



An Investigation of the *In Vitro* Effects of Unconjugated Linoleic Acid and its Potential Impact on Haemostasis.

Submitted by:
Catriona Kielty (B.Sc.)

April 2021

Supervisors:
Dr. Carmel Kealey and Dr. Damien Brady

A Thesis Submitted in Fulfilment of the
Requirements for the Degree of Doctor of
Philosophy (Ph.D).

“I may not have gone where I intended to go, but I think I have ended up where I needed
to be”

Douglas Adams, 1988.

Declaration

I hereby declare that this thesis, submitted to the Athlone Institute of Technology for the degree of Doctor of Philosophy, is a result of my own work and has not in the same or altered form, been presented to this institute or any other institute in support of my degree other than which I am now a candidate.

Catriona Kiely

Date

Acknowledgements

I would like to extend my sincere gratitude to my research supervisors Dr. Carmel Kealey and Dr. Damien Brady, whose constant support and encouragement throughout this entire endeavor made this possible. You have always gone out of your way to make time for me. Without your guidance and unwavering faith in my capabilities, I have no doubt that I would never have made it this far.

Thank you Dr. Kealey for your patience and positivity. In sharing your own personal PhD experiences over the past few years, you inspired in me, the determination to persevere and weather the many storms we have encountered on this journey. As a successful woman thriving in the area of STEM you are a continuous source of inspiration.

Thank you Dr. Brady, for encouraging me to take on the challenge of postgraduate study and for always believing I was up to that challenge. As well as the constant support, your good humor and candor helped brighten many a dark day, and I will be forever grateful.

To Dr. Emma Murphy, there will never be enough thank yous! Your strength and support, as well as your expertise in all things inflammatory, helped me push this over the line. I will be forever grateful for your friendship over the last few grueling months.

Thank you to the wonderful friends I have made throughout this process; Sarah, Lisa and Kiera, a consistent source of motivation/procrastination, there was a comfort in sharing the mutual insanity we endured that can never be matched and I think it will bind our friendship for a life time.

Evert, you have been a true source of strength and support; thank you for the unrelenting love and encouragement.

To my family, thank you. To my parents, Conor and Mary, who have given me everything they possibly could in this life to help me get this far. To my brothers Michael and Austin, and my sisters, Edel and Noelle, who have been there for me through all the ups and downs. There isn't a day that goes by where I don't stop and think of how lucky I was (and still am) to have you guys to play with, to fight with and to grow with. And we can't forget Robert the collie, whose fluffy cuddles were an essential part of the study process.

And finally, all of this started with one brilliant horse, thank you Bracken.

Contents

List of Abbreviations	x
List of Tables	xiv
List of Figures.....	xv
Abstract.....	xxii
Chapter 1	1
Introduction.....	1
1.1. Context and Origin of the Investigation.....	2
Chapter 2	5
Literature Review.....	5
2.1. Lipids -Characterisation and Classification.....	6
2.1.1. Structure and Function of Fatty Acids	8
2.1.2 Essential Fatty Acids – ω6 and ω3 Fatty Acids.....	11
2.2. Linoleic acid	13
2.2.1. Digestion and Intestinal Absorption of Linoleic Acid.....	14
2.2.2. Cellular Transport and Processing of Linoleic acid	16
2.2.3. Cellular Metabolism of Linoleic Acid	19
2.2.4. Linoleic Acid in Mitochondrial Homeostasis and Metabolism.....	21
2.2.5. Linoleic Acid Storage in Intracellular Lipid Droplets - Structure, Biogenesis and Functions	24
2.3. Linoleic Acid and the Production of Pro-Inflammatory Mediators	30
2.3.1. Linoleic Acid and Eicosanoid Production.....	32
2.3.2. Linoleic acid and Cytokine Production.....	38

2.4. Linoleic Acid and the Production of Reactive Oxygen Species.....	42
2.4.1. The Superoxide Anion and Superoxide Dismutase.....	46
2.5. Linoleic Acid and Apoptosis, Necrosis and Necroptosis	49
2.5.1. Linoleic Acid and Apoptosis.....	49
2.5.2. Linoleic acid and Necrosis/Necroptosis	53
2.6. The Haemostatic System.....	56
2.6.1. Endothelium	57
2.6.2. Primary Haemostasis, Secondary Haemostasis and Fibrinolysis	57
2.6.3. Haemostasis and Coagulopathies in the Equine.....	61
2.6.4. Linoleic Acid and Coagulation.....	64
2.7. Summary of the Literature.....	65
2.8. Aims and Objectives.....	67
2.8.1. Aim	67
2.8.2. Objectives.....	67
Chapter 3	68
Methods and Materials	68
3.1. Cell Culture Study	69
3.1.1. HepG2 Cell Culture and Maintenance.....	69
3.1.2. HUVEC Cell Culture and Maintenance.....	69
3.1.3. Sub-culturing HepG2 and HUVEC Cells.....	70

3.1.4. Preparation of Unconjugated Linoleic Acid and Cell Treatment.....	70
3.1.5. Resazurin Cell Viability Assay	72
3.1.6. Tetrazolium Bromide (MTT) Cell Viability Assay	73
3.1.7. Adenosine Triphosphate (ATP) Assay	74
3.1.8. Oil Red O Assay for Intracellular Lipid Accumulation	77
3.1.9. Collection of Cell Lysates.....	79
3.1.10. Fluorescent Microscopy – Nile Red Staining for intracellular Lipid Accumulation.....	80
3.1.11. Quantification of Intracellular Lipid Droplets using Flow Cytometry.....	83
3.1.12. TNF-α Enzyme Linked Immunosorbent Assay (ELISA).	84
3.1.13. Superoxide Dismutase (SOD) Assay.....	88
3.1.14. Thromboxane B₂ (TXB₂) Assay.....	90
3.1.15. Analysis of Apoptosis in HepG2 Cells treated with ULA.....	91
3.2. Analysis of Hepatic Samples.....	93
3.2.1. Hepatic Tissue Sample preparation	93
3.2.2. Unconjugated Linoleic Acid – Storage and Handling	94
3.2.3. Unconjugated Linoleic Acid Standard Preparation	94

3.2.4. HPLC Analysis	95
3.2.5. HPLC Collection of the Fraction	95
3.2.6. Fourier Transform Infrared Spectroscopy for identification of the Hepatic Extract.....	96
3.2.7. Gas Chromatography – Time of Flight – Mass Spectrometry for the Identification of the Hepatic Extract.....	96
3.3. Statistical Analysis.....	97
Chapter 4	99
Analysis of the Hepatic Extract for the Presence of ULA	99
 4.1. Analysis of a Commercial Standard of ULA and the Identification of ULA in Equine Hepatic Samples by HPLC	100
 4.2. Fourier Transform Infrared Spectroscopy for identification of the Hepatic Extract Fraction	102
 4.3. Gas Chromatography-Mass Spectrometry for the Identification of ULA in the Hepatic Extract Fraction	105
 4.4. Discussion	108
4.4.1. HPLC Analysis of ULA	108
4.4.2. Fourier Transform Infrared Spectroscopy for identification of the Hepatic Extract Fraction	109
4.4.3. Gas Chromatography - Time of Flight - Mass Spectrometry for the Identification of the Hepatic Extract.....	111
4.5. Conclusion.....	113
Chapter 5	115
Preliminary Analysis of the <i>In Vitro</i> Effects of ULA	115

5.1 <i>In Vitro</i> Effects of ULA; the Assessment of the effects of ULA over 24 and 48 hours	116
5.1.1. Preliminary Analysis of Cell Viability after ULA Treatment	117
5.2. Adenosine Triphosphate Assay	119
5.2.1. ATP Standard Curve	119
5.2.2. ATP Production in HepG2 Cells treated with Varying Concentrations of ULA 120	
5.3. Cell Viability in HepG2 Cells treated with ULA over 24 and 48 Hours .	121
5.4. Oil Red O for intracellular Lipid Accumulation	127
5.5. Discussion	132
5.5.1. Preliminary Analysis of Cell Viability after ULA Treatment	132
5.5.2. ATP Production in HepG2 Cells treated with Unconjugated Linoleic Acid.	
134	
5.5.3. Comparison of Cell Viability in HepG2 Cells using the Resazurin (alamarBlue®) Assay and the MTT Assay.....	138
5.5.4. Intracellular Lipid Accumulation in HepG2 Cells using Oil Red O.	141
5.6. Conclusion.....	141
Chapter 6	143
The <i>In Vitro</i> Effects of ULA on HepG2 Cells	143
6.1. Cell Viability of HepG2 Cells Treated with ULA over 8 Hours	145
6.2. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using Oil Red O.	148
6.2.1. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using the Nile Red Fluorescent Stain	151

6.3. Assessment of the Inflammatory effects of ULA though the Secretion of TNF-α	156
6.4. The Effects of ULA on Superoxide Dismutase (SOD) in HepG2 Cells.....	157
6.5. The effects of ULA on the Production of Thromboxane in HepG2 cells.	159
6.6. The Effects of ULA on Apoptosis in HepG2 cells.....	162
6.7. Discussion.....	170
6.7.1. Cell Viability in HepG2 Cells Treated with ULA over Shorter Treatment Intervals.	172
6.7.2. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using Oil Red O.....	176
6.7.3. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using the Nile Red Fluorescent Stain.	179
6.7.4. Assessment of the Inflammatory Effects of ULA though the Secretion of TNF-α	184
6.7.5. The Effects of ULA on the activity of Superoxide Dismutase in HepG2 Cells.	
188	
6.7.6. The Effects of ULA on the activity of Thromboxane Production in HepG2 Cells	194
6.7.7. The Effects of ULA on Cell Death in HepG2 cells	198
6.8. Conclusion.....	205
Chapter 7	207

The <i>In Vitro</i> Effects of ULA on HUVEC Cells	207
7.1. Preliminary Analysis of Cell Viability after ULA Treatment	209
7.2. Cell Viability in HUVEC Cells Treated with ULA over Shorter Treatment Intervals.....	211
7.3. Intracellular Lipid Accumulation in HUVEC Cells treated with ULA	214
7.3.1. Analysis of Intracellular Lipid Accumulation using Oil Red O	214
7.3.2. Analysis of Intracellular Lipid Droplets in HUVEC cells treated with ULA using Nile Red Fluorescent Stain.....	217
7.4. Assessment of the Inflammatory Effects of ULA though the Secretion of TNF-α	221
7.5. The Effects of ULA on the activity of Superoxide Dismutase in HUVEC Cells.	223
7.6. The Effects of ULA on Thromboxane Production in HUVEC Cells	225
7.7. Discussion	227
7.7.1. Preliminary Assessment of Cell Viability in HUVEC Cells	229
7.7.2. Cell Viability in HUVEC Cells Treated with ULA over Shorter Treatment Intervals	230
7.7.3. Analysis of Intracellular Lipid Accumulation in ULA treated HUVEC Cells.	233
7.7.3.1. Analysis of Intracellular Lipid Accumulation using Oil Red O.	234

7.7.4. Assessment of the Inflammatory Effects of ULA though the Secretion of TNF-a	239
7.7.5. The Effects of ULA on the activity of Superoxide Dismutase in HUVEC Cells	243
7.7.6. Assessment of Thromboxane B₂ Production in ULA treated HUVEC Cells	249
 7.8. Conclusion.....	255
 Chapter 8	257
 Summary Discussion and Conclusion	257
 8.1. The Effects of ULA on HepG2	258
 8.2. The Effects of ULA on HUVEC	260
 8.3. Concluding Remarks.....	261
 Chapter 9	264
 Future Work	264
 9.1. ULA induced Reactive Oxygen Species Production.....	265
 9.2. The Effects of ULA on the Hepatic Production of Inflammatory Metabolites	265
 9.3. The Effects of ULA on the Induction of Hepatic Inflammation	266
 9.4. The Effects of ULA on Endothelium	267
 9.5. The Effects of ULA on Equine Platelet Aggregation and Thromboxane Production	268
 Chapter 10	270
 Limitations	270
 10.1. Project Limitations.....	271
 10.2. Standardisation of the Experimental Design of Lipid Studies.....	271
 10.3. Limitations of Cell Types used in this Research	272
 10.4. Limitations of Oil Red for Analysis of Intracellular Lipid Droplets.....	274
 10.5. Thromboxane Control	276
 10.6. Conclusion.....	277
 Chapter 11	278

Dissemination of Research	278
11.1. Dissemination of Research.....	279
Chapter 12	280
Bibliography	280
12.1. Bibliography.....	281
Appendices	319
Appendix 1 – Analysis of the Hepatic Extract.....	320
Appendix 2	329
Appendix 3	333
Appendix 4	336
Appendix 5 – Draft Publication 1 - Journal of Lipid Research	337

List of Abbreviations

AC	Adenylyl Cyclase
ACP	Acyl Carrier Protein
APAF-1	Apoptotic Protease Activating Factor 1
ASK-1	Apoptosis Signalling Kinase-1
AT	Antithrombin
ATGL	Adipose Triacylglycerol Lipase
BAK	BCL-2 antagonist/killer
BAX	BCL-2-associated X protein
BCL-2	B Cell Lymphoma – 2
BH	BCL-2 Domain
BID	BH3-Interacting Domain Death Agonist
BIM	BCL-2-interacting mediator of cell death
CACT	Carnitine:Acylcarnitine Transferase
cAMP	Cyclic Adenosine Monophosphate
CAS	Chemical Abstract Number
CO ₂	Carbon Dioxide
CoA	Co-Enzyme A
COX	Cyclooxygenase
CPT1	Carnitine Palmitoyltransferase
DAG	Diacylglycerol
DAGT	Diacylglycerol Transferases
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
EC ₅₀	Half-maximal effective concentration
ELAM-1	Endothelial Leukocyte Adhesion Molecule-1

ELISA	Enzyme Linked Immunosorbent Assay
ELOVL	Elongation of Very Long Chain Fatty Acids Protein
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FA	Fatty Acids
FABP	Fatty Acid Binding Proteins
FAD	Flavin Adenine Dinucleotide
FADD	Fas-Associated Death Domain
FAS	Fatty Acid Synthase
FATP	Fatty Acid Transport Proteins
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GLA	Gamma Linolenic Acid
H ₂ O	Water
HSL	Hormone Sensitive Lipase
ICAM-1	Intracellular Adhesion Molecule-1
IC ₅₀	Half-maximal inhibitory concentration
IEC	Irish Equine Centre
I-FABP	Intestinal-Fatty Acid Binding Protein
IL-6	Interleukin 6
IMM	Inner Mitochondrial Membrane
IMS	Inter Membrane Space
JNK	c-Jun N-terminal kinase
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAG	Monoacylglycerol

MAGL	Monoacylglycerol Lipase
MAPK	Mitogen-Activated Protein Kinase
MFI	Median Fluorescence Intensity
MLKL	Mixed-Lineage Kinase Domain Like
MPT	Mitochondria Permeability Transition
NAD	Nicotineamide Adenine Dinucleotide
NAFLD	Non-Alcoholic Fatty Liver Disease
NF-κB	Nuclear Factor kappa B
NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
OIE	World Organisation for Animal Health
OMM	Outer Mitochondrial Membrane
OXLAM	Oxidized Linoleic Acid Metabolites
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PCTV	Pre-Chylomicron Transport Vesicle
PGE ₁	Prostaglandin E ₁
PI	Propidium Iodide
PLA ₂	Phospholipase A ₂
PLC-β	Phospholipase C-β
PKC	Protein Kinase C
PUFA	Polyunsaturated Fatty acids
PUMA	p53-Upregulated Modulator of Apoptosis
RIPK	Receptor-Interacting Protein Kinase
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TACE	TNF-α Converting Enzyme

TAG	Triacylglycerol
TAGL	Triacylglycerol Lipase
TCA	Tricarboxylic Acid Cycle
TF	Tissue Factor
TNF- α	Tumour Necrosis Factor- α
TNFR	Tumour Necrosis Factor Receptor
TP	Thromboxane Receptor
tPA	Tissue Plasminogen Activator
ULA	Unconjugated Linoleic Acid
UPR	Unfolded Protein Response
VCAM-1	Vascular Cell Adhesion Molecule-1
vWF	von Willebrand factor

List of Tables

Table 2.1	Lipid categories of the comprehensive classification system and the number of structures in the LIPID MAPS database.....	Pg. 8
Table 4.1.	Vibrational frequencies and their corresponding functional groups pertaining to the FTIR spectrum of commercial ULA and the fraction of interest collected from the hepatic extract	Pg. 103
Table 4.2.	Identification of the additional peaks observed in the chromatogram of the derivatised hepatic extract	Pg. 106
Table 5.1.	The half-maximal inhibitory concentration (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period of 24 and 48 hours.....	Pg.11
		8
Table 5.2.	The half-maximal inhibitory concentration (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA.....	Pg. 123
Table 6.1.	The half-maximal inhibitory concentrations (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period of 8 hours.....	Pg. 146
Table 7.1.	The half-maximal inhibitory concentration (IC_{50}) of ULA in HUVEC cells treated with varying concentrations of ULA over a period of 24 and 48 hours.....	pg.211

Table 7.2. The half-maximal inhibitory concentrations (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period of 8 hours.....	Pg. 214
--------------------------------------------------------------------------------------------------------------------------------------------------------------------	----------------

List of Figures

2.1. Chemical structure of fatty acids.....	Pg. 9
2.2. Classification, chemical structure, and primary dietary sources of fatty acids.....	Pg. 10
2.3. The metabolic pathways of the essential fatty acids, the $\omega 6$ linoleic acid and the $\omega 3 \alpha$ -linolenic acid	Pg.11
2.4. Chemical structure of linoleic acid.....	Pg. 13
2.5. Chemical structure of a triacylglycerol	Pg. 15
2.6. The potential metabolic fates of linoleic acid	Pg.19
2.7. Desaturation and elongation of linoleic acid occurs as a result of enzymes produced by the endoplasmic reticulum	Pg. 20
2.8. Lipid droplet formation occurring between the leaflets of the endoplasmic reticulum.....	Pg. 26
2.9. Schematic of the production of eicosanoids from linoleic acid via the cyclooxygenase (COX) metabolic pathway.....	Pg. 33
2.10. Thromboxane A ₂ receptor and signal transduction via G protein coupling.....	Pg. 36
2.11. Schematic diagram of the downstream signalling pathways of TNF- α	Pg. 40

2.12. Schematic representation of ROS-mediated cell death and cell survival signalling pathways.....	Pg.44
2.13. Schematic representation of the extrinsic and intrinsic apoptotic pathways.	Pg. 50
2.14. Schematic representation of TNF receptor signalling and regulation of cell death and cell survival.....	Pg. 55
2.15. Schematic of the coagulation cascade.....	Pg. 59
2.16. Epistaxis associated with exercise induce pulmonary haemorrhage	Pg.63
2.17. Schematic of the possible intracellular fates of linoleic acid.....	Pg.66
3.1. Flow Cytometry gating strategy used to identify Nile Red Positive population in HepG2 cells treated with ULA for 1 hour.	Pg.84
3.2.1. Gating strategy for the analysis of cell death mediated by ULA.....	Pg.90
3.2.2. Example of the extended gate segregating debris from viable cells.....	Pg.92
4.1. A chromatogram of the commercial standard of ULA at a concentration of 80 μ g/ μ l as prepared by the author.....	Pg.101
4.2. Chromatogram of an equine hepatic sample displaying a peak at 6.686 minutes indicative of ULA.....	Pg.101
4.3. Calibration curve for commercial unconjugated linoleic acid obtained using a 250 x 4.6mm Excil C18 5 μ m reverse phase analytical column.....	Pg. 102

4.4.	FTIR spectra, including peak numbers, obtained for a commercial standard of ULA (A) and the fraction of interested collected from the hepatic extract (B).....	Pg.103
4.5.	GC-MS Chromatogram of a derivatised standard of commercial linoleic acid (A) and the derivatised hepatic extract (B).....	Pg.106
5.1.	Percentage of viable HepG2 cells treated with varying concentrations of ULA over a period of 24 and 48 hours compared with an untreated control	Pg.116
5.2.	ATP Standard Curve	Pg.118
5.3.	The concentration (ng/ μ L) of ATP produced by HepG2 cells treated with varying concentrations of ULA for a period of 21 hours	Pg.119
5.4.	Percentage cell viability of HepG2 cells treated with varying concentrations of ULA over a period of 24 hours and 48 hours compared with an untreated control	Pg.121
5.5.	Percentage cell viability of HepG2 cells treated with varying concentrations of ULA over a period of 24 hours and 48 hours compared with a positive growth control.....	Pg.124
5.6.	Percentage lipid accumulation in HepG2 cells treated with varying concentrations of ULA over a period of 24 and 48 hours.	Pg.126
5.7(A).	Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over 24 hours.	Pg.128

5.7(B). Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over 48 hours.....**Pg.129**

6.1. Percentage cell viability of HepG2 cells treated with varying concentrations of ULA over a period of 8 hours compared with a positive growth control.....**Pg.143**

6.2. Concentration-response curves were generated from the MTT cell viability data (presented in figure 6.1) and used to determine the IC50 of ULA in HepG2 cells treated with varying concentrations of the fatty acid over 8 hours.....**Pg.144**

6.3. Percentage lipid accumulation in HepG2 cells treated with varying concentrations of ULA assessed over a period of 8 hours**Pg.146**

6.4. Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over different time intervals.....**Pg.148**

6.5.1. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 1 and 2 hours of supplementation**Pg.150**

6.5.2. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 4 and 6 hours of supplementation.**Pg.151**

6.5.3. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 8 hours of supplementation.....**Pg.152**

6.5.4. Examples of red fluorescence (A) and yellow/gold fluorescence (B) in the same cells treated with 0.1mM of ULA for 6 hours.....**Pg.152**

6.6. Median Fluorescence Intensity of Nile Red determined by flow cytometry in HepG2 cells treated with varying concentrations of ULA over 8 hours.....**Pg.153**

- 6.7.** TNF- α standard curve (A). TNF- α production in HepG2 cells treated with 0.1 and 0.25mM of ULA for 4 hours (B).....**Pg.154**
- 6.8.** Standard curve for Superoxide Dismutase. Statistical analysis was performed using mean absorbance values \pm S.E.M.....**Pg. 156**
- 6.9.** Units of superoxide dismutase produced in HepG2 cells treated with varying concentrations of ULA over 8 hours**Pg.157**
- 6.10.** Standard curve for thromboxane B2. Statistical analysis was performed using mean absorbance values (\pm S.E.M) (n=2).....**Pg159**
- 6.11.** Concentration of thromboxane B2 produced in HepG2 cells treated with varying concentrations of ULA over 8 hours.**Pg.160**
- 6.12.** Flow cytometry dot plots (A) and histograms (B) of HepG2 cells treated 0mM (top) and 2.5mM (bottom) of ULA for 6 hours.....**Pg.162**
- 6.13.** Mean percentage of events or cell (C) population compared to the mean population of debris (D) removed from down-stream apoptosis analysis.....**Pg. 165**
- 6.14.** Flow cytometry dot plots representing HepG2 cells treated with increasing concentrations of ULA.....**Pg.167**
- 6.15.** Induction of ULA mediated cell death in HepG2 cells treated with ULA from 1 to 8 hours.....**Pg.169**
- 7.1.** Percentage of viable HUVEC cells treated with varying concentrations of ULA over a period of 24 and 48 hours compared with an untreated control.....**Pg.209**

- 7.2.** Percentage cell viability of HUVEC cells treated with varying concentrations of ULA over a period of 8 hours compared with a positive growth control.....**Pg.211**
- 7.3.** Concentration-response curves were generated from the MTT cell viability data (presented in figure 7.2) and used to determine the IC50 of ULA in HUVEC cells treated with varying concentrations of the fatty acid over 8 hours.....**Pg.212**
- 7.4.** Percentage lipid accumulation in HUVEC cells treated with varying concentrations of ULA assessed over a period of 8 hours**Pg.214**
- 7.5.** Oil Red O staining maps (x 200) of HUVEC cells treated with varying concentrations of ULA over different time intervals. of**Pg.216**
- 7.6.1.** Qualitative determination of intracellular lipid accumulation in HUVEC cells treated with varying concentrations of ULA after 1 hour using fluorescent microscopy**Pg.218**
- 7.6.2.** Qualitative determination of intracellular lipid accumulation in HUVEC cells treated with varying concentrations of ULA after 2 hours using fluorescent microscopy**Pg.219**
- 7.7.** Median fluorescence intensity of Nile Red determined by flow cytometry in HUVEC cells treated with varying concentrations of ULA over 8 hours.....**Pg.220**
- 7.8.** TNF- α standard curve (A). TNF- α production in HUVEC cells treated with 0.1mM and 0.25mM of ULA for 4 hours (B).....**Pg.221**

- 7.9.** Standard curve for Superoxide Dismutase. Statistical analysis was performed using mean absorbance values \pm S.E.M (n=2).....**Pg.223**
- 7.10.** Units of superoxide dismutase (SOD) produced in HUVEC cells treated with varying concentrations of ULA over 8 hours.**Pg.224**
- 7.11.** Standard curve for thromboxane B2. Statistical analysis was performed using mean absorbance values (\pm S.E.M)**Pg. 226**
- 7.12.** Concentration of thromboxane B2 produced in HUVEC cells treated with varying concentrations of ULA over 8 hours.....**Pg.227**

Abstract

Introduction: Linoleic acid, an essential $\omega 6$ fatty acid, is the most abundant polyunsaturated fatty acid in the Western mammalian diet. Linoleic acid has been linked with an increased inflammatory response. It has also been associated with platelet inhibition and with a reduction in platelet thromboxane. Excessive unresolved inflammation can lead to uncontrolled tissue damage and has been associated with several inflammatory mediated diseases. Plasma free fatty acids are known to increase in several disease states as well as during fasting and strenuous exercise. As such, this investigation aimed to elucidate the potential mechanisms by which linoleic acid at supraphysiological concentrations may impact cell health in the liver and the vasculature.

Methods: *In vitro* investigations were used to determine the effects of increasing concentrations of ULA on the inflammatory response. Assays were performed using the human hepatoma cell line, HepG2 and the human umbilical vein endothelial cell line HUVEC. The effects of supraphysiological concentrations of ULA over 8 hours of exposure were assessed. Both cell lines were assayed for the effects of ULA on cell viability and intracellular lipid accumulation. ELISA was used to determine the effects of ULA on thromboxane A₂ and TNF- α production. Cell lysates were assayed for the presence of superoxide dismutase (SOD). HepG2 cells were also analysed for ULA induced apoptosis and necrosis using flowcytometry.

Results: Treatment concentrations of 1mM and 2.5mM of ULA induced a significant reduction in cell viability even after 1 hour of treatment in both cell lines. The ability of both cell lines to sequester ULA intracellularly at these concentrations was also significantly reduced compared to cells treated with the more physiologically comparable 0.1mM. HepG2 cells treated with 1mM and 2.5mM of ULA presented with a significant reduction in the presence of SOD after 4 hours. However, ULA had no significant effect on the presence of SOD in HUVEC cells. HepG2 cells also presented with increased apoptosis and necrosis at these concentrations after 4 hours of treatment. HUVEC cells treated with 1mM and 2.5mM of ULA showed a significant increase in thromboxane A₂ secretion. However, no significant effect on thromboxane A₂ production was observed in HepG2 cells.

Conclusion: The data suggests that ULA, at supraphysiological concentrations induces cell dysfunction in a number of ways depending on cell type. While Linoleic acid may not directly induce specific disease states, in a system compromised by disease or physiological stress, increasing plasma concentrations of linoleic acid, leading to increasing ROS and diminished antioxidant capabilities may initiate or further exacerbate pathophysiology.

Chapter 1

Introduction

Chapter 1. Introduction

1.1. Context and Origin of the Investigation.

Toxicology is traditionally considered to be the science of poisons (Eaton and Gilbert, 2008). However, as was historically reasoned by the Swiss physician and chemist Paracelsus in the 16th century, “Solely the dose determines that a thing is not a poison” suggesting that all substances have the potential to be toxic, it is merely the dose that determines it. Today, toxicology is defined by Eaton and Gilbert, (2008) as the study of the adverse effects of chemical or physical agents on living organisms. Gallo (2008) describes modern toxicology as going beyond the study of the adverse effects of exogenous agents to the study of molecular biology, using toxicants as tools.

Lipids and their component parts, fatty acids, are found in the tissues of microorganisms, plants, insects and animals, and their biological functions are as diverse as their chemistry (Nelson and Cox, 2017). They are crucial for optimum health and play vital roles as enzyme cofactors, electron carriers, hormones, and intracellular messengers as well as having several other important functions (Nelson and Cox, 2017). Nutritionally, lipids provide a concentrated source of energy (9kcal/gram) as well as essential nutrients such as the essential fatty acids, linoleic acid and linolenic acid, and lipid-soluble vitamins A, D, E and K (Frankel, 2005). However, to revert to the reflections of Paracelsus that the dose determines the poison, over-consumption of fats, or lipids, has been associated with several obesity related diseases, all beginning with central excess adiposity and collectively referred to as metabolic syndrome (Eckel *et al*, 2010, Cameron *et al*, 2008).

Lipotoxicity has been broadly defined as cellular toxicity observed in the presence of an abnormal accumulation of fat (Unger 2003, Mahli and Gores, 2008), particularly when this accumulation occurs in non-adipose tissues (Feldstein *et al*, 2004). It is associated with several obesity related disorders, such as fatty liver, lipid cardiomyopathy, non-insulin-dependent diabetes mellitus and insulin resistance, all falling under the umbrella term of metabolic syndrome (Unger, 2003). According to Feldstein *et al*, (2004), free fatty acids are important mediators of lipotoxicity, both as potential cellular toxins and by leading to excessive intracellular lipid accumulation. On a molecular level, Symons and Abel, (2013) attributes lipotoxicity to impaired tissue homeostasis associated with changes in lipid utilisation or lipid induced changes in intracellular signalling.

In 2009, the Irish Equine Centre (IEC) observed an increase in the occurrences of an idiopathic and fatal haemorrhagic condition in horses. This increase prompted the subsequent investigation carried out by Cooper (2015). While, initially, these occurrences were thought to be related to contaminated feedstuffs, with the suspect contaminant presumed to be an anticoagulant of sorts, chemical analysis of hepatic tissue obtained from affected equines confirmed the presence of a lipid, more specifically, the polyunsaturated fatty acid, linoleic acid, in its dietary form (Cooper, 2015). Cooper (2015) went on to confirm that linoleic acid was present in the hepatic tissues of approximately one thousand animals reported by the IEC to have succumbed to idiopathic fatal haemorrhage.

Linoleic acid, an ω -6 essential fatty acid, is associated with the production of proinflammatory mediators as it is metabolised to arachidonic acid, a substrate for several pro-inflammatory eicosanoids, specifically, prostaglandins, leukotrienes and

thromboxane (Simopoulos, 2008). In contrast, linoleic acid has also been associated with the inhibition of platelet aggregation and a reduction in platelet thromboxane production (Needleman *et al*, 1982, MacIntyre *et al*, 1984) postulated to be as a result of cyclooxygenase inhibition (Zhang *et al*, 1997). Both humans and equines exist on a diet whereby linoleic acid is the most abundant polyunsaturated fatty acid (Hallebeek and Beynen, 2002, Calder, 2012). This particular fatty acid can be found in numerous edible oils such as soybean oil, safflower oil, sunflower oil and sesame oil (James *et al*, 2000, von Hanstein *et al*, 2020), as well as nuts, seeds, meat and eggs (Whelan and Fritzsche, 2013). According to Fritzsche, (2008), and Belury, *et al*, (2018), dietary lipid intake may impact the development of several chronic diseases such as obesity, cardiovascular disease, diabetes and certain cancers, conditions associated with chronic inflammation (Johnson and Fritzsche, 2012).

Based on evidence presented in the literature and the findings of Cooper (2015), an investigation of the potential adverse effects of supraphysiological concentrations of linoleic acid was carried out. Due to recognised links between linoleic acid, inflammation and coagulation, the present investigation sought to examine the relationship between the essential fatty acid, as an exogenous agent of toxicity, and markers associated with inflammation and haemostasis. An initial step in understanding this potential relationship was to examine its cytotoxic or inflammatory effect on an experimental model representative of human tissues. The following investigation will aim to illuminate the effects of the ω -6 essential fatty acid, linoleic acid, with regards to lipotoxicity and inflammation using a human *in vitro* model.

Chapter 2

Literature Review

Chapter 2. Literature Review

2.1. Lipids -Characterisation and Classification.

Lipids are a large, diverse group of naturally occurring organic compounds. According to Hennessy *et al*, (2016), they may be defined as fatty acids, their derivatives and any biosynthetically or functionally related substances. The LIPID Metabolites and Pathway Strategy (MAPS) database defines lipids as hydrophobic or amphipathic compounds which involve either the carbanion-based condensations of thioesters and/or the carbocation-based condensations of isoprene units (Fahy *et al*, 2005, Zhao *et al*, 2015).

Lipids are most commonly found in the tissues of microorganisms, plants, insects and animals, and their biological functions are as diverse as their chemistry. They are primarily stored as fats and oils in many organisms with phospholipids and sterols being significant structural elements of biological membranes. Other lipids, although present in smaller quantities, also play vital roles as enzyme cofactors, electron carriers, hormones, and intracellular messengers as well as having a number of other important functions (Nelson and Cox, 2017). A function of particular interest to this project is the production of eicosanoids, particularly prostaglandins – hormone-like lipid compounds involved in reactions such as the inflammatory response and blood coagulation. Nutritionally, lipids provide a concentrated source of energy (9kcal/gram) as well as essential nutrients such as the essential fatty acids, linoleic acid and linolenic acid, and lipid-soluble vitamins A, D, E and K (Frankel, 2005). According to Hodgson *et al*, (2014) lipids are the major energy substrate used by the horse during low-intensity exercise.

Fahy *et al*, (2005) classified lipids through subdivision into eight categories based on the different hydrophobic and hydrophilic elements that make up lipids. These categories are comprised of fatty acyls, glycerophospholipids, sterols, sphingolipids, glycerolipids, prenols, saccharolipids, and polyketides. Within these categories several sub-categories exist under which over 44,000 structures are classified (Table 2.1).

Table 2.1. Lipid categories of the comprehensive classification system and the number of structures in the LIPID MAPS database as of the 25th of June 2020 (LIPID MAPS® Lipidomics Gateway, Fahy *et al*, 2009).

<i>Category</i>	<i>Curated</i>	<i>Computationally-generated</i>	<i>All</i>
Fatty acyls	7817	1792	9609
Glycerolipids	232	7379	7611
Glycerophospholipids	1616	8312	9928
Sphingolipids	1649	3176	4825
Sterol lipids	2875		2875
Prenol lipids	1373		1373
Saccharolipids	27	1294	1321
Polyketides	6881		6881
TOTAL	22470	21953	44423

Of the eight categories defined by Fahy *et al*, (2005), the category of relevance to this work is that of the fatty acyls, which encompasses the various classes of fatty acids. Fahy *et al*, (2005) state that the fatty acyl structure represents the major constituent of complex lipids. These fatty acyls are characterised by repetition of the methylene groups that result in the hydrophobicity of fatty acids.

2.1.1. Structure and Function of Fatty Acids

Fatty acids, according to Ahern and Rajagopal (2012) are considered to be the most important lipids in the cell, as they are the constituents of all other lipids. Choque *et al*, (2014) stated that fatty acids, particularly polyunsaturated fatty acids (PUFA), that have more than one double bond, are the basic components involved in the architecture and function of cellular membranes. They play an integral role in several biological functions including the production of eicosanoids such as prostaglandins, thromboxanes and leukotrienes.

Structurally, fatty acids consist of a carboxyl group linked to a long hydrocarbon chain ending with a methyl group and can be described as either saturated, meaning they do not contain double bonds, or unsaturated, meaning they contain one or more double bonds (Ahern and Rajagopal, 2012).

Fatty acids rarely occur freely in nature but are found in esterified forms as major components of various lipids. Many of the fatty acid residues of plants and animals are unsaturated or even polyunsaturated. In PUFAs, the double bonds are inclined to be present at every third carbon atom toward the methyl terminus of the molecule and typically form the *cis-cis* configuration, as is apparent for linoleic acid and α -linolenic acid in figure 2.1. With the *cis-cis* configuration, the two hydrogen atoms adjacent to the double bond protrude on the same side of the chain (Voet and Voet, 2011).

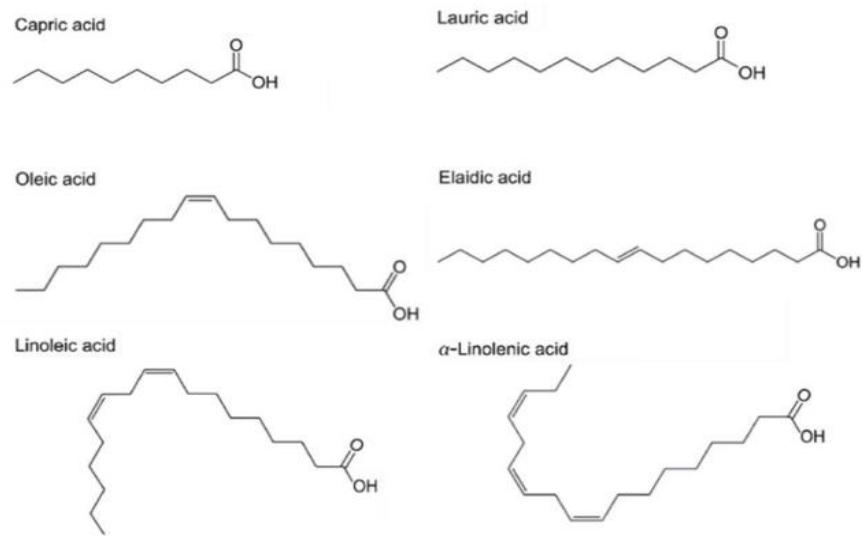


Figure 2.1. Chemical structure of fatty acids. Saturated fatty acids include capric acid (C10:0) and lauric acid (C12:0). Unsaturated fatty acids include oleic acid (C18:1) and elaidic acid (trans-C18:1). Polyunsaturated fatty acids include linoleic acid (C18:2) and α -linolenic acid (C18:3) (adapted from Yoon *et al*, 2018).

This structure causes a rigid 30° bend to occur in the hydrocarbon chain (Voet and Voet, 2011). The more double bonds in a PUFA, the more pronounced the curve in the molecule's chain becomes (figure 2.1). This form of configuration reduces the flexibility of the molecule, therefore limiting its packing efficiency regarding cell membranes. In contrast, in the conjugated forms of certain molecules, the double bonds tend to occur at every second carbon atom (Voet and Voet, 2011). However, according to Nelson and Cox (2017) and Voet and Voet (2011) double bonds in polyunsaturated fatty acids are rarely conjugated. Conjugated fatty acids refer to a mixture of positional and geometric isomers of PUFAs with conjugated double bonds (Nagao and Yanagita, 2005).

Fatty acids can be further defined by mammalian dietary requirements (figure 2.2). While many fatty acids are required for homeostasis several can be synthesised within the body,

and, as such, are considered non-essential fatty acids. Oleic acid (C18:1) (figure 2.2) is an example of a non-essential fatty acid (Cury-Boaventura *et al*, 2003). Fatty acids that cannot be produced by the mammalian body are deemed essential, must be derived from the diet, and are termed as such – essential fatty acids (Frankel, 2005, Di Pasquale, 2009).

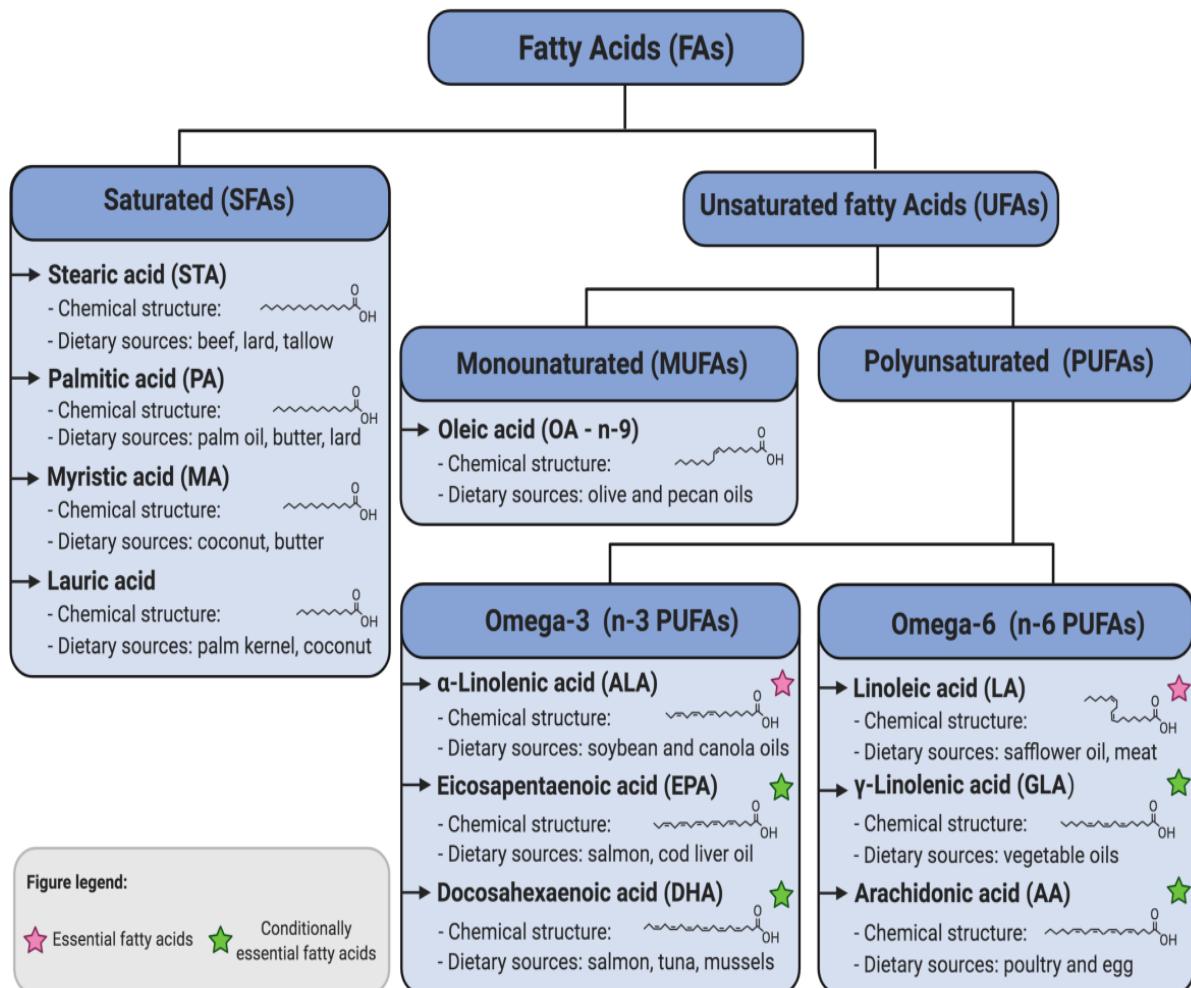


Figure legend:

★ Essential fatty acids

★ Conditionally essential fatty acids

Figure 2.2. Classification, chemical structure, and primary dietary sources of fatty acids. (Radzikowska *et al*, 2019).

2.1.2 Essential Fatty Acids – ω6 and ω3 Fatty Acids

Particular importance is placed on a specific group of polyunsaturated fatty acids, namely linoleic acid and α-linolenic acid. These fatty acids are classed as essential, as during fatty acid metabolism, the mammalian body lacks the endogenous enzyme Δ3- desaturase, and consequently, cannot incorporate double bonds into fatty acids past the ninth carbon from the carboxyl end of the molecule (Lichtenstein, 2005, Di Pasquale, 2009, Whelan and Fritsche, 2013, Simopoulos, 2016). As such, these fatty acids must be derived from the diet (Frankel, 2005, Di Pasquale, 2009).

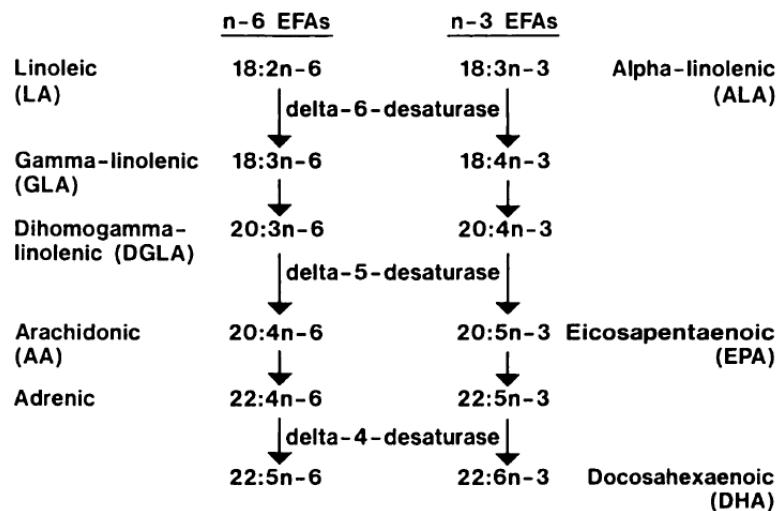


Figure 2.3. The Metabolic pathways of the essential fatty acids, the ω6 linoleic acid and the ω3 α-linolenic acid (Horrobin, 1993).

There are two groups of essential fatty acids; linoleic acid, the ω6 group, which is metabolised to produce gamma-linolenic acid (GLA) and arachidonic acid, and α-linolenic acid, the ω3 group, whose metabolites include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Di Pasquale, 2009). These two classes of essential fatty acids are not interconvertible, are metabolically and functionally distinct, and often have important opposing physiological functions (Simopoulos, 2008). The ω6 fatty acids are

associated with the production of pro-inflammatory agents and signalling molecules while the ω 3 fatty acids are associated with the production of anti-inflammatory mediators (Calder 2005).

While both fatty acids are metabolised by the same desaturase enzyme, namely Δ 6-desaturase, according to Calder (2005), there is competition between the ω 6 and ω 3 fatty acid families for metabolism (figure 2.3). Although the preferred substrate for Δ 6-desaturase is α -linolenic acid, because linoleic acid is much more prevalent in most Western diets when compared with α -linolenic acid, the metabolism of ω 6 fatty acids is quantitatively more important (Calder 2005, Simopoulos, 2008). High intake of ω 6 fatty acids, particularly linoleic acid, and low ω 3 fatty acid intake as a result of a modern Western diet, has been proposed to induce a chronic low grade-inflammatory state within the body (Simopoulos, 2008). In equine nutrition, even more importance could be placed on linoleic acid when it is considered that, according to Robb *et al.* (1972) it is a major component of equine adipose tissue. Consequently, linoleic acid and its potential to induce inflammation are the main focus of this body of research.

2.2. Linoleic acid

Linoleic acid ($\text{C}_{18:2} \Delta^9, \Delta^{12}$) is an eighteen-carbon polyunsaturated fatty acid with two *cis* double bonds after carbon nine and after carbon twelve from the carboxyl end, as portrayed in figure 2.4 (O’Quinn *et al*, 2000). As the final carbon-carbon double bond is six carbons from the most distal (methyl) carbon from the carboxyl end, linoleic acid is referred to as an omega (ω) six fatty acid (Pelley, 2007).

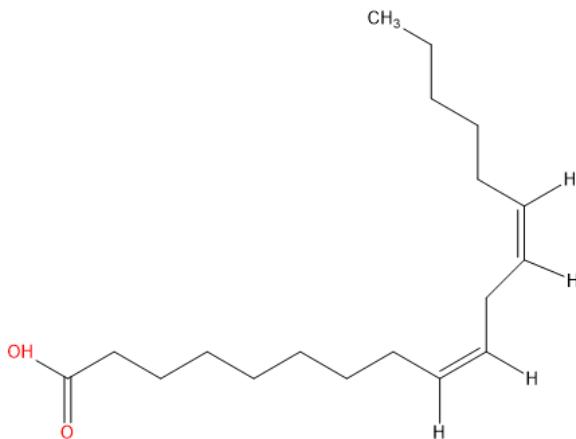


Figure 2.4. Chemical structure of linoleic acid ($\text{C}_{18:2} \Delta^9, \Delta^{12}$) (authors own work, produced using ChemDraw Professional 15.0).

Linoleic acid is the most abundant PUFA in most foods and even foods with very low lipid content, such as vegetable, fruits and grains are predominantly rich in linoleic acid (Whelan and Fritzsche, 2013). Mammalian diets, containing corn, soy, safflower and sunflower oils are abundant sources of linoleic acid (James *et al*, 2000), as are nuts, seeds, meat and eggs (Whelan and Fritzsche, 2013). Diets containing high quantities of such foods have been termed by several authors, as Western diets (James *et al*, 2000, Simopoulos, 2008, Fritzsche, 2015, Innes and Calder, 2018).

While dietary linoleic acid exists in the *cis*-9, *cis*-12 formation, conjugated isomers of linoleic acid also exist. Conjugated linoleic acids are group of positional and geometric conjugated isomers of linoleic acid. Theoretically several exist, however, the most

commonly occurring isomers exist in the *cis*-9, *trans*-11 formation (Chin *et al*, 1992). According to Chin *et al*, (1992), naturally occurring conjugated linoleic acids are most abundant in meat and dairy products, According to Nagao and Yanagita (2005) conjugation of fatty acids occurs through the biohydrogenation of unsaturated fatty acids by the ruminal bacterium *Butyrvibrio fibrisolvens* in ruminant mammals, such as cows, sheep, goats and camels. While conjugated fatty acids are abundant in ruminal meat and dairy products, in contrast, non-ruminal meat products contain much lower quantities of conjugated fatty acids (Chin *et al*, 1992). In monogastric herbivores such as horses, dietary fatty acids reach the small intestine first and are absorbed directly, before any remaining fatty acids are subjected to microbial processes in the hindgut (Clauss *et al*, 2008).

In the equine gut, pasture forage is fermented by microbiota of the colon to produce short chain fatty acids and characterisation of these fatty acids, and their intestinal absorption have been well documented (Nedjadi *et al*, 2014), however, literature on the production of CLA in the non-ruminant, particularly the equine, is scarce.

2.2.1. Digestion and Intestinal Absorption of Linoleic Acid

Whelan and Fritsche (2013) report that linoleic acid follows a similar metabolic fate to other fatty acids. After ingestion of a fat containing meal, catabolism of dietary lipids occurs mainly in the small intestine and begins with the gastrointestinal lipolysis of dietary triacylglycerols (TAG) (Zechner *et al*, 2012). TAGs are esters comprised of glycerol and three fatty acids (figure 2.5) and are quantitatively the most important lipid component in the mammalian diet (Mu and Porsgaard, 2005).

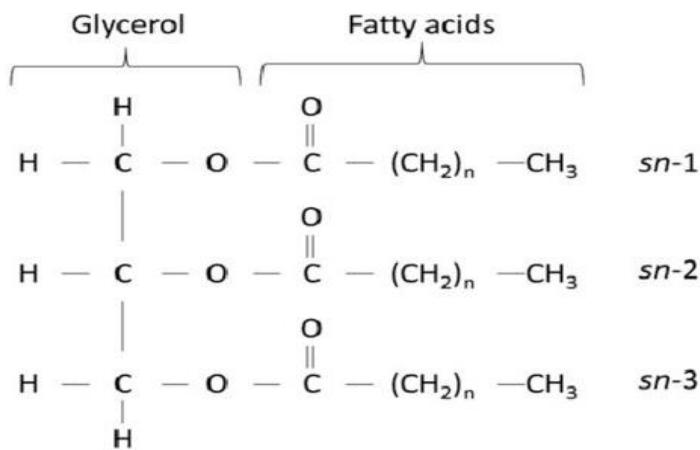


Figure 2.5. Chemical structure of a triacylglycerol (TAG) (Mills *et al*, 2017).

