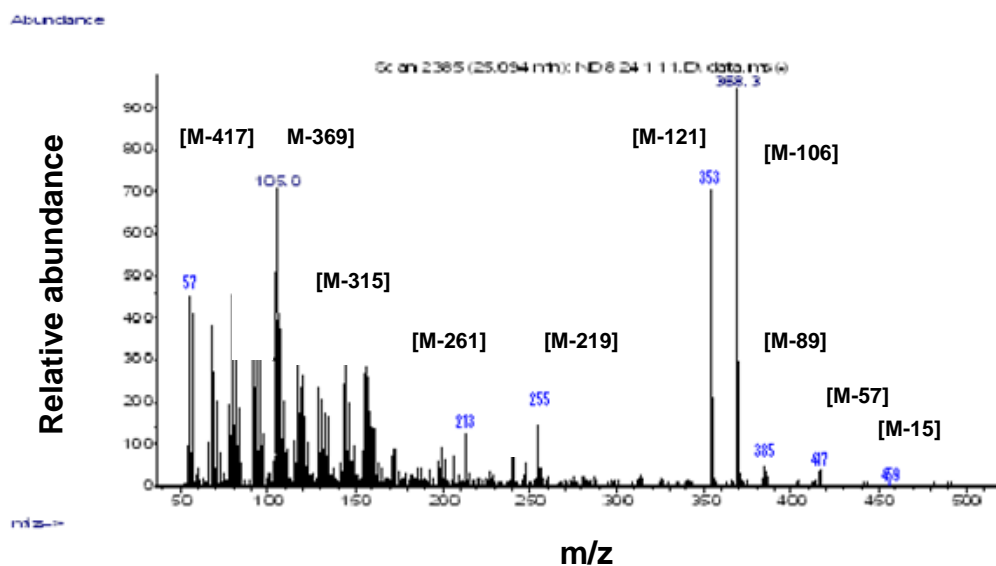


### ALS patients

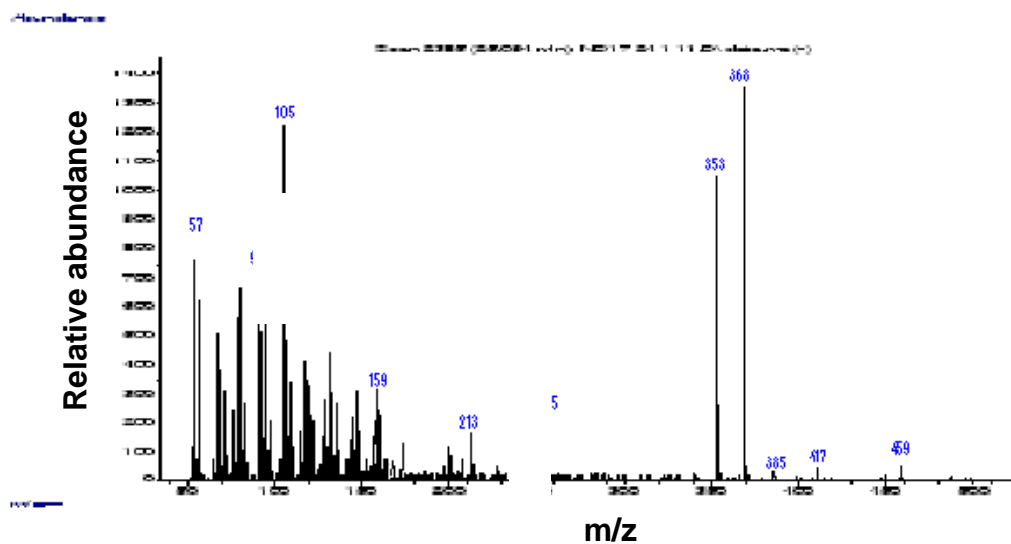


**Figure 4.** EI spectrum of the compound eluting at 25.1 min (see table 6.3) identified as **5,6 $\beta$ -epoxy-cholesterol** (5,6 $\beta$ -epoxy).