Following gastric pre-digestion, the food bolus enters the duodenum where bile salts, synthesised in the liver from cholesterol and stored in the gallbladder, begin emulsification of dietary lipids. Pancreatic lipase is then released into the duodenum and the upper segment of the jejunum in response to the hormone cholecystokinin. This enzyme binds to the surface of drops of dietary lipids and begins to degrade the TAGs (Mu and Porsgaard, 2005, Iqbal and Hussain, 2009). This degradation is region specific and enzyme activity occurs on the *sn*-1 and *sn*-3 positions of the TAG molecule. This results in the release of 2-monoacylglycerol (2-MAG) and free fatty acids. According to Iqbal and Hussain (2009), 2-MAG is the predominant form in which monoacylglycerols are absorbed by the small intestine. According to Carlier *et al*, (1991) linoleic acid is found in the *sn*-2 position of most plant and animal TAGs. Further hydrolysis of 2-MAG by pancreatic lipase results in the release of glycerol and free fatty acids. These free fatty acids can then be taken up from the intestinal lumen into the enterocytes where they can be biosynthesized into neutral lipids (Iqbal and Hussain, 2009). While specific published

data on the digestion and intestinal absorption of linoleic acid is limited, this may suggest that linoleic acid enters the enterocyte in the form of 2-MAG.

2.2.2. Cellular Transport and Processing of Linoleic acid

Mansbach and Gorelick (2007), and Hesse *et al* (2013), indicate that while the movement of fatty acids across the apical membrane of the enterocyte is not well understood, it is believed that both a protein-independent diffusion model and protein-dependent mechanisms such as fatty acid transport proteins (FATPs) are involved.

While the diffusion model has been observed in Caco2 cells and adipocytes, Mansbach and Gorelick (2007) have reported that the cellular uptake mechanisms for fatty acids are saturatable and competitive indicating that the mechanism may be protein mediated. Goré *et al*, (1994) determined that linoleic acid was taken up by intestinal cells *via* plasma membrane fatty acid binding proteins, that were competitive with the ω3 fatty acid α-linolenic acid. A number of candidate proteins have been proposed to take part in the protein-dependent uptake of fatty acids across the cell membrane (Iqbal and Hussain, 2009). FATPs are a family of six different proteins of which FATP2 to FATP4 are expressed in the intestine. FATP4 has also been localised in the intestinal endoplasmic reticulum (Mansbach and Gorelick, 2007).

Once inside the enterocyte, linoleic acid must cross the cytoplasm to reach the endoplasmic reticulum, where it is used to synthesise complex lipids. Specific reports on the intracellular fate of linoleic acid remain elusive, however, Hesse *et al*, (2013) states that once fatty acids and MAGS, which may contain linoleic acid in the *sn*-2 position (Carlier *et al*, 1991), are transported inside the cell, they bind to intracellular fatty acid

binding proteins (FABP) and are delivered to the endoplasmic reticulum for re-esterification. Progressive acylation of MAGs on the membrane of the endoplasmic reticulum results in the re-assembly of TAGs (Iqbal and Hussain, 2009). These TAGs then cross the membrane of the endoplasmic reticulum into the lumen where they are used in the formation of lipoproteins such as pre-chylomicrons (Hesse *et al*, 2013).

These pre-chylomicrons are large (150 – 500nm) and therefore cannot flow freely from the endoplasmic reticulum (Hesse *et al*, 2013). They require a vesicular transport system in the form of a pre-chylomicron transport vesicle (PCTV), which buds off the endoplasmic reticulum membrane and is transported to the Golgi complex, the final site of chylomicron assembly (Hesse *et al*, 2013). It is here that the chylomicrons are carried through the Golgi stack where they progress to the basolateral membrane for exocytosis. They are secreted through the plasma membrane into the lamina propria and finally into the mesenteric lymph vessels that are located inside each of the intestinal villi (Mu and Høy, 2004, Mansbach and Gorelick, 2007). These chylomicrons are initially released into the lymphatic system before entering circulation in the subclavian vein *via* the thoracic duct where they distributed to the various tissues around the body including the liver (Mu and Høy, 2004). While there are no specific reports on the transport or packaging of linoleic acid, it could be postulated that it follows a similar fate to other fatty acids.

When chylomicrons arrive at the liver, they release free fatty acids as a result of lipolysis *via* the action of lipoprotein lipase. These free fatty acids enter through a number of fatty acid transport proteins and CD36. Once inside the hepatocyte, they are package into TAGs or cholesterol esters, where they are either stored in the form of lipid droplets or secreted into circulation as very low-density lipoproteins (Rui, 2014). While specific data on the

mechanism of linoleic acid uptake by hepatocytes was not available, Chambaz *et al*, (1986) determined that linoleic acid was as effective as other fatty acids (palmitic, oleic and α -linolenic acid) in the hepatic synthesis of TAGs. This may suggest that the mechanism by which hepatocytes take up and package linoleic acid is similar to other fatty acids.

According to Whelan and Fritsche, (2013), linoleic acid is packaged into these chylomicrons as well as phospholipids, triacylglycerols, or cholesterol esters and enters the general circulation through the subclavian vein *via* the thoracic duct. These chylomicrons are delipidated to form much smaller remnant particles as they are transported to the liver. This allows for the release and delivery of linoleic acid to hepatic, extrahepatic and adipose tissues where it is stored or released and used as necessary by other tissues, such as the cardiac and skeletal tissues. After cellular uptake, the fate of linoleic acid is determined by the specific needs of the particular tissue (Whelan and Fritsche, 2013).

2.2.3. Cellular Metabolism of Linoleic Acid

Linoleic acid has a number of metabolic fates (figure 2.6), depending on cellular and tissue needs. Like all fatty acids, it can be used as a source of energy. It can be esterified to form neutral and polar lipids such as phospholipids, triacylglycerols, and cholesterol esters. It can form part of cellular and organelle phospholipid membranes, functioning as a structural component to maintain membrane fluidity. In addition, when released from membrane phospholipids, it can be enzymatically oxidized to a variety of derivatives involved in cell signalling (Whelan and Fritzsche, 2013).

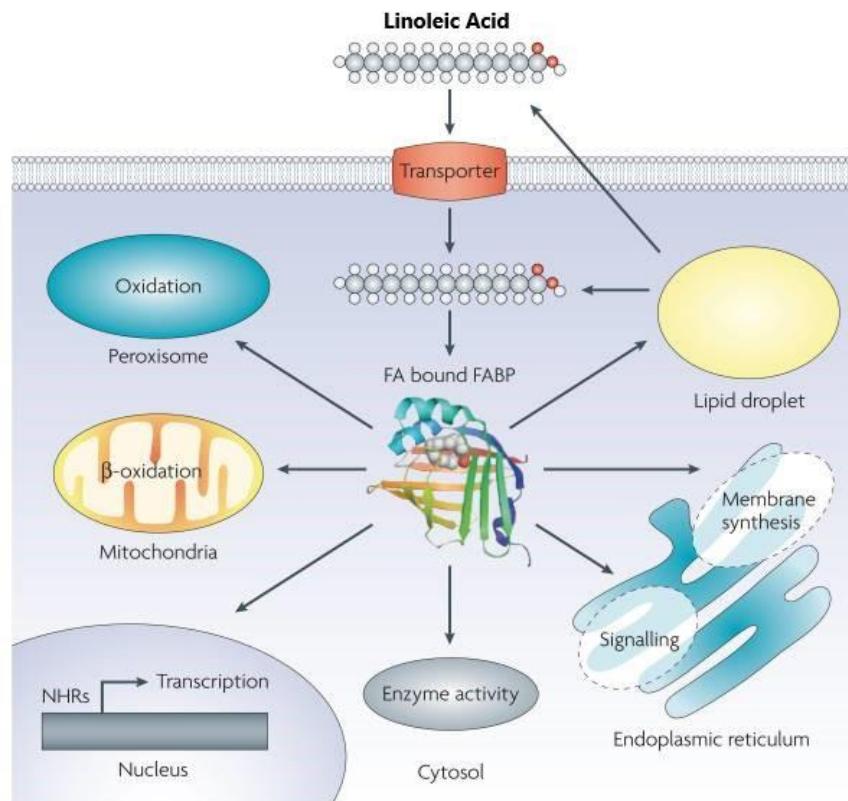


Figure 2.6. Potential metabolic fates of linoleic acid (adapted from Furuhashi and Hotamisligil, 2008)

Linoleic acid is a precursor for several metabolites involved in many physiological and pathophysiological functions including the pro-inflammatory process. It is primarily converted to γ -linolenic acid and dihomogamma-linoleic acid, and finally, to arachidonic acid, a twenty carbon fatty acid with four double bonds. This conversion occurs as a result of microsomal enzymes, $\Delta 6$ desaturase, the elongases ELOVLs (elongation of very long chain fatty acids protein) and $\Delta 5$ desaturase respectively (figure 2.7) (Horrobin, 1993,

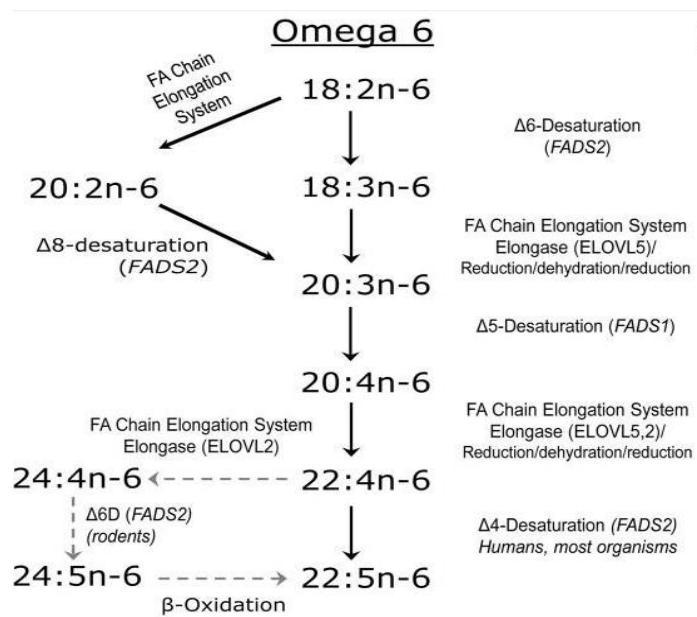


Figure 2.7. Desaturation and elongation of linoleic acid occurs as a result of enzymes produced by the endoplasmic reticulum, namely desaturases and elongases (Fatty acid desaturase; FADS)(adapted from Zhang *et al*, 2016).

Rett and Whelan, 2011, Choque *et al*, 2014, Schuster *et al*, 2018). Both rate limiting enzymes are localised in the endoplasmic reticulum and produced mainly by the liver, however, other tissues have also been reported to produce these enzymes (Skrzypski *et al*, 2009). Both linoleic acid and arachidonic acid can be released from cellular phospholipid membranes by the action of phospholipase A₂, a cytosolic enzyme responsible for cleaving fatty acids from the *sn*-2 position of phospholipids (Burke and Dennis, 2009). Subsequently, arachidonic acid can be converted into several bioactive

compounds referred to as eicosanoids. These eicosanoids are important in the normal metabolic function of cells and tissues, but when persistently produced in excess, they are known to contribute to a number of chronic diseases, such as inflammation and cancer (Whelan and Fritzsche, 2013). According to Whelan and Fritzsche, (2013), it is this possible conversion to arachidonic acid for which linoleic acid has received the most notoriety.

Linoleic acid can also be enzymatically oxidised *via* the actions of lipoxygenases and cyclooxygenases produced by the endoplasmic reticulum and nuclear membrane or the microsomal cytochrome P450 enzyme family as well as by free radical-mediated oxidation in response to oxidative stress (Reinaud *et al*, 1989, Kirpich *et al*, 2016, Schuster *et al*, 2018). Linoleic acid metabolites produced *via* these pathways, referred to as bioactive oxidized linoleic acid metabolites or OXLAMs, include 9- and 13 hydroxy-octadecadienoic acid (9- and 13-HODE) and 9- and 13-oxooctadecadienoic acid (9- and 13-oxoHODE) (Ramsden *et al*, 2012, Choque *et al*, 2014, Ramsden *et al*, 2018). According to Schuster *et al*, (2018), these metabolites are abundant in the liver and, while they may play a role in regulating mitochondrial function, in excess, they can increase oxidative stress and induce mitochondrial dysfunction and apoptosis.

2.2.4. Linoleic Acid in Mitochondrial Homeostasis and Metabolism

While the primary function of the mitochondria is the production of ATP, they are also capable of producing a number of fatty acid products required for cellular metabolism and homeostasis (White *et al*, 2005). Mayr (2014), suggests that the mitochondria provide a major contribution to lipid metabolism, particularly the biosynthesis of some

phospholipids and the β -oxidation of fatty acids as well as the synthesis of unique fatty acids, lipid cofactors and steroid hormones.

Linoleic acid metabolism by the mitochondria has not been completely documented, however, it is reported to play a number of key roles in maintaining mitochondrial homeostasis, particularly as a major fatty acid constituent of the unique mitochondrial phospholipid, cardiolipin, found on the inner mitochondrial membrane (Bradley *et al*, 2016, Fajardo *et al*, 2017). According to Fajardo *et al*, (2017) fluctuations in the linoleic acid content of cardiolipin has been associated with impairments in cytochrome C oxidase activity as well as structural and functional mitochondrial abnormalities and reactive oxygen species (ROS) production. Linoleic acid is enzymatically transferred from donor phospholipids such as phosphatidylcholine *via* tranacylase onto immature forms of cardiolipin to generate the mature cardiolipin in the mitochondrial membrane (Bradley *et al*, 2016).

2.2.4.1. Linoleic Acid and Mitochondrial β -oxidation

Linoleic acid can also be used by the mitochondria as a source of energy production (Whelan and Fritsche, 2013). Upon entering the cell, long chain fatty acids such as linoleic acid are activated through the formation of thioesters with co-enzyme A (CoA) (Aon *et al*, 2014). These thioesters are either esterified to form TAGs that are subsequently stored within intracellular lipid droplets, or they are oxidised *via* β -oxidation within the mitochondria to produce cellular energy

β -oxidation begins when a long chain fatty acid such as linoleic acid, is activated on the outer mitochondrial membrane by long chain acyl-CoA synthetase to form a fatty acyl-

CoA; for example, β -oxidation of linoleic acid, would result in the production of linoleoyl-CoA. However, this activated long chain fatty acyl-CoA cannot permeate the inner mitochondrial membrane. Therefore, it must be converted, *via* carnitine palmitoyltransferase (CPT1), to long-chain acylcarnitine, which can then be transported by the enzyme carnitine:acylcarnitine transferase (CACT) into the inner mitochondrial membrane (Aon *et al*, 2014). Once inside, the long-chain acylcarnitine molecule is converted back to fatty acyl-CoA where β -oxidation occurs *via* the sequential action of four enzyme families (Aon *et al*, 2014). These four enzymes are responsible for cleaving two carbons per cycle from the fatty acyl-CoA resulting in the production of a fatty acyl-CoA that is now two carbons shorter, and a molecule termed acetyl-CoA (Aon *et al*, 2014). The process of β -oxidation will repeat until all available acetyl-CoAs have been produced. In the case of linoleoyl-CoA, this means the formation of 9 molecules of acetyl-CoA. Acetyl-CoA then enters the tricarboxylic acid cycle (TCA), also commonly referred to as the citric acid cycle or Krebs cycle, where it reduces nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) to form NADH and FADH₂. These electron donors drive the mitochondrial oxidative phosphorylation to produce ATP (Aon *et al*, 2014, Jaishy and Abel, 2016).

2.2.4.2. Linoleic Acid and Fatty Acid Synthesis within the Mitochondria

The primary site of cellular lipid synthesis is the endoplasmic reticulum and the Golgi apparatus, as well as the cytosolic fatty acid synthesis pathway (Kastaniotis *et al*, 2017). However, Mayr (2014), suggests that the mitochondria also contribute to lipid metabolism. While mitochondria have the ability to synthesise lipids on their own, they also depend on the transfer and assembly of lipids formed in the endoplasmic reticulum.

This constant supply and exchange of lipids is vital for the maintenance of mitochondrial membrane integrity and overall cellular homeostasis (Horvath and Daum, 2013).

According to White *et al*, (2005), the mitochondria is capable of producing a diverse range of products, such as unsaturated fatty acids, required for cellular metabolism. However, according to Kastaniotis *et al*, (2017), mitochondrial fatty acid synthesis has yet to be completely elucidated. A recently recognised feature of these diverse mitochondrial functions is the ability to synthesise fatty acids in an acyl carrier protein (ACP) (a component of fatty acid biosynthesis) – dependent manner (Kastaniotis *et al*, 2017). One of the most well documented products of mitochondrial fatty acid synthesis is octanoyl-ACP, a precursor for lipoic acid, a co-factor required for the conversion of pyruvate to acetyl-CoA (White *et al*, 2005, Kastaniotis *et al*, 2017). Unfortunately, according to Hiltunen *et al*, (2009) and Kastaniotis *et al*, (2017), the range of fatty acids produced by the mitochondrial fatty acid synthesis pathway have yet to be determined. After an extensive review of the current literature available on mitochondrial fatty acid synthesis, particularly regarding linoleic acid, this researcher finds that this statement still holds true.

2.2.5. Linoleic Acid Storage in Intracellular Lipid Droplets - Structure, Biogenesis and Functions

Linolenic acid that is not required for cellular metabolism or homeostasis may be stored in the form of intracellular lipid droplets. Intracellularly, transit rates within the endoplasmic reticulum are slow, and some TAGs are thought to detach from the membrane and become cytosolic lipid droplets (Mansbach and Gorelick, 2007). They differ from chylomicrons in their size and protein composition (Hesse, *et al*, 2013). These

lipid droplets, previously considered to be cytoplasmic inclusions for intracellular lipids, have recently been given the title of organelle (Olzmann and Carvalho, 2018). They play a key role in intracellular lipid storage and energy homeostasis (Olzmann and Carvalho, 2018), as well as serving as organising centres for synthesising specific lipids (Walther and Farese, 2012). It is also hypothesised that, in cases of intracellular lipid overload, lipid droplets may serve as buffers for potentially toxic lipids, preventing lipotoxicity and oxidative stress (Welte, 2015, Olzmann and Carvalho, 2018). Linoleic acid overload may contribute to intracellular lipid accumulation in the form of lipid droplets. Carro *et al*, (2013) observed linoleic acid incorporation into intracellular lipid droplets in bovine oocytes. Schlager *et al*, (2017) also reported that macrophages also accumulate neutral lipid droplets in the form of cholesterol esters, enriched with linoleic acid as well as arachidonic acid.

Lipid droplets alternate between periods of growth and consumption mediated by lipolysis and lipophagy, the autophagic degradation of intracellular lipid droplets (Olzmann and Carvalho, 2018, Kounakis *et al*, 2019). These processes that closely reflect cycles of nutrient availability as well as the cellular metabolic rate (Olzmann and Carvalho, 2018). While it has been established that lipid droplets are commonly found in most mammalian cells (Welte, 2015), the number, composition and size can vary from cell to cell, as well as within the same cell (Olzmann and Carvalho, 2018).

Despite their ubiquitous nature, the mechanisms by which lipid droplets are produced are still poorly understood (Olzmann and Carvalho, 2018). Lipid droplets consist of a core of hydrophobic neutral lipids, surrounded by a phospholipid monolayer and these neutral lipids are synthesised in the endoplasmic reticulum (Hashemi, and Goodman, 2015, Guo

et al, 2009). It is thought that their formation begins within the endoplasmic reticulum (Welte, 2015), portrayed in figure 2.8. Esterification of activated fatty acids, to diacylglycerols or sterol, is carried out by the enzymes acyl-CoA:diacylglycerol

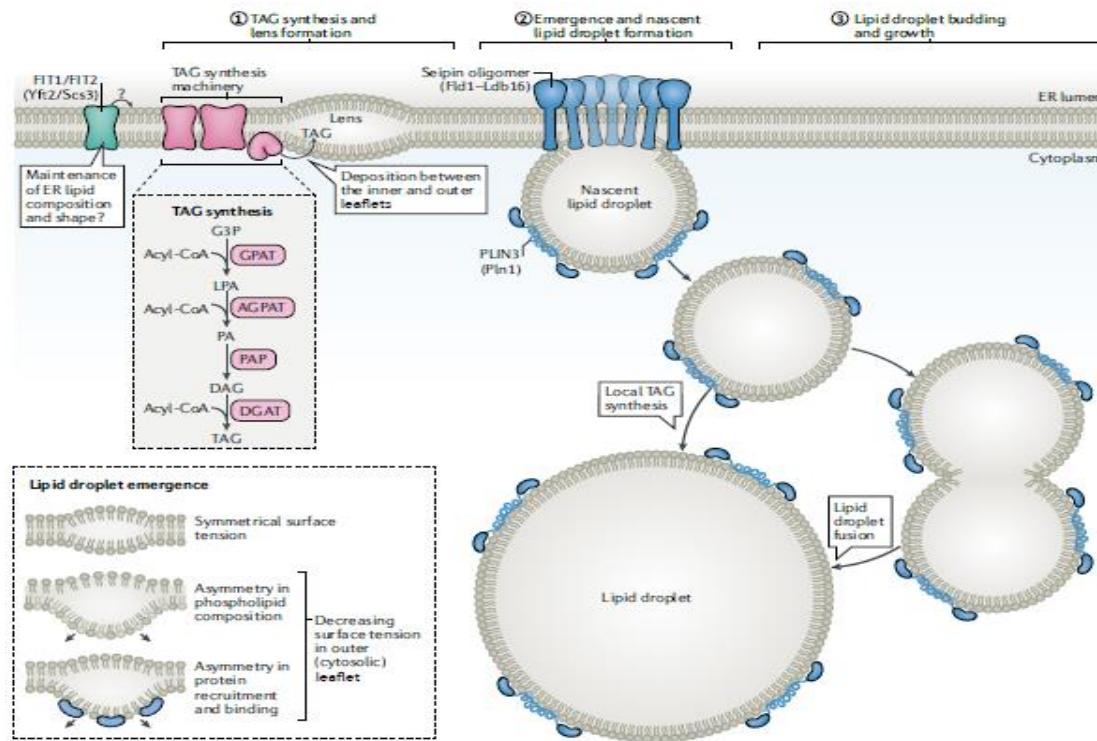


Figure 2.8. Lipid droplet formation occurring between the leaflets of the endoplasmic reticulum. TAG, Triacylglyceride; DAG, diacylglycerol; DGAT, acyl-CoA:diacylglycerol acyltransferase; G3P, glycerol-3-phosphate; GPAT, glycerol-3 phosphate acyltransferase; LPA , lysophosphatidic acid; PA , phosphatidic acid; PAP, phosphatidic acid phosphatase; PLIN, perilipin. (Olzmann and Carvalho, 2018).

transferases (DAGT1 and DAGT2) produced by the endoplasmic reticulum (Welte, 2015). According to Hashemi and Goodman, (2015), low levels of a “few mole percent” of neutral lipids are thought to diffuse freely between the bilayer of the endoplasmic reticulum (Hashemi and Goodman, 2015). As these neutral lipids increase in concentration, an “oil lens” forms and expands, eventually creating a lipid droplet “bud” off the endoplasmic reticular membrane into the cytoplasm (Walther and Farese, 2012, Olzmann and Carvalho, 2018). Expansion of this bud may occur in several ways. Bridges

may form between the lipid droplet bud and the endoplasmic reticulum resulting in the transfer of triacylglycerols (Wilfling *et al*, 2013), or triacylglycerol synthesis may occur directly on the lipid droplet surface (Hashemi, and Goodman, 2015). Expansion may also occur through droplet to droplet fusion (Murphy *et al*, 2010). Eventually, in higher eukaryotes, some, but not all, lipid droplets detach from the endoplasmic reticulum, however, it is not yet fully understood how or why this occurs (Olzmann and Carvalho, 2018).

In addition to the membrane bridges formed with the endoplasmic reticulum, lipid droplets also form contact sites with other organelles, such as the peroxisomes, lysosomes and mitochondria, however the molecular basis for these contact sites is poorly understood (Olzmann and Carvalho, 2018).

Lipid droplets are thought to have multiple functions. During periods of starvation, fatty acids derived from lipid droplet lipolysis or lipophagy are converted at the mitochondrial membrane into acylcarnitine before being transported into the mitochondria for β -oxidation (Olzmann and Carvalho, 2018). These newly released fatty acids can also be used for membrane biosynthesis during periods of cell growth as well as for the synthesis of specific lipids such as TAGs (Walther and Farese, 2015). As, already mentioned, another key function of lipid droplets may be the prevention of lipotoxicity through sequestration of fatty acids and their toxic derivatives in times of lipid overload (Walther and Farese, 2012, Welte, 2015, Olzmann and Carvalho, 2018). Cellular overload of free fatty acids may have several deleterious effects. These include the disruption of membrane integrity and cellular signalling as well as the production of fatty acid derivatives, such as acylcarnitine and ceramide (Olzmann and Carvalho, 2018). Where

excess intracellular linoleic acid occurs, its neutralisation and incorporation into lipid droplets may reduce the production of derivatives and metabolites such as arachidonic acid or OXLAMs.

Impairment of fatty acid sequestration or disruption of lipid droplet biogenesis can result in lipotoxicity related diseases such as type-2 diabetes and non-alcoholic fatty liver disease (NAFLD) (Olzmann and Carvalho, 2018). According to Feldstein *et al*, (2004), free fatty acids are important mediators of lipotoxicity, both as potential cellular toxins and by leading to excessive intracellular lipid accumulation. When lipids accumulate excessively in non-adipose tissue, they may enter deleterious nonoxidative pathways that may induce cell injury and death (Feldstein *et al*, 2004). At a cellular level, this disruption results in endoplasmic reticulum stress, initiating the activation of the unfolded protein response (UPR). The primary function of this UPR is to restore endoplasmic reticulum homeostasis. The accumulation of unfolded proteins triggers cascades that manage the slowing of protein translation and upregulation of genes involved in ER protein folding, protein degradation and lipid biosynthesis (Olzmann and Carvalho, 2018).

2.2.5.1. Lipid Droplet Interactions with Other Organelles

While the synthesis and storage of lipid droplets is an important characteristic in mammalian cells, spatial organisation of this process is poorly understood (Stone *et al*, 2008). As already discussed, the formation of lipid droplets involves several steps that involve the endoplasmic reticulum, including the potential formation of a bridge like structure that allows TAGs and associated proteins move between them (Walther and

Farese, 2012). According to Olzmann and Carvalho, (2018), these two organelles maintain a relationship throughout their life cycle.

However, there is increasing evidence in the literature that lipid droplets interact with other organelles (Guo, *et al*, 2009). While the functions of these interactions have yet to be elucidated, it is hypothesised that they involve the exchange of lipids, either for lipid droplet growth or for their catabolic degradation (Guo *et al*, 2009).

According to Olzmann and Carvalho, (2018), lipid droplets interact with each other. Droplet to droplet interactions are thought to occur to facilitate efficient TAG storage. Olzmann and Carvalho, (2018) state that when lipid droplets interact, neutral lipids are transferred, resulting in the shrinkage of one droplet and the growth of the second. This may result in a reduction of total lipid droplet surface area and in turn, a reduction in potentially unnecessary cytosolic lipolysis (Olzmann and Carvalho, 2018). It had been reported that the cell death–inducing DFFA (DNA fractionation factor alpha)-like effector (CIDE) family of proteins, specifically Fat Specific Protein 27 (FSP27) controls droplet to droplet contact (Gong *et al*, 2011, Jambunathan, *et al*, 2011). However, while the transfer of neutral lipids has been documented between droplets, the fate of the remaining phospholipids and proteins remains unknown (Olzmann and Carvalho, 2018).

During times of nutrient deprivation, starved cells will use fatty acids, such as linoleic acid, released from lipid droplets to fuel β -oxidation, and ATP production (Welte, 2015). The trafficking of these fatty acids is thought to occur through direct contact between lipid droplets and the mitochondria (Olzmann and Carvalho, 2018). Such contact sites would therefore reduce cytosolic free fatty acids, such as linoleic acid, preventing lipotoxicity

and aberrant lipid signalling (Welte, 2015, Olzmann and Carvalho, 2018). It is hypothesised that a perilipin (lipid droplet associated) protein family member PLIN5 may be involved in the tethering of lipid droplets to mitochondria. PLIN5 is associated with the regulation of lipolysis through its interaction with adipose triglyceride lipase (ATGL). However, whether this tethering is direct or whether more specific mitochondrial associated proteins are also involved remains unknown (Olzmann and Carvalho, 2018).

2.3. Linoleic Acid and the Production of Pro-Inflammatory Mediators

A well accepted hypothesis exists that excessive dietary lipids, such as linoleic acid, can negatively impact health, leading to several pathological conditions, including obesity, cardiovascular disease and several conditions associated with chronic inflammation (Fritzsche, 2008). Calder (2013) reported that consumption of a diet rich in saturated fat results in the up regulation of processes related to inflammation. According to Rocha *et al.* (2009) obesity and atherosclerosis are chronic inflammatory processes, mediated by inappropriate lipid accumulation.

Linoleic acid and its direct metabolite, arachidonic acid, are associated with increased production of pro-inflammatory mediators such as prostaglandins, leukotrienes and thromboxane, collectively termed eicosanoids (Simopoulos, 2008). According to Simopoulos (2008), eicosanoids are highly biologically active at low concentrations, therefore, overproduction can contribute to the induction of disorders such as coronary artery disease, diabetes, arthritis, cancer, thrombus formation and several inflammatory disorders. As linoleic acid is a precursor of arachidonic acid, the hypothesis exists that

excessive ingestion of this fatty acid may contribute to chronic inflammation (Johnson and Fritsche, 2012).

The inflammatory process is an important part of the body's immediate response to injury or infection (Henson, 2005). It involves several cell types and is controlled by a host of extracellular and intracellular mediators and regulators, such as cytokines, growth factors and eicosanoids (Calder *et al*, 2009b, Turner *et al*, 2014, Innes and Calder, 2018). Inflammation is initially characterised by swelling, heat, pain and redness at the site of injury or infection, occurring as a result of increased blood flow and increased capillary permeability. Rapid influx of blood granulocytes, typically neutrophils and monocytes that mature into inflammatory macrophages initiate these physiological characteristics (Ricciotti and FitzGerald, 2010, Calder, 2011). This allows for the movement of plasma and large molecules, to cross from the blood through the endothelium as well as the passage of leukocytes from the bloodstream into the surrounding tissue (Calder, 2006).

This process is further promoted by the release of leukocyte chemoattractants from the compromised tissue and the upregulation of adhesion molecules on the endothelium that enable the transient tethering of leukocytes to the endothelium. These leucocytes, now activated, release several mediators of inflammation including lipid signalling molecules, reactive oxygen species and enzymes, depending upon the cell types present as well as the anatomical site of injury and the inflammatory stimulus (Innes and Calder, 2018).

While the primary purpose of these inflammatory mediators is to protect the host by neutralising and eradicating damaging pathogens, these mediators, and the cellular mechanisms involved in their production, can further compromise the host system (Innes

and Calder, 2018). Impaired resolution can lead to the development of chronic inflammation, which may, over time, be detrimental to the host (Bogatchev *et al*, 2005).

2.3.1. Linoleic Acid and Eicosanoid Production

Linoleic acid and its metabolite, arachidonic acid, are associated with increased eicosanoid production. Eicosanoids are short-lived pro and anti-inflammatory mediators that act in close proximity to the site of synthesis. As such, tissue specific responses depend upon which arachidonic acid products are generated at the site of interest and which receptors are present in the responding cells (Bogatcheva *et al*, 2005).

Phospholipase A₂ releases arachidonic acid and potentially linoleic acid from the *sn*-2 position of cellular membrane phospholipids. They are then converted to a range of eicosanoids by the action of three principle classes of enzymes; cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 monooxygenases (Miles *et al*, 2002, Bogatcheva *et al*, 2005). Of these enzymatic pathways, the COX pathway is the most physiologically relevant in the mammalian cell, with two main isoforms being recognised; COX-1, which is present in several cell types and COX-2, which is typically induced by inflammatory stimuli and growth factors (Morita, 2002, Khanapure *et al*, 2007). Both enzymes contribute to the production of eicosanoids during inflammation. (Ricciotti and FitzGerald, 2011).

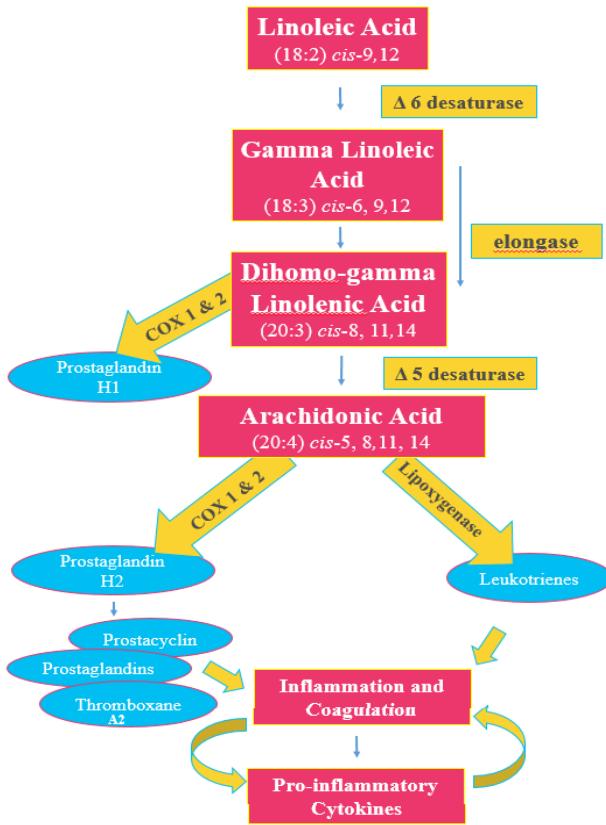


Figure 2.9. Schematic of the production of eicosanoids from linoleic acid *via* the cyclooxygenase (COX) metabolic pathway (authors own work).

The first eicosanoids formed from the cyclooxygenase metabolism of arachidonic acid are the prostanoids, namely prostaglandins and thromboxane (figure 2.9). They regulate several physiological and pathological functions, including the development and resolution of inflammation. They are also powerful mediators of vasoactivity, inducing alterations in barrier function in the endothelium (Bogatvcheva *et al*, 2005). The five bioactive prostanoids produced *in vivo* include prostacyclin (or prostaglandin I₂), prostaglandin D₂, prostaglandin E₂ and prostaglandin F_{2α} and thromboxane (Hata and Breyer, 2004, Ricciotti and FitzGerald, 2011, Bogatvcheva *et al*, 2005).

They are produced ubiquitously in several cell types, with one or two being produced more dominantly than others. They work as paracrine and autocrine lipid mediators to

maintain local homeostasis (Ricciotti and FitzGerald, 2011). They also induce several pharmacological effects regulating many physiological systems including the central nervous system, cardiovascular, gastrointestinal, genitourinary, endocrine, respiratory, and immune systems. In addition, prostaglandin synthesis has been implicated in several diseases such as cancer, cardiovascular disease, and inflammation (Hata and Breyer, 2004). In uninflamed tissues, the levels of prostanoids present is typically low, however, these levels increase dramatically in response to inflammatory stimuli. The tissue prostanoid profile is also dependent upon the differential expression of COX 1 and 2 enzymes within the specific cells present at the site of inflammation (Ricciotti and FitzGerald, 2011).

Prostanoids exert their effect through the activation of G-protein coupled receptors, composed of eight subfamily members; the E prostanoid receptor (EP) of which there are 4 subtypes, the D prostanoid receptor (DP), the F prostanoid receptor (FP), the I prostanoid receptor (IP), and thromboxane receptor (TP) (Bogatcheva *et al*, 2005, Ricciotti and FitzGerald, 2011). Prostanoid receptors couple to a range of intracellular signalling pathways that mediate the effects of receptor activation on cell function (Ricciotti and FitzGerald, 2011). These receptors operate *via* cyclic adenosine monophosphate- (cAMP), Ca^{2+} , or Rho-linked intracellular pathways (Brayer *et al*, 2001, Bogatcheva *et al*, 2005). Thromboxane A₂ and prostacyclin are the two main products of arachidonic acid metabolism *via* the COX pathways in endothelial cells and platelets (Karim *et al*, 1996). Specifically, the thromboxane and prostacyclin receptors, TP and IP respectively, regulate injury induced vascular proliferation (Bogatcheva *et al*, 2005).

2.3.1.1. Linoleic Acid and its impact on Thromboxane

Linoleic acid has been reported to have antithrombotic properties and to affect thromboxane function and production in a number of cell types, including endothelial cells (Griesmacher *et al*, 1989). Srivastava *et al*, (1895), observed a reduction in platelet thromboxane production as a result of linoleic acid (0.2mM) supplementation. Srivastava *et al*, (1895) postulated that this reduction in thromboxane was to be due to the inhibition of COX. Similar results were observed by Needleman *et al*, (1982) and MacIntyre *et al*, (1984), in linoleic acid treated platelets, suggesting that free fatty acids, such as linoleic acid may interfere with prostaglandin production (Needleman *et al*, 1982).

Thromboxane A₂ is produced during the catalysis of arachidonic acid, by COX, to form unstable products referred to as prostaglandin endoperoxides, PGG₂ and PGH₂ (Shen and Tai, 1998). These intermediates are then further metabolised by thromboxane synthase, to form thromboxane. This prostanoid is a short-lived prostanoid with a chemical half-life of approximately thirty seconds (Hamberg *et al*, 1975, Nakahata, 2008). It has been most extensively characterized for its role in modulating hemodynamics and cardiovascular function (Hata and Breyer, 2004). More specifically, it is a potent mediator of vasoconstriction and platelet aggregation, as well as inducing leucocyte adhesion and up-regulating proinflammatory cytokines (Shen and Tai, 1998, Yokoyama, *et al*, 2005)

Thromboxane A₂ is primarily produced in platelets through the action of platelet COX-1, however, it can also be produced by several other cell types, including macrophages and dendritic cells, hepatocytes and endothelial cells (Spolarics *et al*, 1984, Griesmacher *et al*, 1989, Urquhart *et al*, 2002, Kabashima *et al*, 2003, Hata and Breyer, 2004, Ricciotti and FitzGerald, 2011). The thromboxane receptor has also been reportedly expressed in several cell and tissue types, including the liver and the endothelium, suggesting, according to Nakahata (2008), that thromboxane A₂ is involved in a wide range of physiological/pathophysiological conditions.

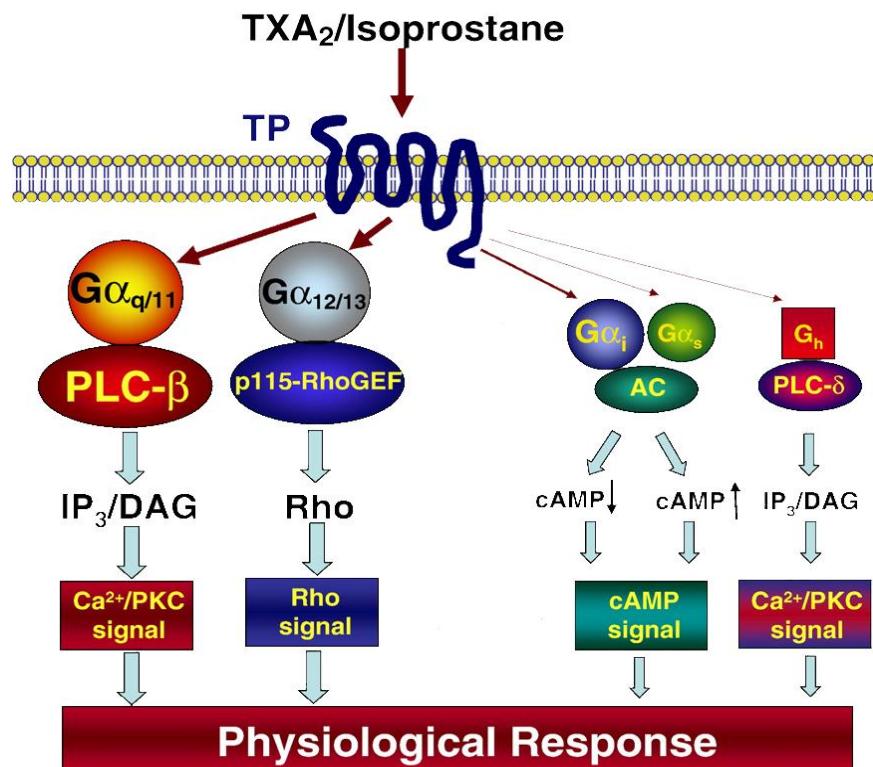


Figure 2.10. Thromboxane A₂ receptor and signal transduction *via* G protein coupling. PLC- β ; phospholipase C- β ; p115-RhoGEF, p115 guanine nucleotide exchange factor for Rho; AC, adenylyl cyclase; PLC- δ , phospholipase C- δ ; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; cAMP, cyclic adenosine monophosphate; PKC, protein kinase C; Ca²⁺ (adapted from Nakahata *et al*, 2008).

The thromboxane receptor (TP) principally mediates the activity of thromboxane A₂, however, it can also be activated by several lipid metabolites, including isoprostanes (peroxidative products of PUFAs) (Ricciotti and FitzGerald, 2011). Mediation occurs through coupling *via* multiple G-proteins, such as Gq/11, G12/13 and multiple small G proteins, as portrayed in figure 2.10. This, in turn regulates several effectors, including phospholipase C-β (PLC-β), small G protein Rho (p115-RhoGEF), adenylyl cyclase (AC) and phospholipase C-δ (Nakahata *et al*, 2008, Ricciotti and FitzGerald, 2011). Induction of these effectors results in the initiation of physiological responses such as platelet activation and vasoconstriction *via* the mobilisation and release of second messengers such as calcium (Ca₂₊) from the endoplasmic reticulum leading to Ca₂₊/protein kinase C (PKC) signalling and cAMP signalling. The Rho signalling pathway also mediates several other events including regulation of the actin cytoskeleton, cell proliferation, and apoptosis (Nakahata *et al*, 2008).

In endothelial cells, thromboxane A₂ augments the surface expression of a number of adhesion proteins, including intracellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Ishizuka, *et al*, 1998). Hunt *et al*, (1992) also demonstrated that thromboxane A₂ induces the release of prostacyclin in bovine aortic endothelial cells. As prostacyclin is a potent vasodilator and an inhibitor of platelet aggregation, (Kawabe *et al*, 2010). Hunt postulated that this represents a negative feedback mechanism to reduce the vasoconstrictive and aggregatory effects of thromboxane A₂. In the liver, thromboxane A₂ is thought to act in an autocrine or paracrine manner (Yokoyama *et al*, 2005). Fisher

et al, (1987) determined that thromboxane A₂ increased portal vein pressure as well as glucose output in isolated rat livers, indicating vasoconstriction.

2.3.2. Linoleic acid and Cytokine Production

Linoleic acid is associated with increased pro-inflammatory cytokine production. Bradley *et al*, (2008) and Li *et al*, (2015) state that obesity is linked with chronic sub-clinical inflammation and increased circulating proinflammatory cytokines such as tumour necrosis factor- α (TNF- α). According to Tripathy *et al*, (2003) and Bradley *et al*, (2008), acute increases in plasma free fatty acids trigger inflammatory and oxidative stress mechanisms that are implicated in the development of cardiovascular and metabolic disorders, such as atherosclerosis and insulin resistance as well as steatohepatitis and cirrhosis (Feldstein *et al*, 2004). Linoleic acid was also reported to induce cytokine expression and secretion in human retinal Müller cells (Capozzi *et al*, 2016).

Cytokines are protein molecules, released by activated cells that function as chemical messengers between cells of the immune, inflammatory and other systems (Brennan *et al*, 1995). They are primarily activated in response to whole bacteria or bacterial endotoxin, such as lipopolysaccharide (LPS), and play a crucial role in the regulation of inflammation and haematopoiesis (Munoz *et al*, 1991) They participate in acute and chronic inflammation *via* a complex, and sometimes contradictory, network of interactions (Turner *et al*, 2014). They are produced and consumed locally at the site of compromised or injured tissues. They are involved in the activation of components of both the innate immune system as well as specific immune responses involved in the rapid neutralization of injury (Miles *et al*, 2002).

According to Tripathy *et al*, (2003), cytokine mediated inflammation is stimulated by the cytoplasmic proinflammatory transcription factor nuclear factor- κ B (NF- κ B) present in the cytosol. Inflammatory signals, such as endotoxin, proinflammatory cytokines and linoleic acid can induce activation of NF- κ B, (Tripathy *et al*, 2003, Hennig *et al*, 2006). Once activated, NF- κ B translocates to the nucleus where it activates the transcription of genes involved in the inflammatory response, including proinflammatory cytokines, adhesion molecules, and enzymes generating reactive oxygen species (ROS) (Tripathy *et al*, 2003).

Overproduction of inflammatory cytokines can result in their accumulation in cells and tissues at the site of injury and chronic overproduction can contribute to increased systemic levels, leading to the development of several inflammatory diseases (Miles *et al*, 2002). According to Bradley *et al*, (2008), chronic low-grade inflammation has been linked to the pathology of several metabolic and cardiovascular diseases including diabetes and atherosclerosis.

2.3.2.1. Linoleic Acid and its impact on Tumour Necrosis Factor- α

One of the major cytokines, and the primary cytokine of interest in this investigation is TNF- α . Free fatty acids, such as linoleic acid, have been shown to induce TNF- α expression and secretion in several cell and tissue types. Bradley *et al*, (2008) demonstrated that palmitic acid, a saturated fatty acid, at concentrations ranging from 0.05 to 0.5mM, initiated an increase in TNF- α production in adipocytes, while Feldstein *et al*, (2004) reported that palmitic and oleic acid at concentrations ranging from 0.5mM to 1mM, induced TNF- α expression in HepG2 cells. Linoleic acid itself, has been reported

to induce TNF- α production in Caco2 intestinal cells at concentrations ranging from 0.25mM to 1mM, according to Li *et al*, (2015), indicating that linoleic acid may contribute to cytokine mediated induction of inflammation.

TNF- α , an endotoxin-induced glycoprotein, is found in a soluble and membrane bound form. The soluble plasma form of TNF-alpha is cleaved from the membrane forms by a metalloproteinase termed TNF-alpha-converting enzyme (TACE) (Josephs *et al*, 2018).

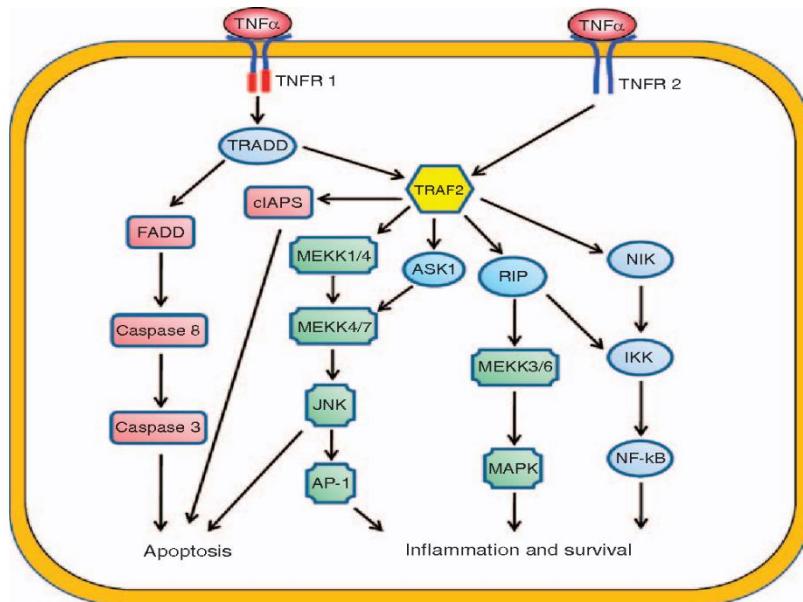


Figure 2.11. Schematic diagram of the downstream signalling pathways of TNF- α . The TNF- α receptors can activate different pathways to induce apoptosis, cell survival or inflammation. TNF- α induces apoptosis by binding caspase-8 to fas activated death domain (FADD) and promotes inflammation and survival, which is mediated through TNF receptor associated factor (TRAF2) via C-Jun-terminal kinase (JNK)-dependent kinase cascade, mitogen activated protein kinase kinases referred to as MEKK kinase cascade and NF- κ B activation by receptor interacting protein (RIP). cIAPS; cellular inhibitors of apoptosis proteins. MAPK; mitogen activated protein kinase AP-1; activator protein-1 (Wu and Zhou, 2010).

It is a potent inflammatory mediator that is central to the inflammatory action of the innate immune system. It has several roles, including stimulating cell proliferation, inducing cytolytic or cytostatic activity against tumour cells, and causing inflammatory, antiviral, and immunoregulatory effects (Turner *et al*, 2014). While TNF- α plays a role in the

activation of NF-κB, one of the key regulators of inflammation (Tripathy *et al*, 2003, Marchix *et al*, 2015), its gene expression is, in turn, regulated at the transcriptional level by NF-κB as well as other factors (Parameswaran and Patial, 2010). Toborek *et al*, (2002) suggest that TNF- α uses NF-κB to amplify its own signalling.

While the cytokine is typically produced by activated macrophages, T-lymphocytes, and natural killer (NK) cells (Parameswaran and Patial, 2010, Josephs *et al*, 2018), its expression and secretion has also been reported in a number of other cell types, including hepatocytes (Stonans, *et al*, 1999, Gutierrez-Ruiz *et al*, 1999), intestinal Caco2 cells (Li *et al*, 2015) and the endothelial cell line HUVEC (Imaizumi *et al*, 2000).

It mediates its affect through two primary cell surface receptors; the pro-apoptotic TNF-R1 and the anti-apoptotic TNF-R2 (Josephs *et al*, 2018). These TNF-R receptors are found on almost all nucleated cell types (Cambien *et al*, 2003). According to Josephs *et al*, (2018), in many systems, TNF-α promotes apoptosis through TNF-R1, which is expressed on various tumour cells, but induces survival signalling through TNF-R2, expressed on various immune cells.

Activation from TNF-R1 is responsible for a several inflammatory responses attributed to TNF-α, including cytotoxicity, NF-κB activation, and upregulation of adhesion and cytokine genes. TNF-R1, is also involved in the activation of several kinases such as apoptosis signalling kinase-1 (ASK-1) and C-Jun-terminal kinase (JNK) as well as pro-apoptotic signalling *via* the Fas-associated death domain (FADD) (Parameswaran and Patial, 2010).

In recent years, new evidence has indicated that the activation of the immune system has strong influences on blood coagulation and thrombus formation (Swystun and Liaw, 2016). Increased TNF- α levels have been associated with the activation of platelets (Page *et al*, 2018). TNF- α has been reported to promote coagulation by increasing the shedding of the protein C receptor and inhibiting thrombomodulin production, inducing complement activation and stimulating the production of tissue factor by endothelial cells (Page *et al*, 2018, Saha and Smith, 2018). Page *et al*, (2018) observed that “low” levels of TNF- α resulted in platelet activation and clumping. While the literature indicates that TNF- α may augment coagulation *via* upregulation of tissue factor expression in cultured endothelial cells (Esmon, 1999, Esmon, 2000), in contrast, it has also been historically reported to inhibit thrombus formation (Cambien *et al*, 2003). Cambien *et al*, (2003) postulated that TNF- α , at concentrations similar to those seen in sepsis (1ng/mL) inhibited thrombus formation *via* the TNF receptors, posing the hypothesis that TNF- α may be procoagulant but not prothrombotic. It may be postulated that linoleic acid induced TNF- α secretion may also have anticoagulatory properties.

TNF- α has also been shown to induce reactive oxygen species (ROS) (Chen *et al*, 2008, Page *et al*, 2018) as well as expression of superoxide dismutase (SOD) in several cell types (Kuratko and Constante, 1998).

2.4. Linoleic Acid and the Production of Reactive Oxygen Species

Long-chain fatty acids, such as linoleic acid, as well as their derivatives and metabolites, namely arachidonic acid, can modify intracellular production of reactive oxygen species (ROS). ROS, mediators of oxidative stress, play an important role in the transduction of

both physiological and pathophysiological signalling and the progression of inflammatory disorders (Mittal *et al*, 2014, Forrester *et al*, 2018). ROS are reactive intermediates of molecular oxygen produced as by-products of numerous enzymatic reactions. They are produced as part of basal metabolic function, in various cell compartments, such as the cell membrane, cytoplasm, mitochondria, endoplasmic reticulum (ER), and peroxisome, (Forrester *et al*, 2018). According to Forrester *et al*, (2018), depending on the source, cell type and tissue environment, ROS may contribute to normal physiological processes or impaired responses that leads to metabolic dysfunction and inflammatory signalling.

Dröge (2001) and Incalza *et al*, (2018) states that at physiological concentrations ROS act as important second messengers, transducing intracellular signals involved in various biological processes, including the maintenance of redox homeostasis. However, elevated ROS levels are thought to contribute to the onset of several diseases associated with inflammatory signalling and metabolic dysfunction such as obesity, atherosclerosis, diabetes mellitus, and stroke (Forrester *et al*, 2018, Incalza *et al*, 2018), as well as NAFLD (Ore and Akinloye, 2019), neurodegenerative diseases and cancer (Das, 2011, Barrera, 2012).

Several cellular organelles are involved in ROS production. The contribution of each organelle to the total cellular ROS production is considerable and varies between cell type (Yoboue *et al*, 2018). Forrester *et al*, (2018), states that cytosolic ROS are primarily produced through the activity of the membrane bound enzyme complex, nicotinamide adenine dinucleotide phosphate [NADPH] oxidase (NOX). Fatty acids such as arachidonic acid have been reported to directly activate NOX production of ROS, while the oxidation of both linoleic acid and arachidonic acid, by LOX and COX, in the

production of eicosanoids is also thought to produce ROS (Hatanaka *et al*, 2006, Morgan *et al*, 2007, Kim *et al*, 2008, Hatanaka *et al*, 2013).

Mitochondrial ROS are produced as typical by-products of the electron transport chain (ETC) during mitochondrial respiration and metabolic enzymatic activity (Forrester *et al*, 2018) and can also be produced during β -oxidation of fatty acids (Schönfeld and Wojtczak, 2008). According to Das (2011) and Masarone *et al*, (2018) they are generated as a result of electron leakage during the electron transport steps of ATP production. Forrester *et al*, (2018) states that increased mitochondrial ROS generation as a result of dysregulated enzymatic activity and cellular stress can influence metabolic pathways such as Krebs cycle, fatty acid synthesis and ATP generation.

Other organelles such as peroxisomes and the endoplasmic reticulum are also major sources of ROS. Peroxisomal ROS are produced as by-products of enzymatic reactions within fatty acid β -oxidation and protein synthesis while endoplasmic reticulum ROS are produced during protein folding, resulting in the production of hydrogen peroxide

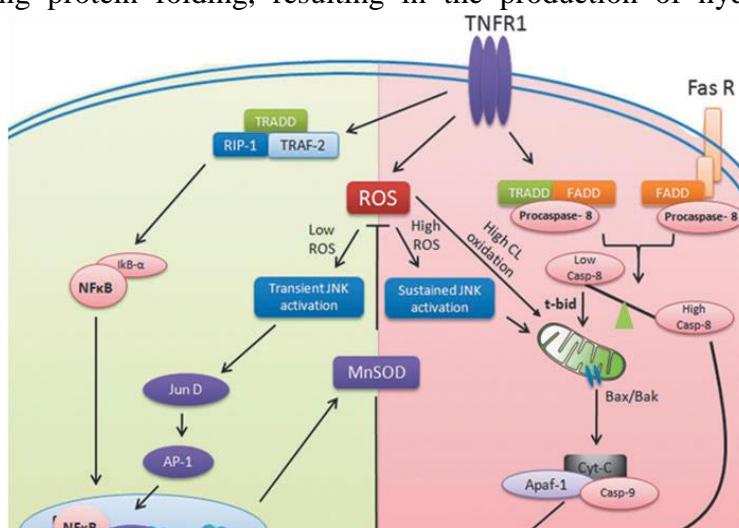


Figure 2.12. Schematic representation of ROS-mediated cell death and cell survival signalling pathways. The balance between apoptosis and cell survival is modulated by intracellular ROS generation. TRADD; TNFR1-associated death domain. RIP-1; receptor-interacting kinase-1. (Mittal *et al*, 2014).

(Forrester *et al*, 2018, Yoboue *et al*, 2018). According to Masarone *et al*, (2018) endoplasmic reticulum ROS accounts for approximately 25% for all cellular ROS generation. Metabolic pathways involving cyclooxygenases and lipoxygenases are also potential sources of ROS (Gloire *et al*, 2006).

Free fatty acid production of ROS have been linked to the initiation, progression, and resolution of the inflammatory response (Artwohl *et al*, 2003, Chelombitko, 2018). These ROS are involved in the activation of several mediators of inflammation, such as NF- κ B, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) in response to various agonists (Artwohl *et al*, 2003, Hatanaka *et al*, 2013).

Canty *et al*, (1999) and Mittal *et al*, (2014) suggest that low levels of intracellular ROS have been shown to activate NF- κ B, initiating the expression of various adhesion molecules and enhancing neutrophil binding on the endothelium (Mittal *et al*, 2014), as well as inducing the expression of genes regulated by NF- κ B (Toborek *et al*, 1996), initiating inflammatory yet survival signalling pathways. ROS are also proposed to play a critical role in TNF- α mediated JNK activation (Shen *et al*, 2006), Gloire *et al*, 2006). TNF- α has been shown to increase intracellular ROS, leading to the impairment of NF- κ B activation and the prolonged activation of JNK, resulting in apoptosis. NF- κ B regulates the expression of a number of antioxidants responsible for the clearance of ROS, while JNK, in turn, further promotes ROS production (Shen *et al*, 2006). This suggests that ROS may enhance TNF- α induced apoptosis through suppression of the antiapoptotic NF- κ B while activating the proapoptotic JNK (Shen *et al*, 2006, Blaser *et al*, 2016).

Free fatty acids have been reported to increase ROS production in several cell types. Inoguchi *et al*, (2000) observed an increase in ROS production in both smooth muscle vascular cells and endothelial cell after supplementation of 0.2mM of palmitate *via* PKC stimulated NOX. Hatanaka *et al* (2006) reported an increase in both intracellular and extracellular ROS in neutrophils supplemented with 0.2mM of either oleic, linoleic or γ -linolenic acids, while Hatanaka *et al* (2013), also observed increased production of ROS, specifically superoxide, in fibroblast cell lines, treated with oleic, linoleic and γ -linolenic acids, also *via* stimulation of NOX. Morgan *et al*, (2007) observed increased ROS in pancreatic islet cells after incubation with 0.1mM of palmitic acid.

While fatty acid metabolism may induce the production of ROS, ROS are also thought to interact with fatty acids, inducing lipid peroxidation. According to Barrera *et al*, (2012) reactive intermediates, produced by oxidative stress, can alter the membrane bilayers and cause the lipid peroxidation of polyunsaturated fatty acids. This peroxidation can induce changes in membrane permeability and fluidity, altering cellular integrity (Barrera *et al*, 2012).

2.4.1. The Superoxide Anion and Superoxide Dismutase

There are three primary species of ROS; the superoxide anion, hydrogen peroxide and the hydroxyl radical (Collin, 2019). ROS, specifically the superoxide anion, is generated rapidly, by one-electron reduction of molecular oxygen through enzymatic catalysis by NOX, xanthine oxidase, or during electron transfer reactions in the electron transport chain (ETC) within mitochondria or *via* cytochrome P450 (Mittal *et al*, 2014).

The superoxide anion is the first to be generated and is responsible for the production of a number of other ROS, including hydrogen peroxide (Incalza *et al*, 2018). Free fatty acids, such as γ -linolenic, linoleic and oleic acids have all been reported to increase production of extracellular superoxide anion. Interestingly, the increase in superoxide production was positively correlated to the number of double bonds present on each fatty acid molecule (Hatanaka *et al*, 2013).

The super oxide anion can react with nitric oxide, an endothelium derived relaxing factor, in the presence of arginine and NOX to form peroxy nitrate, a damaging ROS that can cause lipid peroxidation, protein oxidation, protein nitration and enzyme inactivation (Ighodaro and Akinloye, 2018). The superoxide anion, due to its lipophilicity and redox potential, can also initiate lipid peroxidation (McCord, 2008, Ighodaro and Akinloye, 2018).

Mammalian cells have evolved to reduce and prevent the damaging effects of ROS by developing an array of antioxidant defence systems, referred to as ROS scavengers, that function to remove the oxidants. These include superoxide dismutase (SOD) which dismutes the superoxide anion to hydrogen peroxide, and glutathione peroxidase and catalase which converts hydrogen peroxide to water (Mittal *et al*, 2014, Younus, 2018).

The SOD antioxidants are the first line of defence against ROS mediated tissue injury (Younus, 2018). They are a group of metalloenzymes of which there are three main isoforms, cytosolic or copper zinc SOD (CuZnSOD) (SOD-1), mitochondrial or manganese (MnSOD) (SOD-2) and extracellular CuZnSOD (SOD-3), are essential for the prevention of superoxide anion-induced damage to various tissues (Das, 2011). These

enzymes catalyse the same reaction, converting the superoxide anion through the alternate reduction and re-oxidation of copper for SOD-1 and SOD-3 and manganese in the case of SOD-2 (Mondola *et al*, 2016). Cytosolic SOD is ubiquitous in mammalian cells, not only in the cytosol, but also in the nucleus, peroxisomes and mitochondrial intermembrane space (Choi *et al*, 2011). According to Mondola *et al*, (2016) it is also expressed at relatively high levels in blood vessels. Mondola *et al*, (2016) states that the activity of cytosolic SOD, in normal mouse aorta, accounts for 50 to 80% of total SOD activity, with similar patterns of expression reported in human arteries.

Deficiencies and alterations in SOD have been associated with a number of pathologies observed in both animals and humans (Ighodaro and Akinloye, 2018). Deficiencies in cytosolic SOD have been reported to increase erythrocyte susceptibility to oxidative stress resulting in anaemia and compensatory activation of erythropoiesis (Iuchi *et al*, 2007). According to Choi *et al*, (2011) SOD interactions with lipid molecules, such as dipalmitoyl-phosphatidylcholine, can induce the production of cytotoxic SOD aggregates. Kim *et al*, (2005) reported that unsaturated fatty acids, such as oleic acid, bound directly to SOD-1. Kim *et al*, (2005) proposed that fatty acid binding may affect SOD conformation leading to the formation of cytotoxic granular SOD aggregates. Alterations and aggregation of SOD-1 have been associated with the pathogenesis of amyotrophic lateral sclerosis (Kim *et al*, 2005, Choi *et al*, 2011)

It could be hypothesised that fatty acids, through their effects on ROS generating enzyme complexes such as NOX, may contribute to an increase in intracellular ROS and the pro-oxidant state observed in several lipid mediated inflammatory disorders. Lipid molecules,

such as linoleic acid, may also impact the effectiveness of intracellular antioxidant defence mechanisms.

2.5. Linoleic Acid and Apoptosis, Necrosis and Necroptosis

Over-accumulation of intracellular free fatty acids, such as linoleic acid, can induce cell death (Hawkins *et al* 1998, Wu *et al*, 2008, Zhang *et al*, 2012). Many signalling pathways and factors that are activated by inflammation are involved in the regulation of cell death (Yang *et al*, 2015). Cell death manifests with macroscopic morphological alterations (Galluzzi *et al*, 2018). Two forms of cell death have been well documented in the literature: apoptosis and necrosis. Apoptosis, or programmed cell death, is a form of highly regulated cell death, increasingly recognized for its pathogenic role in several diseases, including liver diseases (Malhi and Gores, 2008). Necrosis is an unregulated form of cell death primarily resulting from acute cell trauma (Singh *et al*, 2019). During apoptosis, cells shrink with integral but “ruffling” plasma membranes, and nuclei are condensed and fragmented, while necrosis involves the swelling of cells, leading to plasma membrane rupture and the release of cellular components (Nagata, 2018).

2.5.1. Linoleic Acid and Apoptosis

Normal physiological conditions require the clearance and replacement of damaged, dysfunctional or unnecessary cells *via* programmed or regulated cell death, referred to as apoptosis (Singh *et al*, 2019). Intracellular lipid signalling can induce fatty acid mediated cell death under normal physiological conditions (Magtanong *et al*, (2016). However, excessive dietary lipids have been reported to induce apoptosis in several cell lines including endothelial cells, hepatic cells, and pancreatic β-cells (Artwohl *et al*, 2003,

Vecchini *et al*, 2004, Zhang *et al*, 2012). Linoleic acid has triggered apoptosis in Human Umbilical Vein Endothelial Cells (HUVEC) and in rat hepatoma cells at concentrations of 0.25mM (Artwohl *et al*, 2003, Zhang *et al*, 2012).

According to Galluzzi *et al*, (2018), apoptosis occurs for two primary reasons. Firstly, it operates as a built-in effector of physiological programs for normal development and tissue turnover. Secondly, disturbance of the intracellular or extracellular microenvironment will initiate apoptosis, when such disturbance is too intense or prolonged for adaptative responses to restore cellular homeostasis (Galluzzi *et al*, 2018)

During apoptosis, cells will present with cytoplasmic shrinkage, chromatin condensation or pyknosis, nuclear fragmentation or karyorrhexis, plasma membrane blebbing and externalisation of phosphatidylserine, concluding with the formation of intact small

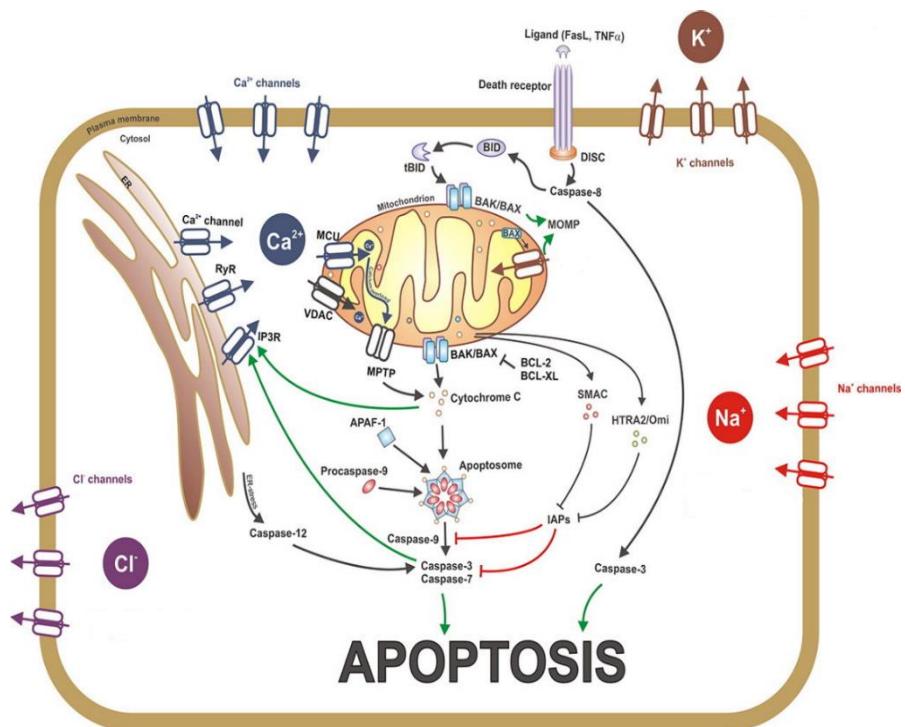


Figure 2.13. Schematic representation of the extrinsic and intrinsic apoptotic pathways. Second mitochondria-derived activator of caspases; SMAC. high-temperature requirement protein A2; HTRA2. Inhibitors of apoptosis proteins; IAPs (adapted from Kondratskyi *et al*, 2015).

vesicles referred to as apoptotic bodies (Yang *et al*, 2015, Galluzzi *et al*, 2018, Kale *et al*, 2018). These apoptotic bodies are then efficiently taken up by neighbouring cells with phagocytic activity and degraded within lysosomes. Initiation, propagation and execution of apoptosis involves a complex array of signal transduction modules that fall under two pathways, intrinsic apoptosis and extrinsic apoptosis, as portrayed in figure 2.13 (Galluzzi *et al*, 2018, Singh *et al*, 2019).

The intrinsic apoptotic pathway is initiated by a variety of microenvironmental perturbations, such as DNA damage, endoplasmic reticulum stress, excessive production of ROS, overload, replication stress and mitotic defects that occur within the cell (Kondratskyi *et al*, 2015, Galluzzi *et al*, 2018). This pathway involves mitochondrial mediated apoptosis, under the control of mitochondrial B cell lymphoma (BCL-2) pro-enzymes (Ouyang, *et al*, 2012). The BCL-2 family of proteins, key regulators in mitochondrial mediated apoptosis, consist of both pro-apoptotic and anti-apoptotic enzymes (Galluzzi *et al*, 2018, Singh *et al*, 2019). They control cell death primarily by direct binding interactions that regulate mitochondrial outer membrane permeabilization leading to the irreversible release of intermembrane space proteins, subsequent caspase activation and apoptosis (Kale *et al*, 2018). Each member of this family contains one or more BCL-2 domains (BH), BH-1 to BH-4.

Apoptosis is induced by BH-3 only activator proteins, such as BCL-2-interacting mediator of cell death (BIM), BH3-interacting domain death agonist (BID) and p53-upregulated modulator of apoptosis (PUMA). These proteins that bind and activate either or both of the pro- apoptotic proteins BCL-2-associated X protein (BAX) or BCL-2 antagonist/killer (BAK). The activation of BAX or BAK at the mitochondrial surface results in the

formation of macropores in the mitochondrial membrane, causing mitochondrial outer membrane permeabilization (Singh *et al*, 2019). Lipids such as sphingolipids are required as co-factors for BAX and BAK mediated mitochondrial outer membrane permeabilization (Magtanong *et al*, 2016). According to Artwohl *et al*, (2003) the pro-apoptotic properties of free fatty acids, including linoleic acid, correlate with the expression of BAK in HUVEC cells. Mitochondrial outer membrane permeabilization results in the release of pro-apoptotic proteins and cytochrome C from the mitochondrial intermembrane space into the cytosol (Kale *et al* 2018). Oxidation of mitochondrial cardiolipins containing, linoleic acid, promotes the release of cytochrome C and other key apoptotic effectors from the mitochondria into the cytosol (Magtanong *et al*, 2016, Fajardo *et al*, 2017). Cytochrome C binds to apoptotic protease activating factor 1 (APAF1), leading to the formation of the apOTOSOME, a caspase activation platform (Tait and Green, 2010, Singh *et al*, 2019).

The apOTOSOME activates the initiator caspase, caspase 9, which together with caspase 8 and 10, process and activate the effector caspases 3, 6 and 7 (Yang *et al*, 2015, Kale *et al*, 2018, Singh *et al*, 2019). Oxidised PUFAs such as docosahexaenoic acid and linoleic acid have been reported to induce apoptosis *via* caspase activation in human monocytic leukaemia cells (Iuchi *et al*, 2019). These effector caspases cleave several substrates required for cellular homeostasis, resulting in the biochemical and morphological features of apoptosis, namely exposure, nuclear condensation, membrane blebbing and genomic DNA fragmentation (Yang *et al*, 2015, Kale *et al*, 2018).

The extrinsic pathway is initiated by extracellular disturbances in the microenvironment and is mediated by pro-apoptotic ligands binding to and activating death receptors (Tait

and Green, 2010, Kondratskyi *et al*, 2015, Galluzzi *et al*, 2018). Death receptors include Fas cell surface death receptor (FAS, also known as CD95 or APO-1), and TNF receptors. Death receptor ligation induces the recruitment of adaptor molecules, such as FAS-associated death domain (FADD) proteins. FADD bind to and activate caspase 8, which then directly activates the executioner caspases 3 and 7, which, in some cell types (type I) is enough to induce apoptosis, in the absence of mitochondrial outer membrane permeabilization. In other cell types (type II), crosstalk between the extrinsic and intrinsic pathways are required, with caspase 8 activating BID, and, in turn, mitochondrial outer membrane permeabilization, leading back to mitochondrial mediated apoptosis (Tait and Green, 2010).

2.5.2. Linoleic acid and Necrosis/Necroptosis

According to Escobar *et al* (2015) and Yang *et al*, (2015), necrosis is the primary form of “accidental” cell death caused by inflammation, occurring as a result of very harsh physicochemical stimuli, including abrupt changes in temperature, osmotic pressure, or pH. Cury-Boaventura *et al*, (2005) states that free fatty acid doses close to physiological serum concentrations (~0.015mM) can cause cell death by apoptosis however, at larger concentrations, they can induce necrosis. Necrosis has been observed in human B-lymphocytes treated with oleic and linoleic acid (0.1mM) (Cury-Boaventura *et al*, 2005).

Necrosis is characterised morphologically by generalized swelling of cell membranes and organelle membranes, leading to the disruption of the plasma membrane integrity and release of intracellular contents into the extracellular space. This induces inflammatory and autoimmune reactions to occurring in neighbouring cells and tissues (Escobar *et al*, 2015).

While necrosis is thought to be an unregulated form of cell death, several authors indicate regulated pathways of necrosis exist, with necroptosis being the most studied to date (Yang *et al*, 2015, Escobar *et al*, 2015, Galluzzi *et al*, 2018).

According to Galluzzi *et al*, (2018), mitochondria permeability transition (MPT) necrosis is a form of regulated cell death, involving the manifestation of morphological characteristics of necrosis. The term MPT refers to an abrupt loss of the integrity resulting in increased permeability of the inner mitochondrial membrane, resulting in the rapid dissipation of mitochondrial membrane potential, osmotic breakdown of both mitochondrial membranes and cell death (Galluzzi *et al*, 2018). According to Cury-Boaventura *et al*, (2005), this can lead to mitochondrial depolarisation, which can be initiated by linoleic acid. Oxidative stress and cytosolic calcium ion overload in the cytosol, mediated by endoplasmic reticular stress, also constitute major etiological determinants of MPT driven necrosis (Galluzzi *et al*, 2018)

Necroptosis, initiated by perturbations of either the extracellular or intracellular microenvironment detected by specific death receptors, is a form of regulated cell death that also demonstrates necrotic morphological characteristics (Galluzzi *et al*, 2018). Necroptosis mediated cell death critically depends on, and is defined by, the sequential activation of the receptor-interacting protein kinase (RIPK) complex RIPK3, induced by death receptors such as TNF-R1, TNF-R2 and Fas (Yang *et al*, 2015, Galluzzi *et al*, 2018).

According to Galluzzi *et al*, (2018), engagement of the death receptor TNF-R1 in particular appears to be the trigger for RIPK3 activation. The biological outcome of TNF-R1 signalling pathways span from cell survival and activation to a number of subroutines of regulated cell death, depending on cellular intrinsic (intracellular protein expression) or extrinsic (receptor stimulation and duration) factors. Some of these pathways are represented in figure 2.14. In necroptosis, TNF-R1 activation of RIPK3 induces

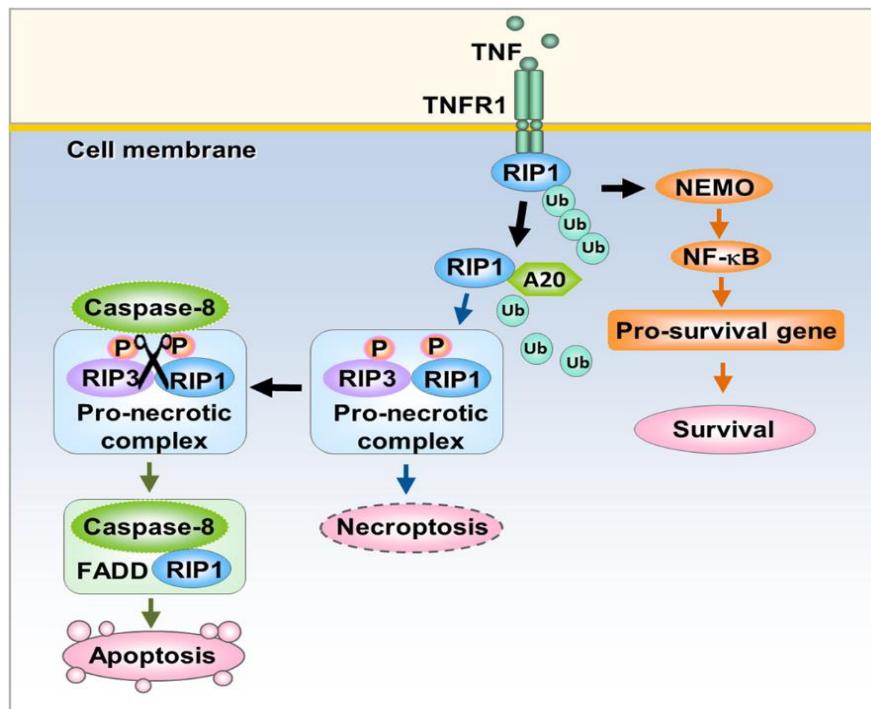


Figure 2.14. Schematic representation of TNF receptor signalling and regulation of cell death and cell survival. (Yang *et al*, 2015).

phosphorylation of an effector referred to as mixed-lineage kinase domain like (MLKL) (Weber *et al*, 2018), MLKL then translocates to the plasma membrane, where it induces permeabilization and modifies sodium and calcium influx, increasing osmotic pressure resulting in membrane rupture and death (Yang *et al*, 2015, Galluzzi *et al*, 2018, Weber *et al*, 2018).

Lipids are essential signalling molecules required for the physiologically normal activity of regulated cell death. However, elevated levels of free fatty acids can be cytotoxic, inducing cell death by both apoptosis and necrosis. Cnop *et al*, (2001) suggests that excessive free fatty acids may destabilise cellular membranes and induce rapid necrosis.

2.6. The Haemostatic System

Haemostasis consists of a complex array of interrelated processes, necessary for the maintenance of blood flow and volume under normal physiological conditions. It is also responsible for the preservation of blood volume and restoration of vascular integrity under physiological duress such as trauma (Austin, 2017). The process involves intricate interplay between four major components; vascular endothelium, platelets, the coagulation cascade and fibrinolysis or regulators of coagulation (Austin, 2017, Lassen and Swardson, 1995). A delicate balance between coagulant, anticoagulant, fibrinolytic and anti-fibrinolytic activities must be maintained for successful clot formation to be achieved. Dysfunction in either, or both, the coagulation or fibrinolytic systems can result in pathological thrombosis or haemorrhage, both a frequent complication of surgery, trauma or illness (Epstein, 2014, Austin, 2017).

In order to understand the potentiation of disorders of the haemostatic system it is essential to understand the “normal” mechanism by which a blood clot is formed (Lassen and Swardson, 1995). Mammals maintain normal haemostasis *via* three interrelated processes. Primary haemostasis involves the adhesion, activation and aggregation of platelets. Secondary haemostasis results in the formation of a fibrin meshwork or clot and finally, fibrinolysis results in the degradation of the fibrin clot (Epstein, 2014).

2.6.1. Endothelium

The role of the endothelium is multifaceted with the vascular endothelium being described by Sira and Eyre (2016) as being a fine cellular monolayer lining the circulatory system. As well as providing a physical barrier between blood components and reactive sub-endothelial structures, endothelial cells produce inhibitors of coagulation and platelet aggregation (Austin, 2017). Upon initial tissue injury, the integrity of the endothelial basement membrane is disrupted and prothrombotic haemostatic factors such as collagen, von Willebrand factor (vWF), fibronectin and tissue factor (TF) are released, initiating the coagulation cascade. The endothelium also modulates fibrinolysis through the production of activators such as tissue plasminogen activator (tPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1) (Austin, 2017, Sira and Eyre, 2016).

2.6.2. Primary Haemostasis, Secondary Haemostasis and Fibrinolysis

Primary haemostasis is the formation of the initial platelet plug (Cox *et al*, 2013, Austin, 2017). Upon vascular injury, P-selectin, a cell adhesion protein produced by the α -granules of platelets (Palta *et al*, 2014), binds to exposed sub-endothelial collagen, laminin or fibronectin and released vWF to promote platelet adhesion and aggregation (Cox *et al*, 2013, Epstein, 2014). Exposure to collagen promotes platelet activation, resulting in the release of various coagulation factors (Epstein, 2014). Phospholipase A₂ is activated, releasing arachidonic acid from the phospholipid membrane leading to its conversion, *via* COX, to thromboxane A₂ (Austin, 2017). Morphological changes also occur upon platelet activation, with cells becoming globular with prominent pseudopodia (Cox *et al*, 2013). Thromboxane A₂ promotes further aggregation resulting in the formation of a temporary

platelet plug (Palta *et al*, 2014). The formation of this plug is limited by maintaining a negatively charged surface to repel negatively charged platelets (Epstein, 2014). Further platelet adhesion and activation are also inhibited by the production of prostacyclin by healthy endothelial cells (Epstein, 2014), providing a counter-balance to the effects of thromboxane A₂ and maintaining vessel lumen patency (Palta *et al*, 2014).

Secondary haemostasis involves a series of enzymatic reactions including various clotting factors that result in the production of thrombin, cleaving fibrinogen to fibrin and forming a fibrin meshwork (Epstein, (2014)). It occurs in three steps: initiation, amplification and propagation (Epstein, 2014, Sira and Eyre, 2016, Austin, 2017).

Initiation (extrinsic) occurs when TF, a glycoprotein expressed on the surface of exposed or inflamed endothelial cells or fibroblasts, binds with circulating or activated factor VIIa forming a TF-VIIa complex. (Epstein, 2014, Sira and Eyre, 2016). This complex then activates factor X and factor IX, in the presence of factor V, resulting in the production of factor Xa and factor IXa. Factor Xa binds to prothrombin to produce a small amount of thrombin (Sira and Eyre, 2016).

Amplification (intrinsic) occurs when this small quantity of thrombin activates more platelets and in turn, more clotting factors (i.e. factors V, VIII and XI are activated to Va, VIIIa and XIa on the platelet surface) resulting in the production of more thrombin (Epstein, 2014, Sira and Eyre, 2016). According to Park and Koh, (2018) appears to have association with inflammation and innate immunity.

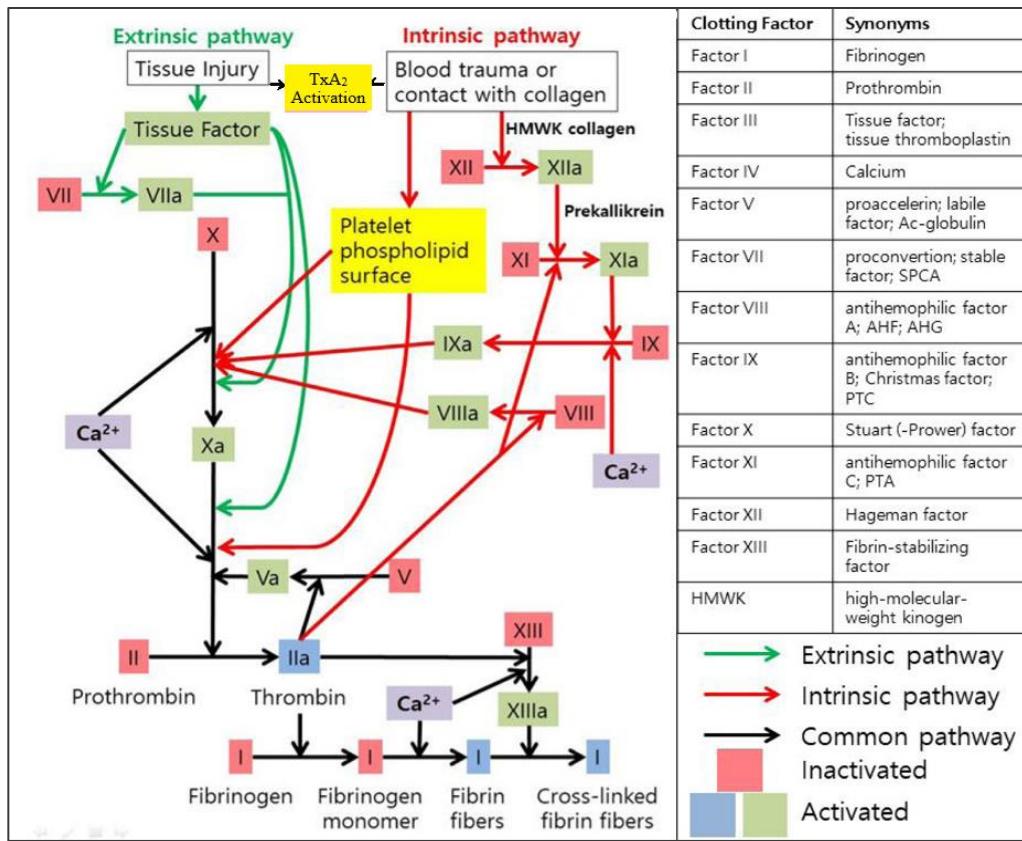


Figure 2.15. Schematic of the coagulation cascade. The main role of extrinsic pathway follows green arrows. The intrinsic pathway follows red arrows. The common pathway follows the black arrows. SPCA, Serum prothrombin conversion accelerator; AHF, antihemophilic factor; AHG, antihemophilic globulin; PTC, plasma thromboplastin component; PTA, plasma thromboplastin antecedent (Park and Koh, 2018).

During the propagation phase, previously generated factor IXa forms a complex with factor VIIIa and together they activate factor X on the platelet surface to produce factor Xa (Epstein, 2014). Factor Va interacts with this newly formed factor Xa to form much greater amounts of thrombin producing what is referred to as the thrombin “burst” in the pathway referred to as the common pathway (Sira and Eyre, 2016). This thrombin is then sufficient to cleave fibrinogen to form fibrin, producing a stable biopolymer that strengthens and adds stability to the initial platelet plug (Sira and Eyre, 2016). A detailed schematic view of the coagulation cascade is provided in figure 2.15.

Secondary haemostasis is also regulated by several circulating and localised anticoagulants (Epstein, 2014), in order to prevent thrombosis at the site of injury (Austin, 2017). Naturally occurring anticoagulants include antithrombin (AT), the main inhibitor, which inhibits the effects of thrombin and factors IXa, Xa, XIa and XIIa (Palta *et al*, 2014). AT activity is greatly potentiated by heparin, located on the surfaces of healthy endothelial cells (Epstein, 2014, Austin, 2017). The TF-Pathway Inhibitor inhibits the TF-VIIa complex formation, in turn preventing the production of thrombin (Sira and Eyre, 2016). Interestingly, thrombin, when complexed to endothelial surface thrombomodulin, an integral membrane protein, acts as a potent anticoagulant by activating Protein C (Austin, 2017). Protein C, in turn, proteolytically inactivates Factors Va and VIIIa, further inhibiting thrombin production.

Fibrinolysis, the final stage in the wound healing process, according to Sira and Eyre (2016) runs parallel to the coagulation cascade, with the primary aim of localising and limiting clot formation. The process involves a series of tightly regulated enzymatic steps in which plasminogen is cleaved by tPA to create plasmin (Epstein, 2014). Plasmin, in turn, hydrolyses the arginine and lysine bonds of fibrinogen and fibrin resulting in clot degradation (Austin, 2017). According to Austin (2017), these degradation products also work to inhibit thrombin and fibrin polymerisation, preventing further clot formation. Fibrinolysis is also regulated with PAI-1 inhibiting the actions of tPA while plasmin is inactivated by α 2-antiplasmin (Austin, 2017).

While haemostasis is tightly regulated, the complexity of the process means that a dysfunction at any level of the coagulation or fibrinolytic systems could result in

haemorrhagic or thrombotic conditions with associated morbidity and mortality (Epstein, 2014, Austin, 2017).

2.6.3. Haemostasis and Coagulopathies in the Equine

Epstein (2014) describes the haemostatic system of the equine very much as is outlined in section (2.6.2). In the equine, platelets are round, oval or elongated and measure 2.5–3.5 μm in diameter. In the normal healthy equine, platelet count is 100,000 platelets/ μL with a count below this considered an indication of thrombocytopenia (Satue *et al*, 2017). Byars *et al*, (2003) indicate that normal values in the equine for PT are between 15 and 20 seconds while aPTT values range from 131 to 199 seconds. In humans, PT values of 10.7 to 13.8 seconds and aPTT values of 25.4 to 27.7 seconds have been reported by Geffre *et al*, (2010) and Zaar *et al* (2014). Comparatively, in the bovine, PT ranges are reported to be 20 to 30 seconds while aPTT ranges are 25 to 45 seconds (Heuwieser *et al*, 1989). In the canine, PT ranges between 6.8 and 9.9 seconds while aPTT ranges between 12.8 and 17.4 seconds (Geffre *et al*, 2010). According to Byars *et al*, (2003) equines harbour a comparatively lethargic coagulation system and have a tendency to produce clots more slowly than other domestic mammals, as indicated by relatively longer aPTT values. As such, haemorrhagic and thrombotic disorders in the equine are of intense importance for the successful management of several primary clinical compromises that affect blood coagulation (Byars *et al*, 2003). Comparison studies were carried out by Bell *et al*, (1955) on horses without any haematological disorders. These studies revealed poor clot reaction times in horses with relatively normal platelet counts by human standards. Bell *et al*, (1955) also indicated that what would be considered a deficiency in anti-haemophilic globulin or coagulation factor VIII by human standards, appears to be normal

in the horse. Interestingly, the prolonged aPTT observed in the horse, according to Lassen and Swardson (1995), is indicative of a factor VIII problem within the coagulation system. Bell *et al*, (1955) also indicated that clotting times and thromboplastin generation in the horses studied in their research correlated to that of moderately severe human haemophiliacs. Spontaneous haemorrhage is unusual in the equine, and, as such, Bell *et al*, (1955) proposed that a compensatory mechanism, for example, an increased thromboplastic activity, must naturally exist in the horse to account for the rarity of haemorrhagic conditions. As the literature implies, haemostasis is a complex process (Lassan and Swardson, 1995), and the anomalies in the equine haemostatic system as described by Bell *et al*, (1955), Lassan and Swardson (1995) and Byars *et al*, (2003) only adds to its complexity. As such, even minor disruptions to the process could result in coagulopathy.

Coagulopathies, or abnormal haemostasis, can occur as a result of hypocoagulation, resulting in a bleeding diathesis, or hypercoagulation, resulting in thrombosis (Epstein, 2014). In equines, hereditary conditions that result in hypocoagulation are rare (Epstein, 2014) and conditions that result in haemorrhage are most likely caused by trauma (Lassen and Swardson, 1995), whereas thrombotic disorders are more common (Ness, 2014, Byars *et al*, 2003).

In cases where trauma is not evident, many clinical findings in horses with coagulopathies, according to Epstein (2014), correlate to an underlying condition such as gastrointestinal diseases. Other conditions that can result in equine haemorrhage include haemophilia (type A), von Willebrand's disease, thrombocytopathies (abnormal platelet

function), primary fibrinolysis (hyperplasminemia) and liver failure, to name a few (Byars *et al*, 2003).

In instances where a hypocoagulopathy has been diagnosed, the clinical signs will vary depending on whether primary or secondary haemostasis has been effected (Epstein, 2014). When primary haemostasis has been affected, according to Epstein (2014) and Ness (2014), bleeding from mucosal surfaces, such as epistaxis (figure 2.16), as well as bruising, petechiae and haematoma formation, as a result of trauma, will be evident. When the hypocoagulopathy effects secondary haemostasis, Epstein (2014) and Ness (2014)



Figure 2.16. Epistaxis associated with exercise induce pulmonary haemorrhage (EIPH) (O'Sullivan, 2016).

both indicate that intracavitary haemorrhage, bruising and haematoma formation without a related trauma is more commonly observed.

Platelet defects have also been observed in the equine (Fry *et al*, 2005, Christopherson *et al*, 2006, Norris *et al*, 2006). Fry *et al*, (2005) indicated that such platelet defects in the equine occur downstream of the platelet collagen receptors but upstream from the thromboxane A₂ receptor. This hypothesis correlates with studies carried out by MacIntyre *et al*, (1984) and Needleman *et al*, (1982) where human platelets were treated

with PUFA. Both documented a diminished thromboxane A₂ release in platelets treated with linoleic acid, with Needleman *et al*, (1982) reporting a reduction of thromboxane A₂ of up to 50% when compared with control platelets.

2.6.4. Linoleic Acid and Coagulation

MacIntyre *et al*, (1984) and Needleman *et al*, (1982) both found that incubating platelets with unsaturated fatty acids, such as linoleic acid, at concentrations ranging from 0.05mM to 0.4mM, inhibited platelet aggregation. It was proposed that this effect was the result of disruption of thromboxane A₂ biosynthesis by an alteration in the phospholipid composition that inhibited phospholipase activity. MacIntyre *et al*, (1984) have suggested that the inhibitory effects of *cis*-unsaturated fatty acids may be the result of the inhibition of fatty acid cyclooxygenase, resulting in the suppression of thromboxane A₂ biosynthesis. However, Zhang *et al*, (1997) determined that, while certain fatty acids do have the ability to inhibit the release of cyclooxygenase metabolites from platelets, none of the 18 carbon fatty acids had any effect.

Another suggestion, especially where linoleic acid is concerned, is that chain elongation and desaturation of the fatty acid to form dihomo-gamma-linolenic acid, which can then be metabolised to Prostaglandin E₁ (PGE₁). PGE₁ is a potent inhibitor of platelet aggregation that works by stimulating platelet adenylyl cyclase. Further work in the area carried out by Zhang *et al*, (1997) determined that incubation of platelets with dihomo-gamma-linolenic acid inhibited A₂₃₁₈₇ stimulated thromboxane release.

MacIntyre *et al*, (1984) state that the effects of long-chain fatty acids on platelet function are mediated by their interaction with, and perhaps their incorporation into the platelet

membrane. The data collated by MacIntyre *et al*, (1984) suggests that fatty acids in the platelet membrane are distributed into specific lipid domains, however, the details of the influence of the organisation of membrane structure on receptor mediated platelet activities requires clarification.

2.7. Summary of the Literature

Free fatty acids, such as linoleic acid, follow a complex metabolic pathway and are essential for several diverse physiological functions, ranging from energy storage and production to intracellular signalling. Linoleic acid, itself, is an essential fatty acid and must be derived from the diet. It is a precursor for numerous molecules involved in inflammation and coagulation. The literature presented here demonstrates that excessive intake of fatty acids, such as linoleic acid, has been linked with various pathological conditions associated with inflammation, oxidative stress, haemostasis and cell death. While it is apparent that the mechanisms and pathways involved are complex and multifaceted, the impact of excessive linoleic acid on *in vitro* mechanisms remains to be elucidated.

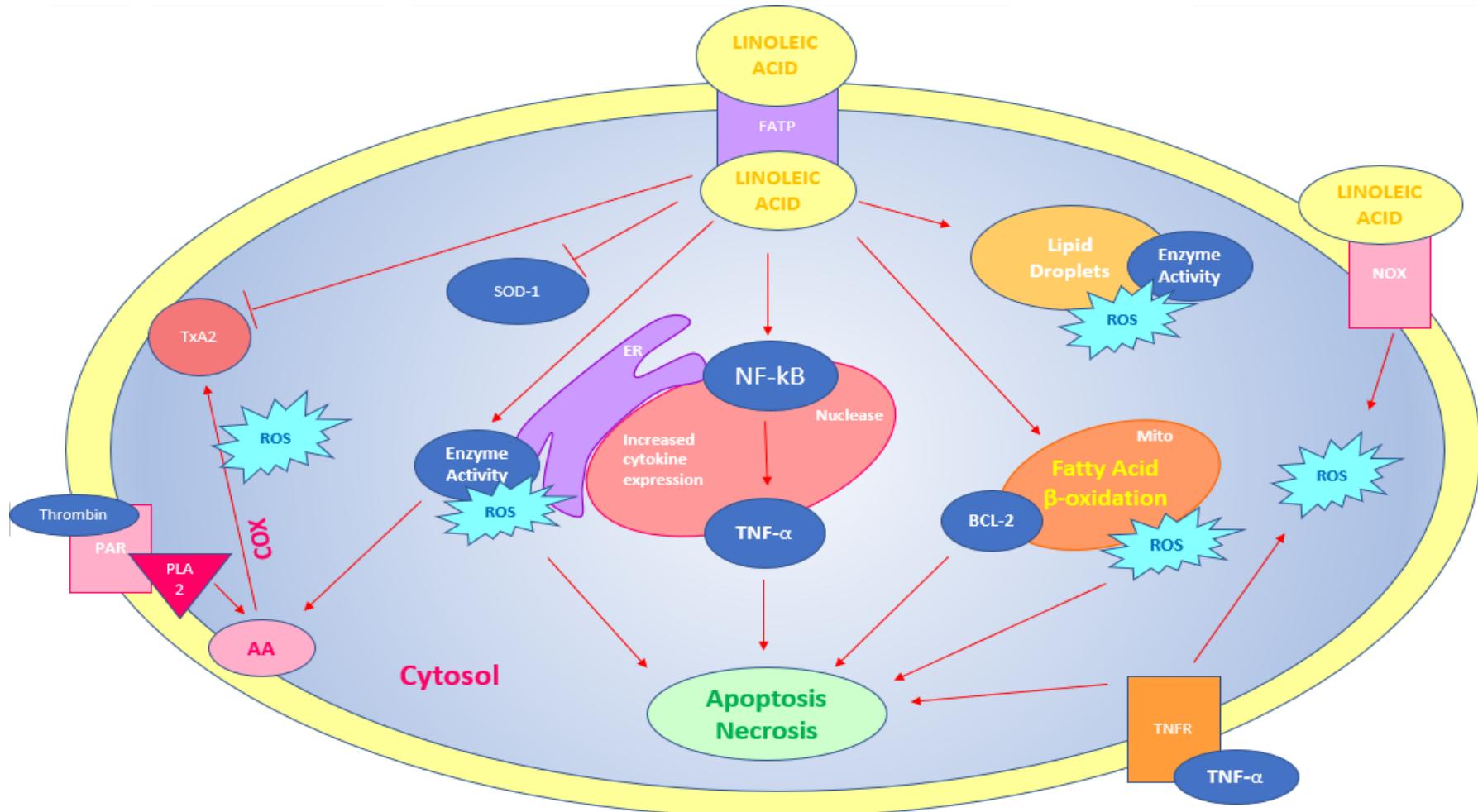


Figure. 2.17. Schematic of the possible intracellular fates of linoleic acid. Once inside the cell it is transported *via* fatty acid binding proteins where it can be packaged into lipid droplets, used for energy production in the mitochondria through β -oxidation or enzymatically transformed into derivative such as arachidonic acid. Excessive intracellular linoleic acid may lead to increased production and secretion of proinflammatory eicosanoids such as thromboxane A₂ (TxA₂) as a result of excess arachidonic acid. Linoleic acid may also inhibit TxA₂ function. Production of excess proinflammatory cytokines such as TNF- α may occur through linoleic acid induced NF- κ B activation. Increased intracellular metabolism of linoleic acid in several organelles can lead to increased production of reactive oxygen species (ROS). Linoleic acid may also inhibit ROS scavenger enzymes such as superoxide dismutase-1 (SOD-1). These factors may induce intracellular stress, leading to apoptosis.

2.8. Aims and Objectives

2.8.1. Aim

The aim of this research is to investigate, using an *in vitro* model, if physiologically high levels of unconjugated linoleic acid potentiates an effect on intracellular homeostasis, focusing on inflammation, oxidative stress and haemostasis.

2.8.2. Objectives

- Investigate the effects of unconjugated linoleic acid cell viability and intracellular lipid accumulation.
- Identify the effects of unconjugated linoleic acid on the secretion of the cytokine, TNF- α , and the eicosanoid thromboxane A₂.
- Identify the effect of unconjugated linoleic acid on intracellular oxidative stress through the analysis of the superoxide anion scavenger enzyme superoxide dismutase.
- Assess the effects of unconjugated linoleic acid on the induction of cell death, namely apoptosis and necrosis.

Chapter 3

Methods and Materials

3. Methods and Materials

3.1. Cell Culture Study

3.1.1. HepG2 Cell Culture and Maintenance

A vial of Human Hepatoma (HepG2) cells was kindly donated by Dr. Emily Crowley (Athlone Institute of Technology). These cells were cultivated in 75cm² cell culture flasks containing Dulbecco's Modified Eagles Medium (DMEM) high glucose (GibcoTM) with Foetal Bovine Serum (FBS) at a concentration of 0.1mL/mL (v/v). The medium was supplemented with 0.01mL/mL (v/v) of an antibiotic solution containing penicillin (10,000U/mL) and streptomycin (10,000µg/mL). This medium is termed here after as complete HepG2 growth medium. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ (Lammi *et al*, 2015 and de Sousa Araujo *et al*, 2015).

3.1.2. HUVEC Cell Culture and Maintenance

A vial of Human Umbilical Vein Endothelial Cells (HUVEC) was kindly donated by Dr. Dan O'Toole (National University of Ireland Galway). These cells were cultivated in 75cm² cell culture flasks containing Hams F12 (Kaighn's modified) medium (GibcoTM) with FBS (0.1mL/mL) (v/v). The medium was supplemented with 0.01mL/mL (v/v) of an antibiotic solution containing penicillin (10,000U/mL) and streptomycin (10,000µg/mL). The medium also contained endothelial cell growth (ECG) supplement (0.05mg/ml) (w/v) (Sigma Aldrich) and heparin (0.1mg/ml) (w/v) (Fisher Scientific) as recommended by the ATCC. This medium is termed here after as complete HUVEC growth medium. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The HUVECs were much

slower to grow than HepG2 cells and optimum growth was achieved when the media was changed every two days.