### Healthy control

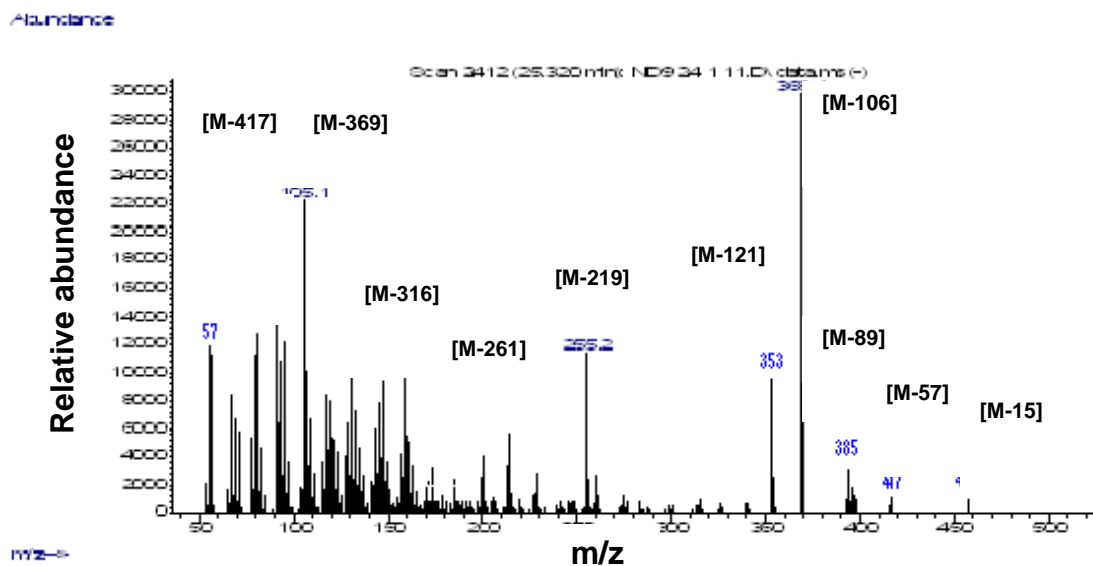


### ALS patients

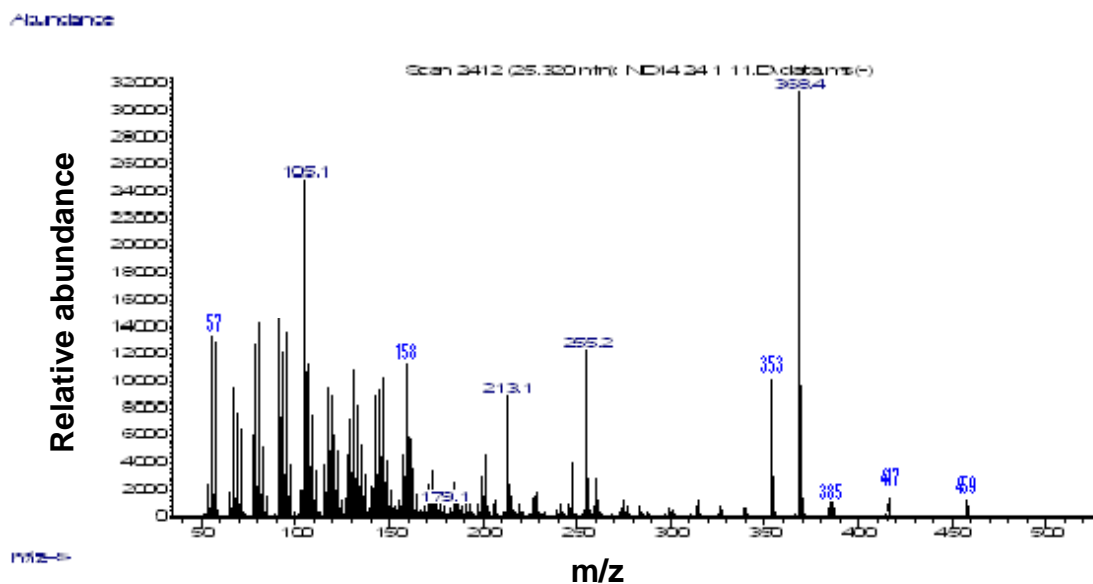


**Figure 5.** EI spectrum of the compound eluting at 25.3 min (see table 6.3) identified as **5,6 $\alpha$ -epoxy-cholesterol** (5,6 $\alpha$ -epoxy).

### Healthy control

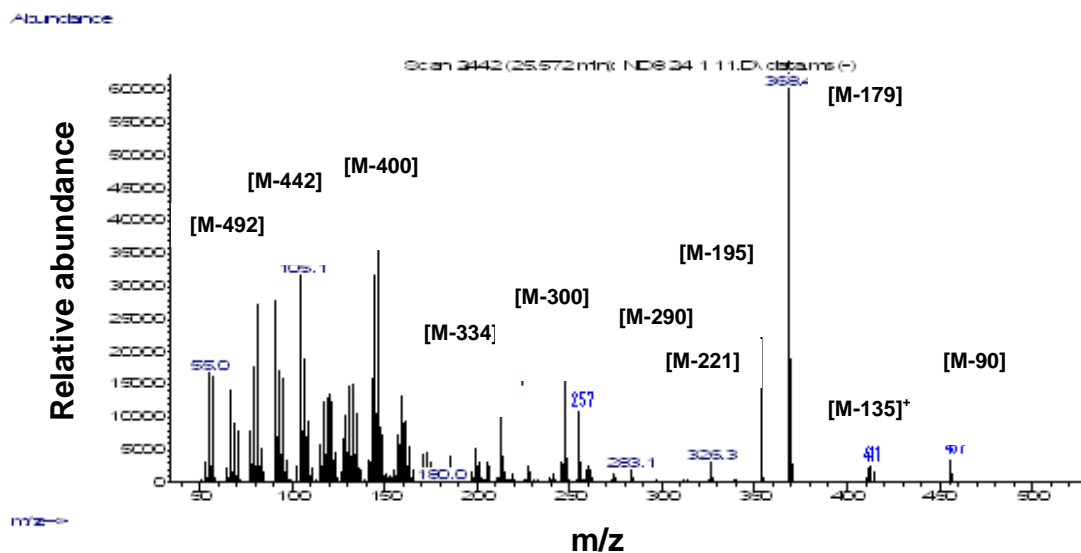


### ALS patients

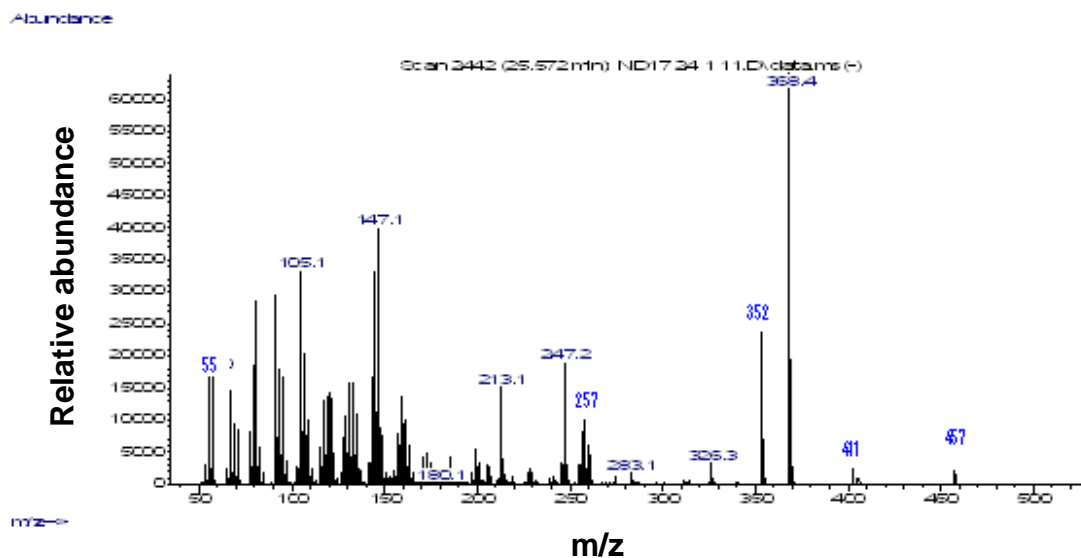


**Figure 6.** EI spectrum of the compound eluting at 25.6 min (see table 6.3) identified as **27-hydroxycholesterol (27-OH)**.