3.1.3. Sub-culturing HepG2 and HUVEC Cells

Waste culture medium was removed from the culture flask and discarded. Phosphate Buffer Saline (PBS) was used to remove further traces of trypsin inhibiting serum before adding 2mL of trypsin. Cells were incubated in trypsin-EDTA (0.25%) (GibcoTM) for approximately 5 minutes to allow the monolayer to separate from the flask. Once cells had detached and dispersed, 4mL of complete growth medium was added to the flask and cell were gently aspirated by gentle pipetting to facilitate complete detachment.

The suspension was then added to a falcon tube and centrifuged. HepG2 cells were centrifuged at 400rcf for 5 minutes while HUVEC cells were centrifuged at 300rcf for 5 minutes to allow for the formation of a cell pellet. The supernatant, containing trypsin, was then removed and the pellet was resuspended in complete growth medium. Aliquots of the cell suspension were then placed in new culture vessels with the appropriate amount of fresh complete growth medium. Cells were incubated at 37°C with 5% CO₂, allowed adhere and were grown until 90% confluency was reached.

3.1.4. Preparation of Unconjugated Linoleic Acid and Cell Treatment

Using a method adapted from work carried out by Di Nunzio *et al*, (2011) a 1M solution of ULA was prepared by adding 140.2mg of the oil to 359.8μL (w/v) of 100% molecular biology grade isopropyl alcohol (IPA) to make a volume of 500μL. The mixture was vortexed for approximately 20 seconds to ensure the oil was

completely dissolved. To 500mL of serum free, antibiotic free high glucose (4.5g/L) DMEM, 2.5mg of bovine serum albumin (BSA), a fatty acid carrier molecule, was added to create a 0.5% solution. This media was then used as the diluent. This ULA solution was further diluted to create a 10mM working solution. Initially, cells were treated with ULA concentrations ranging from 0mM to 10mM.

3.1.4.1. Assay Modifications

After initial cytotoxicity assessment, a number of treatment variables were amended. Dilutions of ULA were made at concentrations of 0.1mM, 0.25mM, 0.5mM, 0.75mM, 1mM, 2.5mM and 5mM using a fatty acid free BSA constituted media as the diluent. High glucose DMEM was replaced with low glucose (1g/L) DMEM. Cells were initially treated over a period of 24 and 48 hours. The final concentration of IPA in the media was kept below 1% (v/v). The solutions were prepared fresh for each assay (Di Nunzio *et al*, 2011). The positive growth control cells were grown in fatty acid free BSA constituted media containing \leq 1% IPA. This method was repeated using the Hams F12 (Kaighn's modification), supplemented with fatty acid free BSA (0.05%), ECG (0.05mg/mL)(w/v) and heparin (0.1mg/mL)(w/v), as the diluent to treated HUVEC cells. The media used in the preparation on treatments from here on is termed treatment media.

3.1.4.2. Amended Treatment Method

Based on data obtained from the above experiments, treatments were then optimised to include concentrations of 0.1mM, 0.25mM, 0.5mM, 1mM and 2.5mM only, over periods of 1, 2, 4, 6 and 8 hours. Both HepG2 and HUVEC cell lines were treated with ULA in this manner.

3.1.5. Resazurin Cell Viability Assay

A resazurin based (AlamarBlue®) cell viability assay was chosen as it is relatively non-toxic and liver specific, however, the assay is compatible with numerous cell lines including HUVEC (McMillian *et al*, 2002, Nourse *et al*, 2007). The alamarBlue® assay relies on viable cells continuously reducing resazurin to resorufin causing an increase in fluorescence and a colour change from blue to red in the media surrounding the cell (McMillian *et al*, 2002).

Both HepG2 and HUVEC cells were trypsinised, centrifuged and resuspended. A small aliquot of cells was taken, and a cell count performed. Cells were seeded into 96 well plates at a density of 2×10^4 cells/well for HepG2 cells and 1×10^4 cells/well for HUVEC cells. Cells were incubated at 37°C in 5% CO₂ overnight to allow them to adhere to the surface of the plate.

Once the cells had reached 70% confluence the media was removed and 100µL of treatment was added to each well. To the positive growth control, 0.05% fatty acid free BSA constituted medium containing IPA at a concentration no greater than 1% was added.

Cells were then incubated at 37°C and 5% CO₂ for a period of either 24 hours or 48 hours. In order to assess the cell viability of HepG2 cells, three hours prior to the specified incubation period 10µL of resazurin stain was added to each well and plates were incubated under the same conditions. After three hours plates were analysed using a Biotek® multiwell plate reader at both 570nm and 600nm. HUVEC cells were assessed in a similar manner, however, cells were seeded at a concentration of 1×10^4 cells/well while the resazurin incubation period was increased from three

hours to four hours (Nourse *et al*, 2007). For each assay, all concentrations were tested in triplicate with three independent tests performed ($n=3$).

3.1.5.1. Amended Resazurin Cell Viability Assay

In order to ensure that the ULA in the media was not affecting optical density and absorbance values obtained, it was decided to remove the media containing the oil from cell, once the time point had been reached. This media was replaced with 100 μ L of fresh treatment media containing resazurin at a 1:10 dilution. One again, HepG2 cells were incubated for 3 hours, while HUVEC cells were incubated for 4 hours before being analysed.

3.1.5.2. Calculations

The percentage difference in the amount of resazurin reduced by treated cells when compared to a positive growth control (untreated cells) was calculated using the following equation:

$$\frac{(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2 \text{ of test agent dilution}}{(\varepsilon_{ox})\lambda_2 A^\circ\lambda_1 - (\varepsilon_{ox})\lambda_1 A^\circ\lambda_2 \text{ of untreated positive growth control}} \times 100$$

Where: ε_{ox} = molar extinction coefficient of alamarBlue® oxidized form (blue)

A = absorbance of test wells

A° = absorbance of positive growth control well

λ_1 = 570nm

λ_2 = 600nm

3.1.6. Tetrazolium Bromide (MTT) Cell Viability Assay

In order to ensure the cell viability results obtained during the resazurin experiments were reliable and accurate, Tetrazolium Bromide (MTT) (Sigma Aldrich) cell viability assays were carried out on HepG2 cells treated with ULA over, 24- and 48-

hour periods. MTT was chosen as it is an effective and popular method of assessing cellular metabolic activities. The water-soluble yellow tetrazolium dye is reduced by the dehydrogenase system of active cells to the water insoluble purple formazan. This formazan can then be measured and quantified spectrophotometrically, with the concentration being directly proportional to the number of metabolically active cells (Wang *et al*, 2010).

Cells were grown and treated as described (3.1.4. and 3.1.5). An initial stock solution of MTT was prepared by dissolving 100mg of MTT (make and supplier) in 20mL of PBS (Sigma Aldrich) (5mg/mL solution)(w/v). From this stock a 1:10 dilution of MTT was made by adding 500 μ L of MTT stock to 4500 μ L of treatment media to produce a working stock. Once the treatment time points had been reached the media containing ULA treatments was removed from all cells and discarded. The MTT working stock was then added to each well at a volume of 100 μ L. HepG2 cells were incubated at 37°C for 3 hours, while HUVEC cells were incubated at 37°C for 4 hours. After incubation, the MTT containing media was removed from all wells and 100 μ l of warm (37°C) dimethyl sulfoxide (DMSO) was added to each well. Cells were then incubated at 37°C for 30 minutes on a shaking incubator to dissolve the purple formazan crystals. Absorbance was then measured at 540nm using a Biotek® multiwell plate reader.

3.1.7. Adenosine Triphosphate (ATP) Assay

Yamashina *et al*, (2009), stated that the majority of the cellular energy carrier Adenosine Triphosphate or ATP is produced by the mitochondria of most mammalian cells. As such, cellular production of ATP can be used as an indicator of cellular health. In order to determine if high concentrations of ULA affects the ATP

producing ability of the mitochondria of HepG2 cells, an ATP assay was carried out.

The ATP assay kit was purchased from Sigma Aldrich (Arklow, Co. Wicklow)

3.1.7.1.Assay Component Preparation

The ATP assay was carried out according to the manufacturer's instructions. All vials were centrifuged briefly before being opened and ultrapure water was used for the preparation of all reagents. The ATP assay buffer and the ATP probe were equilibrated to room temperature. The ATP converter and developer mix were each reconstituted in 220 μ L ATP assay buffer. The ATP standard was prepared using 100 μ L of water to generate a 10mM ATP standard solution. This solution was kept on ice until required for use.

3.1.7.2. Sample Preparation

HepG2 cells were seeded in a 6-well plate at a seeding density of 5×10^5 cells/mL with 3mL per well and incubated for 24 hours to allow cell adherence. After 24 hours the used media was removed and cells were then treated with ULA for a period of 21 hours at the following concentrations: 0mM, 2mM, 4mM, 6mM, 8mM, and 10mM. These dilutions were prepared as previously described in section 3.1.4.

After the 21-hour incubation period, the ULA treatment was removed from each well. Cells in each well were washed with PBS and trypsinized. The cells from each well were then transferred into separate Falcon tubes and centrifuged at 400rcf for 5 minutes to form cell pellets. These pellets were resuspended in 500 μ l of ATP assay buffer to induce cell lysis and release intracellular ATP. Two dilutions, a 1:5 and a 1:10 dilutions were then prepared from the suspensions using the ATP assay buffer as the diluent. All samples and sample dilutions were kept on ice.

3.1.7.3. ATP Standard Preparation

In order to prepare the ATP standards, 10 μ l of the 10mM ATP standard solution was diluted with 90 μ l ultrapure water to generate an initial standard stock of 1mM. Volumes of 2 μ l, 4 μ l, 6 μ l, 8 μ l and 10 μ l of this stock were then added in duplicate to the wells of a 96 well plate. The final volumes of these wells were made up to 50 μ l using the ATP assay buffer. This resulted in the generation of 0, 2, 4, 6, 8, and 10nmoles/ μ l of ATP standard.

3.1.7.4. ATP Assay Procedure

The colorimetric reaction mix to be used on both the standards and the samples was prepared by adding 3,520 μ l of ATP assay buffer, 160 μ l of ATP probe, 160 μ l of ATP converter and 160 μ l of developer mix to a Falcon tube. The contents were then centrifuged briefly to ensure homogeneity throughout the mixture. A separate reaction mix was prepared by adding 920 μ l of ATP assay buffer, 40 μ l of ATP probe and 40 μ l of developer mix to a separate Falcon tube and briefly centrifuging. The ATP converter was omitted for this preparation.

The samples and their dilutions were then removed from the ice. Each sample was added to the well of a separate 96-well plate in triplicate, with 50 μ l of the sample blanks being added singularly. The relevant reaction mix was then added at a volume of 50 μ l to all wells and the plates were incubated for a period of 30 minutes at room temperature. Following this, the absorbance was read at 570nm using a Biotek Synergy HT plate reader equipped with Biotek Gen5 version 2.01 software.

The concentration of ATP in cell samples was calculated by subtracting the sample blank values from the sample values using the following equation:

$$C = \frac{S_a}{S_v}$$

Where: C is the concentration of ATP in the sample (nmole/ μ L) obtained using the standard curve.

S_a is the amount of ATP in the unknown sample well (nmole/ μ L).

S_v is the sample volume added to the well (50μ L).

3.1.8. Oil Red O Assay for Intracellular Lipid Accumulation

3.1.8.1. Preparation of 10% Neutral Buffered Formalin

A standard stock of Formalin (Formalin 37% - 40%, Sigma Aldrich) was used to prepare a 10% neutral buffered formalin solution. To make 100mL, 10mL of the standard stock was pipetted into a 100mL volumetric flask. The solution was then made up to the 100mL mark on the flask using distilled water. The pH of the solution was then adjusted with the addition of 400mg of sodium phosphate monobasic (NaH_2PO_4 , SigmaAldrich, CAS No. 7558-80-7) and 650mg of sodium phosphate dibasic (Na_2HPO_4 , Sigma Aldrich, CAS No. 7558-79-4).

3.1.8.1.1. Preparation of 60% Isopropanol

A volume of 60mL of molecular biology grade isopropanol (IPA) ($\text{C}_3\text{H}_8\text{O}$, Sigma Aldrich, CAS No. 67-63-0) was pipetted into a 100mL volumetric flask and distilled deionised water was added to make 100mL of solution.

3.1.8.1.2. Preparation of Oil Red O Stock Solution

A stock solution of Oil Red O was prepared by dissolving 360mg of Oil Red O powder ($\text{C}_{26}\text{H}_{24}\text{N}_4\text{O}$, Sigma Aldrich, CAS No. 1320-06-5) in 100mL of molecular

biology grade IPA (w/v). The solution was stirred at room temperature overnight, after which it was filtered using a 0.2um filter and stored at 4°C until in use.

3.1.8.1.3. Preparation of Oil Red O Working Solution

A volume of 6mL of Oil Red O stock solution was diluted with 4mL deionised distilled water (ddH₂O). This solution was then filtered using a 0.2um syringe filter and left to stand for 20 minutes at room temperature. The working solution was freshly prepared for each assay.

3.1.8.1.4. Oil Red O Staining for Intracellular Lipid Accumulation

The following modified Oil Red O staining procedure was adapted from Lin *et al*, (2007), Kim *et al* (2013) and Cao *et al*, (2016). HepG2 cells were seeded in 24 well plates at a density of 1×10^5 cells/well, while HUVEC cells were seeded at a density of 5×10^4 cells/well. Cells were incubated overnight to allow for cell adhesion, after which they were treated with varying concentrations of ULA. After the appropriate treatment period, all media was removed from each well and cells were washed once with ice cold PBS before being incubated for 10 minutes in 10% formalin at a volume of 500µL/well. This was removed and fresh formalin (10% at 500µL/well) was added to each well. Cells were then incubated for one hour at room temperature. After fixation, cells were washed twice with ddH₂O. They were then incubated at room temperature for 5 minutes with 60% IPA. The IPA was removed, and cells were allowed to dry completely. Oil Red O working solution at a volume of 400µL/well was then added to each well, with great care taken not to stain the sides of each well. Cells were then incubated at room temperature for 10 minutes, after which the staining solution was removed. Cells were washed immediately 4 times

with ddH₂O. After the fourth wash 500µL of ddH₂O was left on the cells and microscopic images were acquired.

In order to quantify intracellular lipid accumulation, the ddH₂O was then removed and cells were allowed to dry completely. The stained cells were incubated at room temperature for 10 minutes in 100% IPA at a volume of 500µL/well. After incubation, the solution in each well was gently pipetted up and down several times to ensure optimum Oil Red O elution. Subsequently, 100µL of the solution from each well was added to the well of a 96 well plate. Using 100% IPA as the blank, the absorbance of each solution was measured at 500nm using Biotek® multiwell plate reader. Data was expressed as the percentage of lipid accumulation when compared to a positive growth control, cells that were grown in fatty acid free BSA constituted media, containing ≤1% IPA.

3.1.9. Collection of Cell Lysates

HepG2 cells were seeded at a density of 4×10^5 cells/well in 6 well plates and allowed to adhere overnight. As HUVEC cells were considerably difficult to grow and as a result, much more limited than HepG2 cells, they were seeded at a density of 3.5×10^4 cells/well in a 12 well plate and allowed to grow for 48 hours. Cells were treated with varying concentrations of ULA previously described in section 3.1.4. To make cell lysis buffer, protease inhibitor cocktail tablets (cOmplete™ ultra tablets, mini, EDTA free from Roche) were added to a commercial lysis buffer (Sigma Aldrich) at a rate of one tablet per 10mL of buffer.

Once the specified treatment time point had been reached, the media was removed from each well. This media was centrifuged and saved in individual Eppendorf tubes

before being stored at -80°C. Cells were then washed with warm (37°C) PBS twice, after which 150µL of ice-cold lysis buffer was added to each well. Cells were then incubated on ice for 15 minutes and gently agitated using a laboratory rocker. Cells were then scraped from each well using a sterile cell scraper. The resultant suspension was then agitated by pipetting using a 100µL micropipette in order to mechanically aid cell lysis, after which it was transferred into a sterile Eppendorf tube. This suspension was then incubated on ice for a further 30 minutes before being centrifuged at 4000xg for 15 minutes at 4°C. The supernatant from each tube was then aliquoted into a fresh tube and stored at -80°C. The lysis pellet was resuspended in ice cold sterile PBS and stored at -80°C.

3.1.10. Fluorescent Microscopy – Nile Red Staining for intracellular Lipid Accumulation

3.1.10.1 Preparation of Reagents and Buffers

A solution of 4% paraformaldehyde was prepared by dissolving 4g of paraformaldehyde powder (Sigma Aldrich, CAS No. 30525-89-4) in 50mL of warm (~60°C) PBS. Sodium hydroxide (NaOH) (Sigma Aldrich, CAS No. 1310-73-2) at a concentration of 1N was added drop by drop until the paraformaldehyde appeared completely dissolved and the solution became clear. This solution was then filtered to remove particulate and made up to 100mL with PBS. The pH was then adjusted to 6.9 using a few drops of concentrated hydrochloric acid.

A permeabilization buffer of 0.1% (v/v) Triton X-100 PBS buffer was prepared by dissolving 0.1mL of Triton X-100 (Sigma Aldrich, CAS No. 9002-93-1) in 100mL of 1xPBS.

A 0.1mg/mL (w/v) stock solution of Nile Red was prepared by dissolving 1mg of Nile Red Powder (Sigma Aldrich, CAS No. 7385-67-3) in 10mL of molecular biology grade dimethyl sulfoxide (DMSO, Sigma Aldrich, CAS No. 67-68-5). From this stock solution a working solution of 0.02 μ g/mL of Nile Red was prepared by diluting 10 μ L of the stock in a total of 50mL PBS. This stock was made fresh on the day of use.

A working solution of DAPI (4, 6-diamidino-2-phenylindole) (Waterborne Inc., CAS No. 28718-90-3) was prepared by diluting 10 μ L of 5000x stock solution in a total of 50 μ L of PBS, according to the manufacturer's specifications.

3.1.10.2. Preparation of Collagen and Collagen Coated Plates

In order for HUVEC cells to grow successfully on glass coverslips in 6 well plates, the coverslips and wells required collagen coating. Glacial acetic acid 99.9% (Sigma Aldrich) was diluted 1:1000 using sterile ddH₂O to produce a 17.5mM solution. Lyophilised collagen Type I, extracted from rat tail (Sigma Aldrich) was dissolved in 17.5mM of glacial acetic acid at a concentration of 1mg/mL (w/v). This initial stock was then aliquoted and stored at -20°C prior to use. From this stock, a working stock of 15 μ g/mL was prepared.

3.1.10.3. Preparation of cells for Fluorescent Microscopy

HepG2 cells were cultured on glass cell culture slides in 6 well plates at a seeding density of 1 x 10⁶ cells/well. HepG2 cells were allowed to adhere overnight prior to being treated with varying concentrations of ULA as previously described (3.1.4.). HUVEC cells were seeded at a density of 2x10⁴ cells/well in 6 well plates that had been coated with collagen. HUVEC cells were allowed to grow to 90% confluency over 2 to 3 days with one media change prior to use.

Once the treatment time point had been reached, treatment media was removed from all wells and cells were washed five times with warm PBS to remove any surface lipids and reduce background fluorescence. After washing, cells were fixed with 500 μ L of cold 4% paraformaldehyde for 15 minutes. After fixation, cells were washed twice with cold PBS before being incubated in 500 μ L of cold permeabilization buffer for 5 minutes. Again, cells washed twice with cold PBS before 1mL of Nile Red working stock was added to each well. The following steps were then carried out in a dark laboratory to avoid sample degradation. Cells were incubated in Nile Red for 15 minutes in the dark. After staining, Nile Red was removed, and cells were washed five times in cold PBS with the last wash being left on cells to allow for more manageable removal of cover slips from wells. Cover slips were then mounted with Fluoroshield™ mounting media containing DAPI.

Slides were stored at -20°C overnight. Fluorescent 12-bit images were obtained using a Leica DM 2000 fluorescent microscope fitted with a Leica DFC425 C digital camera and Leica Application Suite software. Fluorescence was viewed at two spectral settings. Yellow/gold fluorescence was viewed in the blue excitation range using a 450 – 490nm band pass exciter filter, a 510nm dichromatic mirror beam splitter and a 515nm long pass suppression filter. Red was viewed in the green excitation range using a 515 – 560nm band pass exciter filter, a 580nm dichromatic mirror beam splitter and a 590nm long pass suppression filter. Minimal image processing was performed using ImageJ software.

3.1.11. Quantification of Intracellular Lipid Droplets using Flow Cytometry

3.1.11.1. Preparation of Buffers and Reagents

Flow Buffer was prepared by adding 292.24mg of EDTA (Sigma Aldrich) to 1L of 1 x PBS to create a 1mM (w/v) solution. Tween® 20 (Sigma Aldrich) was then added at 2mL to create a 0.2% (v/v) solution and finally 1g of sodium azide (Sigma Aldrich) was added to create a 0.1% (w/v) solution. The buffer was filtered using a 0.2 μ m to remove particulate. A Nile Red working stock at a concentration of 0.3 μ g/mL (w/v) was prepared in PBS from an initial stock solution of 0.1mg/mL (w/v) of DMSO (section 3.1.10.).

3.1.11.2. Preparation of cells for Flow Cytometry

Flow cytometry was used to quantify the accumulation of intracellular lipid droplets with both HepG2 cells and HUVEC cells. HepG2 cells were seeded in 24 well plates at a density of 1×10^5 cells/well and allowed to adhere over night before being treated as previously described (section 3.1.4.). After treatment, media was removed, and cells were washed once with 1mL of warmed PBS before 200 μ L of trypsin was added to each well. Cells were incubated for approximately 5 minutes until all cells appeared to be detached. Media, at a volume of 400 μ L was then added to each well, after which, the complete contents of each well was transferred to a 1.5mL centrifuge tube. Cells were centrifuged at 400rcf for 5 minutes at 4°C. After centrifugation, the supernatant was discarded, and cell pellets were re-suspended in cold PBS. Cells were centrifuged and the supernatant was removed. Cells were re-suspended in 500 μ L of Nile Red and incubated in the dark at room temperature for 15 minutes. After incubation in Nile Red, cells were centrifuged as above, supernatant removed, and the cell pellet was washed twice in cold PBS. After the final wash step, the cell

pellet was re-suspended in 500 μ L of flow buffer and analysed using a MACSQuant® Analyse 10 flow cytometer (Miltenyl Biotec, Germany). Intracellular lipid accumulation was detected by fluorescence activated cell sorting (FACS). The fluorescence signals of both unstained cells and stained cells were obtained using a laser with an excitation of 488 nm and an emission of 585/40 nm. Unstained cells were used as an auto-fluorescence control. Data was obtained using FlowJo v10 software and was expressed using the arbitrary unit, median fluorescence intensity (MFI) (Wolins *et al*, 2018). As per Wolins *et al*, (2018) data was normalised by dividing the MFI observed at different fatty acid concentrations by the MFI observed of the cells grown with 0 μ M of fatty acid. (2018),

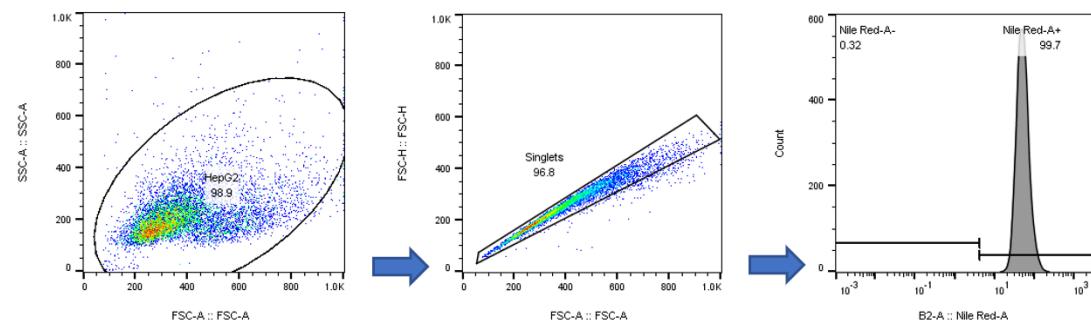


Figure 3.1. Flow Cytometry gating strategy used to identify Nile Red Positive population in HepG2 cells treated with ULA for 1 hour.

3.1.12. TNF- α Enzyme Linked Immunosorbent Assay (ELISA).

In order to ascertain the inflammatory impact of ULA on both epithelial and endothelial cells, an enzyme linked immunosorbent assay was performed on cells that were treated with varying concentrations of the fatty acid. Lipopolysaccharide (LPS), also referred to as endotoxin, is known to stimulate TNF- α in several different cell types. Both HepG2 cells and HUVEC cells were treated with LPS at a concentration of 10 μ g/mL (Saad *et al*, 1995). This was used as a positive control for TNF- α secretion. Ethanol at a concentration of 80mM was also used to induce TNF-

α secretion in HepG2 cells (Neuman *et al*, 1998). An Invitrogen Human TNF- α uncoated ELISA kit was sourced from Bio-sciences Ltd, Dublin, Ireland. The kit was stored according to the manufacturer's instructions at 4°C.

3.1.12.1. Preparation of Samples, Standards and Reagents

3.1.12.1. Wash Buffer Preparation

A wash buffer of 0.05% PBST (v/v) was prepared by dissolving 500 μ L of Tween™ - 20 (Sigma Aldrich, CAS no. 9005-64-5) in total volume of 1L of 1 x phosphate buffered saline (PBS). This was stored at 4°C until required for use.

3.1.12.2. Stop Solution Preparation

A solution of 1M phosphoric acid (H₃PO₄) was prepared by diluting 34mL of 85% (w/v) phosphoric acid (Company here, CAS no. 7664-83-2) in 466mL of deionised, distilled water to make 500mL of stop solution. This was stored at 4°C until required for use.

3.1.12.3. Coating Buffer Preparation

To prepare 120mL 1x coating buffer (v/v), 12mL of 10x coating buffer provided in the kit was added to 108mL of deionised, distilled water.

3.1.12.4. ELISPOT Diluent Preparation

A concentration of 1x ELISPOT (v/v) diluent was prepared by diluting 10mL of 5x ELISPOT diluent provided in the kit with 40mL deionised, distilled water. This was prepared freshly on the day of use.

3.1.12.5. Capture Antibody Preparation

To prepare sufficient capture antibody for one 96 well plate, 48 μ L of anti-human TNF- α purified capture antibody (x250), provided in the kit, was added to 12mL of 1x coating buffer (v/v). This was prepared freshly on the day of use.

3.1.12.6. Detection Antibody Preparation

Anti-human TNF- α biotin (x250) provided in the kit, was added at a volume of 48 μ L to 12mL of 1x ELISPOT diluent (v/v). This provided sufficient antibody for one 96 well plate. This was prepared freshly on the day of use.

3.1.12.7. Enzyme Preparation

Avidin- horseradish peroxidase (HPR) (x250) provided in the kit, was added at a volume of 48 μ L to 12mL of 1x ELISPOT diluent (v/v). This provided sufficient enzyme for one 96 well plate. This was prepared freshly on the day of use.

3.1.12.8. TNF- α Standards Preparation

Initially, lyophilised Human TNF- α , provided in the kit, was reconstituted with 1mL of deionised, distilled water, agitated gently and allowed to sit for 15 minutes. The concentration of this solution was 15ng/mL (w/v). A stock solution of TNF- α , at a concentration of 500pg/mL was prepared by diluting 100 μ L of the initial standard solution in 2900 μ L of 1x ELISPOT diluent (v/v). From this stock solution, 2-fold serial dilutions were prepared to produce an eight-point standard curve.

3.1.12.9. Sample Preparation

Cells were seeded in 24 well plates at a density of 1 x 10⁵ cells/well and incubated at 37°C in 5% CO₂ overnight, after which they were treated with varying concentrations of ULA. The culture media was removed from these cells after the appropriate treatment period and transferred into 1.5mL Eppendorf tubes. These

cultures were centrifuged at 4°C for 10 minutes at 220xg. The supernatant was transferred to fresh Eppendorf tubes and immediately stored at -80°C until analysis.

THP-1 cells (ATCC, Rockville, MD, USA) (used at passage 20) were kindly donated by Dr. Emma Murphy. Cells were cultured in RPMI-1640 (Sigma-Aldrich), supplemented with 10% FBS (Sigma-Aldrich), 1% penicillin G (100 U/mL) and streptomycin (100 µg/mL) solution (Sigma-Aldrich). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. For differentiation into macrophages, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) (Peprotech EC, London, UK) at a concentration of 100 ng/mL for 48 h. THP-1 cells were seeded at a density of 4×10^5 cells/well in 96 well plates and 24 h later injured with LPS (100 ng/mL) in RPMI supplemented with 1% penicillin/streptomycin. After 24 hours cells were washed in PBS and serum free media was added to cells for further 24 hours. After 24hrs cell supernatant was collected for analysis.

3.1.12.10. TNF- α ELISA Experimental Procedure

Corning™ Costar™ 9018 ELISA plates (provided with the kit) were coated with 100µL/well of diluted capture antibody. Plates were sealed and incubated at 4°C overnight. All coated wells were then washed three times with 250µL/well of wash buffer, with a soaking time of approximately 1 minute allowed between washes. Plates were blotted on absorbent paper to ensure all residual wash buffer was removed.

In order to prevent non-specific protein binding, all wells containing capture antibody were blocked. A volume of 200µL ELISPOT diluent was added to each well and plates were incubated for one hour at room temperature. After incubation, plates were washed once with wash buffer.

Standards and samples were then added at a volume of 100µL/well and plates were incubated at room temperature for overnight at 4°C. After incubation, standards and samples were aspirated and wells were washed with three to five times with wash buffer, as previously described, before diluted detection antibody was added at a volume of 100µL/well. Plates were sealed and incubated at room temperature for 1 hour.

The detection antibody was aspirated and, once again, plates were washed with wash buffer three to five times. Diluted Avidin-HRP was added to each well at a volume of 100µL/well, after which, plates were sealed and incubated for a further 30 minutes at room temperature. The Avidin-HRP was aspirated and the wash step was repeated once more, this time, for five to seven washes.

Tetramethylbenzidine (TMB) substrate solution was added to each well at a volume of 100µL/well. Plates were sealed and incubated at room temperature for 15 minutes. Stop solution, at a volume of 50µL/well, was added to each well, after which, plates were analysed using a Bioteck® multiwell plate reader at 450nm.

3.1.13. Superoxide Dismutase (SOD) Assay

3.1.13.1. Preparation of Reagents and Samples.

A Superoxide Dismutase Assay –WST-1 kit was obtained from Sigma Aldrich. The following stock solutions and buffers were provided in the kit. WST working solution (v/v) was prepared by mixing 1mL of the WST stock solution with 19mL of the buffer solution. The enzyme stock solution was centrifuged high speed for 5 seconds before being gently pipetted several times to ensure homogenisation. The enzyme working solution (v/v) was then prepared by adding 15µL of the enzyme stock solution to 2.5mL of dilution buffer. The lysates prepared previously (section

3.1.9.) were used for the analysis of cytosolic SOD (CuZnSOD). Cytosolic SOD was extracted from these lysates by adding two times the volume of ethanol:chloroform (at a ratio of 2:1). This mixture was then vortexed for a minimum of 30 seconds before centrifugation at 3000rpm at 4°C for 10 minutes. After centrifugation the supernatant was removed and stored on ice prior to analysis. A standard of SOD was prepared by diluting a standard of superoxide dismutase from bovine erythrocytes (Sigma Aldrich) with dilution buffer.

3.1.13.2. SOD Assay Protocol

In accordance with the protocol, 200µL of WST working solution was added to every well. Then three blanks were prepared as follows; to Blank 1, 20µL of ddH₂O and 20µL of enzyme working solution was added. To Blank 2, 20µL of sample solution (lysate extract collected from cells treated with 0.25mM for 4 hours) and 20µL of dilution buffer was added. To Blank 3, 20µL of ddH₂O and 20µL of dilution buffer was added. To all other wells, 20µL of the appropriate standard or sample and 20µL of the enzyme working solution was added. The assay was the incubated at 37°C for 20 minutes before being analysed using a Biotek® multiwell plate reader at 450nm. SOD activity was then calculated using the following equation provided in the protocol:

$$SOD\ activity(inhibition\ rate\ %) = \frac{(A_{blank1}-A_{blank3})-(A_{sample}-A_{blank2})}{(A_{blank1}-A_{blank3})} \times 100$$

3.1.14. Thromboxane B₂ (TXB₂) Assay

The production of thromboxane B₂ was assessed using the ParameterTM Thromboxane B2 Assay kit from R and D Systems.

3.1.14.1. Sample Preparation

Cells were seeded in 24 well plates at a density of 1 x 10⁵ cells/well and incubated at 37°C in 5% CO₂ overnight, after which they were treated with varying concentrations of ULA. The culture media was removed from these cells after the appropriate treatment period and transferred into 1.5mL Eppendorf tubes. These cultures were centrifuged at 4°C for 10 minutes at 220xg. The supernatant was transferred to fresh Eppendorf tubes and immediately stored at -80°C until analysis. Prior to analysis, samples were thawed completely, and a 2-fold dilution was made.

3.1.14.2. Preparation of Reagents and Standards

The following stock solutions and buffers were provided in the kit. The wash buffer was warmed to room temperature and gently mixed to ensure any pre-formed crystals were completely dissolved prior to use. The wash buffer was then diluted by adding 100mL of the 10X concentration to 900mL of dionised, distilled H₂O. The substrate solutions were mixed together in equal parts, protected from light and used within 15 minutes of mixing.

Initially, lyophilised Thromboxane B₂, provided in the kit, was reconstituted with 1mL of deionised, distilled water, agitated gently and allowed to sit for 15 minutes. The concentration of this solution was 200ng/mL (w/v). A stock solution of Thromboxane B₂, at a concentration of 20ng/mL was prepared by diluting 100µL of the initial standard solution in 900µL of the calibrator diluent (v/v). From this stock

solution, 2-fold serial dilutions were prepared to produce a seven-point standard curve.

3.1.14.3. Thromboxane B₂ Assay Protocol

Once all reagents, working standards and samples were prepared appropriately, 150µL of the calibrator diluent was added to the non-specific binding wells, while 100µL of the calibrator diluent was added to the zero standard wells. The standards or samples (100µL) were added to the appropriate remaining wells. The primary antibody solution (50µL) was then added to all wells except the non-specific binding wells. The plate was covered with an adhesive strip and incubated at room temperature on a horizontal orbital microplate shaker at 500rpm ± 50rpm for 2 hours. After incubation, 50µL of Thromboxane B₂ conjugate was added to all wells. The plate was covered with a fresh adhesive strip and incubated as before for 1 hour. All wells were aspirated and washed with wash buffer for a total of four washes. The plate was blotted dry before 200µL of substrate solution was added to each well. The plate was incubated for a further 30 minutes at room temperature in the dark, after which 100µL of stop solution was added to all wells. Optical density was determined immediately using a Biotek® multiwell plate reader at 450nm.

3.1.15. Analysis of Apoptosis in HepG2 Cells treated with ULA

Apoptosis was assessed using the Tonbo Biosciences® Annexin V-FITC kit obtained from R & D Systems. Cells were cultured in 24 well plates at a density of 5 x 10⁵ cells/well and left to adhere overnight before being treated as previously described (section 3.1.4.). Once the treatment time point was reached, cells were harvested using trypsin-EDTA (0.25%) supplemented with BSA (0.2%) (w/v) to minimise enzymatic damage to cellular membranes. Cell culture media, containing dead and

dying cells, was also collected. Once all cells were fully detached and collected, they were centrifuged to remove trypsin. Cells were then washed three times with 500 μ L of warm (37°C) PBS and centrifuged once more to create a pellet. The supernatant was discarded, and cells were resuspended in 97.5 μ L flow buffer containing 2.5 μ L Annexin V reagent (v/v). Cells were incubated at room temperature for 30 minutes in the dark, after which 400 μ L of flow buffer was added to each sample. Immediately before analysis propidium iodide (Miltenyl Biotec, Germany) was added to each sample at a rate of 5 μ L per 500 μ L (v/v) of cell suspension. Samples were then analysed, at a rate of 10,000 events per sample by flow cytometry using a MACSQuant® Analyse 10 flow cytometer (Miltenyl Biotec, Germany).

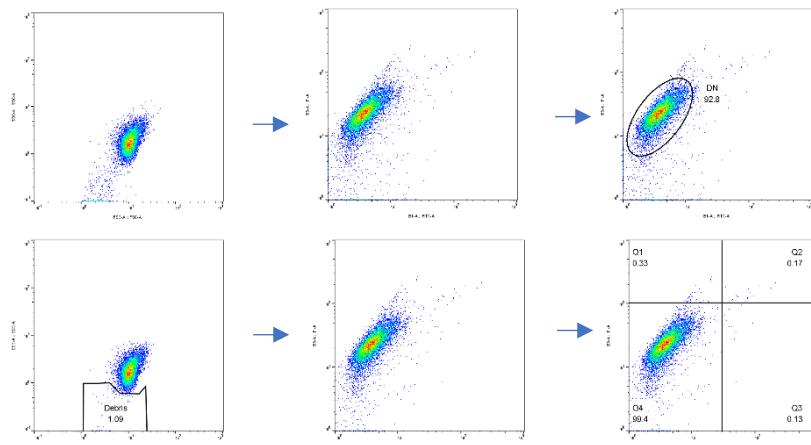


Figure 3.2. Gating strategy for the analysis of cell death mediated by ULA.

Initial analyses were performed on a stained untreated control. From left to right (figure 3.2.), in the first dot plot, the first gate used side scatter on the y axis to assess cell granularity or internal complexity while forward scatter on the x axis was used to determine cell size. The second dot plot displayed the propidium iodide channel on the y-axis versus the Annexin V-FITC channel on the x-axis on the entire ungated population. Within this dot plot, the double negative region (DN) was gated as can be seen in the third dot plot. This gate ensured the segregation of events would

include whole cells that remained unstained as well as unstained cellular debris. The events in this double negative segregation were then viewed SSC on the y axis and (FFC) on the x axis as displayed in the fourth dot plot. Cells with low FSC were segregated and labelled as debris. This debris gate was inverted creating a population which was considered to be not debris (whole cells). This action was then applied to the whole population. The inversion of the debris gate allowed for analysis of the rest of the population using the propidium iodide channel on the y-axis versus the Annexin V-FITC channel on the x-axis as seen in the fifth dot plot. The sixth dot plot segregated cells into four quadrants based on stain uptake; live cells , negative for both AV-FITC and PI (Q4); early apoptotic cells, positive for AV-FITC and negative for PI (Q3); late apoptotic cells, positive both for AV-FITC and PI (Q2) while cells positive only for PI were considered as necrotic (Q1).

3.2. Analysis of Hepatic Samples

3.2.1. Hepatic Tissue Sample preparation

Hepatic samples were excised during post-mortem examination of equines presented at the IEC. Samples were stored in a 50mL Falcon tube and stored at -20°C. Samples were transported from the IEC to AIT on ice and stored at -20°C on arrival. The hepatic sample to be analysed was removed from frozen storage and allowed to thaw completely. The sample was then prepared by adding 1g of hepatic tissue to a mortar with 0.5g of sodium sulphate (anhydrous) (Sigma Aldrich, CAS No. 7757-82-6) (w/w). The tissue and the sodium sulphate were ground together for approximately 10 seconds using a pestle after which 2mL of 100% v/v HPLC grade Acetonitrile (Manufacturer and CAS no.) was added (w/v). The components were ground together for a further 20 seconds. The liquid part of the mixture was then removed

and transferred to a centrifuge tube after which it was vortexed for approximately 30 seconds. It was centrifuged at 9000 x g for 2 minutes to remove the organic layer. The supernatant was then placed in a glass vial and stored at -20°C for approximately 12 hours (overnight).

The sample was then removed from cold storage and transferred to a centrifuge tube before once again being centrifuged at 9000 x g for 2 minutes. The supernatant was removed and transferred to an amber glass HPLC vial before being immediately analysed using HPLC.

3.2.2. Unconjugated Linoleic Acid – Storage and Handling

A commercial standard of ULA (CAS No. 60-33-3) that was reported to be ≥99% was obtained from ACROS Organics™ and stored, according to the manufacturer's guidelines, at -20°C. Prior to use the vial of oil was opened under a stream of nitrogen in order to prevent degradation by oxidation and 1mL aliquots were transferred into amber vials that were then sealed air tight. These vials were then stored at -20°C until required for use.

3.2.3. Unconjugated Linoleic Acid Standard Preparation

A commercial standard of ULA was used to prepare the standards to be used to create a calibration curve. As the linoleic acid was in liquid form, the density of the liquid was used to perform the calculations for the standards. At 25°C the density of the linoleic acid is considered to be 0.902g/mL. Using this information, a stock solution of 100µg/µL (v/v) was prepared.

The initial stock solution was prepared in 100% HPLC grade acetonitrile. From this stock solution, standards were made at concentrations of 80 μ g/ μ L, 60 μ g/ μ L, 40 μ g/ μ L, 20 μ g/ μ L and 10 μ g/ μ L using acetonitrile 100% as the diluent. These standards were analysed using HPLC at the parameters described above.

3.2.4. HPLC Analysis

The HPLC system used for chromatographic separation of both standards and samples consisted of a Waters 1515 isocratic chromatographic pump, a Waters 717 Autosampler with a 250 μ L sample loop and a Waters Dual λ Absorbance spectrophotometer detector. Data collection, integration and calibration was carried out using Empower 2 (Waters Corporation) software. Chromatographic separation was accomplished using a 250 x 4.6mm Excil C18 5 μ m reverse phase analytical column.

The mobile phase consisted of 100% methanol (CAS No. 67-56-1) which was degassed and filtered before use. This mobile phase was then pumped at a flow rate of 0.5mL/min at room temperature. Standards and samples were injected at a volume of 20 μ L and the final run time per injection was 10 minutes. A UV detector set at a wavelength of 305nm was used to determine peaks.

3.2.5. HPLC Collection of the Fraction

Using the previously described method (3.2.1. and 3.2.4.) hepatic samples were prepared and analysed using HPLC. The fraction was collected manually by placing a glass bottle at the end of the waste tubing when absorbance value relating to the peak of interest began to appear on the detector.

3.2.6. Fourier Transform Infrared Spectroscopy for identification of the Hepatic Extract

Fourier Transform Infrared (FTIR) spectroscopy was carried out on both a commercial standard of ULA as well as the fraction collected from the hepatic extract. Spectra were obtained using a Spectrum One FTIR spectrometer (Perkin Elmer) coupled with Attenuated Total Reflectance (ATR) sampling accessory.

All samples were analysed at ambient temperature with a total of 32 scans per sample cycle being performed. Spectra were recorded in transmission mode in the spectral region of 4000 to 650cm^{-1} 4 cm^{-1} resolution.

3.2.7. Gas Chromatography – Time of Flight – Mass Spectrometry for the Identification of the Hepatic Extract

Initially 200mg of ULA standard was added to 6mL of Boron Trifluoride -methanol derivatising reagent (BF_3 , CAS No. 373-57-9) while 20mg of the hepatic extract was added to 2mL of BF_3 (w/v). This was then heated in a tightly sealed glass vial at 100°C in a heating block for 60 minutes before being allowed to cool to an ambient temperature. Once cool, 2mL of n-Hexane was added to the derivatised ULA standard to produce a solution at a concentration of 100mg/mL and 1mL of n-hexane was added to the hepatic extract to produce a solution at a concentration of 20mg/mL.

Saturated sodium sulphate at a volume of 10mL was added to both solutions and the mixtures were vortexed for 2 minutes before being left to settle to allow separation of the two phases. The upper organic phase, the n-hexane, containing the methyl esters was removed to a clean, dry GC-MS vial. The ULA standard was diluted

further with n-hexane to produce five varying concentrations – 1000 μ g/mL, 750 μ g/mL, 500 μ g/mL, 250 μ g/mL and 100 μ g/mL. Once these standard dilutions were prepared, all solutions, including the derivatised hepatic extract, were placed in clean, dry GC-MS vials, sealed and analysed.

An Agilent 7890A Gas Chromatograph equipped with a Kronus SA Time-of-flight mass spectrometer (SAI) was employed. The separation was obtained on a HP-5 column, 30m x 0.25mm x 0.25 μ m. The carrier gas was helium and had a flow rate of 1ml/minute. Injection volume was 1 μ l. The gradient temperature program consisted of 50°C for 3 minutes, increasing at 20°C per minute for 10 minutes until 250°C was reached with a final hold time at this temperature for 17 minutes.

3.3. Statistical Analysis

Data are presented as the mean \pm standard error of the mean (S.E.M). Statistical analysis was performed on scientific data using GraphPad Prism software. All graphs were created using the GraphPad Prism software. All data is expressed as the mean with error bars representing S.E.M. Initially, Statistical significance was analysed by the one-way ANOVA with Dunnett's test used to compare the treatments to the positive growth control in 24 hour and 48 hour experiments. (McMillian *et al*, 2002) In the case of experiments that were conducted over five different time points, as each experiment contained more than two groups, one-way-ANOVAs were used to identify if significance existed between the means of all groups. This analysis was followed by Bonferroni's *post hoc* tests in order to establish where significance lay between groups. Bonferroni's was selected as it is considered conservative, and

therefore reduces the possibility of interpreting data as falsely significant (Kao and Green, 2008). Criterion for statistical significance was set at $P < 0.05$.

Concentration-response curves were generated to determine the half maximal inhibitory concentration (IC_{50}), the concentration of the inhibitor, in this case ULA, producing 50% inhibition.

Chapter 4

Analysis of the Hepatic Extract for the Presence of ULA

Chapter 4 Analysis of Hepatic Extract for the Presence of ULA

The original study involved the identification of an unknown signal observed in the HPLC chromatograms of an analysed extract obtained from the hepatic tissues of equines that had presented at the IEC with an idiopathic and fatal haemorrhagic condition. This signal was identified as linoleic acid in its *cis*-9, *cis*-12, unconjugated form (ULA). In order to ascertain if the observation made by the IEC regarding idiopathic haemorrhage in horses was a continued concern, equine hepatic tissue samples excised during necropsy by Dr. Ursula Fogarty at the IEC were analysed for the presence of linoleic acid in its unconjugated form.

4.1. Analysis of a Commercial Standard of ULA and the Identification of ULA in Equine Hepatic Samples by HPLC

HPLC was the primary analytical technique used in the original investigation, with a validated method having already been developed by Cooper (2015). As such, the method was applied to the analysis of new hepatic samples obtained from the IEC, in order to ascertain if the phenomenon was ongoing. It also facilitated the collection of desired fractions of separated sample components for further analysis

HPLC analysis, using the method established and validated by Cooper (2015), allowed for the qualitative assessment of ULA in equine hepatic tissue obtained from the IEC. ULA standards were prepared using a commercially purchased fatty acid with an indicated purity of 99%. The fatty acid was opened under a stream of nitrogen to prevent degradation due to oxidation. The main peak observed at 6.328 is that associated with ULA, however a shoulder was observed at approximately 5.8 minutes that is believed to be an isomer of linoleic acid (figure 4.1).

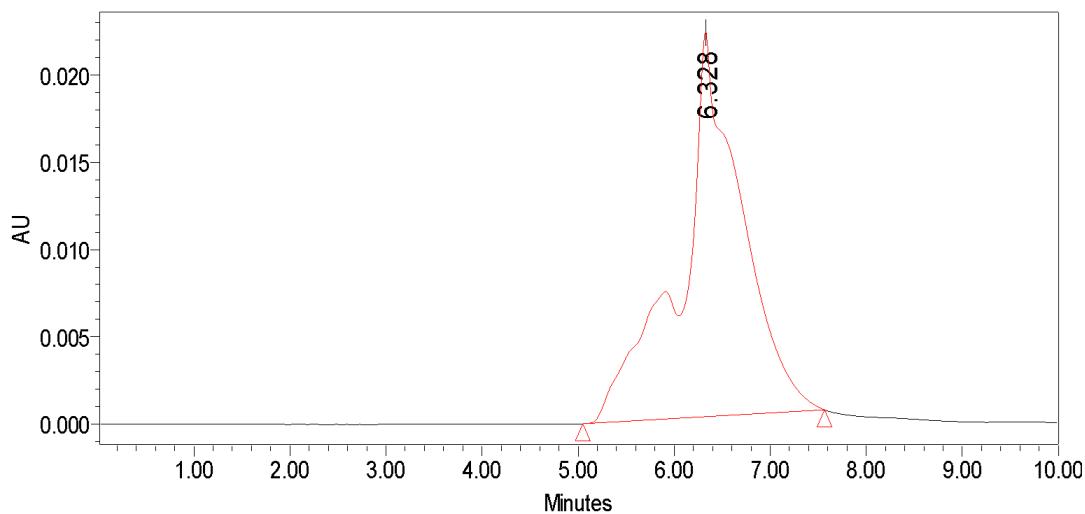


Figure 4.1. A chromatogram of the commercial standard of ULA at a concentration of 80 $\mu\text{g}/\text{ml}$ as prepared by the author.

In the current investigation, 217 equine hepatic tissue samples were analysed for the presence of ULA. The chromatogram displayed in figure 4.2 is an example of the results synonymous with the presence of ULA. The obvious peak displayed at 6.686 minutes is comparable to the peak obtained during the HPLC analysis of a commercial standard of ULA (Figure. 4.1).

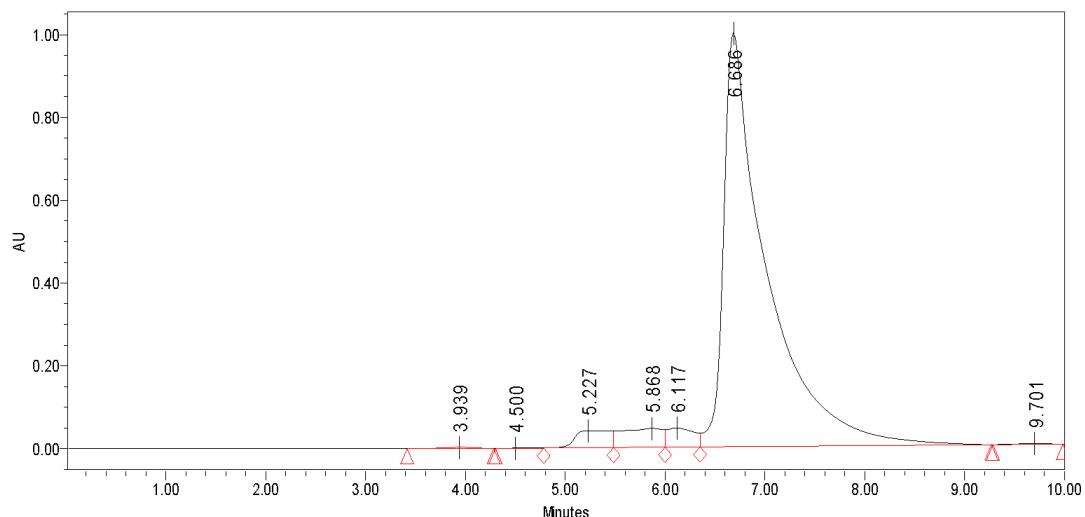


Figure 4.2. Chromatogram of an equine hepatic sample displaying a peak at 6.686 minutes indicative of ULA.

A calibration curve was created using the commercial standard of Linoleic Acid as presented in figure 4.3.