### Healthy control

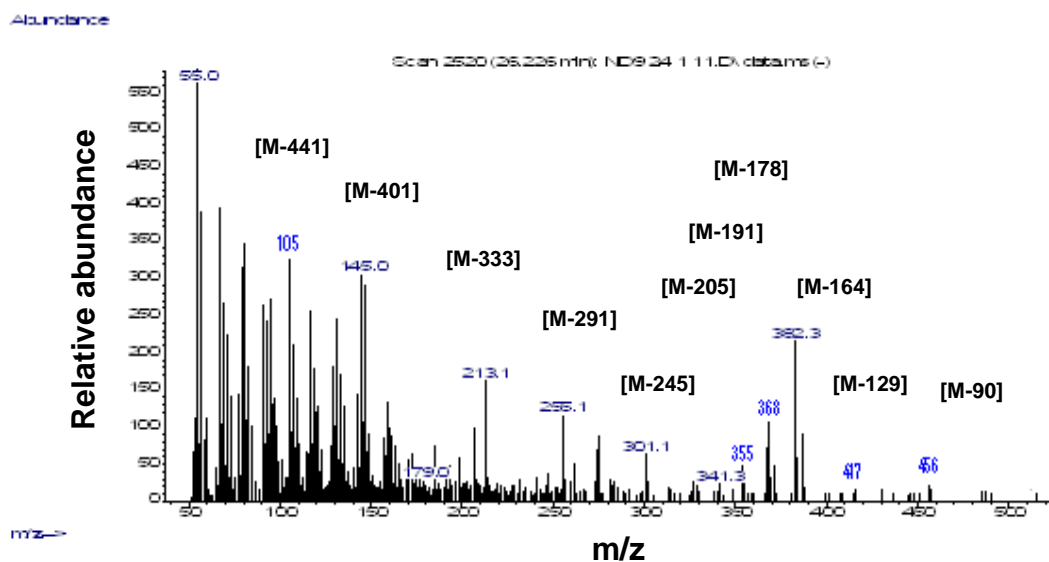


### ALS patients

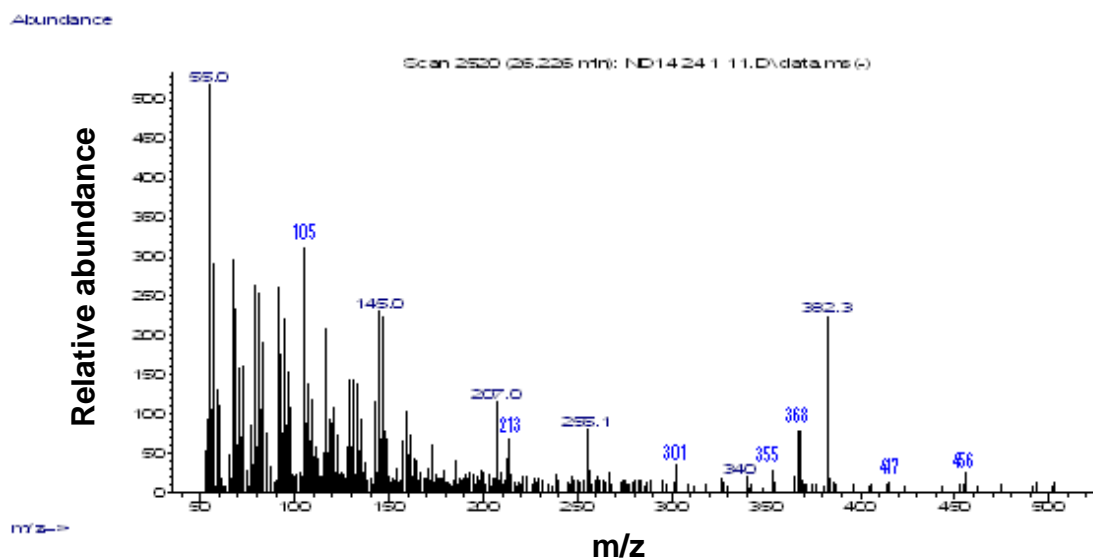


**Figure 7.** EI spectrum of the compound eluting at 26.2 min (see table 6.3) identified as **26-hydroxycholesterol (26-OH)**.

### Healthy control

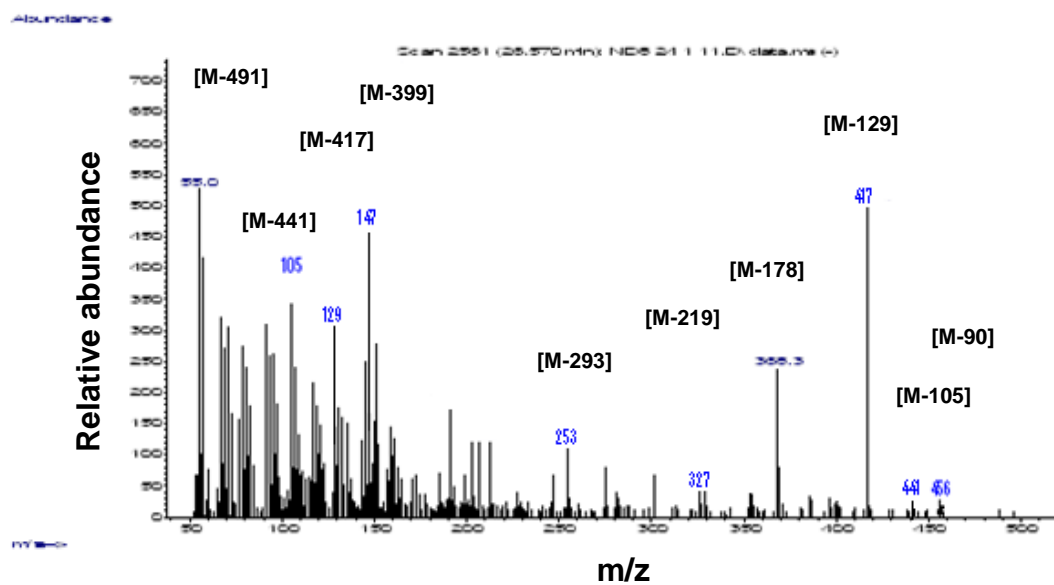


### ALS patients

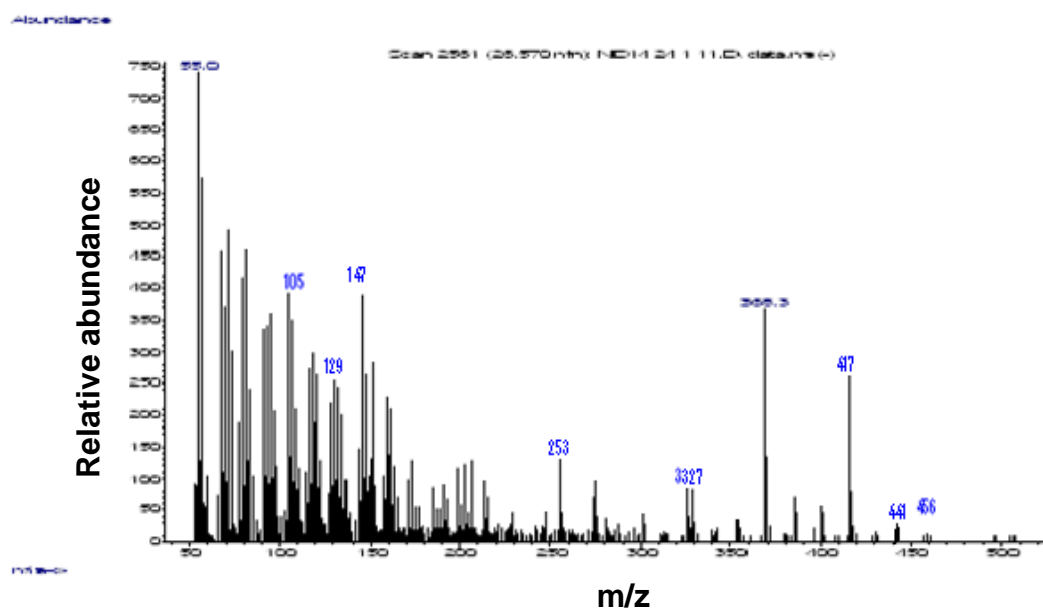


**Figure 8.** EI spectrum of the compound eluting at 26.6 min (see table 6.3) identified as **4 $\beta$ -hydroxycholesterol** (4 $\beta$ -OH).

### Healthy control

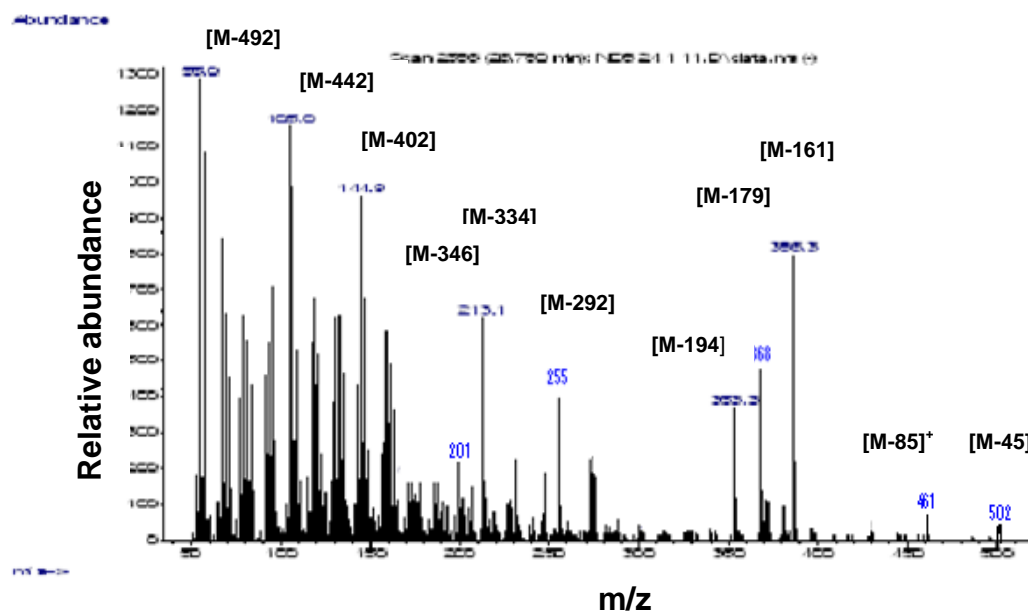


### ALS patients

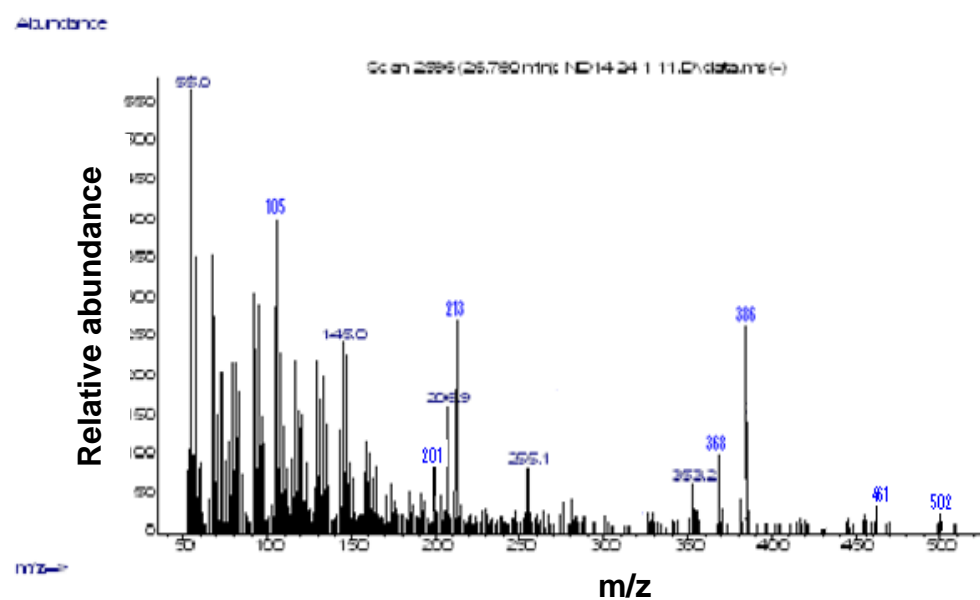


**Figure 9.** EI spectrum of the compound eluting at 26.8 min (see table 6.3) identified as **20 $\alpha$ -hydroxycholesterol** (20 $\alpha$ -OH).

### Healthy control

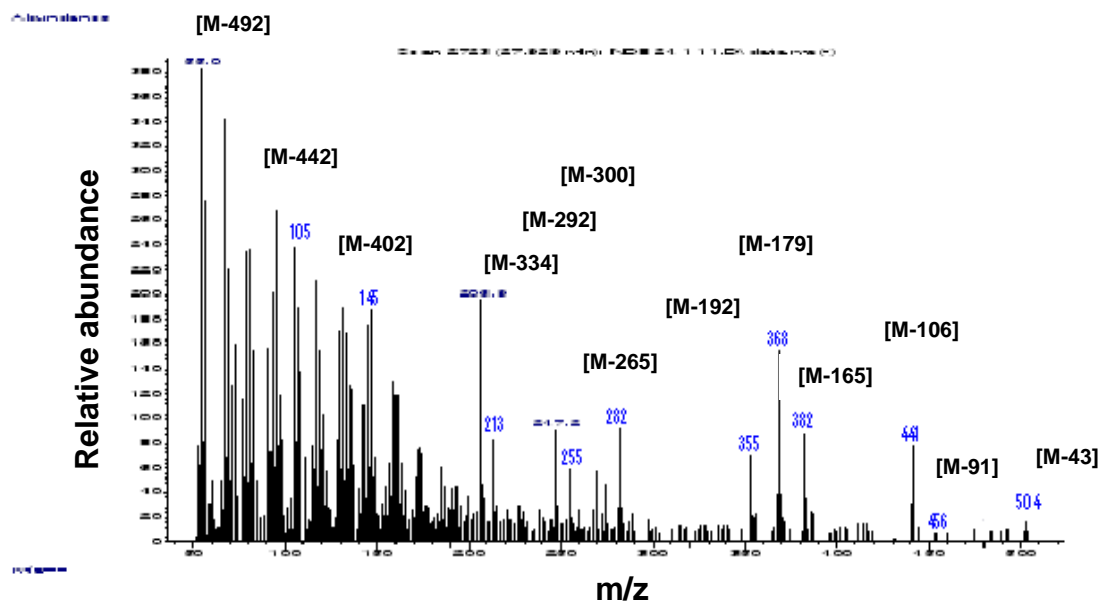


### ALS patients

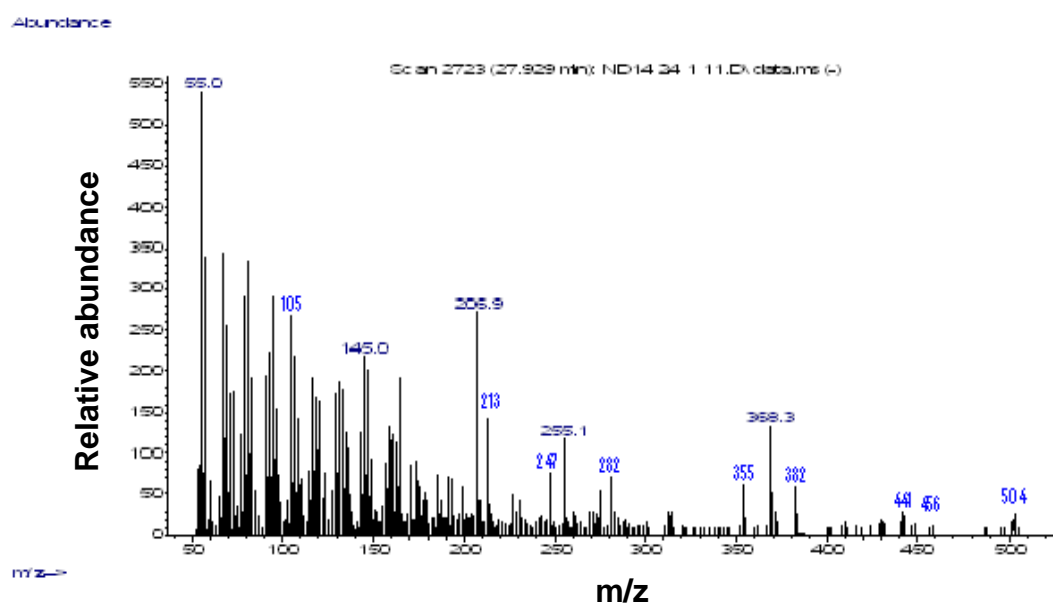


**Figure 10.** EI spectrum of the compound eluting at 27.9 min (see table 6.3) identified as **22-hydroxycholesterol** (22-OH).