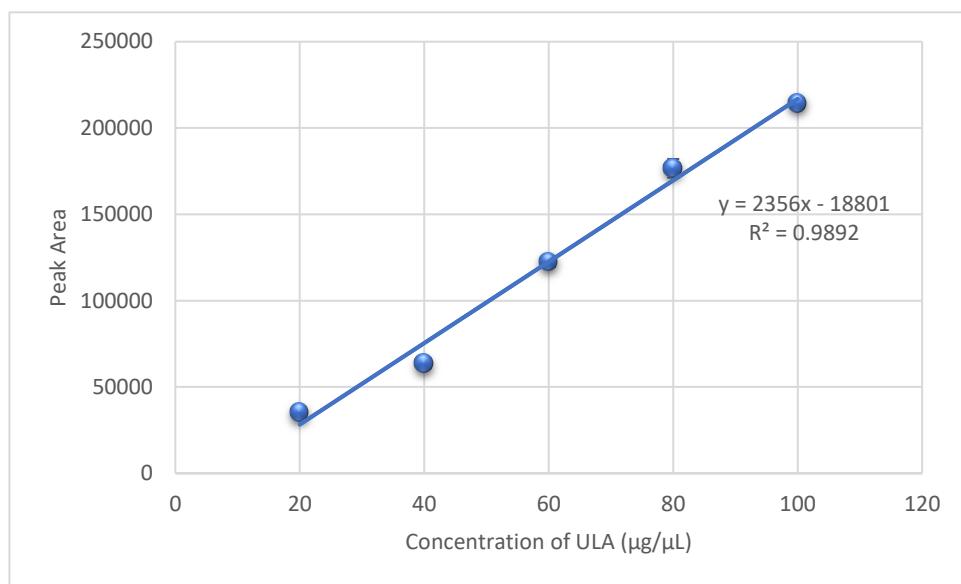


Figure 4.3. Calibration curve for commercial unconjugated linoleic acid obtained using a 250 x 4.6mm Excil C18 5 μm reverse phase analytical column.

This curve was used to quantify the concentration of linoleic acid in each of the 217 hepatic samples analysed, with a mean concentration of 2.6mg (± 0.28) per gram of Equine hepatic tissue identified.

4.2. Fourier Transform Infrared Spectroscopy for identification of the Hepatic Extract Fraction

FTIR was carried out on a sample of commercial ULA (Figure 4.4 A) as well as the fraction of interest collected from the hepatic extract (Figure 4.4 B). FTIR is an efficient low-cost analytical technique that provides structural and kinetic information on very small quantities of a sample while eliminating the need for potentially destructive sample preparation (Salehpour and Dubé, 2012). According to Forfang *et al*, (2017) FTIR spectroscopy is widely used for efficient and economical detection of the main components of biological material such as lipids.

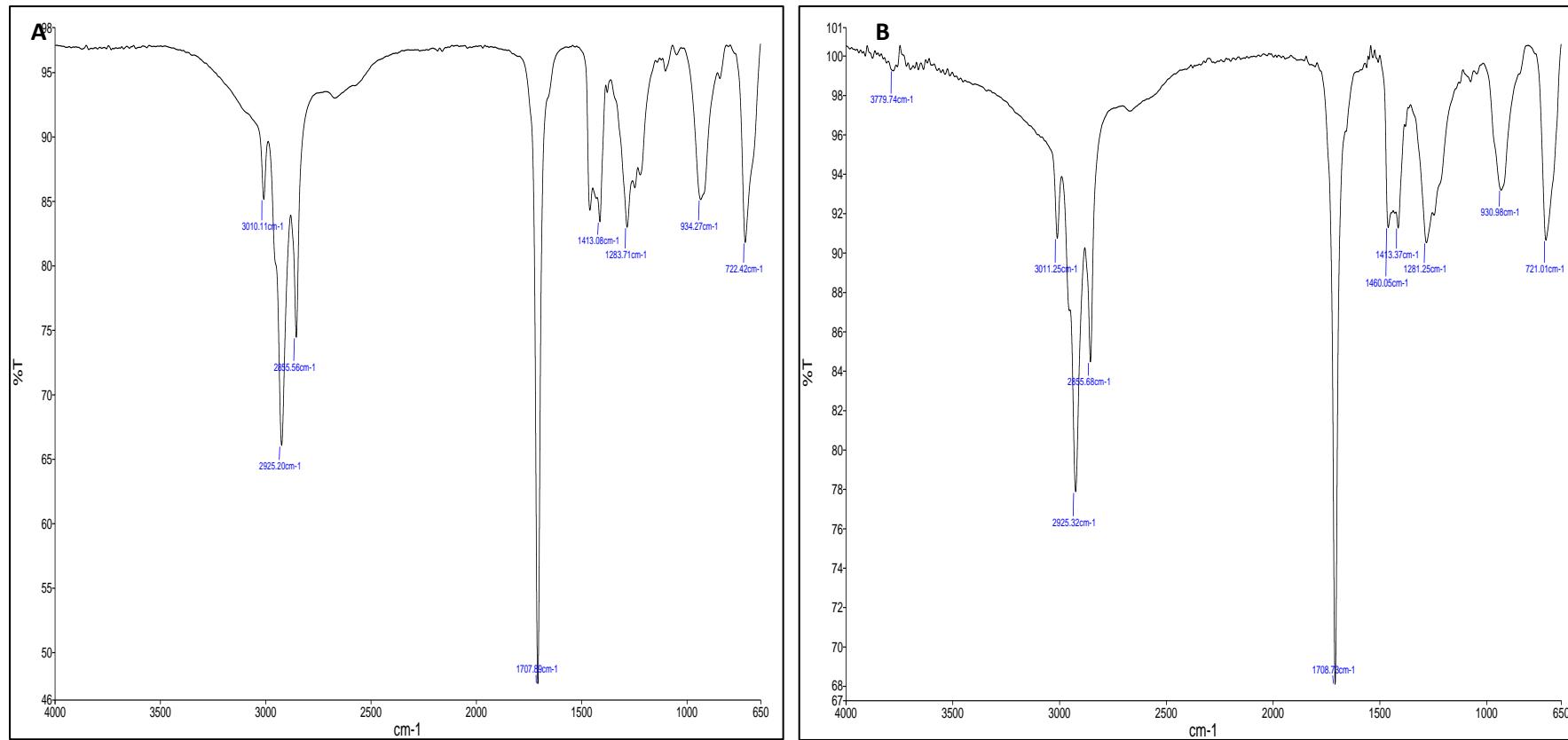


Figure 4.4. FTIR spectra, including peak numbers, obtained for a commercial standard of ULA (A) and the fraction of interested collected from the hepatic extract (B).

The various functional groups that were identified with the commercial standard of ULA and in the fraction of hepatic extract using FTIR are provided within Table 4.1.

Table 4.1. Vibrational frequencies and their corresponding functional groups pertaining to the FTIR spectra obtained for a commercial standard of ULA and the fraction of interest collected from the hepatic extract (Fig.4.4.).

Group frequency for Commercial ULA (cm^{-1})	Group frequency for Hepatic Extract Fraction (cm^{-1})	Strength	Functional Group
3010	3011	Strong	<i>Cis</i> double bond stretching ^a
2925 & 2855	2925 & 2855	Strong	C-H stretching in $-\text{CH}_3$ and CH_2 ^a
1707	1708	Strong	Carboxylic acid ^c
1460 (not labelled)	1460	Medium	Bending vibrations of the CH_2 and CH_3 aliphatic groups ^b
1413	1413	Medium	Rocking vibrations of <i>cis-di</i> -substituted alkenes ^b
1283	1281	Medium	C-O stretching ^{b, d}
934	930	Weak	Bending vibrations of CH functional groups of <i>trans</i> alkenes ^{b, c}
722	721	Weak	<i>Cis</i> C-H out of plane bend

(^aForfang *et al*, 2017, ^bRohman and Che Man, 2010, ^cCoates, 2000, ^dSafar *et al*, 1994).

When a spectral library search was performed on both of the above samples, a match of 99.68% for the commercial ULA while the hepatic extract fraction returned a match of 98.80% with linoleic acid (HR Nicolet Sampler Library).

4.3. Gas Chromatography-Mass Spectrometry for the Identification of ULA in the Hepatic Extract Fraction

In order to further confirm that the fraction collected during HPLC analysis of the hepatic extract was unconjugated linoleic acid, a crude, a derivatized sample of both commercial ULA and the hepatic extract was analysed using GC-MS. According to Quehenberger *et al* (2011), GC has become widely adopted as a reliable tool for the analysis of complex mixtures of fatty acids.

Fatty acid methyl esters of linoleic acid in the hepatic extract were identified by conducting comparisons of similar peak retention times using the pure commercial ULA standard. The total ion chromatogram for the both the commercial standard of ULA (chromatogram A) and the analyte extracted from equine hepatic tissue samples (chromatogram B) is displayed in Figure 4.5. The major peak observed in chromatogram A of figure 4.5 had a retention time of 13.42 minutes corresponding to a methyl ester of linoleic acid. This retention time corresponds with the main peak observed at 13.40 minutes in chromatogram B. Mass spectrum and library match for both the standard of ULA and the hepatic extract are illustrated in Appendix A. The remaining peaks correspond to additional fatty acid methyl esters, all of which were tentatively identified using NIST library searches and are detailed in table 4.2.

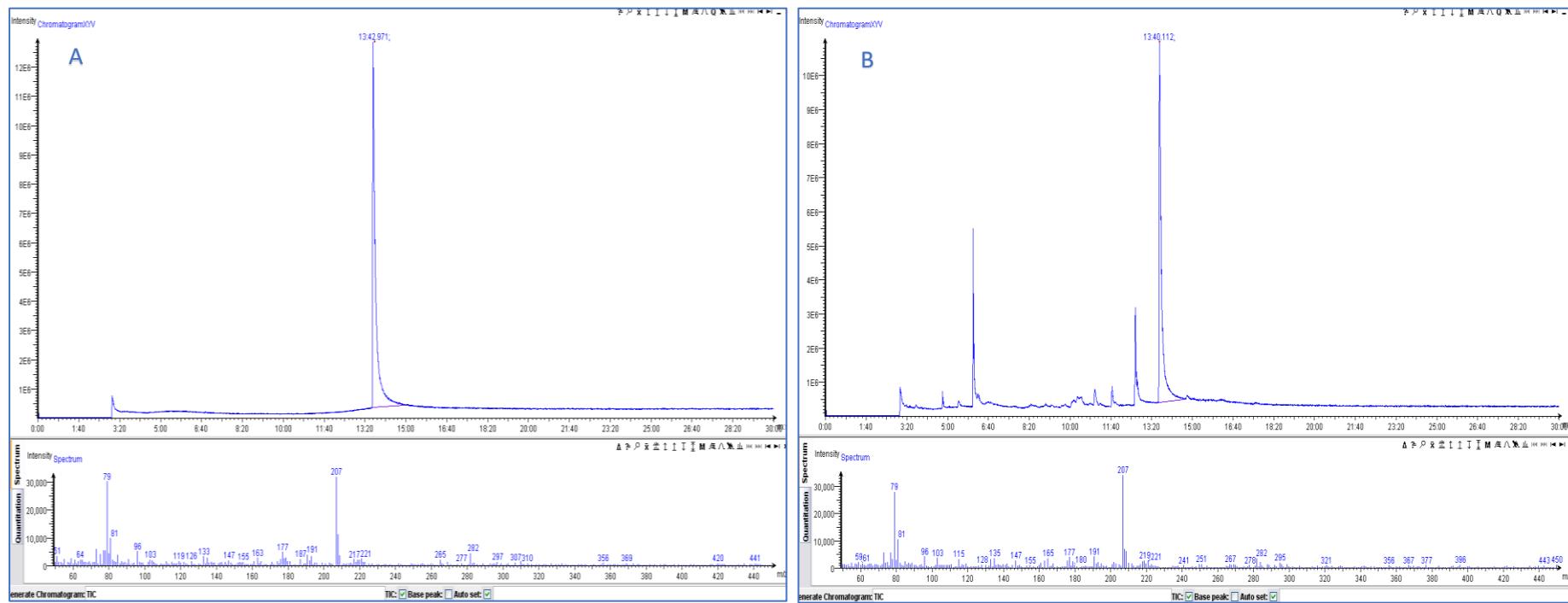


Figure 4.5. GC-MS Chromatogram of a derivatised standard of commercial linoleic acid (A) and the derivatised hepatic extract (B). The main peak in each chromatogram, appearing at 13.42 and 13.40 respectively, have been identified, based on the NIST spectral database, as linoleic acid in its *cis* 9, *cis* 12 formation. In chromatogram B, a number of other peaks were tentatively identified (table 4.2).

The additional peaks observed in chromatogram B representing the hepatic extract.

Although it falls outside the scope of this investigation, these peaks were tentatively identified using the NIST spectral library and are presented in table 4.2.

Table 4.2. Identification of the additional peaks observed in the chromatogram of the derivatised hepatic extract (chromatogram B in figure 4.4).

Retention time (minutes)	NIST MS library match
3.03	Hexane
4.47	1-Hexadecanol, 2 methyl
5.26	Pentanoic acid, 4-methyl, methyl ester
6.01	Hexanal dimethyl acetal
11.00	9, 12-octadecadienal, dimethyl acetal
11.43	Octadecanoic acid, 9, 10-dichloro-, methyl ester
12.40	7-Hexadecanoic acid, methyl ester (Z)

4.4. Discussion

4.4.1. HPLC Analysis of ULA

A typical chromatographic result obtained from HPLC analysis of a hepatic tissue sample is presented in figure 4.2. The peak can be compared to the peak obtained during analysis of a commercial standard of ULA as displayed in figure 4.1, confirming the positive identification of the fatty acid in the tissue sample.

A shoulder observed at 5.8 minutes on the peak obtained for the commercial standards of ULA (Fig. 4.1), thought to be an isomer of linoleic acid, indicates that the commercial standard is not isomerically pure. As the purity of the standard was stated as $\geq 99\%$, the presence of an additional unresolved peak was unexpected. Cooper (2015) highlighted the same issue with commercially produced standards of ULA. Both nuclear magnetic resonance analysis and gas chromatography-time-of-flight-mass spectrometry indicated the presence of conjugated isomers of the fatty acid in the commercial standards of ULA (Cooper, 2015).

The retention time for the peak obtained for the commercial standard was 6.322 ± 0.006 ($n = 14$) minutes (figure 4.1) while the retention time of the hepatic sample was 6.733 ± 0.007 ($n = 217$) minutes (figure 4.2). According to the Commission Decision on implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC) the retention times must be identical within a margin of 5%. Through application of this logic, the hepatic samples and the extract were considered identical to within a margin of 6%. This indicated that further analysis of the hepatic extract was required to confirm the presence of ULA in the equine tissue. As such, further analysis by fourier transform infrared spectroscopy (FTIR) and gas chromatography - time of flight - mass

spectrometry (GC-TOF-MS) were also performed to ensure accurate structural and molecular weight determination.

A standard curve also determined that the mean concentration of linoleic acid in hepatic tissue was 2.6mg (\pm 0.28) per gram of hepatic tissue analysed from 217 specimens. According to unpublished data by Cooper (2015) concentrations observed to be higher than 5mg per 1 gram of tissue analysed were considered high. Of the 217 hepatic samples analysed, 10.6% contained concentrations of 5mg per gram of tissue analysed or higher. Of this 10.6%, the mean concentration of linoleic acid was determined to be 12.1mg (\pm 1.56) per gram of tissue analysed. Among these, one individual case presented with a ULA concentration as high as 30mg per gram of tissue analysed, equating to 0.11mM of ULA, while two more cases reported with concentrations higher than 27mg per gram of tissue analysed (0.10mM of ULA). Recent studies conducted by Adolph *et al* (2019) reported that the n-6 polyunsaturated fatty acids, such as linoleic acid, were the most frequently found fatty acids in the hepatic tissues of equines.

4.4.2. Fourier Transform Infrared Spectroscopy for identification of the Hepatic Extract Fraction

A commercial standard of ULA and a sample of the fraction of interest collected from the hepatic extract were analysed and the resulting spectral peaks were identified in Table 4.1. The peak appearing at \sim 3010cm $^{-1}$ was attributed to the CH stretching of *cis* double bonds (Ayora-Cañada *et al*, 2010). According to Coates (2000), band structures observed between 3150cm $^{-1}$ and 3000cm $^{-1}$ are almost exclusively indicative of unsaturation. The appearance of a strong peak in the region of 2925cm $^{-1}$ and 2855cm $^{-1}$ were attributed to the stretching vibrations of aliphatic CH in the methylene and

terminal methyl groups associated with the structure of a fatty acid chain (Ayora-Cañada *et al*, 2010).

A strong single peak observed at $\sim 1708\text{cm}^{-1}$ corresponded to the C=O stretching vibrations of carbonyl groups (Ayora-Cañada *et al*, 2010) such as carboxylic acid (Coates, 2000). The peaks observed between the regions of 1400cm^{-1} and 1200cm^{-1} were attributed to the bending vibrations of CH₂ and CH₃ aliphatic groups while the peak observed at the $\sim 722\text{cm}^{-1}$ region corresponded to CH₂ rocking vibrations as well as the out-of-plane vibrations of *cis* di-substituted alkenes (Ayora-Cañada *et al*, 2010).

According to Coates (2000), a strong methylene/methyl band (1470cm^{-1}) and a weak methyl band (1380cm^{-1}), plus a band at $725\text{--}720\text{cm}^{-1}$ (methylene rocking vibration) is indicative of a long-chain linear aliphatic structure. The functional groups identified in this analysis corresponds to the functional groups present in ULA (Figure 4.1).

However, the peak observed in the region of $\sim 934\text{cm}^{-1}$, according to Ayora-Cañada *et al*, (2010), corresponded to *trans* alkenes. When a spectral library search was performed on the commercial standard of ULA, a match of 99.6% was made when the spectrum was compared to that of linoleic acid as provided by the HR Nicolet Sampler Library. While there is no published data regarding the acceptable margin of the percentage difference between spectral library standards and commercial standards, the difference between the spectral results obtained in this case may be due to the presence of *trans* alkenes. This may also explain the presence of the shoulder observed in the HPLC analysis of the commercial standard of ULA (Figure 4.1).

Comparisons were then made between the peaks observed in the spectrum obtained for the commercial standard and the spectra obtained for hepatic extract fraction. As indicated by figure 4.4, the majority of bands observed are present in both spectra.

However, a peak appearing in the hepatic extract fraction at 3779cm^{-1} corresponds to a hydroxyl alcohol (Coates, 2002). The appearance of this peak would not be considered unexpected, as the mobile phase used in the HPLC analysis and collection method was methanol. This correlates with the findings of Cooper (2015), who positively identified the presence of *cis*-9, *cis*-12, ULA in similar equine hepatic extracts.

4.4.3. Gas Chromatography - Time of Flight - Mass Spectrometry for the Identification of the Hepatic Extract

Gas Chromatography-Time of Flight-Mass Spectrometry (GC-TOF-MS) was used to identify the presence of ULA in the hepatic extract collected in the HPLC fraction. GC-TOFMS techniques allow for the determination of the molecular weight and analysis of trace amounts of fatty acid methyl esters (Mohd, 2012).

In nature, fatty acids of animal and plant origin generally have even numbers chains containing sixteen to twenty-two atoms with zero to six double bonds in the *cis* configuration. However, numerous exceptions also occur with countless odd and even numbered fatty acids, containing up to 100 carbon atoms, with both *cis* and *trans* double bonds existing (Christy, 1998). Gas chromatography coupled with mass spectrometry is a widely used technique for the analysis of lipids components such as fatty acids (Salimon *et al*, 2017). However, carboxyl groups associated with fatty acids have a tendency to form intermolecular hydrogen bonds, which in turn affect their ability to interact with column packing and thermal stability (Orata, 2012). As such, successful analysis of fatty acids requires the conversion of said molecule to their methyl ester derivatives. The derivatisation technique employed to analyse both the commercial ULA and the hepatic extract involved the use of boron trifluoride (BF_3)

in methanol. According to Aldai (2004) BF_3 has been commonly used as an efficient esterification reagent, converting fatty acids to their methyl ester derivatives while also effectively methylating fatty acids directly from their acyl-glycerol parent lipid. This indicates that, should the hepatic extract contain lipid formations such as acyl-glycerols, these compounds should also be converted directly to methyl esters of their fatty acid components, reducing sample loss as a result.

Once both the standard of ULA and the hepatic extract were analysed, comparisons were made between the chromatograms obtained for both to definitively identify the presence of ULA in the hepatic extract.

Chromatogram A in figure 4.5 represented the derivatised standard of commercial ULA while chromatogram B in figure 4.5 represented the derivatised hepatic extract. The major peak observed in chromatogram A of figure 4.5 had a retention time of 13.42 minutes corresponding to a methyl ester of linoleic acid. This retention time corresponds with the main peak observed at 13.40 minutes in chromatogram B.

When mass spectral data of both samples were compared both samples shared a similar fragmentation pattern, with the same molecular ion peak ($m/z = 282$) and the same base peak ($m/z = 207$). The compounds were also identified by the comparison of the mass spectra and retention indices for both compounds with their references standards in the National Institute of Standards and Technology (NIST) library (Lu *et al*, 2008). The mass spectrum and library match for both the standard of ULA and the hepatic extract are illustrated in Appendix A.

Although it falls outside the scope of this investigation, the remaining peaks observed in chromatogram B are presented in table 4.2. The presence of additional analytes is

not entirely unexpected as the extract was sourced from multiple biological tissues and was not purified other than the initial collection of individual fractions.

4.5. Conclusion

The three analytical techniques, HPLC, FTIR and GC-TOF-MS, used in this chapter confirmed the presence of ULA in hepatic extracts obtained from the tissue of Equines presenting at the IEC with an idiopathic and fatal haemorrhagic condition. According to Adolph *et al*, (2019) n-6 PUFAs, such as linoleic acid are the most frequently observed lipid class found in the hepatic tissues of equines, followed by the saturated fatty acids. Based on the historical findings of Cooper (2015), concentrations of 5mg per gram of tissue analysed were considered high. In the current investigation 10.6% of 217 specimens analysed contained concentrations of 5mg of ULA or higher with some individual specimens returning concentrations of 27 to 30mg of ULA per gram of hepatic tissue analysed. Unfortunately the unavailability of epidemiological data pertaining to each equine sample analysed in the current investigation meant that a pathological dose in the equine could not be determined. However, in the initial study, Cooper (2015) determined that, in 832 Equines analysed, 162 were diagnosed with a coagulopathy. In these animals, linoleic acid, in its unconjugated form, was present at a mean concentration of 6.2mg per gram of hepatic tissue analysed. The other 670 animals without a coagulopathy diagnosis, had a mean concentration of 2.91mg per gram of hepatic tissue analysed. Statistical analysis performed by Cooper (2015) indicated that equines with higher concentrations of unconjugated linoleic acid (mean concentration \geq 10.00 mg/g) had a significantly higher risk of haemorrhaging ($P<0.001$) than animals with lower concentrations (mean concentration of 0.50 mg/g).

Based on this, it could be hypothesised, as Cooper (2015) indicated, that concentrations of unconjugated linoleic acid higher than 5mg per gram of hepatic tissue

analysed could be considered supraphysiological and therefore pathological. It could also be hypothesised that supraphysiological concentrations of ULA in the hepatic tissue of affected equines may be associated with the idiopathic haemorragic condition observed by the IEC. Using these data, further *n vitro* studies were performed to aid in the elucidation of this phenomenon.

Chapter 5

Preliminary Analysis of the *In Vitro* Effects of ULA

Chapter 5. Preliminary Analysis of the *in vitro* Effects of ULA

The following chapter details the preliminary examination of the *in vitro* effects of ULA over a varying range of concentrations and its impact on cell viability, mitochondrial health and intracellular lipid accumulation.

5.1 *In Vitro* Effects of ULA; the Assessment of the effects of ULA over 24 and 48 hours

In order to determine if ULA has the potential to be lipotoxic at physiologically high concentrations, a commercial standard of the fatty acid was purchased from Sigma Aldrich and cell viability assays were carried out using the Human Hepatoma cell line, HepG2. These cells were grown to 70 – 80% confluence before being treated with varying concentrations of ULA ranging from 0mM to 10mM (section 3.1.4).

Cell viability was evaluated by measuring the conversion of the blue non-fluorescent resazurin, the primary reporter dye in alamarBlue®, to resorufin, a red compound that is highly fluorescent. This assay was initially considered as it is highly sensitive and relies on the ability of live cells with functioning mitochondria to enzymatically convert this dye into the measurable fluorescent or colour product, resorufin (McMillian *et al*, 2002, Czekanska, 2011).

In the following alamarBlue® assays, the absorbance of the colour change was measured as an endpoint using absorption spectroscopy. Absorbance was measured at 570nm and 600nm. The values obtained were used to determine the percentage reduction of resazurin in test wells compared with that of a positive growth control, cells treated with 0mM of ULA. The quantity of resorufin produced is proportional to the percentage of viable cells (Nourse *et al*, 2007, Riss *et al*, 2016).

5.1.1. Preliminary Analysis of Cell Viability after ULA Treatment

Initially, a preliminary cell viability study was carried out, in order to assess the lipotoxic effects of high concentrations ULA. HepG2 cells were treated with varying concentrations of ULA over a period of 24 and 48 hours

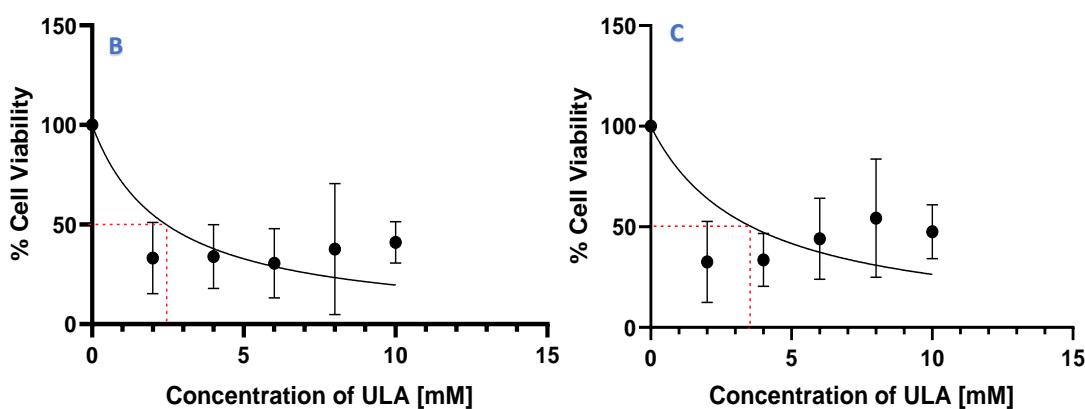
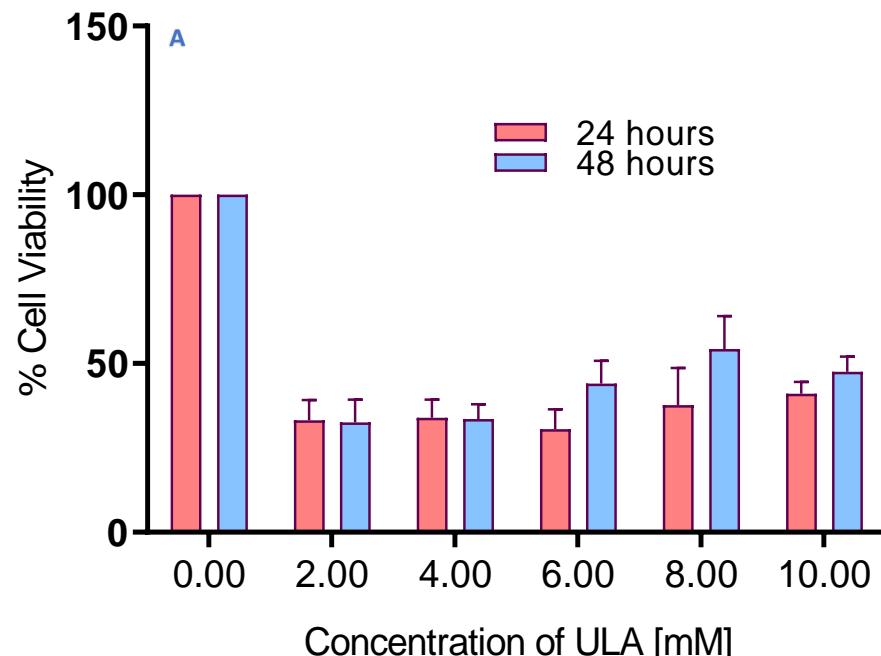


Figure 5.1. Percentage of viable HepG2 cells treated with varying concentrations of ULA over a period of 24 and 48 hours (A) compared with an untreated control ($n=3$). AlamarBlue® was used as an endpoint. Viability levels are expressed as a percent of the control (assigned as 100%). Statistical analysis was performed using mean absorbance values (\pm S.E.M.). One-way ANOVA using Dunnett's test as a post-test to compare all means. Concentration-response curves for 24 hours (B) and 48 hours (C) were generated to establish IC₅₀ values for ULA using GraphPad Prism software.

The results portrayed in figures 5.1 indicate that ULA significantly inhibited the ability of HepG2 cells to reduce resazurin to resorufin when compared with the untreated control ($P=0.0001$). This indicates that the fatty acid had an inhibitory effect on cell proliferation.

Table 5.1. The half-maximal inhibitory concentration (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period or 24 and 48 hours. AlamarBlue® was used as an endpoint ($n=3$). Statistical analysis was performed using mean concentration values ($\pm S.E.M.$).

Time Point	IC ₅₀ Concentration ($\pm S.E.$)
24 hours	2.44mM ± 0.395
48 hours	3.58mM ± 0.621

Concentration-response curves were generated using the cell viability data presented in figure 5.1. These curves represent the relationship between ULA and the inhibition of cell viability in HepG2 cells. An IC_{50} value of 2.44mM (± 0.395) was observed in cells treated for 24 hours while the IC_{50} increased to 3.58mM (± 0.621) in cells treated for 48 hours.

5.2. Adenosine Triphosphate Assay

As previously mentioned, the mitochondria play a vital role in lipid metabolism, particularly the biosynthesis of some phospholipids and the β -oxidation of fatty acids as well as the synthesis of unique fatty acids, lipid cofactors and steroid hormones (Mayr, 2014). They are also responsible for various other functions such as regulation of cell death and cellular energy homeostasis. According to Yamashina *et al.*, (2009), the majority of ATP is produced by the mitochondria of most mammalian cells. In order to determine if high concentrations of ULA affects the ATP producing ability of the mitochondria of HepG2 cells, an ATP assay was carried out.

5.2.1. ATP Standard Curve

A standard curve was prepared to accurately measure ATP levels in HepG2 cells treated with varying concentrations of ULA (Figure 5.2).

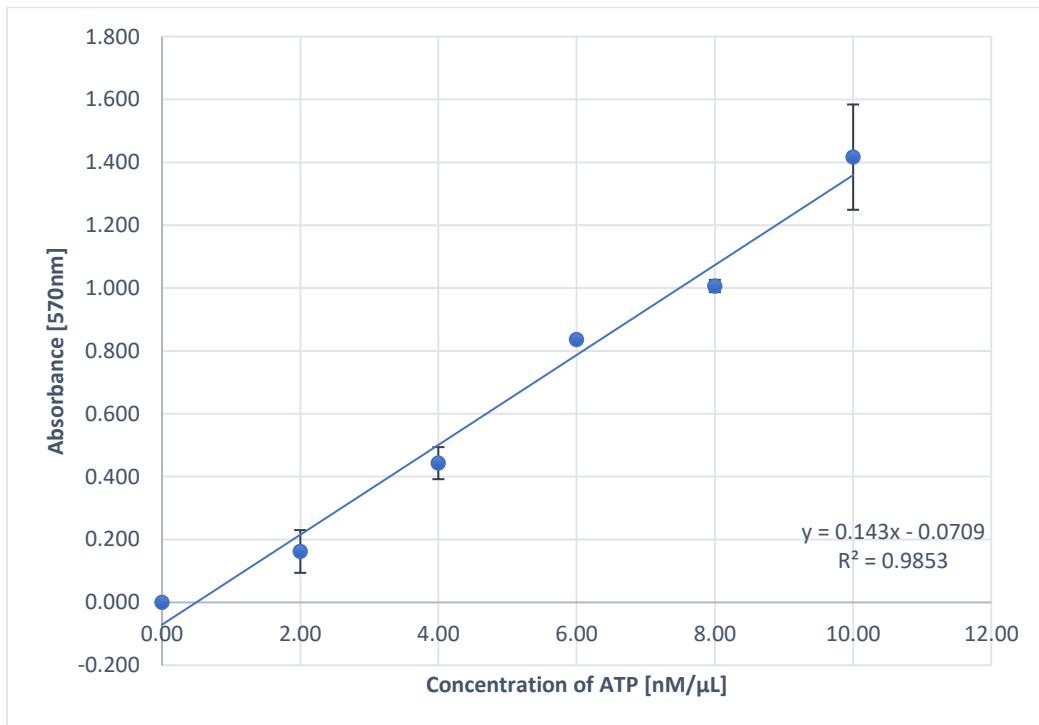


Figure 5.2. ATP Standard Curve

5.2.2. ATP Production in HepG2 Cells treated with Varying Concentrations of ULA

Figure 5.3 details the concentration of ATP produced by HepG2 cells exposed to increasing concentrations of ULA for a period of 21 hours.

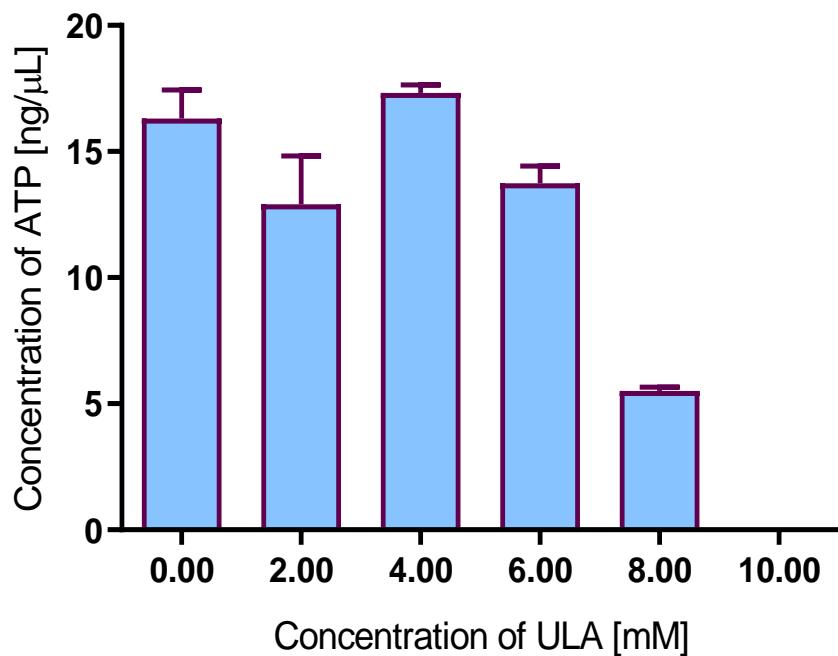


Figure 5.3. The concentration (ng/μL) of ATP produced by HepG2 cells treated with varying concentrations of ULA for a period of 21 hours ($n=3$). Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA with Dunnett's test as a post-test was used to compare all means.

The results displayed in figure 5.3 indicate that, while ATP production was not dose-dependent, a reduction in ATP production occurred as ULA concentrations exceeded 4mM.

5.3. Cell Viability in HepG2 Cells treated with ULA over 24 and 48 Hours

After initial analysis, further cell viability assays were carried out on HepG2 cells treated with ULA ranging from 0mM to 5mM over 24 and 48 hours. Physiological concentrations of free fatty acids have been reported to be between 0.3mM and 1mM (Tikanoja *et al*, 1989, Shultz, 1991, Artwohl *et al*, 2003). These assays were carried out to determine the effects of lower, more physiologically relevant concentrations of the fatty acid on hepatic cells. The treatment method was also altered to include the use of a fatty acid free BSA as well as media with a low glucose content (section 3.1.4.1.) (Yao *et al*, 2011). The resazurin assay was amended to ensure that the optical density values obtained were not affected by the presence of ULA in the treatment media (section 3.1.5.1).

5.3.1. HepG2 Cells Viability after ULA Treatment using Resazurin (alamarBlue®) Assay.

To ensure that ULA was not affecting the optical density, treatment media containing ULA was removed and fresh media containing resazurin was added to each well before incubation. A sample control of 0.1mM of ULA was also assayed to confirm that ULA was not completely inhibiting cell viability.

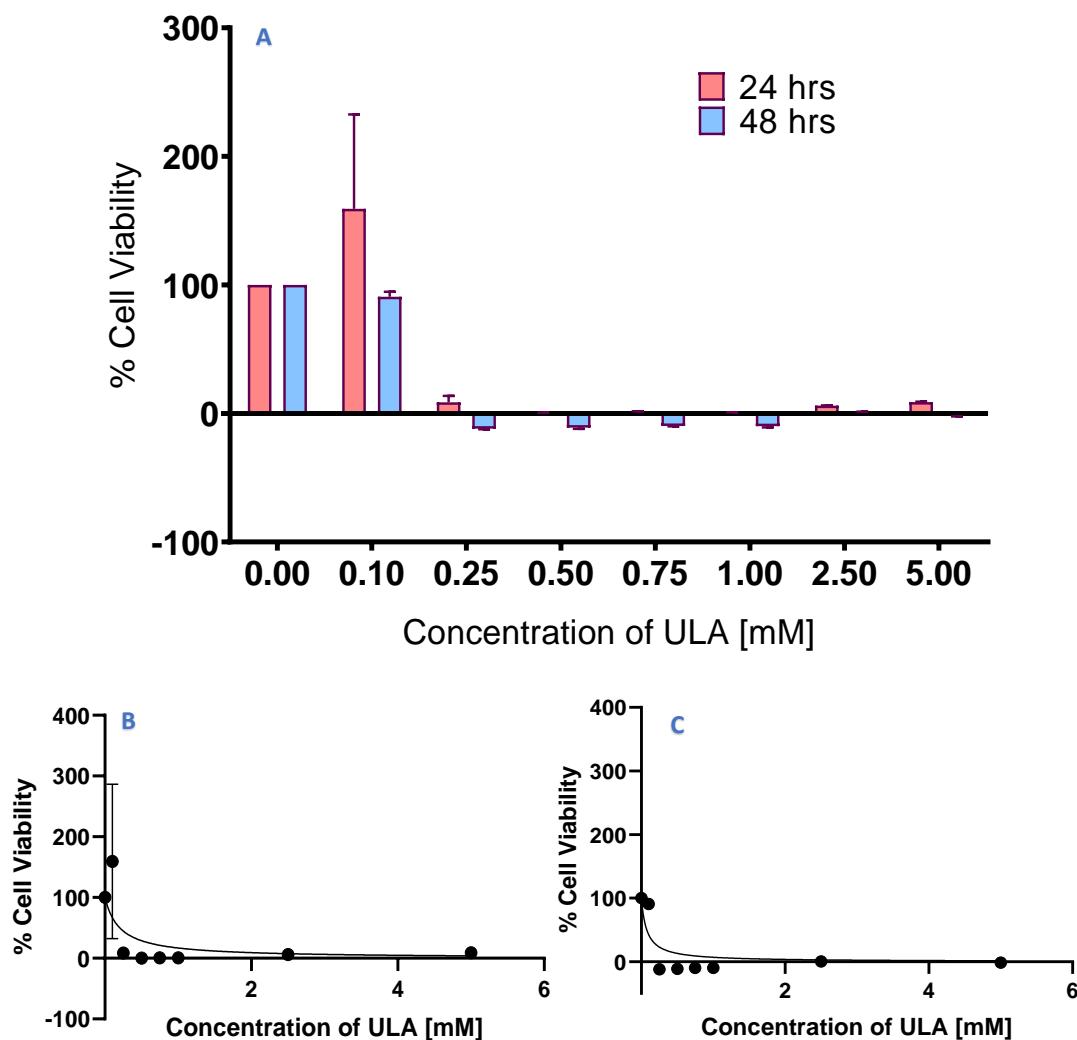


Figure 5.4. Percentage cell viability of HepG2 cells treated with varying concentrations of ULA over a period of 24 hours and 48 hours (A) compared with an untreated control ($n=1$). AlamarBlue® was used as an endpoint. Viability levels are expressed as a percent of the control (assigned as 100%). Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Dunnett's test as a post-test to compare the means of all treated samples to the untreated controls. Concentration-response curves for 24 hours (B) and 48 hours (C) were generated to establish IC₅₀ values for ULA using GraphPad Prism software.

The results portrayed in figure 5.4. illustrate that ULA at all concentrations, except for 0.1Mm, significantly inhibited the ability of HepG2 cells to reduce resazurin to resorufin when compared with the positive growth control after 48 hours ($P=0.0001$). This indicates that the fatty acid had an inhibitory effect on cell proliferation.

Table 5.2. The half-maximal inhibitory concentration (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period or 24 and 48 hours. AlamarBlue® was used as an endpoint ($n=1$). Statistical analysis was performed using mean concentration values ($\pm S.E.M$).

Time Point	IC_{50} Concentration ($\pm S.E$)
24 hours	$0.20\text{mM} \pm 0.139$
48 hours	$0.08\text{mM} \pm 0.032$

Concentration-response curves were generated and an IC_{50} for ULA of 0.20mM (± 0.139) was established for cells treated over 24 hours while an IC_{50} value of 0.08mM (± 0.032) was established for cells treated over 48 hours.

5.3.2. HepG2 Cell Viability after ULA Treatment using the MTT Assay

An MTT assay for cell viability was also performed on HepG2 cells treated with varying concentration of ULA over 24 and 48 hours. The MTT assay is considered to be one of the most widely used and simplest assays for the measurement of cytotoxic effects of compounds as well as the assessment of cell viability (Wang *et al*, 2010, Sumantran, 2011). The concept of the MTT assay, like alamarBlue®, relies on the ability of viable cells to reduce yellow tetrazolium into purple formazan, which can then be measured spectrophotometrically (Wang *et al*, 2010, Riss *et al*, 2016). The quantity of formazan produced is considered directly proportional to the number of viable cells (Riss *et al*, 2016).

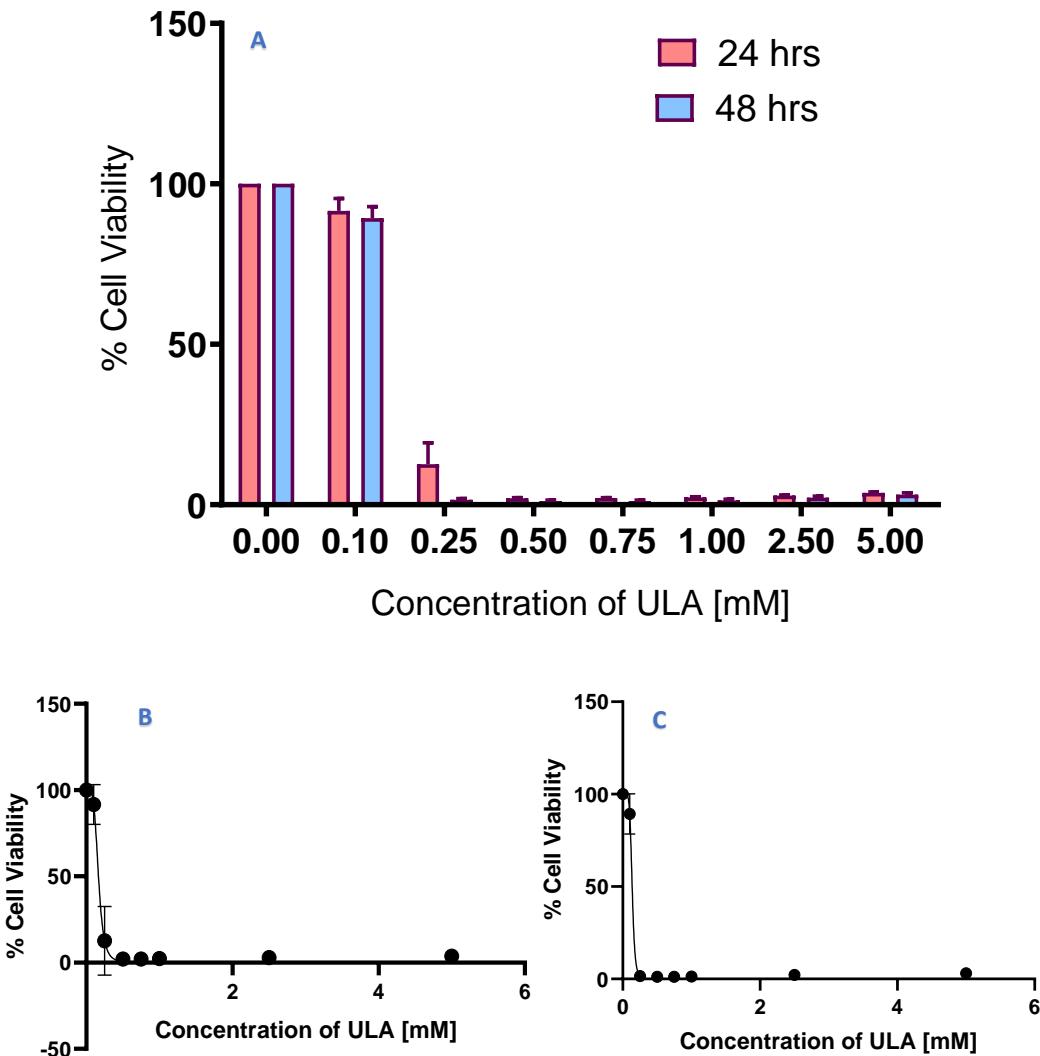


Figure 5.5. Percentage cell viability of HepG2 cells treated with varying concentrations of ULA over a period of 24 hours and 48 hours (A) compared with a positive growth control. MTT was used as an endpoint ($n = 3$). Viability levels are expressed as a percent of the control (assigned as 100%). Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Dunnett's test as a post-test to compare the treated cells to the untreated control. Concentration-response curves for 24 hours (B) and 48 hours (C) were generated to establish IC₅₀ values for ULA using GraphPad Prism software.

The results portrayed in figure 5.5 determined that ULA significantly inhibited the ability of HepG2 cells to reduce tetrazolium into formazan, supporting the results obtained from the alamarBlue® assay. The treatment control, namely, cells treated with 0.1mM of ULA, did not reduce cell viability considerably when compared to the positive growth control.

Table 5.3. The half-maximal inhibitory concentration (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period of 24 and 48 hours. MTT was used as an endpoint ($n=3$). Statistical analysis was performed using mean concentration values ($\pm S.E.M.$).

Time Point	IC_{50} Concentration ($\pm S.E.$)
24 hours	$0.17\text{mM} \pm 0.007$
48 hours	$0.14\text{mM} \pm 0.006$

Concentration-response curves were generated and an IC_{50} for ULA of 0.17mM (± 0.007) was established for cells treated over 24 hours while an IC_{50} value of 0.14mM (± 0.006) was established for cells treated over 48 hours.

5.4. Oil Red O for intracellular Lipid Accumulation

Oil Red O is a hydrophobic lipid soluble diazo dye, with a maximum absorption of 518nm, which is often used in conjunction with brightfield microscopy to visualise and quantify intracellular lipid stores (Mehlem *et al*, 2013, Daemen *et al* 2015). Oil Red O stains neutral lipids and cholestryl esters but not biological membranes. The principle for staining is that Oil Red O is minimally soluble in IPA, with solubility further decreasing upon dilution in water. When the stain is applied to lipid containing cells, the stain will move from the solvent to associate with the lipids (Mehlem *et al*, 2013).

5.4.1. Intracellular Lipid Accumulation in HepG2 Cells

HepG2 cells were treated with varying concentrations of ULA over a period of 24 and 48 hours. Cells were then assessed for intracellular lipid accumulation. Untreated cells, grown in media alone, were considered to have 0% intracellular lipid accumulation and were termed the untreated control group. All treated cells were compared to this control group.

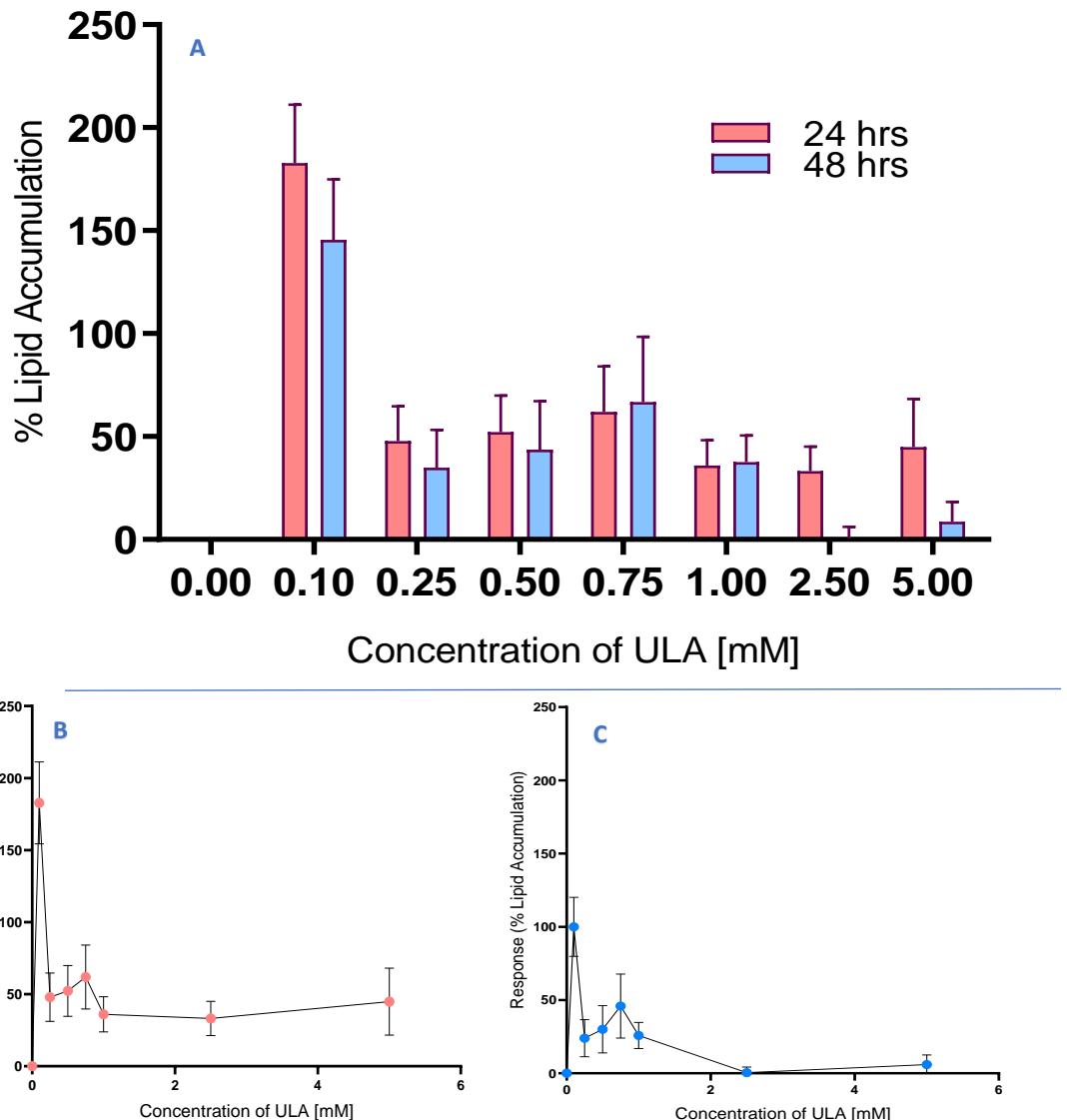


Figure 5.6. (A) Percentage lipid accumulation in HepG2 cells treated with varying concentrations of ULA over a period of 24 and 48 hours. Treated cells were compared to a positive growth control in which cells were grown in fatty acid free BSA constituted media containing $\leq 1\%$ IPA ($n=4$). Lipid accumulation levels are expressed as a percent of the control (assigned as 0%). Statistical analysis was performed using mean absorbance values (\pm S.E.M.). One-way ANOVA using Dunnett's test as a post-test to compare the treated cells to the untreated control. (B) Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA over a period of 24 and 48 hours. Data was also presented in the form of dot plots for 24 hours (B) and 48 hours (C) using GraphPad Prism Software to present the relationship between the concentration and response.