### Health control

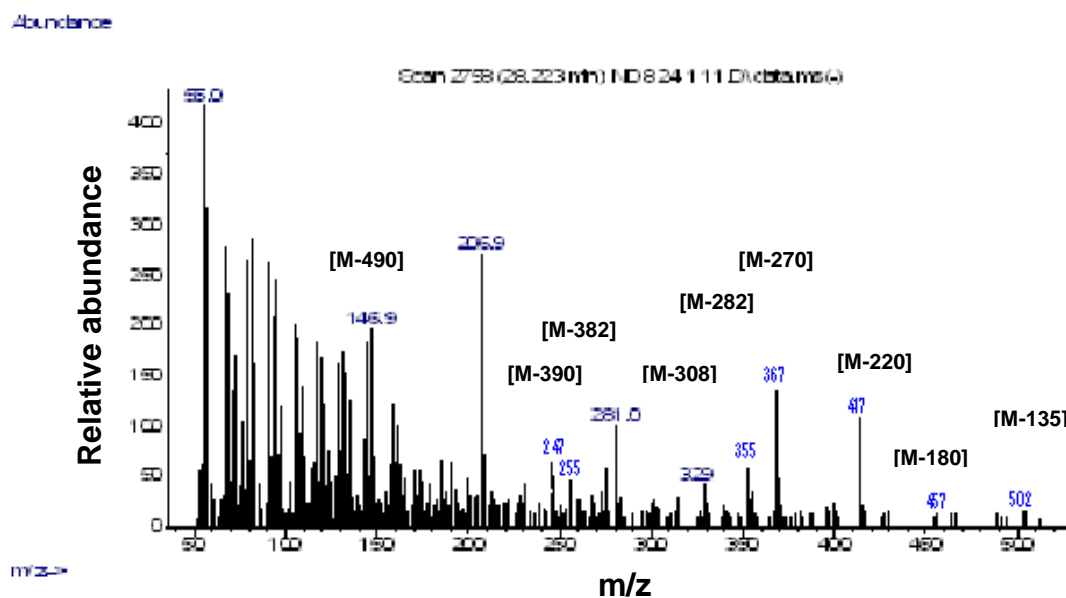


### ALS patients

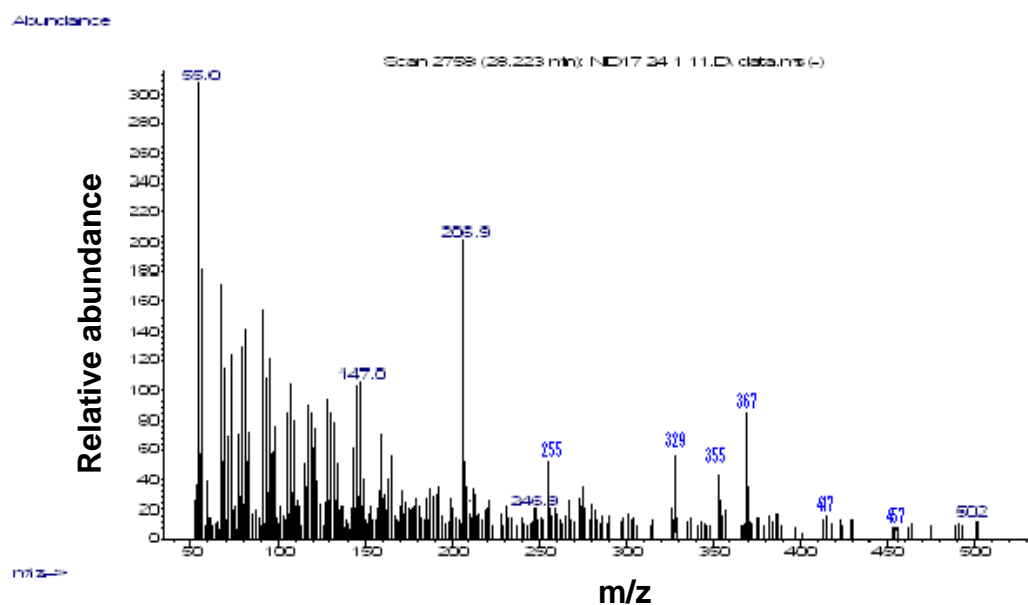


**Figure 11.** EI spectrum of the compound eluting at 28.2 min (see table 6.3) identified as **Cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol** (Chol.triol).

### Health control

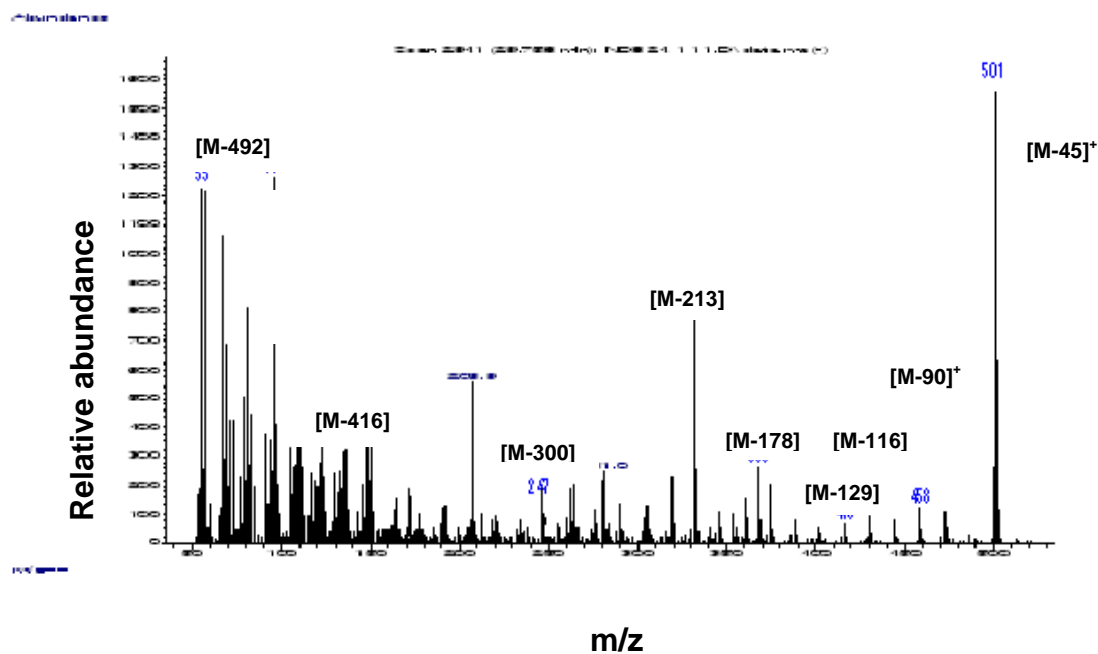


### ALS patients

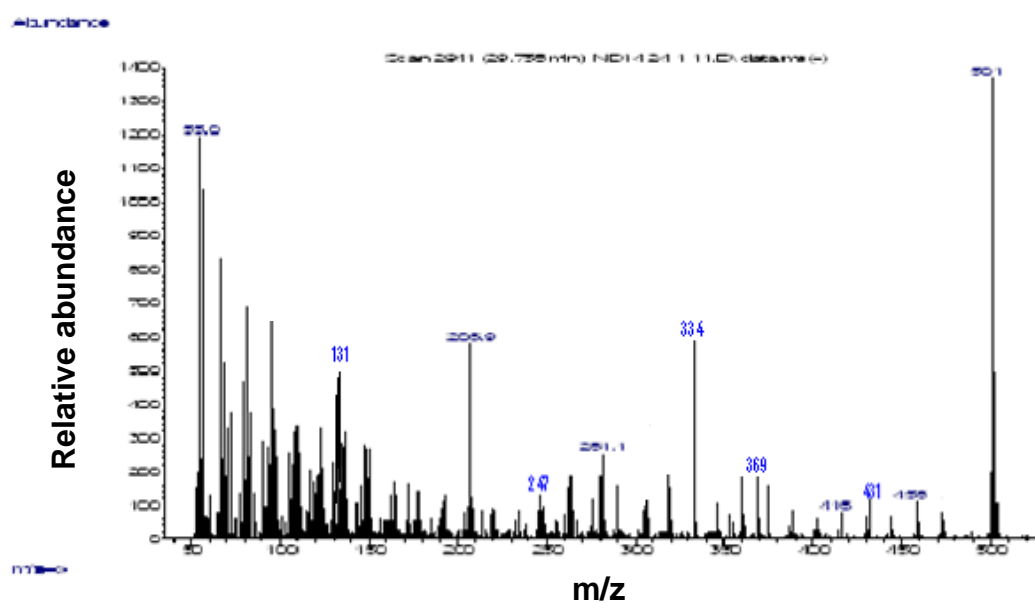


**Figure 12.** EI spectrum of the compound eluting at 29.8 min (see table 6.3) identified as **6-Ketocholesterol** (6-Keto).

### Healthy control

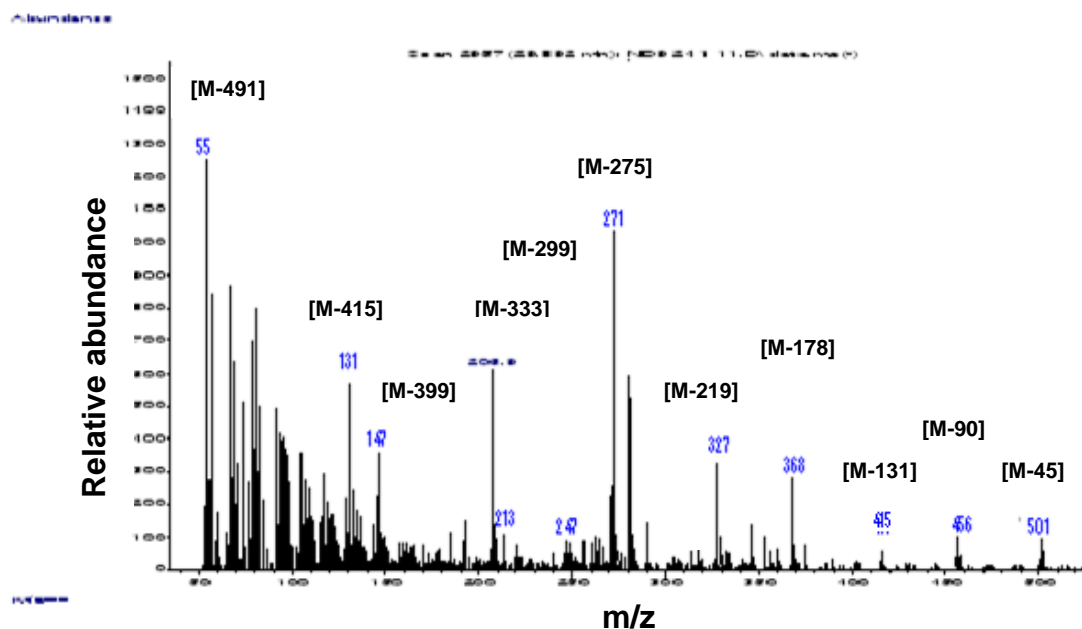


### ALS patients

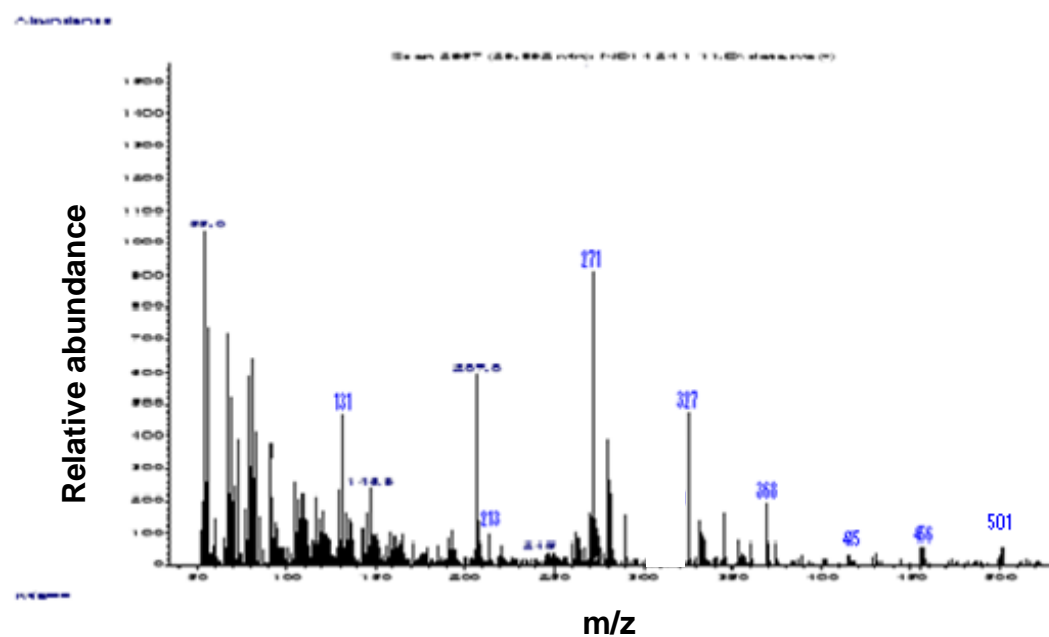


**Figure 13.** EI spectrum of the compound eluting at 29.9 min (see table 6.3) identified as **25-hydroxycholesterol (25-OH)**.