The results indicated a significant increase in intracellular lipid accumulation after 24 and 48 hours of treatment with 0.1mM of ULA ($P = <0.0001$). A mean absorbance value of 0.045 (± 0.007) corresponding to 0% intracellular lipid accumulation was obtained for untreated cells while cells treated with 0.1mM presented with a mean

absorbance value of 0.115 (± 0.008) after 24 hours, indicating an approximately two-fold increase in intracellular lipid accumulation. While there was an increase in intracellular lipid accumulation in all other treatment groups compared, these increases were not statistically significant.

Oil Red O was used to determine intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA over 24 and 48 hours.

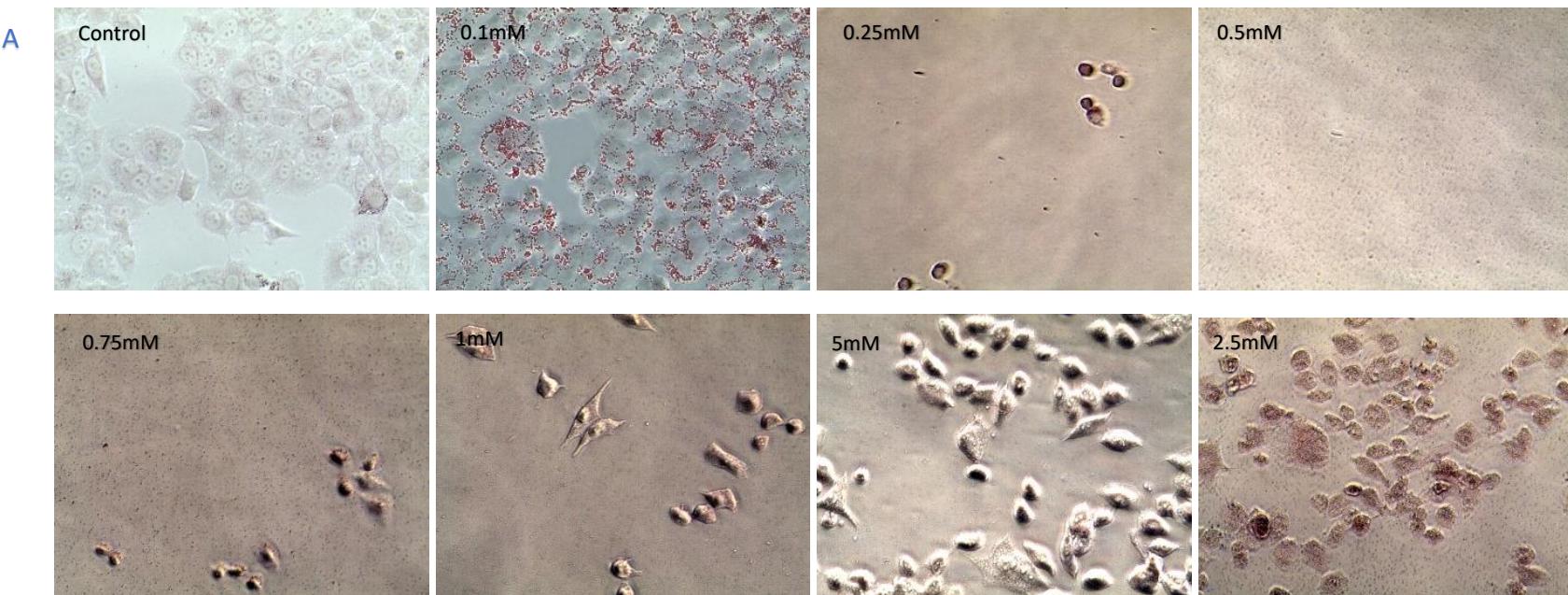


Figure 5.7. (A)Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over 24 hours. Control cells were grown in media containing fatty acid free BSA and 1% IPA.

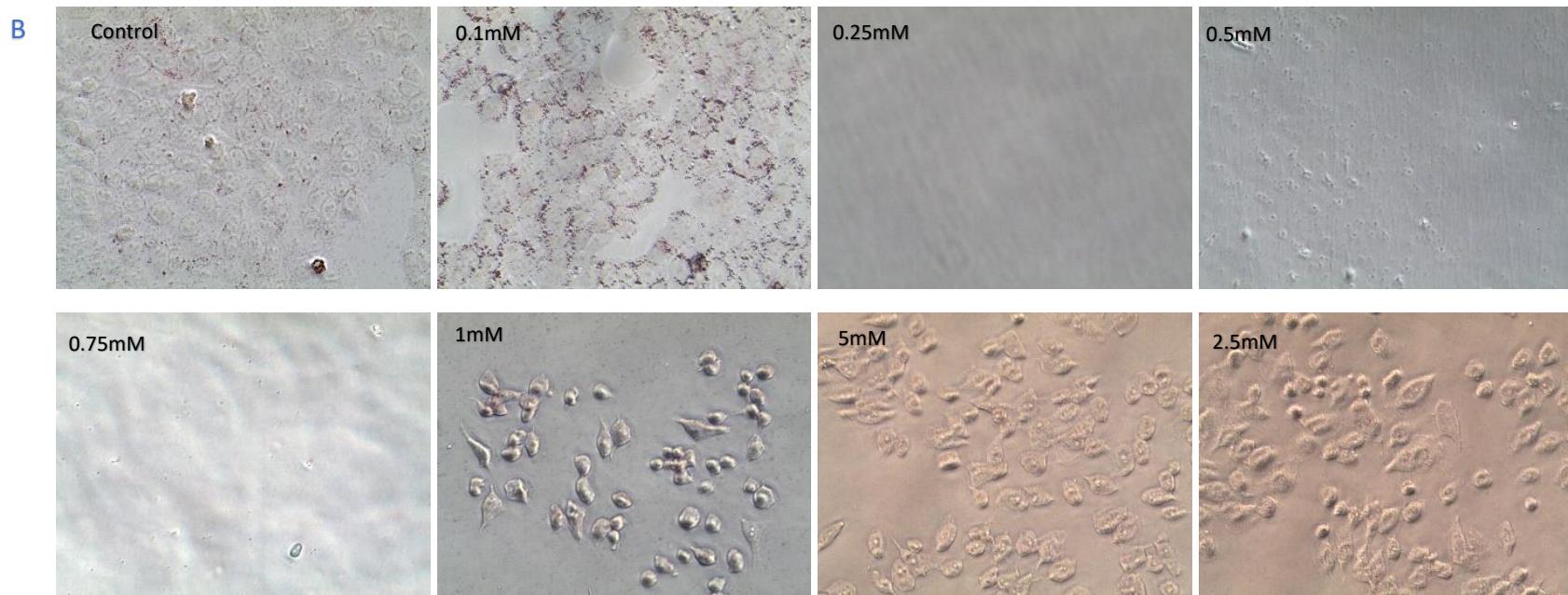


Figure 5.7. (B) Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over 48 hours. Control cells were grown in media containing fatty acid free BSA and 1% IPA.

The micrographs presented in figure 5.7 show the presence of Oil Red O stained lipid droplets in cells treated with 0.1mM of ULA for both time points. The red stain appears more intense in these cells compared to the untreated control, indicated that treatment with 0.1mM of ULA increased intracellular lipid accumulation. This corresponds with the data presented in figure 5.6, where cells treated with 0.1mM presented with the highest percentage of intracellular lipid accumulation. However, as the concentration reached 0.25mM of ULA, cell morphology appears negatively impacted, with cell number in the visual field reduced.

5.5. Discussion

Fatty acids are essential substrates required for numerous functions, including the production of energy and the synthesis of lipids such as membrane lipids and lipids essential for cellular signalling (Zechner *et al*, 2012).

However, despite their physiological importance, excessive amounts of fatty acids can have negative consequences. Zechner *et al*, (2012) postulate that an oversupply of non-esterified fatty acids can disrupt the integrity of the cell membrane, alter cellular homeostasis and initiate the production of harmful bioactive lipids. This can result in the impairment of normal membrane function, endoplasmic reticular stress, mitochondrial dysfunction and eventual cell death.

5.5.1. Preliminary Analysis of Cell Viability after ULA Treatment

According to Yao *et al* (2011), the addition of free fatty acids to cells *in vitro* may lead to a significant increase in the accumulation of lipid droplets in the cytoplasm of the cell. This accumulation of high concentrations of lipid droplets within the cell may induce a lipotoxic response such as apoptosis or necrosis, *via* pro-inflammatory signalling. Lipotoxicity is the broad term given to impaired tissue homeostasis that is attributed to changes in lipid utilisation or lipid induced changes in intracellular signalling (Symons and Abel, 2013).

A preliminary study was initially carried out in order to determine if ULA has the potential to be lipotoxic at increasing concentrations. Cell viability assays were carried out using a commercial standard of linoleic acid and the hepatic epithelial cell line, HepG2. Initially cells were treated with concentrations of ULA ranging from 0mM to 10mM (Figure 5.1.). These concentrations were considered to be much higher than the physiological norm of 0.3mM to 1mM, as indicated by Tikanoja, *et*

al, 1989, Shultz, 1991 and Artwohl *et al*, 2003. However, several studies have determined that physiological plasma concentrations of fatty acids can range from 0.09mM to 1.2mM, with values reaching as high as 2.5mM in stressful conditions such as diabetes, fasting or strenuous exercise (Høstmark, 1995, Toborek *et al*, 1996, Young *et al*, 1998).

It has also been established that, in horses, the normal metabolic rate can be altered through pain, stress or anorexia. Gomaa, *et al* (2009) determined that horses suffering from impaction colic demonstrated serum free fatty acid concentration of as high as 1.638mM. As such, in order to ascertain if high concentrations of ULA may have a toxic effect on hepatic cells or vascular endothelial cells, the fatty acid was initially applied in the ranges of 0mM to 10mM for a period of either 24 or 48 hours.

HepG2 cells treated with varying concentrations of ULA showed a significant decrease in cell viability when compared with cells that had been treated with DMEM (HepG2) with an IPA concentration of $\leq 1\%$ (Figure 5.1.). This was considered to be the untreated control. The performance of a one-way-ANOVA indicated that all group means were significantly different. In order to establish where the significance lay, Dunnett's Multiple Comparison *post hoc* test was carried out. The *post hoc* test determined that the significance lay between each of the treatment groups when compared to the positive growth control ($P=0.0001$). An IC₅₀ of 2.44mM (± 0.395) was also determined for cells treated over 24 hours while, after 48 hours, an IC₅₀ of 3.58mM (± 0.621) was established.

Yao *et al* (2011) indicated that exposure of HepG2 cells to increasing concentrations of free fatty acids (2mM to 3mM) initiated a dose dependent reduction in cell viability. While these results do not directly reflect those obtained by Yao *et al*

(2011), they do indicate that doses of free fatty acids in the range of 2mM to 3mM reduced cell viability significantly.

5.5.2. ATP Production in HepG2 Cells treated with Unconjugated Linoleic Acid.

Mitochondria are essential cytoplasmic organelles responsible for a number of vital cellular mechanisms. As previously mentioned, they are responsible for lipid metabolism, particularly the biosynthesis of some phospholipids and the β -oxidation of fatty acids as well as the synthesis of unique fatty acids, lipid cofactors and steroid hormones (Mayr, 2014). They are also involved in amino acid metabolism and ion homeostasis as well as playing a role in the regulation of cell death pathways *via* the production of reactive oxygen species (Yamashina *et al*, 2009).

However, one of their major functions is the production of Adenosine Triphosphate (ATP). According to Yamashina *et al*, (2009), in healthy mammalian cells, the majority of the cellular energy carrier, ATP, is produced by the mitochondria. These essential cytoplasmic organelles generate ATP through the processes of glycolysis and lipolysis.

One method of assessing mitochondrial health is through the measurement of cellular ATP production. In order to determine if cellular ATP production was affected by high concentrations of ULA, an ATP assay was carried out on HepG2 cells treated with varying concentrations of the fatty acid over a period of 21 hours. The concentrations of ULA ranged from 0mM to 10mM and were selected based on initial cell viability tests carried out using alamarBlue® as an endpoint (section 5.1.1). Statistical data was generated using a one-way ANOVA to compare variance across all means and Dunnett's post-test to compare treatment means to the control.

As figure 5.3 demonstrates, the effect of ULA on the ability of HepG2 cells to produce ATP was not dose dependent. This correlates with the results observed in the cell viability assay performed on ULA (figure 5.1). While the results of the one-way-ANOVA indicated that the variance between group means was significant ($P=0.0001$), Dunnett's Multiple Comparison post hoc test, which was used to compare all means to the positive growth control, indicated that the statistical significance lay between the positive growth control and the cells treated with 8mM and 10mM.

Cells treated with 4mM of the fatty acid produced the most ATP with a mean value of 17.33ng/ μ L (± 0.309) compared with cells treated with 2mM (12.9 ng/ μ L ± 1.929) or the untreated control, 0mM (16.31ng/ μ L ± 1.129). As ATP is generated from the products of fatty acid β -oxidation, the increase in ATP produced by cells receiving higher concentrations of linoleic acid is not surprising. Abdul-Ghani *et al* (2008) stated that free fatty acid metabolites did indeed stimulate ATP synthesis. Cells treated with 6mM of ULA also produced ATP to a volume that was not significantly different when compared with the control, with 13.74ng/ μ L (± 0.681) of ATP being generated, however, these cells did produce less ATP than those treated with 4mM and even the untreated control. Similarly, as the concentration of ULA increased further, there was a significant reduction in ATP production. Cell treated with 8mM of ULA produced a mean concentration of 5.5ng/ μ L (± 0.153) of ATP while cells treated with 10mM did not produce ATP.

The primary role of the mitochondria is the generation of ATP by oxidative phosphorylation. They are also responsible for several other processes including the β -oxidation of fatty acids as well as the synthesis of unique fatty acids, lipid cofactors and steroid hormones (Mayr, 2014).

However, intracellular lipid overload can result in reduced mitochondrial oxidative phosphorylation and ATP production (Jaishy and Abel, 2016). Jaish and Abel (2016), stated that ectopically stored lipids are mostly directed towards non-oxidative pathways, resulting in the generation of potentially toxic products, such as diacylglycerol, ceramides, acyl carnitines and long chain fatty acyl-CoA, to name a few. Such products can contribute to several deleterious effects such as impaired cellular signalling, mitochondrial dysfunction and cell death (Jaishy and Abel, 2016). Abdul-Ghani *et al* (2008) found that human and murine skeletal mitochondria exposed to elevated levels of fatty acid metabolites such as fatty acid carnitine and fatty acid CoA, specifically palmitoyl carnitine, palmitoyl CoA and oleoyl CoA, showed a “precipitous” decline in ATP synthesis. Weinberg (2006) postulated that ATP production can be decreased in pancreatic β -cells as a result of acute lipotoxicity.

Sparks *et al*, (2005) stated that conditions linked with lipid overload and lipotoxicity, such as obesity and type 2 diabetes, have been associated with a reduced mitochondrial mass and function. Studies carried out by Sparks *et al* (2005) involved analysis of the skeletal muscle mitochondria of insulin sensitive human patients fed a high fat diet over a period of three days. Observations made by Sparks *et al* (2005) indicated that consumption of a high fat diet resulted in the down regulation of genes involved in oxidative phosphorylation. Further analysis by Sparks *et al* (2005) suggested that high-fat flux through the mitochondria may impede mitochondrial biogenesis through the reduction of the expression of nuclear genes encoding mitochondrial proteins and transcription factors. Similar work carried out by Richardson *et al* (2005) also found that nuclear encoded mitochondrial gene expression was decreased in response to increased plasma free fatty acids over a

period of 48 hours. As such, the reduction and eventual non-production of ATP observed in ULA treated HepG2 cells in this study may be indicative of lipotoxicity induced mitochondrial dysfunction.

Contrary to these findings however, work carried out by Brands *et al*, (2011) indicated that short term (6 hours) elevation of plasma free fatty acids did not disturb mitochondrial function in human skeletal muscle taken from study participants who received intravenous lipid infusions. These findings corroborate observations made by Brehm *et al* (2009) in human skeletal muscle, also taken from study participants receiving intravenous lipid infusions. While Brehm *et al*, (2009) stated that, while free fatty acid elevation may affect mitochondrial function and ATP synthesis, their observations determined that ATP production was not affected by acute (3 hours) elevated lipid levels.

Further work carried out by Chavez *et al*, (2010) on participants who received intravenous lipid infusions over 8 hours also found that, while mitochondrial membrane potential was reduced after short term plasma free fatty acid elevation, there was no significant change in gene expression, total ATP content or mitochondrial morphology. However, Chavez *et al* (2010) postulated that the lack of effect may indicate that the short duration of lipid infusion in their study may not have allowed sufficient time to detect changes in mitochondrial gene expression.

In this study, the reduction in ATP production was observed in HepG2 cells treated with high concentrations of ULA for 21 hours. This may signify that the deleterious effect of ULA, at very high concentrations, on the ability of HepG2 cells to produce ATP may be time dependent or due to chronic free fatty acid exposure.

5.5.3. Comparison of Cell Viability in HepG2 Cells using the Resazurin (alamarBlue®) Assay and the MTT Assay.

5.5.3.1. HepG2 Cell viability using the Resazurin (alamarBlue®) Assay.

While previously discussed observations made by Yao, *et al* (2011) reflect the results obtained in this study at the lower scale of the concentration gradient, higher concentrations, namely 6mM to 10mM, were considered too high to reflect realistic physiological parameters. The identification of IC₅₀ values for ULA of 2.44mM (± 0.395) (24 hours) and 3.58mM (± 0.621) (48 hours) further strengthened this conclusion. As such, the decision was made to continue analysis of the lipotoxic effects of ULA at a reduced concentration range between 0mM and 5mM. After a number of modifications to the treatment method (section 3.1.4.1. and 3.1.5.1.), cell viability was assessed in HepG2 cells using alamarBlue®.

A second experiment was performed ($n=1$), which included cells treated with 0.1mM ULA. The identification of concentrations of ULA of ~ 0.1mM in a number of equine hepatic tissue samples analysed in Chapter 4, was also considered during this particular experimental design. While Copper (2015) suggested that this concentration of ULA in equine hepatic tissue may be considered high, a thorough review of the literature indicated that concentrations in this range would be considered physiologically normal (Tikanoja, *et al*, 1989, Shultz, 1991, Artwohl *et al*, 2003). This experiment was performed to confirm that ULA at physiologically realistic concentrations does not affect cell viability negatively. One-way-ANOVA and Dunnett's as the *post hoc* test confirmed that there was no significant difference between the untreated control cells and cells treated with 0.1mM ULA after 24 hours of exposure. It was decided that this concentration would serve as a sample control.

Cell viability in treated cells after the treatment method and alamarBlue® assay amendments (figure 5.4.) appeared much lower than the results previously obtained (figure 5.1.). It was postulated that cells treated with media containing a higher glucose concentration may have had a higher cell viability than cells treated with media containing lower glucose concentrations. However, while not statistically significant, it has been found that rat hepatocytes grown in DMEM containing low glucose displayed a higher cell viability and higher total cell count when compared with the same cell line grown in DMEM containing high glucose (Na *et al*, 2014).

The results observed in figure 5.4. may also suggest that the presence of ULA in the media was affecting the optical density, resulting in higher absorbance values. While alamarBlue® is reported to be more sensitive and reliable than other viability assays, there are several factors that have to be considered prior to use (Präbst *et al*, 2017). According to Präbst *et al*, (2017), factors including temperature, pH and initial resazurin concentration must be kept constant during incubation and measurement in order to avoid the formation of artefacts, which may impact optical density readings. Based on these factors, it was decided to repeat cell viability assays on treated HepG2 cells using the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT.

5.5.3.2. HepG2 Cell viability using the MTT Assay.

The MTT assay is one of the most widely used and simplest assays for the assessment of cell viability (Wang *et al*, 2010, Sumantran, 2011). As such, it was decided to perform MTT assays on HepG2 cells treated with varying concentrations of ULA in order to corroborate the findings from the alamarBlue® assays. The trend observed in treated HepG2 cells with the MTT assay (figure 5.5.) was indeed similar to that

observed with the alamarBlue® assay (figure 5.4.). One-Way ANOVA established a statistically significant difference between all means ($P = <0.0001$). Dunnett's Multiple Comparison *post hoc* test, further confirmed that the significance lay between each of the treatment groups when compared to the positive growth control ($P=0.0001$).

Using this preliminary data, a number of further amendments were made to the experimental design. As there appeared to be very little difference among the treatment groups (excluding 0.1mM treated cells) and the treatment times of 24 and 48 hours, it was decided to study the effects of ULA over a shorter period. Time points of 1, 2, 4, 6 and 8 hours were selected. The decision was also made to eliminate the analysis of the effects of 5mM ULA as this was deemed to be unrealistically high even when compared to what would be considered physiologically high. While this experiment returned IC₅₀ values of 0.17mM (±0.007) for 24 hours and 0.14mM (±0.006) for 48 hours (table 5.3), it was decided that cells would be assessed using concentrations of ULA ranging from 0.1mM to 2.5mM going forward. This decision was based on the literature findings, detailing potential plasma fatty acid concentrations ranging from 0.09mM to 2.5mM during physiologically stressful conditions (Tikanoja, *et al*, 1989, Shultz, 1991, Høstmark, 1995, Toborek *et al*, 1996, Young *et al*, 1998, Artwohl *et al*, 2003). The determination of an IC₅₀ value for ULA of 2.44mM (±0.395) in section 5.1.1 (table 5.1) was also considered during the determination of a more appropriate and physiologically relevant dose range for the *in vitro* analysis of ULA.

5.5.4. Intracellular Lipid Accumulation in HepG2 Cells using Oil Red O.

Oil Red O was used to visualise and quantify intracellular lipids in HepG2 cells treated with ULA over 24 and 48 hours (figure 4.6.). Oil Red O has previously been used to evaluate intracellular lipid accumulation caused by exposure of HepG2 cells to free fatty acids (Yao *et al*, 2011). It has also been successfully used to macroscopically visualise adipose cell colonies (Ramirez-Zacarias, *et al*, 1992).

In this study, untreated control cells were assigned as 0% lipid accumulation and all treated cells were expressed as a percent of the control. Cells treated with 0.1mM of ULA displayed the highest percentage of lipid accumulation, presenting a mean value of 182.8% (± 28.43) after 24 hours with a decrease to 145.4% (± 28.38) after 48 hours. Photomicrographs of HepG2 cells treated with varying concentrations of ULA over 24 and 48 hours appear to corroborate this data (figure 5.7). Images of cells treated with 0.1mM ULA over both time points clearly display intracellular lipid droplets in the form of small red intracellular bodies. This result correlates with the data obtained in the cell viability assays, where cells treated with 0.1mM of ULA displayed the highest cell viability.

5.6. Conclusion

This chapter has detailed the preliminary analysis on the *in vitro* effects of high concentrations of ULA. Initial cell viability assays using ULA concentrations ranging from 0mM to 10mM determined that ULA, at concentrations deemed much higher than physiologically relevant (2.5mM), resulted in significantly reduced cell viability. The findings indicate that cell viability, mitochondrial health and the ability of hepatic cells to form protective intracellular lipid droplets are all negatively impacted after 24 and 48 hours of exposure.

This preliminary analysis allowed for assay optimisation and re-evaluation of experimental parameters. The following investigation will detail the *in vitro* effects of ULA at concentrations ranging from physiologically normal (0.1mM) to physiologically high (2.5) over shorter periods of exposure (1 to 8 hours), with particular focus on pro-inflammatory pathways.

Chapter 6

The *In Vitro* Effects of ULA on HepG2 Cells

6. The *In Vitro* Effects of ULA on HepG2 Cells

In the following chapter, the effects of supraphysiologic concentrations of ULA on HepG2 cells were assessed over time points ranging from 1 to 8 hours. Cell viability, intracellular lipid accumulation, oxidative stress, the production of inflammatory mediators and the induction of cell death were all analysed.

Cooper (2015) indicated that ULA in concentrations of or above 5mg per gram of tissue analysed was considered high. The current investigation (Chapter 4) observed this concentration or higher in 10.6% of 217 specimens analysed, approximately 1 in every 20 equines, with some specimens reporting concentrations as high as 27mg to 30mg per gram of tissue analysed. The concentrations of 30mg of ULA identified in the analysis of the hepatic tissue in Chapter 4 would equate to 0.11mM of linoleic acid. However, an extensive review of the literature determined that physiological plasma concentrations of fatty acids can range from 0.09mM to 1.2mM, with values reaching as high as 2.5mM in stressful conditions such as diabetes, fasting or strenuous exercise (Tikanoja *et al*, 1989, Shultz, 1991, Høstmark, 1995, Toborek *et al*, 1996, Young *et al*, 1998, Artwohl *et al*, 2003). It has also been established that, in horses, the normal metabolic rate can be altered through pain, stress or anorexia. Gomaa, *et al* (2009) determined that horses suffering from impaction colic demonstrated serum free fatty acid concentration of as high as 1.638mM. Assays, such as MTT and Oil Red O, which have been previously used to assess the effects of higher concentrations of ULA over longer periods, were carried out on the revised experimental parameters. Fluorescent microscopy and flow cytometry, as well as ELISA to test for biomarkers of inflammation, were also used as investigative tools in this research.

The findings presented here demonstrate the lipotoxic effects of excessively high concentrations of ULA, specifically concentrations surpassing 0.5mM, on hepatocytes.

6.1. Cell Viability of HepG2 Cells Treated with ULA over 8 Hours

The data obtained from both the alamarBlue® and MTT 24 and 48 hour cell viability assays indicated that high concentrations of ULA resulted in a decrease in cell viability before 24 hours. As such, the viability of HepG2 cells treated with ULA for shorter time periods, specifically, after 1, 2, 4, 6 and 8 hours was assessed. Figure 6.1 represents the percentage cell viability in HepG2 cells exposed to varying concentrations of ULA over 8 hours.

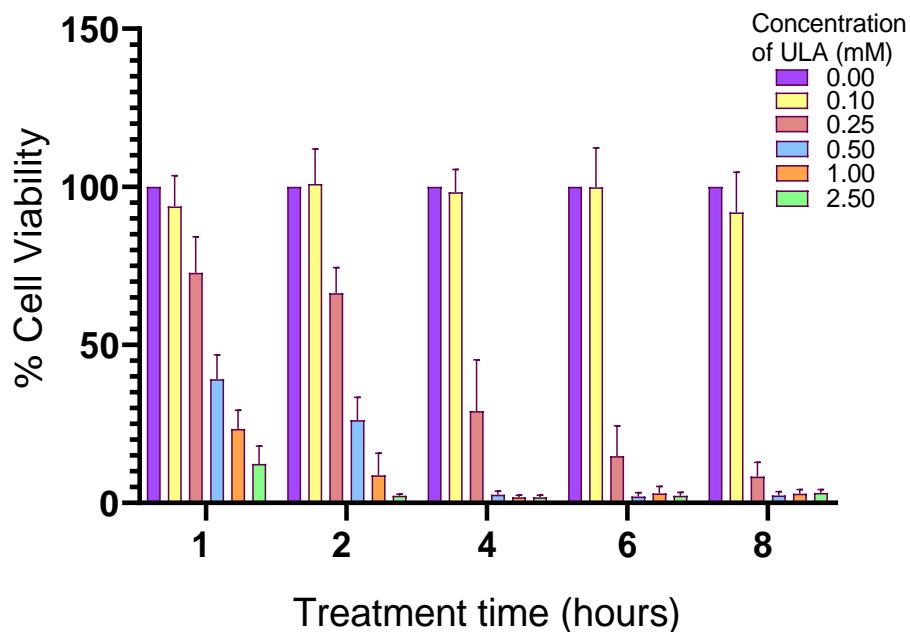


Figure 6.1. Percentage cell viability of HepG2 cells treated with varying concentrations of ULA over a period of 8 hours compared with a positive growth control. MTT was used as an endpoint ($n=3$). Viability levels are expressed as a percent of the control (assigned as 100%). Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's test as a post-test to compare all means.

The results portrayed in figure 6.1 indicate that increasing exposure times and increasing concentrations of ULA initiated a significant reduction in the ability of HepG2 cells to reduce tetrazolium into formazan ($P = <0.0001$). This suggests that both the concentrations of ULA and the length of time HepG2 cells are exposed to ULA significantly reduces cell viability.

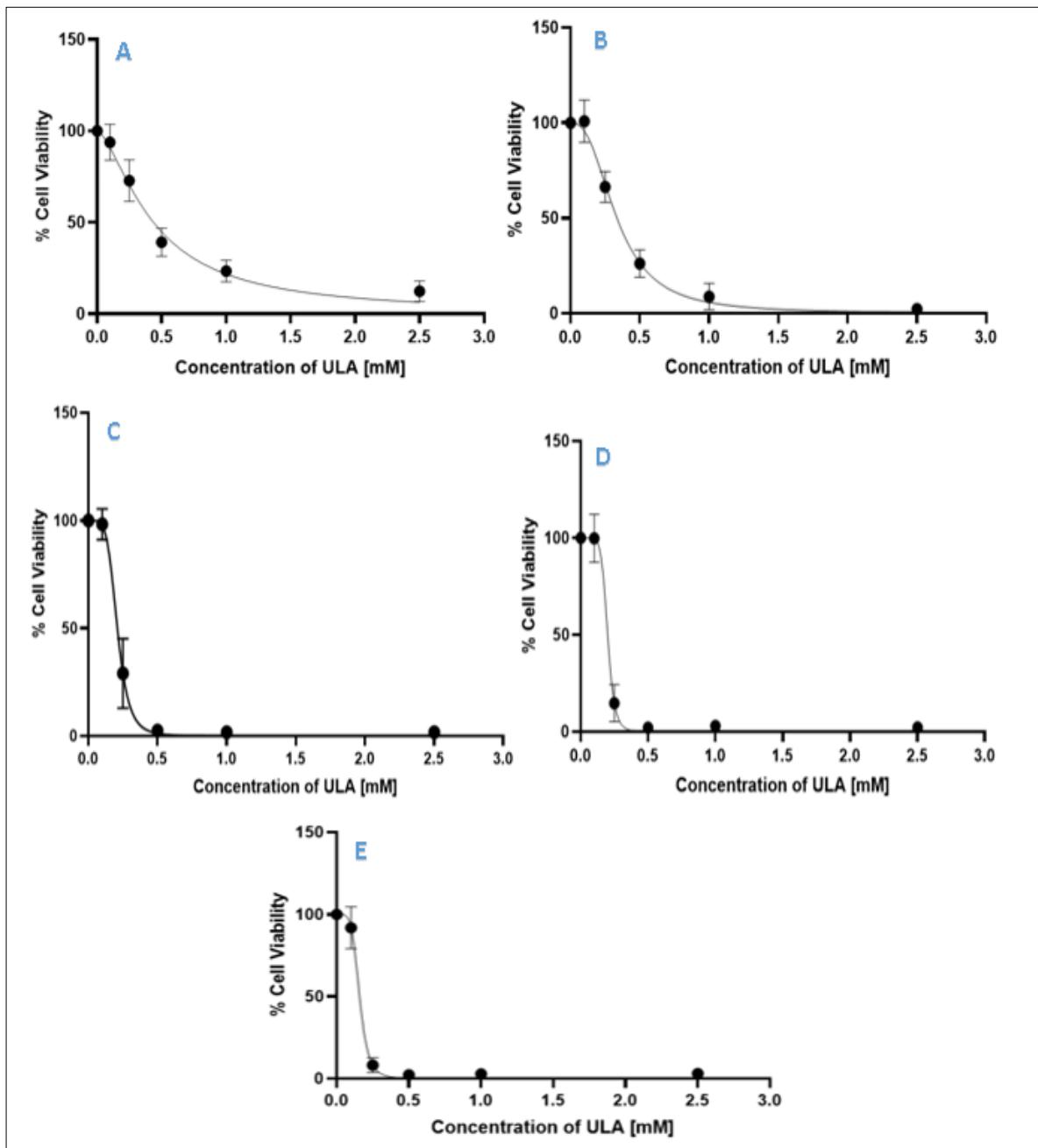


Figure 6.2. Concentration-response curves were generated from the MTT cell viability data (presented in figure 6.1) and used to determine the IC_{50} of ULA in HepG2 cells treated with varying concentrations of the fatty acid over 8 hours (A=1 hour, B=2 hours, C=4 hours, D=6 hours, E=8 hours). Statistical analysis was performed using mean concentration values (\pm S.E.M.).

The half-maximal (50%) inhibitory concentration (IC_{50}) for ULA at each time point was also determined through the use of concentration-response curves (figure 6.2) and are presented in table 6.1.

Table 6.1. The half-maximal inhibitory concentrations (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period of 8 hours. MTT was used as an endpoint ($n = 3$). Statistical analysis was performed using mean concentration values (\pm S.E.M).

Time point	IC_{50} Concentration (\pm S.E)
1 hour	0.44mM \pm 0.021
2 hours	0.34mM \pm 0.010
4 hours	0.21mM \pm 0.009
6 hours	0.20mM \pm 0.030
8 hours	0.16mM \pm 0.005

Concentration-response curves were generated to allow for the continuous representation of the relationship between ULA and the inhibition response observed. At 1 hour an IC_{50} of 0.44mM (± 0.021) was observed, however, as the length time that HepG2 cells were exposed to ULA, a decrease in the tolerance of the cells to ULA was observed, with an IC_{50} of 0.16mM (± 0.005) observed at 8 hours.

6.2. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using Oil Red O.

Oil Red O was used to assess the accumulation of intracellular lipids in HepG2 cells treated with increasing concentrations of ULA over different time points.

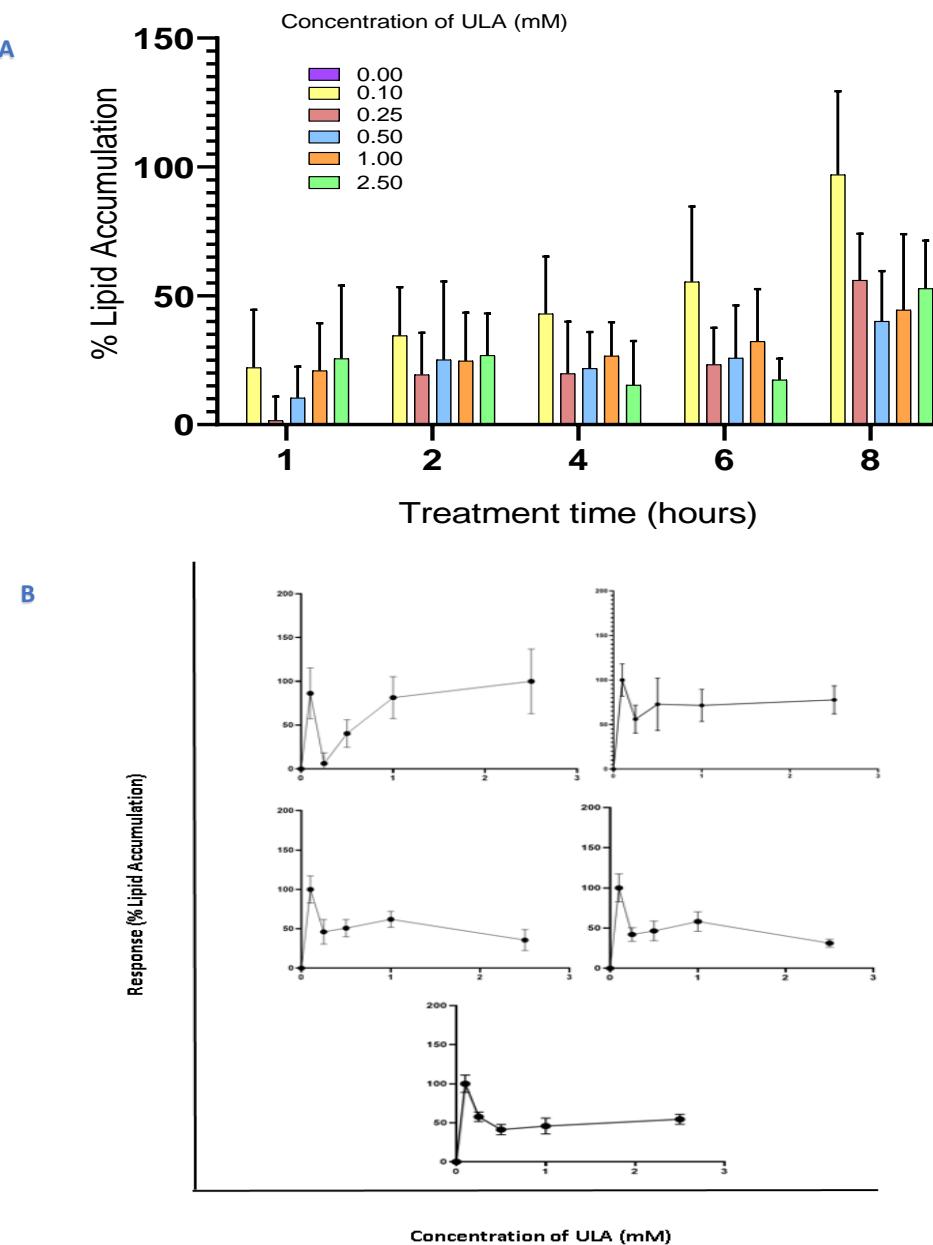


Figure 6.3. (A) Percentage lipid accumulation in HepG2 cells treated with varying concentrations of ULA assessed over a period of 8 hours. Treated cells were compared to a positive growth control in which cells were grown in fatty acid free BSA constituted media containing $\leq 1\%$ IPA ($n=3$). Lipid accumulation levels are expressed as a percent of the control (assigned as 0%). Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's test as a post-test was used to compare all means. (B) Data was also presented in the form of dot plots using GraphPad Prism Software present the relationship between each concentration.

The data portrayed in figure 6.3 indicated that as exposure time increased, intracellular lipid accumulation increased, particularly in cells treated with 0.1mM of ULA. However, as the concentration of ULA increased, a non-monotonic dose response was observed. This suggested that intracellular lipid accumulation was not dose dependent. The mean % lipid accumulation for each ULA concentration, over all five time points, was calculated. Cells treated with 0.1mM of ULA had a mean % lipid accumulation of 50.4% (± 12.85) while cells treated with 2.5mM of ULA had a mean % lipid accumulation of 27.6% (± 6.70).

Light micrographs of Oil Red O stained HepG2 cells indicate the presence of intracellular lipid accumulation. An increase in the presence of intracellular lipids can be seen (stained red), particularly in cells treated with 0.1mM and 0.25mM of ULA between 1 hour and 8 hours of exposure. As the concentration of ULA increased, cell numbers appear to decrease, particularly at 8 hours.

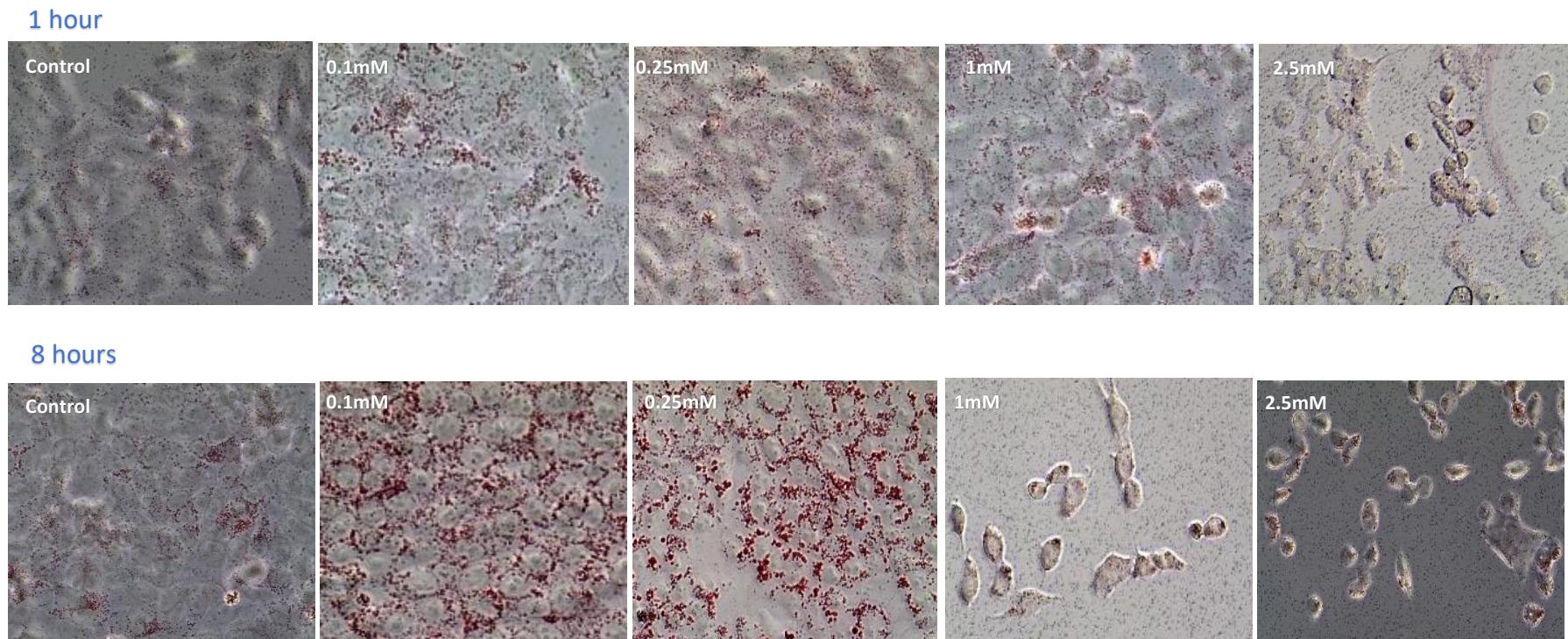


Figure 6.4. Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over different time intervals. Control cells were grown in media containing fatty acid free BSA and $\leq 1\%$ IPA.

6.2.1. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using the Nile Red Fluorescent Stain

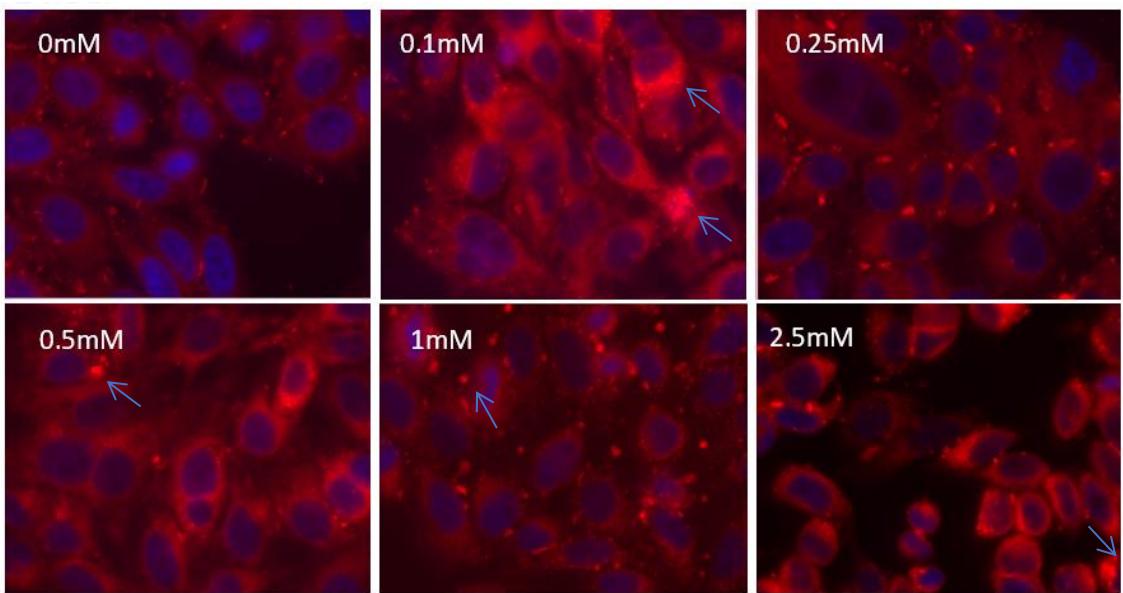
The importance of intracellular lipid accumulation in the induction of lipotoxicity in numerous non-adipose cell types has been well documented (Feldstein *et al*, 2004, Abdul-Ghani *et al*, 2008, Schie *et al*, 2011, Yan *et al*, 2018). Mashek *et al*, (2015) identified excessive lipid droplet accumulation in hepatocytes as a defining feature of hepatic steatosis.

While fat accumulation in the liver can be harmless and reversible, the formation of excessive intracellular lipid droplets in hepatocytes can lead to cellular dysfunction (Schie, *et al* 2011). As such, intracellular lipid accumulation in HepG2 cells, as a result of increasing concentrations of ULA was further investigated using the fluorescent stain Nile Red.

Oil Red O is commonly used to visualise intracellular lipid droplets in a variety of cells, however, according to Aldrich *et al*, (2013) Nile Red based flow cytometry is considered more quantitative and less subjective than Oil Red O.

Nile red was used in conjunction with fluorescent microscopy to visualise intracellular lipid droplets in HepG2 cells exposed to increasing concentrations of ULA over 8 hours. This stain was also used to determine, by flow cytometry, the effects of increasing ULA concentrations on intracellular lipid accumulation, supporting the findings in section 6.2.

1 hour



2 hours

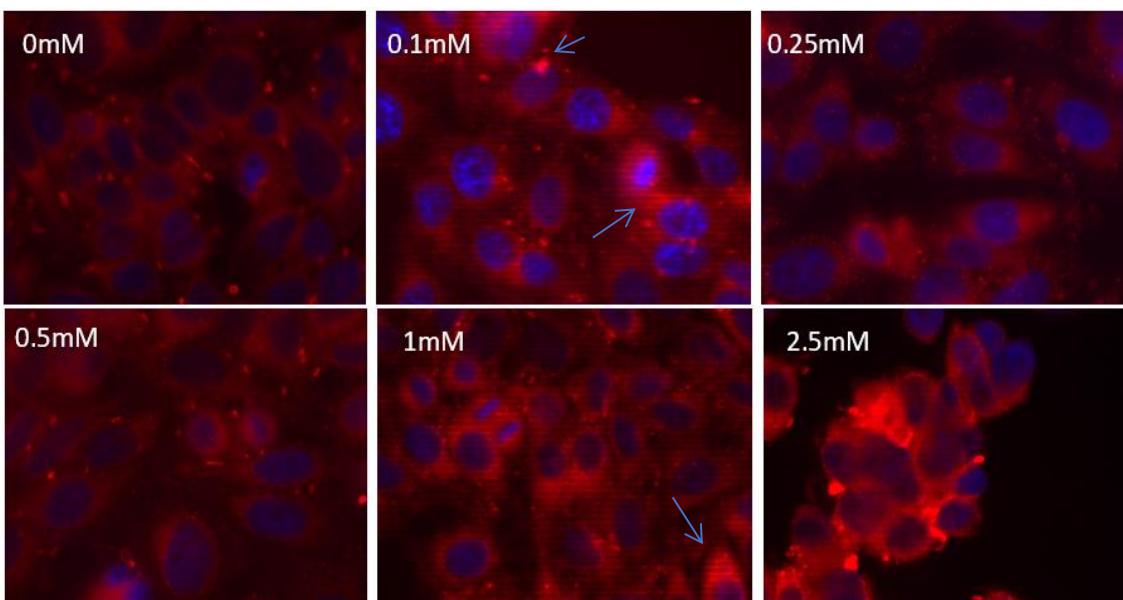
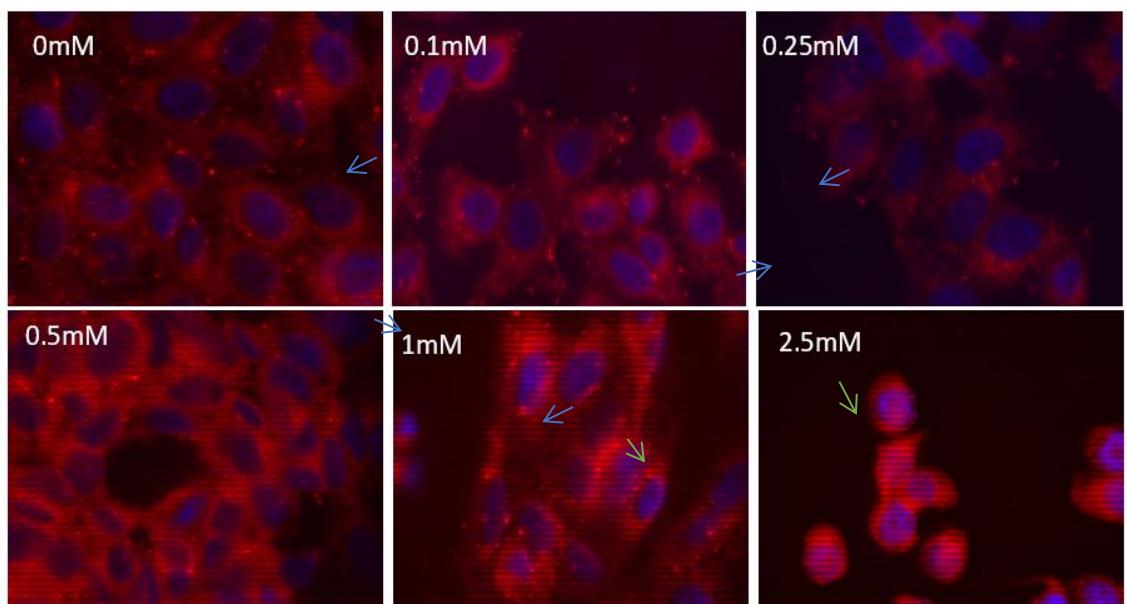


Figure 6.5.1 Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 1 and 2 hours of supplementation. Intracellular lipid accumulation was qualitatively determined using fluorescent microscopy. Fluorescent images (x1000) were obtained using the lipophilic stain Nile Red for intracellular lipid imaging (red) and the nuclear stain DAPI for nucleus imaging (blue).