### Healthy control

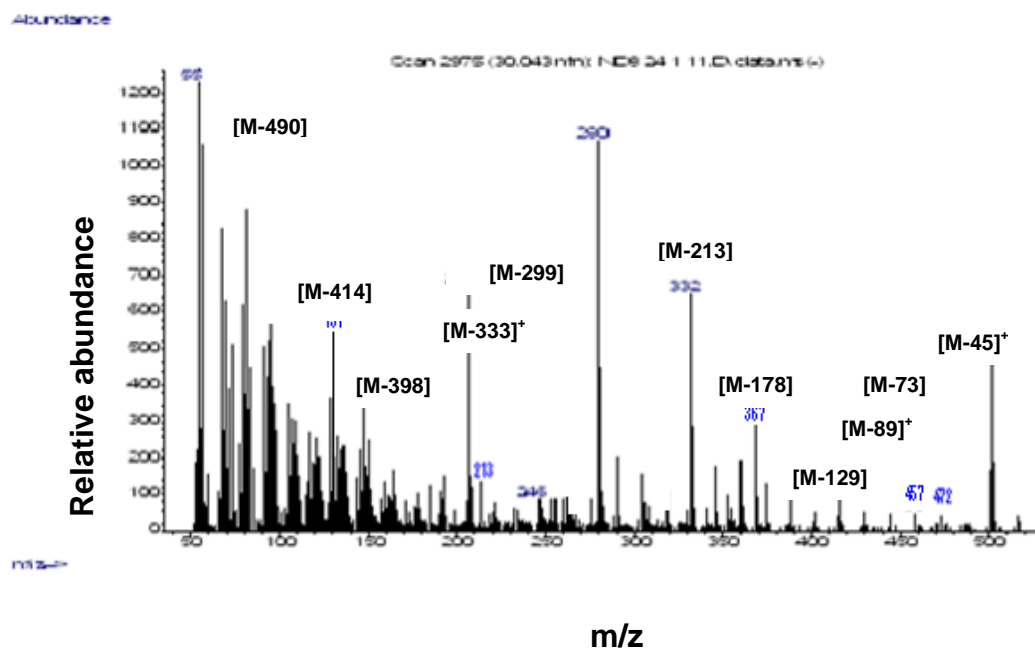


### ALS patients

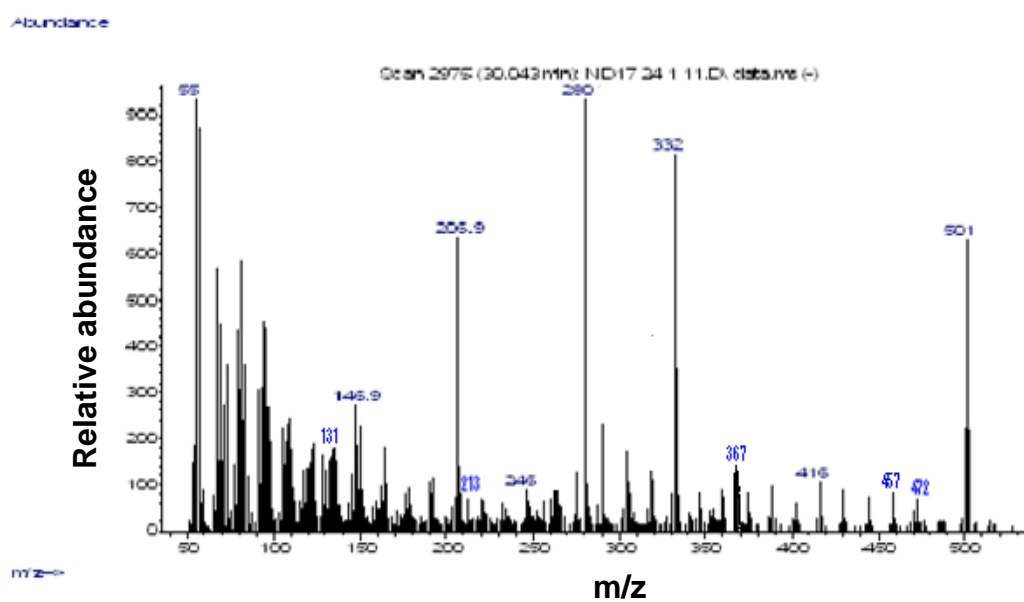


**Figure 14.** EI spectrum of the compound eluting at 30.0 min (see table 6.3) identified as **7-Ketocholesterol (7-Keto)**.

### Healthy control



### ALS patients



### Appendix - 3: Fragmentation patterns of oxysterol-TMS

**Table 1.** Fragmentation patterns of oxysterols with side chain (SC) free of TMS group; SC m/z=113

Fragmentation pattern	7 $\alpha$ -OH-TMS	7 $\beta$ -OH-TMS	5,6 $\beta$ -epoxy-TMS	5,6 $\alpha$ -epoxy-TMS	4 $\beta$ -OH-TMS	Chol-triol-TMS	6-Keto-TMS	7-Keto-TMS
<b>M</b>	<b>546</b>	<b>546</b>	<b>474*</b>	<b>474*</b>	<b>546</b>	<b>637**</b>	<b>547</b>	<b>545</b>
[M-43], [M** -135], [M-45] <sup>+</sup> , [M-43] <sup>+</sup>	503	503	-	-	-	502	<b>501</b>	<b>501</b>
[M-73]	-	-	-	-	-	-	-	<b>472</b>
[M-90], [M* -15], [M** -180], [M-90] <sup>+</sup> , [M-89] <sup>+</sup>	<b>456</b>	<b>456</b>	<b>459</b>	<b>459</b>	<b>456</b>	<b>457</b>	<b>458</b>	457
[M-105], [M-116]	-	-	-	-	441	-	<b>431</b>	-
[M-135], [M* -57], [M-129], [M** -220]	<b>411</b>	<b>411</b>	417	417	<b>417</b>	417	418	416
[M-160], [M* -89]	-	<b>386</b>	<b>385</b>	-	-	-	-	-
[M-178], [M* -106], [M** 270]	<b>368</b>	-	368	368	368	<b>367</b>	<b>369</b>	367
[M-195], [M* -121], [M** -282]	351	351	353	353	-	355	-	-
[M-221], [M-219], [M** -308], [M-213]	<b>325</b>	-	-	-	327	<b>329</b>	<b>334</b>	<b>332</b>
[M-291], [M* -219], [M-293], [M** -382]	<b>255</b>	-	255	255	<b>253</b>	255	-	-
[M-325], [M** -390], [M-300], [M-299]	-	<b>221</b>	-	-	-	247	247	246
[M* -261], [M-333] <sup>+</sup>	-	-	213	213	-	-	-	213
[M-387], [M* -315], [M* -316]	159	159	<b>159</b>	<b>158</b>	-	-	-	-
[M-399], [M-395], [M** -490], [M-398]	147	147	-	-	<b>147</b>	<b>147</b>	-	147
[M-416] <sup>+</sup> , [M-414]	129	129	-	-	129	-	<b>131</b>	<b>131</b>
[M-441], [M* -369], [M** -532]	105	-	105	105	105	-	-	-
[M-473]	<b>73</b>	<b>73</b>	-	-	-	-	-	-
[M-490] <sup>+</sup> , [M* -417], [M-491], [M-492]	57	57	57	57	55	-	55	55

SC; CH<sub>3</sub>CH(CH<sub>2</sub>)<sub>3</sub>CH(CH<sub>3</sub>)<sub>2</sub> → 113, for Ions (A to R) see Figure 6.2.