4 hours



6 hours

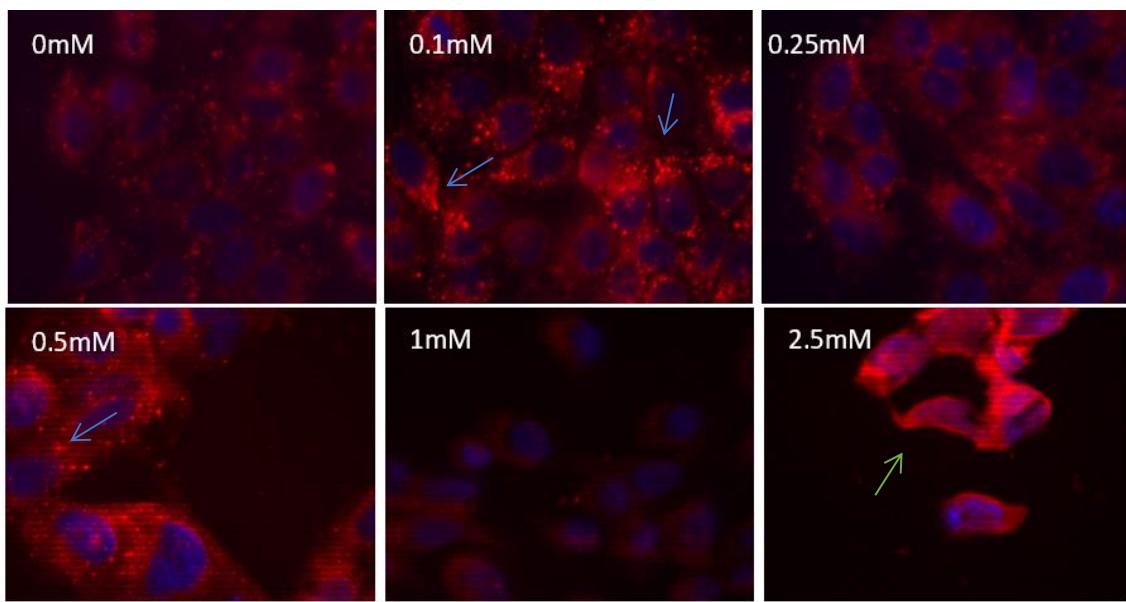


Figure 6.5.2 Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 4 and 6 hours of supplementation. Intracellular lipid accumulation was qualitatively determined using fluorescent microscopy. Fluorescent images ($\times 1000$) were obtained using the lipophilic stain Nile Red for intracellular lipid imaging (red) and the nuclear stain DAPI for nucleus imaging (blue).

8 hours

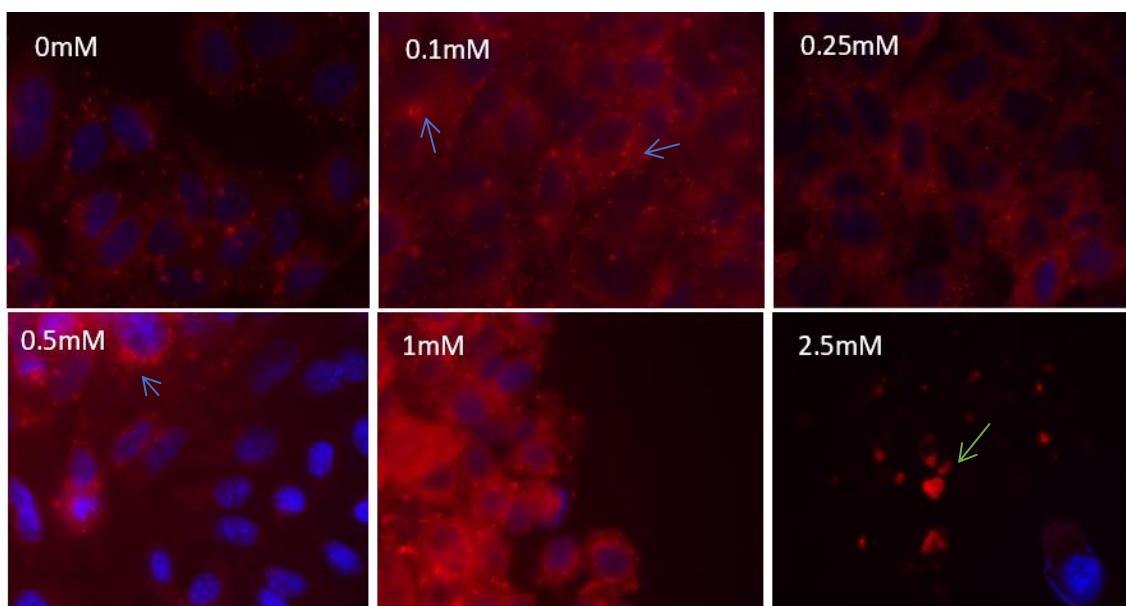


Figure 6.5.3 Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 8 hours of supplementation. Intracellular lipid accumulation was qualitatively determined using fluorescent microscopy. Fluorescent images ($\times 1000$) were obtained using the lipophilic stain Nile Red for intracellular lipid imaging (red) and the nuclear stain DAPI for nucleus imaging (blue).

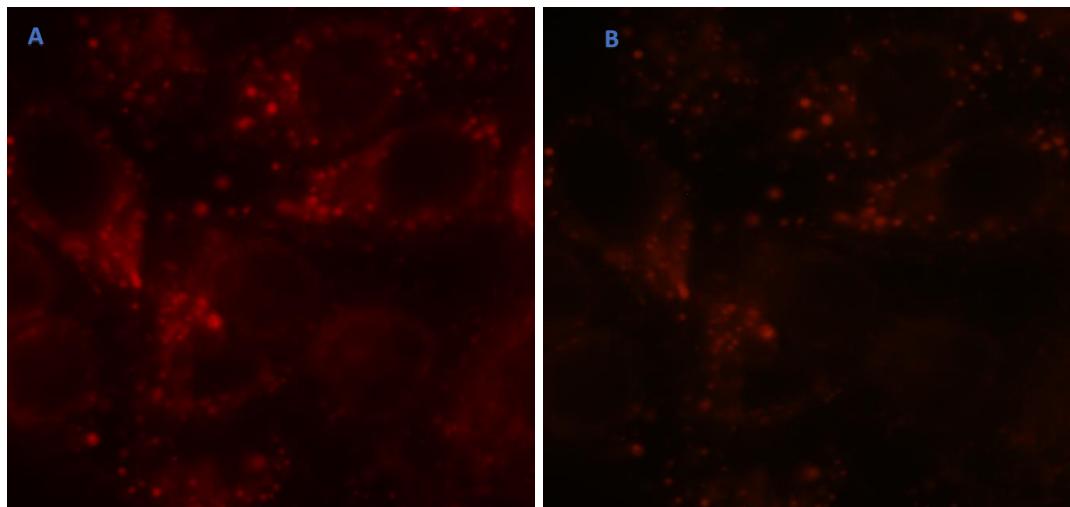


Figure 6.5.4. Examples of red fluorescence (A) and yellow/gold fluorescence (B) in the same cells treated with 0.1mM of ULA for 6 hours.

Fluorescent micrographs allowed for the visualisation of intracellular lipid accumulation in HepG2 cells. As Nile Red is reported to fluoresce yellow gold in the presence of neutral lipids and red in the presence of polar lipids (Diaz *et al*, 2008), micrographs were captured using two spectral settings and then merged to increase

lipid body resolution, as described in section 3.1.10. Distinct lipid droplets can be seen in ULA treated cells, as indicated by the blue arrows, particularly at 0.1mM as can be seen in figure 6.5.1 in cells treated for 1 hour. As the concentration of ULA and exposure time increases, cell morphology changes dramatically, particularly in cells treated with 2.5mM, as indicated by green arrows (figure 6.5.3, 8 hours), suggesting that higher concentrations impacted cellular integrity.

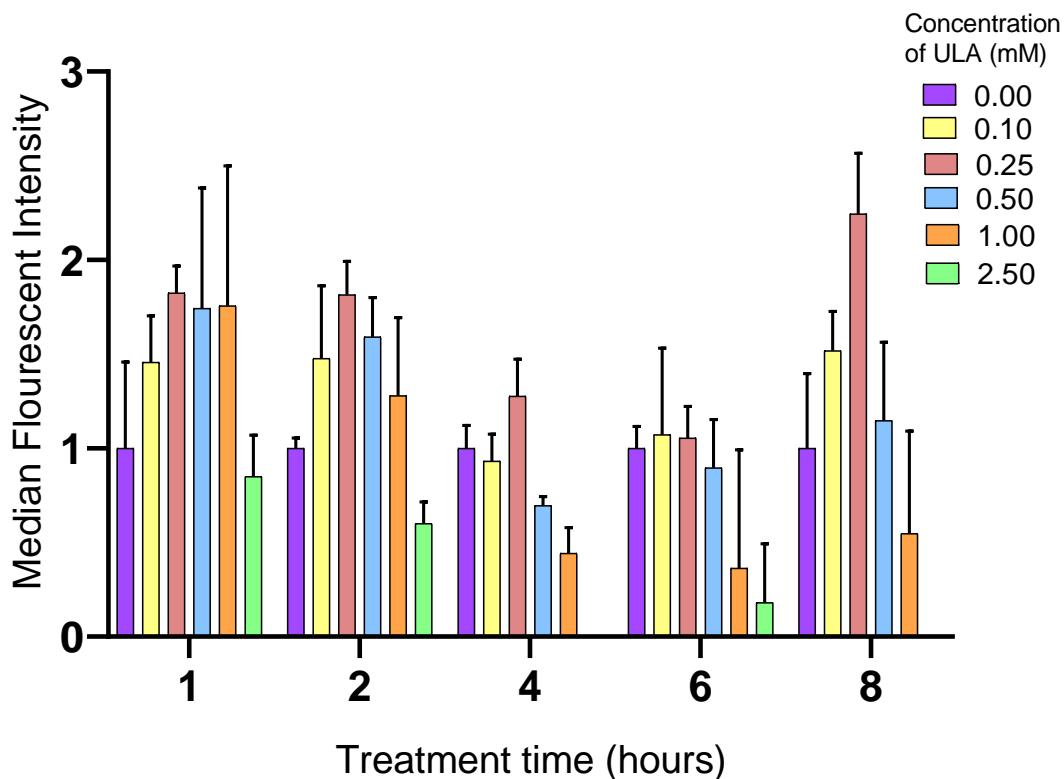


Figure 6.6. Median Fluorescence Intensity of Nile Red determined by flow cytometry in HepG2 cells treated with varying concentrations of ULA over 8 hours. Data has been normalised as described by Wolins *et al*, (2018).

Flow Cytometry was used to detect intracellular lipid accumulation through cellular uptake of the fluorescent lipophilic stain Nile Red. An initial increase in the median fluorescence intensity (MFI) was observed in cells treated with 0.1mM and 0.25mM of ULA. Increasing concentrations of ULA did not induce a dose dependent increase in MFI.

6.3. Assessment of the Inflammatory effects of ULA though the Secretion of TNF-

a

Linoleic acid has been associated with a pro-inflammatory response (Choque *et al*, 2014). TNF- α , soluble paracrine factor, is a pro-inflammatory cytokine that has been widely used to study various elements ranging from cell proliferation and apoptosis to inflammation and lipid haemostasis (Chen *et al*, 2009). In order to ascertain the inflammatory effects of ULA, media from ULA treated HepG2 cells was assessed using ELISA for TNF- α secretion.

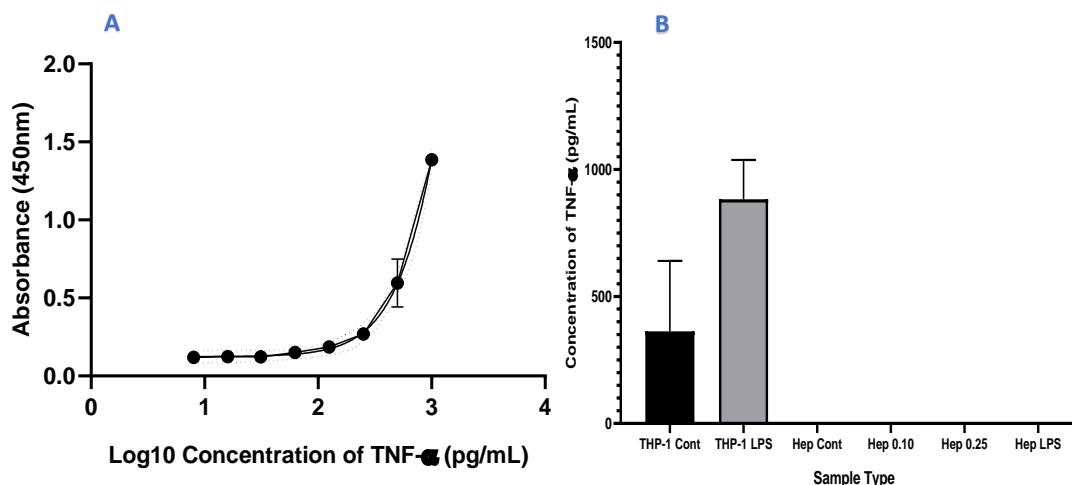


Figure 6.7. TNF- α standard curve (A). TNF- α production in HepG2 cells treated with 0.1 and 0.25mM of ULA for 4 hours (B). LPS injured THP-1 (THP-1 LPS) cells and HepG2 cells (Hep LPS) were used as positive controls while untreated THP-1 (THP-1 Cont) and HepG2 cells (Hep Cont) were used as a negative control (THP-1 supernatant = 1:5 dilution).

While a successful standard curve was obtained using ELISA (Figure 6.7, A), TNF- α was not secreted in detectable quantities by HepG2 cells in response to ULA treatment. A sigmoidal 4 parametric logistic curve was used to calculate the concentrations of TNF- α secreted into media by ULA treated HepG2 cells. Absorbance values for samples were considerably lower than those obtained for the lowest standard concentration (4pg/mL TNF- α), therefore concentrations of TNF- α from treated HepG2 cells could not be interpolated. This indicated that TNF- α levels in the media of treated cells were so low that they were below the limit of detection produced by

the standard curve, suggesting that ULA did not induce any substantial TNF- α secretion in HepG2 cells. Both lipopolysaccharide (LPS) and ethanol were applied separately to HepG2 cells, to serve as positive controls, as both compounds have been reported to induce TNF- α secretion (Gutierrez-Ruiz, *et al*, 1999). However, no detectable quantities of TNF- α were produced by HepG2 cells in response to these compounds. A third positive control, LPS injured THP-1 cells (figure 6.7., B), successfully resulted in TNF- α secretion and was used to confirm the integrity of the assay.

6.4. The Effects of ULA on Superoxide Dismutase (SOD) in HepG2 Cells.

Intracellular free fatty acid overload is reported to enhance the production of ROS such as the superoxide anion (Masarone *et al*, 2018). An increase in superoxide may lead to increased scavenging activity *via* SOD. The WST SOD assay involves the

reduction of the water-soluble tetrazolium (WST-1), by the superoxide anion, to produce a yellow formazan dye. SOD catalyses the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen (Younus, 2018), therefore the presence of SOD inhibits WST-1 reduction. Figure 6.9 represents the units of SOD enzyme present in ULA treated HepG2 cells.

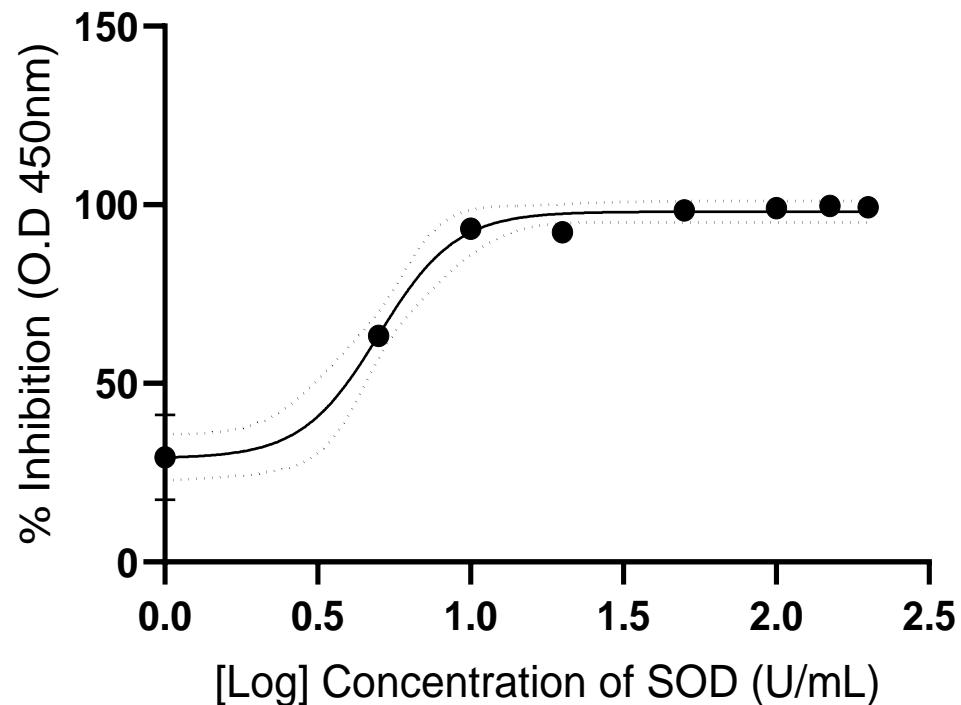


Figure 6.8. Standard curve for Superoxide Dismutase. Statistical analysis was performed using mean absorbance values \pm S.E.M ($n=2$).

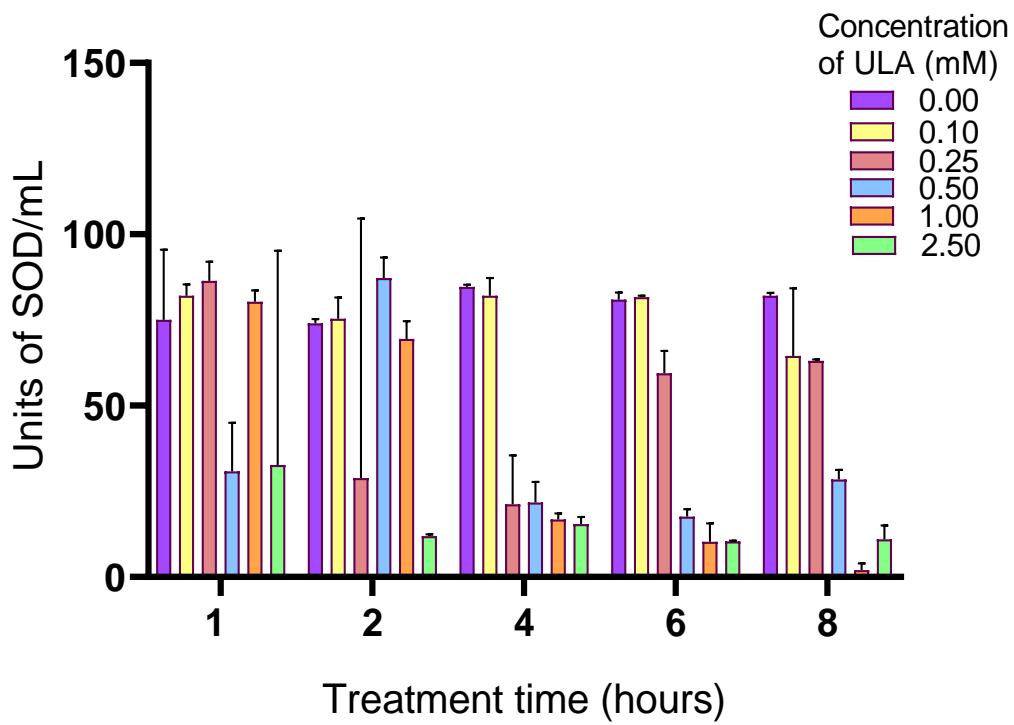


Figure 6.9. Units of superoxide dismutase produced in HepG2 cells treated with varying concentrations of ULA over 8 hours.

content. A decrease in SOD produced by HepG2 cells was observed as the concentration of ULA increased, however, the trend was not dose or time dependent. This decrease was particularly apparent as the treatment time increased past 4 hours. SOD in cells treated with 2.5mM of ULA appeared to be significantly reduced when compared to SOD in cells treated with 0.1mM of ULA ($P = 0.0009$).

6.5. The effects of ULA on the Production of Thromboxane in HepG2 cells.

HepG2 cells were analysed for the production of thromboxane B₂ (the stable metabolite of thromboxane A₂), in response to ULA supplementation.

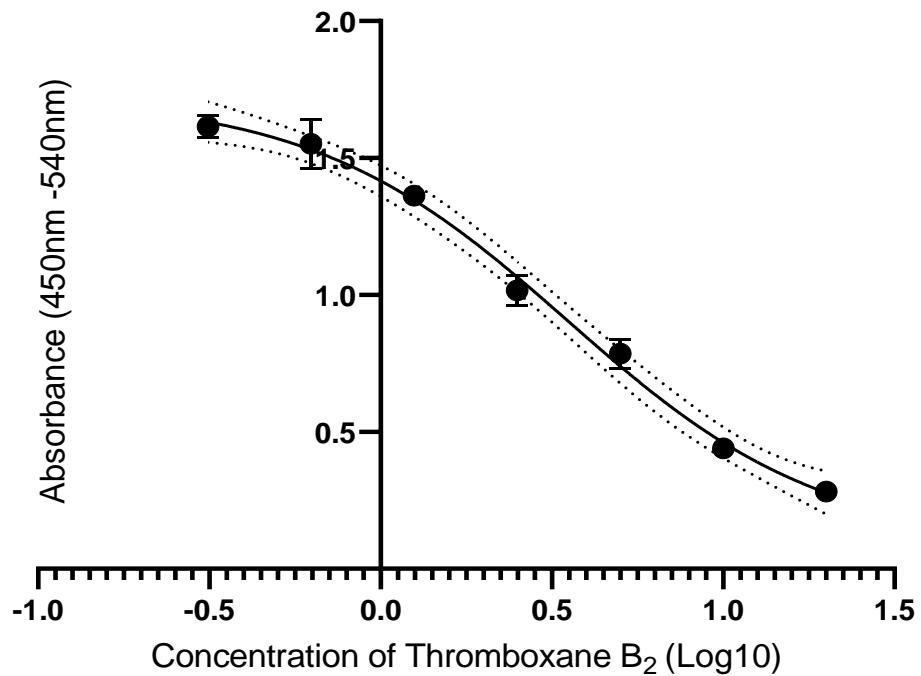


Figure 6.10. Standard curve for thromboxane B₂. Statistical analysis was performed using mean absorbance values (\pm S.E.M) ($n=2$).

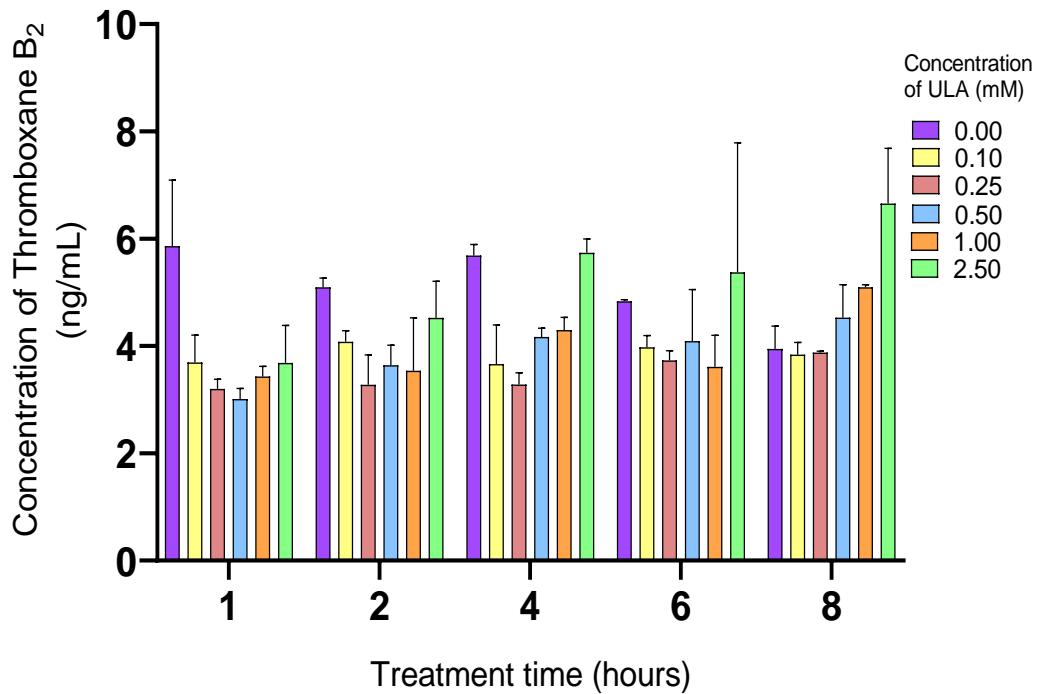


Figure 6.11. Concentration of thromboxane B₂ produced in HepG2 cells treated with varying concentrations of ULA over 8 hours. Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's *post-hoc* test was used to compare all means ($n=2$).

Figure 6.11 demonstrates thromboxane B₂ production in HepG2 cells treated with varying concentrations of ULA. ULA did not induce a time or dose dependent release of thromboxane B₂ in HepG2 cells. Cells treated with 0.1mM and 0.25mM produced significantly less thromboxane B₂ than the untreated control ($P = 0.0195$ and 0.0078 respectively) or cells treated with 2.5mM ($P = 0.0172$ and 0.0070 respectively). This observation occurred after 4 hours of exposure. Cells treated with 2.5mM of ULA produced the highest concentration of thromboxane B₂. Cells treated with this concentration produced significantly more thromboxane B₂ than the untreated control ($P=0.0318$) and those treated with 0.1mM and 0.25mM of ULA ($P=0.0172$ and $P=0.007$ respectively).

6.6. The Effects of ULA on Apoptosis in HepG2 cells

HepG2 cells were stained with Annexin V-FITC (AV-FITC) and propidium iodide (PI) in order to detect cells with disrupted membranes. According to the cell staining, cells were categorized as; live cells, negative for both AV-FITC and PI (Q4); early apoptotic cells, positive for AV-FITC and negative for PI (Q3); late apoptotic cells, positive both for AV-FITC and PI (Q2) while cells positive only for PI were considered as necrotic (Q1).

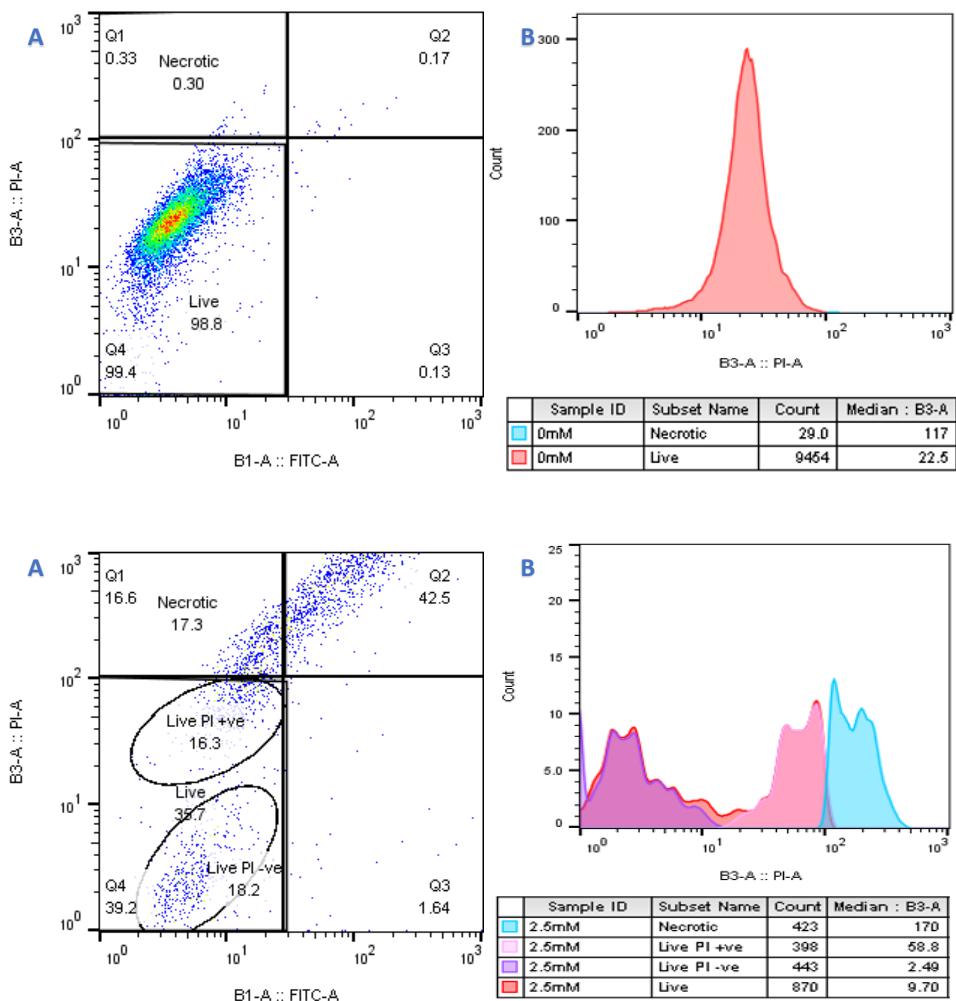


Figure 6.12. Flow cytometry dot plots (A) and histograms (B) of HepG2 cells treated 0mM (top) and 2.5mM (bottom) of ULA for 6 hours. Cells were stained with PI and AV-FITC. The dot plot details the gating strategy used to determine between different cell populations, with each event on the dot plot representing a single cell. The histogram details the amount of PI stain taken up by different cell populations signified by the median fluorescent intensity.

AV-FITC selectively binds to phosphatidylserine, a phospholipid that is exposed on the outside of cells undergoing apoptosis and can be used to label apoptotic cells specifically expressing this phospholipid (Crowley *et al*, 2016). PI is a highly water-soluble fluorescent compound that cannot pass through intact membranes and, as such, is generally excluded from viable cells (Lima *et al*, 2002). It can, however, pass through reversibly damaged cell membranes and induce staining (Rosenberg *et al*, 2019). It binds to DNA and RNA by intercalating between the nucleic acid bases of exposed nuclei with little or no sequence preference (Lima *et al*, 2002). In the current investigation, untreated cells were used as the control, with one tightly packed population being observed, as demonstrated in the top dot plot (A) in figure 6.12. While these cells showed some positivity for PI uptake, this is expected, as sample preparation, such as trypsinization and pipetting, can cause reversible cell damage (Lima *et al*, 2002). In the current experiments, even though untreated cells appeared positive for PI staining, the median fluorescence intensity (MFI) of this uptake was lower (MFI of 22.5) than cells analysed as PI positive (necrotic) (MFI of 117) (figure 6.12 (B) top). These considerations were used to define the gating strategy applied to all samples in order to differentiate between live cell, early apoptotic cells, late apoptotic cells and necrotic cells.

As the concentrations reached 1mM and 2.5mM of ULA after 4 hours the cell population dispersed dramatically. Two distinct populations were observed in Q4 of the dot plots for cells treated at these concentrations, particularly after 6 and 8 hours. These populations can be seen in the dot plots representing the 8-hour experiment in figure 6.14. These populations were gated and analysed separately for PI uptake as demonstrated in figure 6.12 ((A) bottom). These cells were divided into two sub-populations; live cells that were negative for PI (MFI of 2.49) and live cells displaying

some PI uptake (MFI of 58.8). This uptake was still substantially lower than cells considered to be necrotic (MFI of 170).

Cellular debris were gated out as described in section 3.1.15. to ensure the frequency of both live and dead cells was appropriately defined. As the purpose of the experiment was to investigate the effects of ULA on cell death, it was necessary to include dead cells in the population to be analysed. However, cell debris (fluorescent negative particles) needed to be avoided to prevent incorrect statistical values being obtained (Wallberg, *et al*, 2016). As such, cellular debris required appropriate consideration, as this population, having neither a nucleus nor an intact membrane, do not fluoresce and may end up in the population defined as live.

The exclusion of events considered to be debris based on a gating strategy using SSC and FSC alone was not appropriate. According to Reardon *et al*, (2014), traditional use of light scatter threshold - FFC and SSC - would lead to the exclusion of dying or damaged cells of interest. While this would ensure the removal of events with low FSC, events that were considered smaller than viable whole cells, this strategy would not take into consideration small events that belonged to apoptotic or necrotic populations. Reardon *et al*, (2014) suggests that due to the similarities in forward light scatter of damaged cells and debris it is difficult to accurately distinguish damaged cells from debris using forward light scatter alone. An alternative to forward light scatter is to use the fluorescence signal intensity to discriminate both healthy and damaged cells from debris (Reardon *et al*, 2014). Therefore, initial gating was performed while viewing the total population for AV-FITC and PI fluorescence. A double negative population was defined, and this population was then analysed using SSC and FSC, to eliminate events that presented with low FSC and were not fluorescent for AV-FITC or PI. These events were defined as debris and were removed

from down-stream analysis of apoptosis and necrosis. A comparison of the events available for down-stream apoptosis analysis compared to the events removed as debris is presented in figure 6.13.

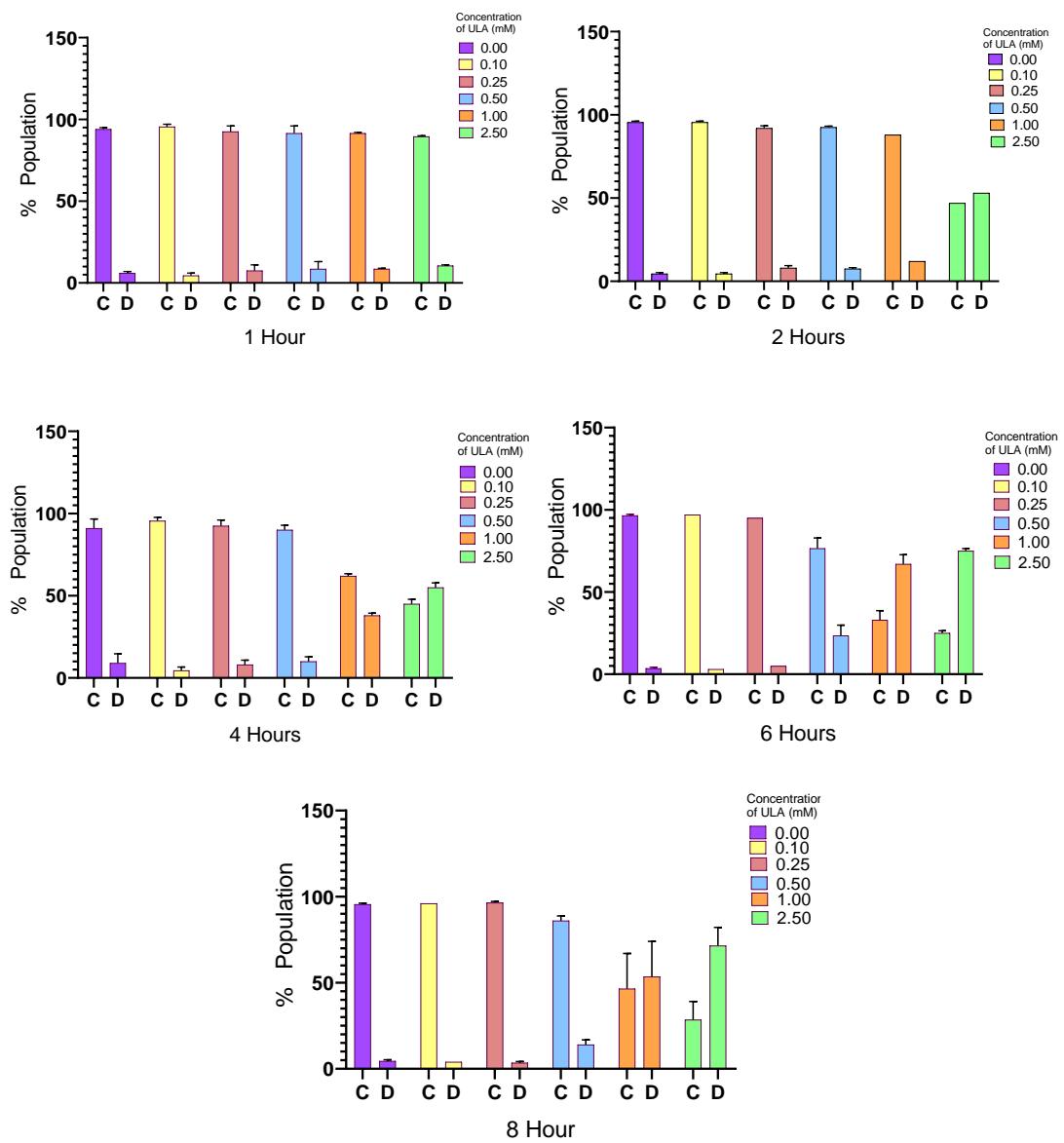


Figure 6.13. Mean percentage of events or cell (C) population that was to be considered for down-stream apoptosis analyses compared to the mean population of debris (D) removed from down-stream apoptosis analysis.

The exclusion of cell debris from the whole cell populations is visually represented in figure 6.13. For each sample a count of 10000 events were analysed. As the concentration of ULA and the length of exposure time increased, the population that was to be considered for down-stream apoptosis analysis began to decrease while the population defined as debris increased. Cells treated with 1mM of ULA for 4 hours showed a mean of 3817 (± 128) events (38% ± 1.29) that were deemed to be cellular debris and a mean of 6184 (± 129) events (62% ± 1.29) that were then analysed for apoptosis. A similar effect was observed in cells treated with 2.5mM after only 2 hours of exposure, while after 8 hours of exposure to this concentration, a much greater reduction in the number of events available for apoptosis analysis was observed. After 8 hours of exposure to 2.5mM of ULA, HepG2 cells presented with a mean of 2849 (± 729) events (28% ± 7) that could be used for analysis of apoptosis (figure 6.13).

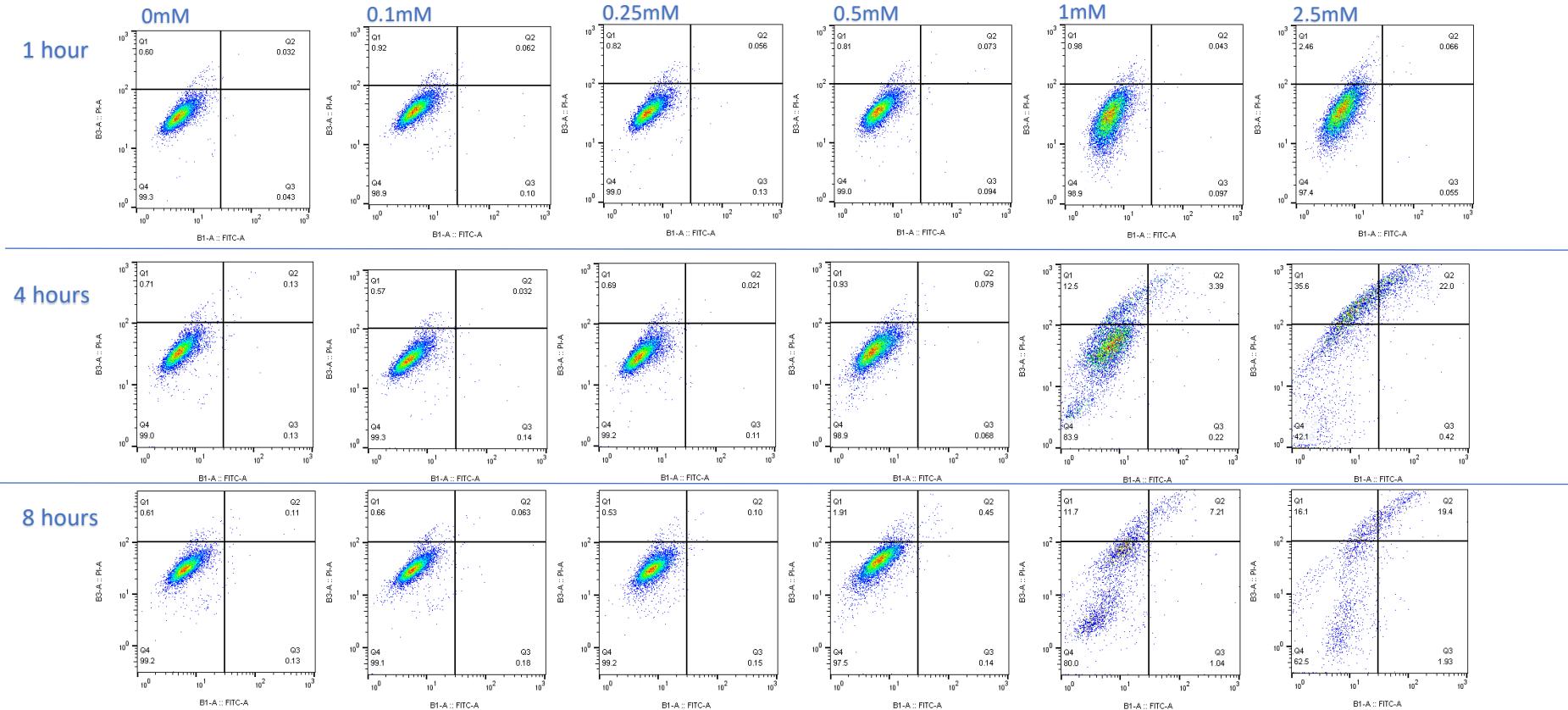


Figure 6.14. Flow cytometry dot plots representing HepG2 cells treated with increasing concentrations of ULA. Viable, apoptotic and necrotic cells in the whole cell population were measured by AV-FITC/PI flow cytometry. The whole cell population was divided into different quadrants (Q); Q 1 were considered necrotic, Q2, late apoptotic, Q3, early apoptotic and Q 4 were considered live.

The dot plots presented in figure 6.14 provide a visual representation of the effects of increasing concentrations of ULA on HepG2 cells. Data was extrapolated from representative dot plots and presented in figure 6.15 as the effects of ULA on the percentage of cells, at each treatment time point in each state; live, early apoptotic, late apoptotic, and necrotic. Statistically, cells treated with 2.5mM showed the most significant decrease in live cells, particularly after 6 and 8 hours of treatment ($P=0.0389$ and $P=0.0374$ respectively) and the most significant increase in both early and late apoptosis, and necrosis compared to all other concentrations.

The results presented in figure 6.15 represent the effects of ULA on cell death in HepG2 cells over a period of 8 hours.

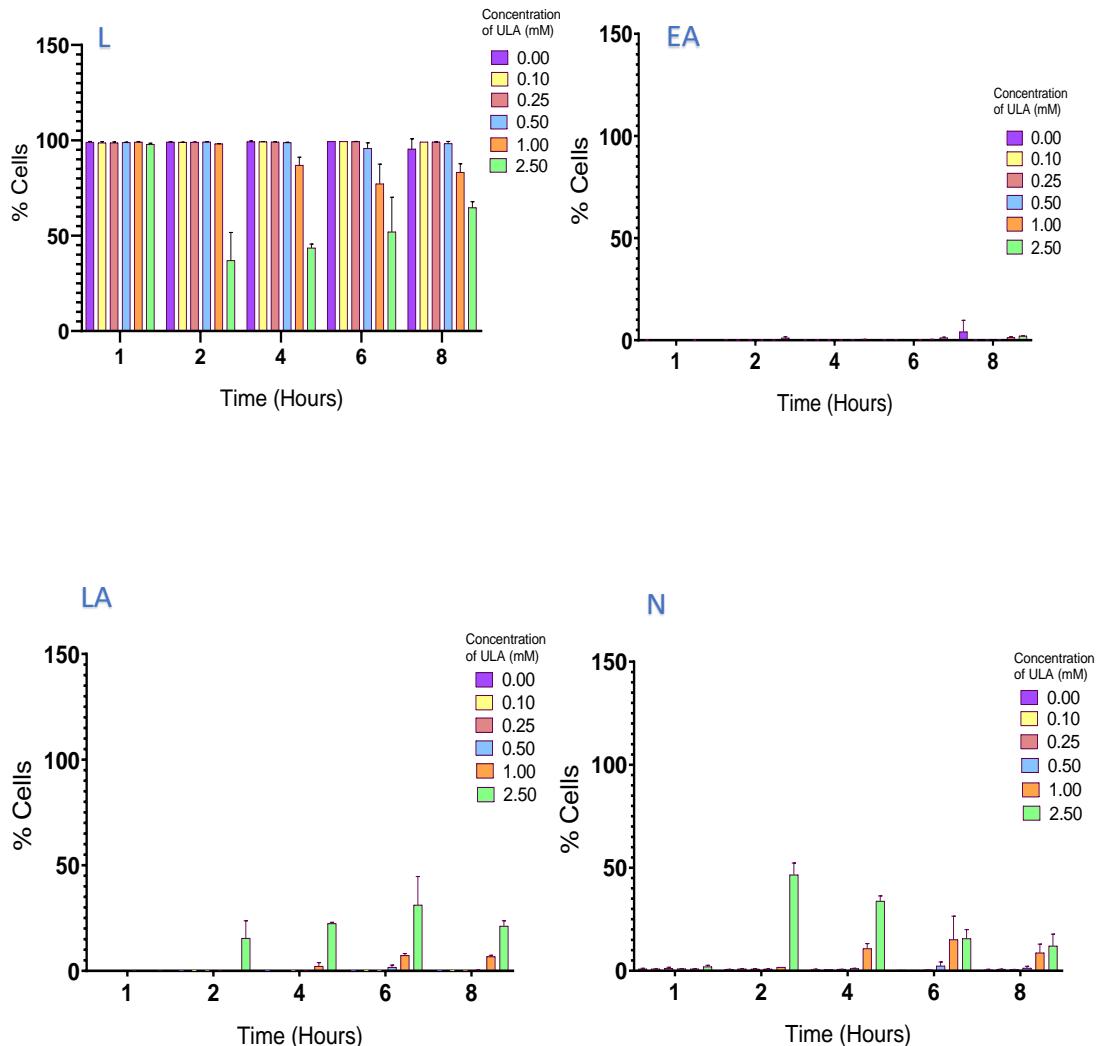


Figure 6.15. Induction of ULA mediated cell death in HepG2 cells treated with ULA from 1 to 8 hours. Data is presented as the effects of increasing concentrations of ULA on the percentage of cells in each cellular state, live, early apoptotic, late apoptotic, and necrotic cells for each time point. Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's *post-hoc* test was used to compare all means ($n=2$). L; live. EA; early apoptosis. LA; late apoptosis. N; necrosis.

6.7. Discussion

Lipids, particularly, fatty acids are among the most abundant cellular metabolites, with immense diversity in structure and function. They play an essential role in cellular membrane assembly and architecture, provide anhydrous stores of energy and serve as signalling molecules in an array of cellular processes (Haberkant and Holtuis, 2014).

Linoleic acid, an ω -6 essential fatty acid and its metabolite, arachidonic acid, have been reported to promote a strong inflammatory response, through the production of pro-inflammatory eicosanoids such as thromboxane and prostaglandin E₂ (Choque *et al*, 2012). In contrast, the ω -3 PUFAs such as α -linolenic acid, have been implicated in anti-inflammatory mechanisms (Calder, 2012). Both α -linolenic acid and linoleic acid are metabolised by the same enzyme, Δ 6-desaturase (Calder, 2012). While the preferred substrate for this enzyme is α -linolenic acid, the abundance of linoleic acid in the western human diet means that its metabolism, according to Calder (2012) is quantitatively more important.

In the equine diet, horses receiving a cereal based diet also have a balance whereby linoleic acid is more abundant. This was highlighted by Hallebeek and Beynen (2002), who determined that an equine receiving a diet containing 7kg of hay and 3kg of cereal-based concentrates would ingest approximately 70g of linoleic acid and 4g of α -linolenic acid. This was in contrast to grazing or browsing horses who were reported to have a higher intake of α -linolenic acid (Hallebeek and Beynen, 2002). Linoleic acid has been reported to lower the rate of metabolism of ω -3 PUFAs, resulting in a decrease their availability and in turn the physiological functions (Choque *et al*, 2012). Based on the current evidence linking linoleic acid to a strong inflammatory response (Choque *et al*, 2012), the effects of the fatty acid, in its unconjugated form, on the

production of markers of inflammation, such as reactive oxygen species, TNF- α and thromboxane, was assessed.

The liver is the main metabolic organ in the body, playing a vital role in lipid metabolism. It is also the organ of interest in this study, as initial studies carried out by Cooper (2015) detected the presence of linoleic acid, in its unconjugated form, in equine liver samples. According to Marchesini *et al* (2008), obesity associated liver disease is recognised as the most prevalent liver disease in the Western world. Emerging data has identified that disturbed hepatic lipid metabolism is associated with elevated circulating free fatty acids, such as palmitic and oleic acid (Asrih *et al*, 2015). As such, the human hepatoma - derived cell line, HepG2, was chosen for this study. Historically, HepG2 cells have been used to investigate the correlation between fatty acids and several diseases associated with the liver, such as NAFLD, Type 2 Diabetes Mellitus and Non-Alcoholic Steatohepatitis (Yao *et al*, 2011, Asrih *et al*, 2015, Zeng *et al*, 2020). These cells have been reported to retain the function of fully differentiated primary hepatocytes, including normal hepatic metabolic functions and are therefore widely used in hepatotoxicity studies (Lima *et al*, 2006, Asrih *et al*, 2015). They have also been reported to retain normal lipoprotein and apolipoprotein synthesis (Dokko *et al*, 1998, Dixon and Ginsberg, 1993). For these reasons, they were considered to be a suitable *in vitro* model for this investigation.

Advancing the work carried out in Chapter 5, HepG2 cells were treated over five different time points, 1, 2, 4, 6 and 8 hours, with varying concentrations of ULA. Concentrations ranged from physiologically normal (0.1mM) to high (2.5mM) (Tikanoja, *et al*, 1989 Shultz, 1991, Toborek *et al* 1996, Artwohl *et al*, 2003), with untreated controls referred to as 0mM. Using these parameters, the effects of shorter-term exposure of hepatic cells to free fatty acids was investigated.

6.7.1. Cell Viability in HepG2 Cells Treated with ULA over Shorter Treatment Intervals.

Cell viability assays, in the form of MTT assays, were used to identify the effects of concentration and the effects of shorter periods of exposure of HepG2 cells to ULA.

As already mentioned, free fatty acids are essential substrates required for numerous functions (Zechner *et al*, 2012). However, fatty acid overload can result in the impairment of normal membrane function, endoplasmic reticular stress, mitochondrial dysfunction and eventual cell death (Borradaile *et al*, 2006).

Studies carried out by Sparks *et al*, (2005) demonstrated the impact of elevated plasma free fatty acid levels over 3 days in the form of the down regulation of genes involved in oxidative phosphorylation. However, contrary to this, Brands *et al*, (2011) and Brehm *et al* (2009) observed no significant mitochondrial disruption after shorter periods of elevated plasm free fatty acids (6 hours and 3 hours respectively) indicating free fatty acid mediated mitochondrial disruption may occur as a result of longer term exposure.

6.7.1.1. The Effects of ULA Concentration on the Cell Viability of HepG2 Cells.

Figure 6.1 represents the effects of varying concentrations of ULA on the cell viability of HepG2 cells treated over five different time points. A decrease in cell viability, as the concentration of ULA increased, was evident. Cells treated with 0.1mM of ULA had a similar cell viability percentage as the untreated groups across all time points. A significant decrease in the cell viability of the 0.1mM treatment group, compared to the untreated cells, was only observed after 8 hours of exposure ($P = 0.0496$). The IC₅₀ of ULA after 8 hours of treatment was determined to be 0.16mM (± 0.005), as presented in table 6.1.