CH<sub>3</sub> → 15, CH(CH<sub>3</sub>)<sub>2</sub> → 43, 3CH<sub>3</sub> → 45, CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub> → 57, Ion N → 57, TMS → 73, TMSO → 89, TMSOH → 90, TMSOH-CH<sub>3</sub> → 105, TMSOH-CH<sub>3</sub>-H → 106, (CH<sub>2</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>-3CH<sub>3</sub> → 116, Ion I → 116, TMS-2CH<sub>3</sub>-H<sub>2</sub>O → 121, SC-CH<sub>3</sub>-H → 129, TMSO-CH(CH<sub>3</sub>)<sub>2</sub>-3H → 135, SC-3CH<sub>3</sub>-H → 159, TMSO-(CH<sub>2</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub> → 160, 2TMSO → 178, 2TMSOH → 180, TMSO-2CH<sub>3</sub>-TMS-2H → 195, Ion F → 213, TMSOH-SC-CH<sub>3</sub>-H → 219, TMSOH-SC-CH<sub>3</sub>-2H → 220, TMSOH-SC-CH<sub>3</sub>-3H → 221, Ion H-TMSOH-2CH<sub>3</sub>-2H → 261, 2TMSOH-CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>-2CH<sub>3</sub>-3H → 270, 2TMSOH-CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>-3CH<sub>3</sub>-H → 282, 2TMSO-SC → 291, 2TMSOH-SC → 293, Ion P-CH<sub>3</sub>-(CH<sub>2</sub>)<sub>3</sub>CH(CH<sub>3</sub>)<sub>2</sub>-H → 299, Ion P-CH<sub>3</sub>-(CH<sub>2</sub>)<sub>3</sub>CH(CH<sub>3</sub>)<sub>2</sub>-2H → 300, 2TMSOH-SC-CH<sub>3</sub> → 308, Ion E → 333, 334, 335, Ion O-TMS-3CH<sub>3</sub>-H → 369(474), Ion B- H<sub>2</sub>O → 382(637), Ion B-2H<sub>2</sub>O-H → 398(545), Ion P-CH<sub>3</sub> → 390(637), Ion M (SC) → 417(474), Ion D → 473(546), Ion P-SC-2H → 490(637), Ion M-2H → 490(545), 491(546), 492(547), Ion O-3TMSOH → 532(637),

**Table 2.** Fragmentation patterns of oxysterols with side chain (SC\*) attached to a TMS group; SC\* m/z=202

Fragmentation pattern	24-OH-TMS	27-OH-TMS	26-OH-TMS	20 $\alpha$ -OH-TMS	22-OH-TMS	25-OH-TMS
<b>M</b>	<b>546</b>	<b>547</b>	<b>546</b>	<b>547</b>	<b>547</b>	<b>546</b>
[M-43], [M-45]	503	-	-	502	<b>504</b>	501
[M-90], [M-91], [M-85] <sup>+</sup>	<b>456</b>	<b>457</b>	<b>456</b>	<b>461</b>	456	<b>456</b>
[M-133], [M-135] <sup>+</sup> , [M-129], [M-106], [M-131]	<b>413</b>	<b>411</b>	<b>417</b>	-	<b>441</b>	<b>415</b>
[M-165], [M-161]	-	-	<b>382</b>	386	<b>382</b>	-
[M-178], [M-179]	368	368	368	368	368	368
[M-194], [M-195], [M-191], [M-192]	352	352	355	353	355	-
[M-220], [M-221], [M-206], [M-219]	326	326	<b>341</b>	-	-	<b>327</b>
[M-247] <sup>+</sup> , [M-265]	-	-	301	-	<b>282</b>	-
[M-290], [M-291], [M-292], [M-275]	255	<b>257</b>	255	255	255	<b>271</b>
[M-299], [M-300]	247	247	-	-	247	247
[M-333], [M-334]	213	213	213	213	213	213
[M-346]	-	-	-	<b>201</b>		
[M-401], [M-400], [M-402], [M-399]	<b>145</b>	<b>147</b>	145	145	145	147
[M-417], [M-415]	<b>129</b>	-	-	-	-	<b>131</b>
[M-442], [M-441]	-	105	105	105	105	-
[M-492], [491]	-	55	-	55	55	55

SC\*; CH<sub>3</sub>CH(CH<sub>2</sub>)<sub>3</sub>C(CH<sub>3</sub>)<sub>2</sub>TMSOH → 202, for ions (A to R) see Figure 6.2.

CH(CH<sub>3</sub>)<sub>2</sub> → 43, 3CH<sub>3</sub> → 45, (CH<sub>2</sub>)<sub>3</sub>CH(CH<sub>3</sub>)<sub>2</sub> → 85, TMSOH → 90, TMS-H<sub>2</sub>O → 91, TMSOH-CH<sub>3</sub>-H → 106, Ion Q → 129, TMSO-C(CH<sub>3</sub>)<sub>2</sub> → 131, TMSO-CH(CH<sub>3</sub>)<sub>2</sub>-H → 133, TMSO-CH(CH<sub>3</sub>)<sub>2</sub>-3H → 135, TMSO-CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>-CH<sub>3</sub> → 161, TMSOH-TMS-2H → 165, 2TMSO → 178, 2TMSO-H → 179, TMSO-CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>-3CH<sub>3</sub> → 191, TMSO-CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>-3CH<sub>3</sub>-H → 192, TMSO-2CH<sub>3</sub>-TMS-H → 194, TMSO-2CH<sub>3</sub>-TMS-2H → 195, TMSOH-TMS-CH(CH<sub>3</sub>)<sub>2</sub> → 206, TMSOH-SC-CH<sub>3</sub>-H → 219, SC\*-H<sub>2</sub>O → 220, SC\*-H<sub>2</sub>O-H → 221, Ion E- H → 247, Ion E-H<sub>2</sub>O-H → 265, Ion E-2CH<sub>3</sub> → 275, Ion E-3CH<sub>3</sub> → 290, Ion E-3CH<sub>3</sub>-H → 291, Ion E-3CH<sub>3</sub>-2H → 292, Ion F-2CH<sub>3</sub>-2H → 333, Ion F-2CH<sub>3</sub>-3H → 334, Ion F-3CH<sub>3</sub> → 346, Ion M → 399, 400, 401, Ion M-H → 402, Ion M-CH<sub>3</sub> → 415, Ion M-CH<sub>3</sub>-2H → 417, Ion M-TMSOH-2H → 491, Ion M-TMSOH-3H → 492.