As the concentrations of ULA increased, cell viability continued to decrease. Once the concentration of ULA surpassed 0.25mM, a significant decrease in cell viability was observed ($P = <0.0001$). This decrease was evident among all groups treated with 0.25mM of ULA or higher, when they were compared with both the untreated control and the 0.1mM treatment group. Similarly, Yao *et al* (2011) observed a dose dependent reduction in the viability of HepG2 cells treated with increasing concentrations of free fatty acids.

6.7.1.2. The Effects of ULA Treatment Time on the Cell Viability of HepG2 Cells.

Increasing treatment times resulted in a marked decrease in cell viability in cells treated with 0.25mM of ULA and higher (figure 6.1). Cells treated with 0.25mM of ULA exhibited a significant decrease in cell viability after 4 (29.0%), 6 (14.8%) and 8 hours (8.3%) of treatment, when compared with 1 hour (72.8%) and 2 hours (66.3%) of treatment ($P = <0.0001$). In cells treated with varying concetration of ULA for 4 hours, the IC₅₀ was determined to be 0.21mM (± 0.009). There was also a significant decrease observed between cells treated with 0.25mM for 4 hours and 8 hours ($P = 0.0018$). Cells treated with 0.5mM of ULA displayed a significant decrease in cell viability when the treatment time surpassed 2 hours ($P = <0.0001$). Cells treated with 1mM and 2.5mM showed a significant reduction in cell viability after 1 hour of exposure to ULA, when compared to all other time points ($P = <0.0001$).

As the concentration of ULA surpassed 0.25mM of ULA, a significant decrease in cell viability was observed. While a significant decrease in cell viability at 0.25mM after 1 hour of exposure was not anticipated, an IC₅₀ of 0.44mM (± 0.021) of ULA was established in cells treated with the fatty acid for this timepoint. Interestingly, several

authors describe normal plasma free fatty acid concentrations ranging from 0.3 to 0.6mM in the overnight fasting state (Tikanoja, *et al*, 1989, Shultz, 1991, Toborek *et al*, 1996, Artwohl *et al*, 2003). However, the most common plasma free fatty acids are palmitic acid and oleic acid (Zhou and Grill, 1994). These results (figure 6.1) suggest that the fatty acid type, structure or degree of saturation may affect cell viability.

The deleterious effects of free fatty acids on cell viability have been reported in numerus cell types (Di Nunzio *et al*, 2011). According to Wu *et al*, (2008) free fatty acids are the important mediators of lipotoxicity. Lipid overload in the hepatocyte can have a detrimental effect on the healthy function of the organ. Circulating free fatty acids are the main contributors to liver triacylglycerol and a correlation has been determined between plasma levels of free fatty acids and disease severity in human patients suffering from non-alcoholic fatty liver disease (NAFLD) (Wu *et al*, 2008). High concentrations of circulating free fatty acids as well as *de novo* lipogenesis, when not counterbalanced by appropriate metabolic processes, such as fatty acid oxidation, can result in various disorders associated with hepatic steatosis (Alsabeh *et al*, 2018). Hepatic lipidosis, as the condition is referred to in veterinary terms, is considered, according to Divers (2015) to be the most common disorder causing equine liver failure.

Increasing exposure time to ULA impacted cellular health. The evidence of this, presented in figure 6.1, is further strengthened by the decreasing IC₅₀ values observed in table 6.1, indicating that HepG2 tolerance to ULA exposure diminishes over time. Cells treated with 0.1mM, the lowest ULA concentration, initiated a decrease in cell viability after 8 hours of exposure. At this timepoint an IC₅₀ of 0.16mM (± 0.005) was observed (table 6.1). According to Karpe *et al*, (2011), and Eaton *et al*, (1969), free

fatty acid turn-over in plasma is extremely rapid, with a half-life of 2 to 4 minutes. Several authors (Zhou and Grill, 1994, Unger, 1995, Bollheimer, *et al*, 1998) have implied that the deleterious effects of free fatty acids on homeostasis are as a result of persistently elevated plasma free fatty acid levels.

Molecular structure may also play a role in how a particular fatty acid impacts cellular activity. Ricchi *et al*, (2009) states that fatty acids are classified as saturated and unsaturated and their structure influences their biological effects. Hawkins (1998) suggests that it is related to the number of fatty acid double bonds and their arrangement, whether conjugated or not. Iuchi *et al*, (2019) demonstrated the anti-proliferative effects of various PUFAs, including linoleic acid, on human cultured cells. Iuchi *et al* (2019) observed a correlation between increasing number of double bonds and decreasing cell viability. Like Hawkins *et al*, (1998), Iuchi *et al*, (2019) proposed that PUFAs with more double bonds are more easily oxidised into highly active and potentially cytotoxic products such as hydrogen peroxides and aldehydes.

Zhang *et al*, (2012) found that free fatty acid induced apoptosis was significantly greater in rat hepatocytes treated with 0.25mM of linoleic acid, a long chain PUFA, than with the same concentration of palmitic acid, a long chain saturated fatty acid. However, when palmitic acid (0.25mM) and linoleic acid (0.125mM) were combined, there was a significant decrease in apoptosis in the same cell line (Zhang *et al*, 2012). Di Nunzio *et al*, (2011), also observed a reduction in cell viability in HepG2 cells treated with linoleic acid as well as other polyunsaturated fatty acids. According to Hawkins *et al*, (1998) linoleic acid, along with a number of other PUFAs, caused an inhibition of growth, *in vitro*, in a pancreatic cancer cell line, hypothesising that the mechanism of action may be due to the disruption of cellular membrane fluidity as

well as the production of by-products of lipid metabolism, such as reactive oxygen species.

6.7.2. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using Oil Red O

Intracellular lipid accumulation is an accumulation of triacylglycerol-containing lipid droplets within the cytosol (Cury-Boiventura *et al*, 2004). In recent years lipid droplets have been recognised as organelles with key functions in lipid and energy homeostasis. They are considered to work as universal storage organelles for neutral lipids in most mammalian cells (Olzmann and Carvalho, 2018). However, intracellular lipid overload resulting in the accumulation of high concentrations of intracellular lipid droplets may induce toxicity, resulting in cellular dysfunction and death (Yao *et al*, 2011, Jaishy and Abel, 2016).

The Oil Red O assay was used to assess intracellular lipid accumulation in HepG2 cells exposed to increasing concentrations of ULA over a period of 8 hours (figure 6.3). The untreated control was considered to have an intracellular lipid accumulation value of 0% as these cells were grown in fatty acid free BSA constituted media. For the determination of the percentage of intracellular lipid accumulation, all other treatment groups were compared to the untreated control.

Light micrographs, presented in figure 6.4, show an increase in the presence of intracellular lipids stained with Oil Red O, when treated cells were compared with untreated cells, particularly in those treated with 0.1mM and 0.25mM of ULA. However, as the concentration of ULA increased, cell morphology appeared to be impacted with HepG2 cells losing their integrity. The number of visible cells in the microscopic field appeared reduced.

6.7.2.1. The Effects of Concentration of ULA on intracellular Lipid Accumulation in HepG2 Cells.

Intracellular lipid accumulation did not increase in ULA treated HepG2 cells, in a dose dependent manor, indicating a non-monotonic dose response (figure 6.3). When untreated cells were compared with all other concentration groups, the most significant increase in intracellular lipid accumulation occurred in cells treated with 0.1mM of ULA, the lowest treatment concentration ($P = <0.0001$). A significantly higher percentage of intracellular lipid accumulation was also observed in this treatment group, when it was compared to all other treatment groups. Cells treated with 0.1mM of ULA presented with a significantly higher percentage of intracellular lipid accumulation, compared with cells treated with 0.25mM ($P = 0.0065$), 0.5mM treatment ($P = 0.016$) and 2.5mM treatment ($P = 0.0007$).

It was hypothesised that as the concentration of ULA increased, so too, would the percentage of intracellular lipid accumulation. However, the results determined that as the concentration of ULA increased, the percentage of intracellular lipid accumulation did not increase in a dose dependent manner.

6.7.2.2. The Effects of Exposure Time of HepG2 cells to ULA on Intracellular Lipid Accumulation.

The length of time that HepG2 cells were exposed to each concentration was also analysed. While cells treated with 0.1mM of ULA increased in a time dependent manner, the only significant increase in intracellular lipid accumulation, in cells treated with this concentration, occurred between cells treated for 8 hours and all other time points ($P = <0.0001$). The same trend was observed in cells treated with 0.25mM of ULA ($P = <0.0001$).

In cells treated with 0.5mM the only significant increase in intracellular lipid accumulation occurred between cells treated for 1 hour and cells treated for 8 hours ($P = 0.0358$). Cells treated with 2.5mM of ULA for 8 hours showed a significant increase in intracellular lipid accumulation when compared to cells treated with the same concentration for 1 hour, 4 hours and 6 hours.

At lower concentrations, 0.1mM and 0.25mM of ULA, intracellular lipid accumulation did increase in a time dependent manner, however, as the concentrations reached and surpassed 0.5mM, a non-monotonic dose response was observed.

Hepatocytes have the greatest capacity, next to adipocytes, to store lipid droplets, however, excessive accumulation of intracellular lipids within the hepatocyte, referred to as hepatic steatosis (Walther and Farese, 2012), has been associated with cell death (Ipsen *et al*, 2018).

As demonstrated by Di Nunzio *et al*, (2011) and illustrated in figure 6.1, increasing concentrations of ULA reduce cell viability in HepG2 cells. Light micrographs portrayed in figure 6.3 showed that, with increasing ULA concentrations and longer treatment times, there were fewer cells present. This suggested that the decrease in intracellular lipid accumulation observed in figure 6.3 may be due to reduced cell number at the time of assay. When non-adipose cells are chronically overloaded with fatty acids, inappropriate lipid accumulation can lead to cellular dysfunction and cell death (Unger, 2003, Borradaile *et al*, 2006, Wei *et al*, 2007). Apoptosis appears to be a principal route of cell death, however necrosis may also occur (Unger and Orci, 2002). As excessive intracellular lipids can lead to imbalances in cellular homeostasis, resulting in dysfunction and death, further investigation into the accumulation of linoleic acid within HepG2 cells was warranted. This was carried out using flow

cytometry combined with the fluorescent lipophilic stain Nile Red. This stain was also used to visualise intracellular lipid accumulation using fluorescent microscopy.

6.7.3. Analysis of Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using the Nile Red Fluorescent Stain.

The importance of intracellular lipid accumulation in the form of cytosolic lipid droplets was discussed in detail in section 2.2.5. It was decided to further investigate the effects of increasing concentrations of ULA on intracellular lipid accumulation.

Nile Red was used as a lipophilic stain for the visualization of intracellular lipid accumulation in HepG2 cells, as well as the analysis of intracellular lipid accumulation using flow cytometry. Nile Red is intensely fluorescent and can serve as a sensitive vital stain for the detection of intracellular lipids (Greenspan *et al*, 1985). According to Aldridge *et al*, (2013) the use of Nile Red in flow cytometry allows for more quantitatively accurate and less subjective analysis of intracellular lipids than Oil Red O.

6.7.3.1. Fluorescent Microscopy

In figures 6.5.1 to 6.5.3, an increase in red stain was observed between treated and untreated cells, particularly those treated with 0.1mM of ULA. Small circular bodies (indicated by blue arrows) were observed in all micrographs including the untreated controls and appeared to surround the nucleus. These circular bodies were more abundant in treated cells. As lipid droplets are thought to form off the endoplasmic reticulum (Guo *et al*, 2009, Hashemi and Goodman, 2015), it may be postulated that these stained circular bodies are lipid droplets. Cells exposed to 2.5mM also showed a distinct change in morphology (indicated by green arrows), even after 1 hour of treatment.

Nile red is reported to fluoresce yellow/gold in the presence of neutral lipids and red in the presence of polar lipids and phospholipids (Greenspan *et al*, 1985, Weller *et al*, 1989, Diaz *et al*, 2008). Consequently, in order to optimize intracellular lipid visualization, micrographs were captured using two spectral settings, and then merged to increase lipid body resolution and visualisation (Durandt *et al*, 2016), as detailed in section 3.1.10. However, prior to the overlaying of these images, fluorescent micrographs, as presented in the figure 6.5.4, demonstrated a stronger red fluorescence with a weaker yellow/gold fluorescence. This suggested that ULA induced the formation of neutral lipid droplets as well as increasing intracellular polar lipid content and phospholipid content (Weller *et al*, 1989). According to Greenspan *et al*, (1985) when viewed for yellow/gold fluorescence, intracellular lipid bodies appeared as small discrete circular structures distributed throughout the cytoplasm, with some being clustered around the nucleus. When cells were viewed for red fluorescence, the same intensely stained structures remained visible, however, the individual bodies were not as readily resolved (Greenspan *et al*, 1985). Greenspan *et al*, (1985) also suggested that a diffuse general staining of the cytoplasm becomes more apparent, which may represent the staining of intracellular membranes and other organelles.

6.7.3.2. Flow Cytometry

Nile red was also used in conjunction with flow cytometry to determine if increasing concentrations of ULA resulted in an increase in intracellular lipids. The median fluorescence intensity (MFI) can be used as an indicator of intracellular lipid accumulation (Greenspan *et al*, 1985, Wolins *et al*, 2018). Greenspan *et al* (1985) and Wolins *et al*, (2018) showed that lipid loaded cells could be differentiated by an increased MFI relative to untreated cells.

6.7.3.3. The Effects of Concentration of ULA on intracellular Lipid Accumulation in HepG2 Cells

As before, a non-monotonic dose response was observed (figure 6.6). The lower concentrations (0.1mM and 0.25mM of ULA) induced an increase in MFI, correlating to an increase in intracellular lipid accumulation. Cells treated with 0.25mM of ULA, induced the greatest increase in MFI, with an MFI value of 2.2 (± 0.19) compared to an MFI of 1.0 (± 0.28) for untreated cells over the same treatment period. Once the concentrations reached and surpassed 0.5mM of ULA, a reduction in MFI was observed, indicating that MFI, and consequently, intracellular lipid accumulation, was not dose dependent. These findings corroborate the previous results obtained using Oil Red O (figure 6.3).

Statistically, cells treated with 0.1mM of ULA induced a significant increase in MFI when compared with cells treated with 1mM ($P = 0.0065$) or 2.5mM ($P = <0.0001$). Cells treated with 0.25mM induced the most substantial increase in MFI compared with all other treatment groups. The MFI was found to be significantly increased in this treatment group, when compared with cells treated with 0.5mM ($P = 0.0015$), 1mM ($P = <0.0001$) and 2.5mM of ULA ($P = <0.0001$). Cells treated with 0.25mM also generated a significantly higher MFI than the untreated control cells ($P = 0.0370$). Cells treated with 0.5mM of ULA had a significantly higher MFI than cells treated with 2.5mM with a P value of 0.0003.

These results indicate lower concentrations, namely 0.1mM and 0.25mM of ULA increased MFI, and consequently, intracellular lipid accumulation, while higher concentrations resulted in a reduction in intracellular lipid accumulation.

6.7.3.4. The Effects of Time of ULA on intracellular Lipid Accumulation in HepG2 Cells

The effects of treatment time on MFI in ULA treated HepG2 cells was also analysed. Increasing treatment times resulted in an increased MFI in cells treated with the lower concentrations of ULA, namely, 0.25mM.

Cells treated with 0.25mM of ULA for 8 hours, showed the most significant increase in MFI when compared with cells treated for 4 hour ($P = 0.0022$) and 6 hours ($P = 0.0004$). Cells treated with this concentration for 1 hours had a significantly higher MFI than those treated for 6 hour ($P = 0.0118$). Cells treated with 2.5mM had a significantly higher MFI after 1 hour and 2 hours of treatment when compared to 4 hours and 8 hours.

These results (figure 6.5) demonstrated that lower concentrations, specifically 0.25mM of ULA significantly increased MFI, indicating an increase in intracellular lipid accumulation. However, as the concentration of ULA surpassed 0.5mM, a decrease in MFI was observed, even after 1 hour of treatment.

As the concentration of ULA reached and surpassed 0.5mM, images obtained from both Oil Red O staining (figure 6.3) and fluorescent microscopy (figure 6.5.1 to 6.5.3), show a distinct change in cellular morphology, as well as a reduction in the number of cells visible within the microscopic field. Cell counts carried out by flow cytometry also indicated a reduction in cell numbers, as concentration and treatment time increased. These data suggested that the ectopic over-accumulation of lipids resulted in a loss of cellular integrity and eventual cellular destruction. Over accumulation of lipids in non-adipose cells may enter non-oxidative pathways, such as unnecessary

cellular signaling pathways (Koutsari *et al*, 2011), leading to cell injury and death (Feldstein *et al*, 2004, Unger *et al*, 2010).

As previously mentioned, Hawkins *et al* (1998), demonstrated that PUFAs, including linoleic acid, induced cell death by apoptosis. Work carried out by Zhang *et al*, (2012) determined that linoleic acid, at concentrations of 0.25mM, induced apoptosis in rat hepatoma cells. Zhang *et al*, (2012) suggested that fatty acid induced cell death may occur through alternate apoptotic pathways, depending on the fatty acid type. Wu *et al*, (2008) postulated that, while the mechanisms by which fatty acids induce cell death have yet to be elucidated, multiple mechanisms are involved.

Due to the close association of lipid droplets with the endoplasmic reticulum, lipotoxicity in hepatocytes may induce endoplasmic reticulum stress and associated apoptosis (Wei *et al*, 2007). Wei *et al* (2007) demonstrated that saturated free fatty acids, at physiological concentrations (~0.1mM) induced endoplasmic reticulum stress within four hours in a rat hepatoma cell line. This was followed by apoptosis within 6 hours (Wei *et al* 2007). Zhang *et al*, (2012) also observed endoplasmic reticulum stress, in the form of elevated calcium flux, induced by linoleic acid in rat hepatoma cells. In the current investigation, a decrease in HepG2 cell viability was observed in cells treated with 0.25mM of ULA after 1 hour (figure 6.1), while intracellular lipid accumulation decreased, once the treatment reached this concentration after 4 hours of exposure (figure 6.6). Borradaile *et al*, (2006) also reported endoplasmic reticulum stress, associated with increased reactive oxygen species, and cell death in cardiomyocytes and Chinese hamster ovary cells treated with 0.5mM of saturated fatty acids.

As well as this, free fatty acids are also thought to induce tumor necrosis factor (TNF) expression through a lysosomal pathway (Feldstein *et al*, 2004, Wu *et al*, 2008). Both Feldstein *et al*, (2004) and Wu *et al* (2008) observed that free fatty acids induced the release of cathepsin B, a lysosomal protease associated with the induction of apoptosis, into the cytosol. Cathepsin B, according to Wu *et al*, (2008) promotes the production of reactive oxygen species and is also believed to play a pivotal role in TNF- α induced apoptosis and tissue injury. As already mentioned, PUFAs, particularly linoleic acid, may influence the production of several metabolic products, such as prostaglandins, leukotrienes and reactive oxygen species that can lead to cellular dysfunction and death (Hawkins *et al*, 1998). Therefore, the effects of increasing concentrations of ULA on the production of inflammatory cytokines, eicosanoids and reactive oxygen species in HepG2 cells was investigated further.

6.7.4. Assessment of the Inflammatory Effects of ULA though the Secretion of TNF- α

TNF- α is reported to be one of the most important cytokines, exerting a series of biological effects on several cell and tissue types (Chen *et al*, 2009). It has been titled a master regulator in several biological process, including apoptosis and necroapoptosis and, as such, has been extensively studied (Blaser *et al*, 2016). Stonans *et al*, (1999) demonstrated that the human hepatoma cell line, HepG2, expressed a variety of cytokine genes, including TNF- α , indicating that cells of hepatocellular linages may be potential producers of these cytokines.

Previous reports have indicated that TNF- α expression is markedly increased in the adipocytes of obese mammals (Kern *et al*, 1995, Li *et al*, 2015). Increased serum TNF- α has also been reported in ponies presenting with equine metabolic syndrome, a

pathological obesity characterised by abnormal body fat accumulation and inflammation (Basinska *et al*, 2015). Free fatty acids, such as palmitic and oleic acid (0.5mM to 1mM), have been shown to induce TNF- α expression in HepG2 cells (Feldstein *et al*, 1998).

Systemic administration of TNF- α has also been reported to decrease platelet activation and inhibit thrombi formation in mice (Cambien, *et al*, 2003). Cambien *et al* (2003) documented the effects of TNF- α on thrombus formation in an *in vivo* mouse model. According to Cambien *et al* (2003), thrombus formation in mice treated with 1ng/ml of TNF- α was approximately three times longer than untreated animals. Furthermore, thrombi in treated mice were unstable and failed to resist shear stress while growing to a larger size, constantly dissociating into single platelets or very small emboli (Cambien *et al*, 2003).

Therefore, the effects of ULA on the production of this pro-inflammatory cytokine in HepG2 cells was investigated using ELISA.

In this investigation, there was no detectable secretion of TNF- α in either the untreated control cells, or HepG2 cells treated with ULA. These results are in agreement with the findings of Gutierrez-Ruiz *et al*, (1999), who reported that, while a number of pro-inflammatory compounds, including LPS, induced TNF- α expression in HepG2 cells, there was no effect on its secretion. Work carried out by Ajuwon and Spurlock (2005) also determined that, while palmitate treatment induced TNF- α expression in adipocytes, cytokine release into culture media was attenuated as the fatty acid concentration increased. These results also correlate with the findings of Suagee *et al* (2013) and Holbrook *et al*, (2015) who determined no difference between the plasma TNF- α concentrations of healthy and obese horses. These results indicate that, while

free fatty acids, such as linoleic acid may induce TNF- α expression, secretion of the cytokine may not always occur. According to Josephs *et al* (2018), TNF- α is found in a soluble and membrane bound form. The soluble plasma form of TNF-alpha is cleaved from the membrane form by TNF-alpha-converting enzyme (TACE). While TACE expression has been reported in HepG2 cells (Gao *et al*, 2015), the impact of linoleic acid on this converting enzyme remains unknown.

Two positive controls, LPS (figure 6.7, B), a potent activator of cytokine production according to Gutierrez-Ruiz *et al*, (1999), and ethanol (80mM), also both failed to induce TNF- α secretion in HepG2 cells. While Saad *et al*, (1995) observed TNF- α secretion in cultured rat hepatocytes treated with LPS at a concentration of 10 μ g/mL, this was not observed in HepG2 cells treated with the same concentration of LPS in this study. According to Neuman *et al*, (1998), treatment of HepG2 cells with 80mM of ethanol induced TNF- α secretion (31.5pg/mL). Contrary to the findings in this investigation, Neuman *et al*, (1998) also reported a TNF- α concentration of 8pg/mL in untreated HepG2 cells. A third positive control in the form of LPS injured macrophages (differentiated THP-1 cells) resulted in the production of TNF- α (figure 6.7), confirming assay integrity and reinforcing the current findings. Imaizumi *et al*, (2000) consider monocytes/macrophages to be one of the most important sources of TNF- α .

While macrophage cells have been the most widely studied cell type for the production of TNF- α , several other cell types have been reported to produce the cytokine, including HepG2 cells (Gutierrez-Ruiz *et al*, 1999). Linoleic acid at concentrations of 0.25mM to 1mM, have been shown to stimulate TNF- α secretion in a number of cell lines, including Caco2 intestinal cells (Li *et al*, 2015). However, in this investigation, TNF- α secretion was not stimulated in HepG2 cells in response to increasing

concentrations of ULA. This response may be due to the induction of other cytokines by linoleic acid, with negative autoregulatory effects on TNF- α secretion (Gutierrez-Ruiz *et al*, 1999). Miles *et al*, (2003) determined that prostaglandin E₂, a pro-inflammatory eicosanoid derived from arachidonic acid, a metabolite of linoleic acid, acts as an inhibitor of LPS induced TNF- α in whole blood. Ajuwon and Spurlock (2005), suggested that the release of interlukin-6 (IL-6) by adipocytes, into culture media, in response to palmitate treatment may have resulted in the suppression of TNF- α secretion. They also postulated the possibility that TNF- α degradation in the media may have affected the measurable concentrations. Paul and Mukherjee (2019) have reported the secretion of IL-10, an anti-inflammatorcyt cytokine, in HepG2 cells, which they state may have a suppressive effect on pro-inflammatory cytokine responses in Hepg2 cells.

Inflammatory responses result in local and systemic production of numerous soluble markers, such as TNF- α , IL-6 and plasminogen activator inhibitor-1 (Fritsche, 2015). Nevertheless, there is no consensus regarding which inflammatory biomarker will provide the best results and the selection of multiple biomarkers does not guarantee that treatment effects will be observed (Fritsche, 2015). However, TNF- α is a pro-inflammatory cytokine that has been widely used to study various elements ranging from cell proliferation and apoptosis to inflammation and lipid homeostasis (Chen *et al*, 2009), and as such, it was chosen as a biomarker of interest in this investigation. Future work may include the effects ULA on TNF- α expression in HepG2 cells, as well as the production of other inflammatory cytokines, such as Il-6.

6.7.5. The Effects of ULA on the activity of Superoxide Dismutase in HepG2 Cells.

Reactive oxygen species (ROS) are by-products of various enzymatic reactions in several cellular compartments, such as the cell membrane, cytoplasm, endoplasmic reticulum and mitochondria (Forrester *et al*, 2018). At physiological concentrations, they regulate cellular homeostasis and participate in normal physiological processes, serving as second messengers and transducing complex intracellular signals involved in several biological functions (Dröge, 2001, Forrester *et al*, 2018, Incalza *et al*, 2018). However, at pathological concentrations, ROS can contribute to maladaptive responses leading to metabolic dysfunction, inflammatory signaling and cellular damage resulting in disease pathophysiology (Forrester *et al*, 2018).

ROS are oxidizing agents generated during cellular metabolism when the chemical reduction of oxygen forms unstable free radicals (Huang *et al*, 2015). They are produced as byproducts of cellular metabolism through the electron transport chain in mitochondria as well as *via* the cytochrome P450 family of enzymes (Mittal *et al*, 2014). According to Thannickal and Fanberg, (2000), ROS can occur as by-products of electron transfer reactions, as a result of any electron-transferring protein or enzymatic system. Excessive production of ROS can result in oxidative stress, leading to macromolecular damage, resulting in various disease states (Ray *et al*, 2012). Thannickal and Fanberg, (2000) and Haung *et al*, (2015) defines oxidative stress as cellular damage caused by an imbalance between pro-oxidants such as ROS and antioxidants. According to Das (2011), ROS can induce oxidative stress leading to cell death by apoptosis or necrosis.

In equines, increases in oxidative stress and ROS have been reported following exercise (Williams *et al*, 2004). While literature detailing oxidative stress in the equine is limited (Siqueira *et al*, 2014), a review carried out by Kirschvink *et al* (2008), detailed the impact of oxidative stress in the equine, with oxidant/antioxidant disequilibrium being attributed to several pathological conditions including exercise-induced pulmonary haemorrhage, chronic obstructive pulmonary disease, colic and laminitis.

Several studies have reported a link between obesity, fatty acid metabolism and increased ROS (Videla *et al*, 2004, Haung *et al*, 2015). Lipid peroxidation, the oxidative degradation of lipids, is itself, a free radical-generating process which occurs on every membranous structure of the cell (Olusi, 2002). Intracellular free fatty acid overload is reported to lead to increased inner mitochondrial membrane permeability, resulting in mitochondrial dysfunction and enhanced ROS production (Masarone *et al*, 2018). The endoplasmic reticulum is also a potent source of ROS. According to Masarone *et al*, (2018), in healthy cells, the oxidative folding of proteins performed in the endoplasmic reticulum, results in the production of ROS, accounting for approximately 25% for all cellular ROS generation. Induction of endoplasmic reticulum stress may lead to an increase in ROS generation (Masarone *et al*, 2018). Free fatty acids are thought to disrupt endoplasmic reticulum calcium flux leading to mitochondrial membrane permeabilization and the release of cytochrome C and increased ROS resulting in apoptosis (Masarone *et al*, 2018). Videla *et al*, (2004) suggests that chronic oxidative stress may be important in the progression of NAFLD.

The superoxide anion, considered to be the primary type of ROS produced in the body (Homma and Fujii, 2019), is continually produced during normal metabolism (Ighodaro and Akinloye, 2018). According to Incalza *et al*, (2018) it is one of the first

reactive species to be generated and is constantly produced in the inner membrane of mitochondria during electron transfer in the electron transport chain (Mittal *et al*, 2014, Togo *et al*, 2018).

In order to protect against the potentially damaging effects of ROS, cells possess several antioxidant enzymes such as superoxide dismutases (SOD) (Thannickal and Fanberg, 2000). SODs are a group of enzymes that represent an important antioxidant defense against oxidative stress in the mammalian body. They are the only known enzyme to directly scavenge the superoxide anion (Borgstahl *et al*, 2018). They catalyse the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen (Younus, 2018). According to Ighodaro and Akinloye, (2018) SOD is a powerful endogenous antioxidant enzyme that acts as a component of first line defense system against ROS. As such, their role in the protection of cells against toxic oxygen derivatives is considered vital (Sun *et al*, 1988).

Based on previous reports in the literature, free fatty acids increase oxidative stress and ROS production both *in vitro* and *in vivo* (Toborek *et al*, 1996, Young *et al*, 1998, Hatanaka *et al* 2006, Morgan *et al*, 2007, Hatanaka *et al*, 2013, Siqueira *et al*, 2014). It was postulated that an increase in ROS in response to fatty acid exposure would initiate an increase in the antioxidant enzymes responsible for their intracellular clearance, a hypothesis shared by Vincent *et al*, (1999). This hypothesis was strengthened by the findings of Kuratko and Constante (1998) who observed an increase in MnSOD in normal rat intestinal epithelial cells in response to supplementation with 0.04mM various free fatty acids, including linoleic acid. In the equine, several authors report that, in trained performance animals, while ROS may increase in response to strenuous exercise, as a result of lipid peroxidation, or in response to disease, so to, do the antioxidants responsible for their neutralisation

(Kinnunen *et al*, 2005, Siqueira *et al*, 2014, Neidzwiedz *et al*, 2014). As the superoxide anion is one of the first ROS produced (Incalza et al, 2018), it was decided to assess the response of SOD, its specific antioxidant, in ULA treated HepG2 cells.

6.7.5.1. The Effects of Concentration of ULA on SOD in HepG2 Cells

Increasing concentrations of ULA reduced SOD production in HepG2 cells (figure 6.9). Cells treated 0.1mM of ULA produced similar concentrations of SOD to the untreated controls over the 8-hour treatment period. As treatment time and concentration of ULA increased, SOD concentration decreased. However, this decrease was not dose or time dependent, with a non-monotonic response being observed.

Both the untreated controls and cells treated with 0.1mM of ULA induced significantly higher concentrations of SOD than those treated with 0.25mM of ULA and higher ($P = \leq 0.0012$). Cells treated with 0.25mM showed a significantly higher concentration of SOD when compared to cells treated with 0.5mM of ULA or higher ($P = \leq 0.0004$). Cells treated with 2.5mM of ULA showed the most marked reduction in SOD production compared to all other ULA concentrations.

6.7.5.2. The Effects of Time on SOD in HepG2 Cells

Increasing treatment times resulted in reduced cytosolic SOD in ULA treated HepG2 cells. Cells treated at concentrations higher than 0.25mM of ULA for 4 hours or longer showed a marked decrease in SOD production.

The effects of increasing treatment times induced a non-monotonic dose response. Cells treated with 0.5mM of ULA over 2 hours had a significantly higher concentration of SOD when compared to cells treated with the same ULA

concentration over 1 hour ($P = 0.0066$). However, as the treatment time reached and surpassed 4 hours a significant reduction in SOD production was observed for this treatment concentration ($P = \leq 0.0054$). Cells treated with 1mM of ULA also showed a significant reduction in SOD production once the treatment period reached and surpassed 4 hours ($P = \leq 0.0004$)

Increasing concentrations of ULA induced a non-monotonic decrease in cytosolic SOD in HepG2 cells. Similarly, Videla *et al*, (2004) reported that SOD activity was decreased in the livers of human patients suffering from NAFLD. Videla *et al*, (2004) found that NAFLD patients exhibited a pro-oxidant state in the liver, however, this pro-oxidant condition occurred concomitantly with a significant decrease in hepatic SOD. Videla *et al*, (2004) postulated that the progressive functional deficiency associated with the development of liver injury, particularly protein synthesis, may contribute to the lower activity of SOD observed in patients suffering from NAFLD. This may explain the reduction in SOD observed in ULA treated HepG2 cells in the current investigation. Investigations carried out by Olusi (2002) also determined a significant decrease in erythrocyte cytosolic SOD in obese human subjects compared to those with a healthy BMI. Olusi (2002) similarly postulated that this decrease may be attributed to progressive tissue damage. In contrast, Kohjima *et al*, (2009) reported that treatment of HepG2 cells with α -linolenic acid, an ω -3 polyunsaturated fatty acid, enhanced SOD expression in HepG2 cells.

Several studies have hypothesised that lipid molecules such as free fatty acids may be positive modulators for misfolded protein aggregations leading to diseases such as amyotrophic lateral sclerosis (ALS) (Kim *et al*, 2005, Choi *et al*, 2010, Appolinário *et al*, 2015). Kim *et al*, (2005) suggested that misfolded proteins may overwhelm the protein handling systems, resulting in the formation of potentially cytotoxic

aggregates. Appolinário *et al* (2015) found that PUFAs such as docosahexaenoic acid induced oligomerisation of cytosolic SOD. Similarly, Kim *et al* (2005) determined that incubation of cytosolic SOD with various unsaturated fatty acids, including linoleic acid, promoted oligomerisation and aggregation of the enzyme. Kim *et al*, (2005) established that during oligomerisation, a protein undergoes a conformational rearrangement, which endows it with a tendency to aggregate and form deposits within affected tissues. These SOD oligomers were found to induce cytotoxicity in a mouse neuroblastoma cell line (Kim *et al*, 2005).

Choi *et al*, (2010) hypothesised that as cytosolic SOD is frequently in contact with lipids, lipid-protein interactions could potentially promote the lipid molecule mediated abnormal assembly of SOD. However, Choi *et al*, (2010) determined that incubation of cytosolic SOD with saturated fatty acids, did not form aggregates. Kim *et al* (2005) observed similar results when cytosolic SOD was incubated with steric acid, a saturated fatty acid, with minimal SOD oligomerisation occurring. This suggested that SOD oligomerisation may require at least monounsaturated fatty acids, implicating the involvement of double bonds in the process (Kim *et al*, 2005).

The reduction in cytosolic SOD in ULA treated HepG2 cells observed in the current investigation may be attributed to a reduction in protein synthesis in injured cells, as proposed by Videla *et al*, (2004). There may also be PUFA induced oligomerisation of the SOD protein by linoleic acid overload, resulting in the production of potentially cytotoxic aggregates, as postulated by Kim *et al*, (2005) and Appolinário *et al* (2015). This may have led to the inactivation of SOD. As fatty acids are proposed to increase ROS production (Videla *et al*, 2004, Haung *et al*, 2015), the combination of this increase alongside the potential inhibition of scavenging enzymes such as SOD, by

ULA, as demonstrated in the current investigation, may lead to cellular dysfunction and death.

6.7.6. The Effects of ULA on the activity of Thromboxane Production in HepG2 Cells

Free fatty acids are potent promotores of inflammation, stimulating the release of pro-inflammatory mediators, such as cytokines (Choque *et al*, 2012, Chen *et al*, 2018). Other key mediators of inflammation include fatty acid derived signalling molecules, collectively referred to as eicosanoids (James *et al*, 2000). Linoleic acid has been reported to effect eicosanoid production in a number of cells types, including human breast cancer cells and the hepatic carcinoma cell line, HepG2 cells (Eder, *et al*, 2002).

The eicosanoid, thromboxane A₂ is a potent platelet aggregator and vasoconstrictor, that is also thought to play a role in mitogenesis and up-regulation of pro-inflammatory cytokines as well as apoptosis (Shen and Tai, 1998, Yokoyama *et al*, 2005, Nakahata, 2008). While it is primarily produced by activated platelets (Hamberg *et al*, 1975, Torres-Duarte and Vanderhoek, 2003, Ander *et al*, 2003), it has been reported that several other cells and tissues also produce thromboxane A₂, including hepatic tissue (Shen and Tai, 1986, Nakahata, 1998, Yokoyama *et al*, 2005).

Yokoyama *et al*, (2005) states that thromboxane plays a pivotal role in producing hepatic liver injury. According to Nanji *et al* (1993) and Yokoyama *et al*, (2005), thromboxane levels in the liver increase during hepatic stress such as alcoholic liver injury and cirrhosis. Thromboxane has also been reported to promote hepatic inflammation and induce hepatic vasoconstriction and leucocyte adhesion in sinusoids (Fisher *et al*, 1986, Katagiri *et al*, 2004, Yokoyama *et al*, 2005). The liver plays an important role in numerous systemic functions including coagulation and immunity

(Ambrojo *et al*, 2013). More specifically, it is responsible for the production of almost all coagulation factors and has a role in the clearance of activated coagulation products, anti-thrombin and plasminogen (Divers, 2015). Diseases of the liver are strongly linked to coagulopathies mainly due to its role in haemostasis (Ambrojo *et al*, 2013).

Previously linoleic acid was reported to have antithrombotic properties with a number of authors reporting a reduction in thromboxane production in thrombin-stimulated platelets treated with the fatty acid. Needleman *et al*, (1982) observed a 50% reduction in thromboxane released from linoleic acid (0.2mM) enriched platelets compared with untreated controls, while MacIntyre *et al*, (1984) observed partial inhibition of thromboxane in platelets treated with 0.035mM of linoleic acid. In this study, the potential for ULA to impact haemostasis was hypothesised. As it has been postulated by Yokoyama *et al*, (2005), that thromboxane A₂ plays a key role in producing hepatic injury after various types of hepatic stress, the effects of ULA on the hepatic production of thromboxane was of particular interest. In this investigation the production of thromboxane B₂ (an inactive stable metabolite of thromboxane A₂) by ULA treated HepG2 cells was analysed.

6.7.6.1. The effects of Concentration on Thromboxane Production.

Thromboxane B₂ production in ULA treated HepG2 cells increased as the concentration of ULA increased. Untreated cells produced significantly more thromboxane B₂ than cells treated with either 0.1mM ($P=0.0195$) or 0.25mM of ULA ($P=0.0078$). Cells treated with 2.5mM of ULA produced significantly more thromboxane B₂ than the untreated control ($P=0.0318$) and those treated with 0.1mM and 0.25mM of ULA ($P=0.0172$ and $P=0.007$ respectively).

6.7.6.2. The effects of Time on Thromboxane Production

Increasing treatment time did not significantly impact the production of thromboxane in ULA treated HepG2 cells. While cells treated with 2.5mM of ULA appeared to have the greatest increase in thromboxane with $3.7 (\pm 0.49)$ ng/mL of thromboxane produced after 1 hour of treatment compared to $6.7 (\pm 0.73)$ ng/mL produced after 8 hours, this increase was not considered statistically significant.

HepG2 cells produced significant amounts of thromboxane B₂ when treated with 2.5mM of ULA compared to cells treated with the lower concentrations of 0.1mM and 0.25mM of ULA. Untreated cells also produced significantly more thromboxane than cells treated with the lower concentrations of ULA. The data obtained from cells treated with 0.1mM and 0.25mM of ULA are consistent with work carried out by Needleman *et al*, (1982) and MacIntyre *et al*, (1984) who observed an inhibition of thromboxane B₂ in human platelets treated with 0.2mM and 0.035mM of ULA respectively, when compared to untreated control cells. Needleman *et al*, (1982) determined that enrichment of platelets with linoleic acid at concentrations as high as 0.2mM reduced the arachidonic acid content of the platelet phospholipids. Needleman *et al*, (1982) hypothesized that modification of the fatty acid composition of the phospholipid membrane may be related to the inhibition of thromboxane B₂ observed after linoleic acid supplementation. According to Vallee *et al*, (1980) if PUFAs other than arachidonic acid, are incorporated in the phospholipid membrane, biosynthesis of its aggregating metabolites, such as thromboxane A₂, may be impaired as a result of substrate deficiency.

However, in contrast to the findings of the current investigation, Needleman *et al*, (1982) only observed inhibition of thromboxane B₂ after 24 hours of linoleic acid

supplementation, whereas, after 4 hours of supplementation, Needleman *et al*, (1982) observed no effect on thromboxane B₂ production in linoleic acid supplemented platelets. The observations made in the current study determined a significant decrease in thromboxane B₂ production in cells treated with 0.1mM and 0.25mM, compared with the untreated control after 1 hour of treatment, as well as after 4 hours as is displayed in figure 6.11. This compares to the observations of MacIntyre *et al*, (1984), where thromboxane B₂ inhibition was observed in linoleic acid (0.035mM) treated platelets after 5 minutes of incubation. Needleman *et al*, (1982) suggested that fatty acid modification of phospholipids required incubation periods of longer than 4 hours, however, according to Ibarguren *et al*, (2014), insertion of free fatty acids into phospholipid membranes and other complex molecules can occur within 3 minutes.

While the lower concentration of ULA, namely 0.1mM and 0.25mM, showed reduced thromboxane B₂ production compared to the untreated control, cells treated with higher concentrations, specifically 2.5mM of ULA, showed a significantly higher production of the eicosanoid. As arachidonic acid release from the phospholipid membrane relies on the activation of phospholipase A₂, it may be hypothesized that linoleic acid induced the activation of phospholipase A₂ in ULA treated HepG2 cells. This, in turn, initiated the release of arachidonic acid from the cell membranes, consequently leading to its conversion to thromboxane. Suh *et al*, (2008) observed the release of arachidonic acid from the phospholipid membranes of primary chicken hepatocytes as a result of linoleic acid (0.1mM) induced phosphorylation of cytosolic phospholipase A₂, after 12 hours of supplementation. While comparable concentrations of ULA did not lead to increased thromboxane production in supplemented HepG2 cells in the current study, higher concentrations may have

initiated more rapid intracellular reactions in response to lipid overload, the disparity may also be attributed to cell type.

A second hypothesis proposed here is that intracellular conversion of ULA to arachidonic acid *via* Δ6, elongases such as ELOVL5 and Δ5 desaturase lead to the COX induced production of thromboxane. According to Whelan and Fritzsche, (2013), linoleic acid can be elongated and desaturated to other bioactive ω6 PUFAs, such as δ-linolenic acid and arachidonic acid. Subsequently, arachidonic acid can be converted to a number of bioactive compounds including eicosanoids, such as thromboxane.

6.7.7. The Effects of ULA on Cell Death in HepG2 cells

Lipids, such as fatty acids, are essential signalling molecules required for the physiologically normal activity of regulated cell death. However, elevated plasma free fatty acid concentrations can disturb the regulation of lipid metabolism, contributing to lipotoxicity (Oh *et al*, 2018). According to Lima *et al*, (2002), elevated levels of free fatty acids, particularly PUFAs such as linoleic acid, can induce cell death by apoptosis and, as concentrations increase, by necrosis.

Several authors have demonstrated over accumulation of intracellular free fatty acids such as linoleic acid, induced cell death by apoptosis (Hawkins *et al* 1998, Wu *et al*, 2008, Zhang *et al*, 2012). Wu *et al*, (2008) postulated that, while the mechanisms by which fatty acids induce cell death have yet to be elucidated, multiple mechanisms are involved.

Hawkins *et al*, (1998) demonstrated morphological evidence that PUFA induced cell death is by apoptosis. Hawkins *et al*, (1998) proposed that PUFAs, such as linoleic acid, disrupt cellular membrane structure and fluidity. The fatty acid composition of the cell membrane has a strong influence on cell response and function, with

membrane phospholipids influencing membrane order and assembly (Innes and Calder, 2018). Alterations in the lipid bilayer, resulting in disruption of membrane structure, function and fluidity may increase cellular susceptibility to apoptosis (Hawkins *et al*, 1998).

In order to determine the effect of ULA on cell death, HepG2 cells were treated with varying concentrations of ULA over a period of time ranging from 1 to 8 hours. While the initial aim of the experiment was to determine the effects of ULA on stages of apoptosis in HepG2 cells, necrosis was also analysed. All cells were analysed using flow cytometry combined with the fluorescent stains AV-FITC, specific for detecting the apoptosis related phospholipid, phosphatidylserine (PS), and PI, a water-soluble compound that intercalates between nucleic acid bases of exposed nuclei. These stains allowed for the determination of early apoptosis *via* PS externalization, or late apoptosis or necrosis *via* PI uptake as a result of loss of membrane integrity and DNA fragmentation (Cury-Boaventura *et al*, 2004).

6.7.7.1. The Effects of ULA Concentration on Cell Death in HepG2 Cells.

Concentrations of 0.1mM and 0.25mM of ULA along with untreated cells did not have any significant impact on the type of cell death induced by ULA (figure 6.15). Cells treated with 1mM and 2.5mM had the most significant impact on all stages of cell death in this investigation.

Cells treated with 1mM of ULA presented with a significant decrease in the percentage of live cells with a mean value of 86.9% (± 3.00) when compared with untreated cells ($P = 0.0103$), cells treated with 0.1mM ($P = 0.0103$), cells treated with 0.25mM ($P = 0.0110$) and cells treated with 0.5mM ($P = 0.0126$). Cells treated with 1mM of ULA also presented with a significant increase in the percentage of cells exhibiting late

apoptosis when compared to untreated cells ($P = 0.0132$) and cells treated with 0.1mM ($P = 0.0127$), 0.25mM ($P = 0.0126$) and 0.5mM ($P = 0.0149$) of ULA. Cells treated with 1mM of ULA also presented with a significant increase in the percentage of necrotic cells ($10.8\% \pm 1.75$) when compared to lower concentrations ($P = 0.0062$ when compared to untreated cells and $P = 0.0064$ when compared to cells treated with 0.1mM).

Cells treated with 2.5mM showed a significant decrease in the percentage of live cells ($37.0\% \pm 10.4$) when compared to other concentrations ($P = 0.0007$) when compared to untreated cells and cells treated with 0.1mM). Significant late apoptosis was also observed in cells treated with this concentration, when compared with those treated with ULA at concentrations lower than 0.5mM ($P = <0.0001$). Necrosis was also significantly higher in cells treated with 2.5mM of ULA when compared to cells treated with lower concentrations ($P = <0.0001$).

6.7.7.2. The Effects of Treatment Time on the Cell Death in HepG2 Cells.

Increasing the length of time that HepG2 cells were exposed to ULA, specifically, those treated with concentrations of 1mM and 2.5mM, did impact cell death (figure 6.15).

Cells treated with 1mM of ULA over 6 hours and 8 hours presented with significant late apoptosis when compare to those treated with the same concentration of ULA over 1 hour ($P = 0.0082$), 2 hours ($P = 0.0085$) or 4 hours ($P = 0.0448$). Cells treated with the same concentration showed a significant increase in cells presenting with early apoptosis after 8 hours of treatment when compared with all other time points ($P = 0.0116$).

After 2 hours, cells treated with 2.5mM showed a significant reduction in the number of cells presenting as live, when compared with those treated at the same concentration for 1 hour ($P = 0.0222$). As treatment time increased, particularly after 8 hours, cells treated with 2.5mM of ULA demonstrated a significant increase in early apoptosis (2.04% ± 0.11) when compared to cells treated with the same concentration for 1 hour (0.04% ± 0.01) ($P = 0.0175$). Necrosis in cells treated with this concentration was most significant after 2 hours of exposure, with 46.6% (± 4.05) of cells presenting as necrotic ($P = 0.0014$ when compared with cells treated with the same concentration for 1 hour).

In the present study, cells treated with 1mM and 2.5mM of ULA had the most significant impact on all types of cell death. Cells treated at these concentrations induced statistically more apoptosis and necrosis than any other treatment concentration. According to Lima *et al*, (2002) concentrations of certain fatty acids, particularly PUFAs can induce apoptotic cell death at close to physiological concentrations (0.1mM), however as concentrations increase, necrosis may be observed.

Cury-Boaventura *et al*, (2004) determined that linoleic acid was a potent inducer of apoptotic cell death. Cury- Boaventura *et al*, (2004) demonstrated that linoleic acid as well as oleic acid at concentrations ranging from 0.025mM to 0.2mM, induced apoptosis in Jurkat cells (immortalised T-lymphocytes), with evidence of DNA fragmentation and chromatin condensation. Linoleic acid also caused PS externalization, indicating induction of apoptosis (Cury- Boaventura *et al*, 2004). In the current research, HepG2 cells treated with 0.1mM, 0.25mM or 0.5mM of ULA did not show a significant increase in apoptosis when compared to all other concentrations. However, cells treated with 1mM of ULA had a significantly higher percentage of late apoptotic cells (6.7 % ± 0.49) than untreated cells (0.1% ± 0.02) or cells treated 0.1mM

(0.1% \pm 0.02), 0.25mM (0.1% \pm 0.03) and 0.5mM (0.3% \pm 0.18), after 8 hours of supplementation. In comparison, Pompeia *et al*, (2002) determined that arachidonic acid, a direct metabolite of linoleic acid, induced apoptosis in leucocytes at concentrations ranging from 0.01mM to 0.4mM after 6 hours of supplementation.

Pompeia *et al*, (2002) also determined that concentrations of arachidonic acid ranging from 0.4mM to 1.6mM induced necrosis. Pompeia *et al*, (2002) observed an almost immediate loss of membrane integrity (within 15 minutes) in leucocytes treated with arachidonic acid once concentrations exceeded 0.4mM. Pompeia *et al*, (2002) also observed increased cell surface irregularity, rapid loss of membrane integrity and abundant cell debris in arachidonic acid treated leucocytes. These are patterns of cell death associated with necrosis. Interestingly, in the current study, cells treated with 2.5mM of ULA presented with a rapid and substantial increase in necrosis after 2 hours of treatment, when compared to all other concentrations, which appeared to reduce after 4 hours. Lima *et al*, (2002) postulated that the cytotoxicity of certain fatty acids was related to the carbon chain length and number of double bonds, with increasing chain length and number of double bonds correlating to increasing cytotoxicity. This may explain why, Pompeia *et al*, (2002) observed the potential onset of arachidonic acid mediated necrosis within 15 minutes, while it did not appear to occur until cells had been treated with ULA for 2 hours in the current study.

Cells, or events, that fell outside the live population but were not positive for any stain were considered to be cellular debris (figure 6.13). In the current investigation, there was an increase in such events in cell groups treated with 1mM and 2.5mM of ULA, particularly after 6 and 8 hours of supplementation (figure 6.13). This cell debris was eliminated from further downstream analysis. This elimination of cell debris reduced the total population of events available for final analysis of cell death. As such, it is

recommended, for future work, that the initial event population would need to be increased from 10,000 events to allow for more statistically accurate analysis of the effects of ULA on cell death.

It was postulated that these cells, or events, were unable to take up either AV-FITC or PI as they presented with both membrane damage preventing AV-FITC uptake and nucleic damage to a degree that PI could not intercalate between the nucleic acid bases. According to Crowley *et al*, (2016) cells can die in the absence of nuclear fragmentation. As such, lack of nuclear fragmentation, or as is the case in this investigation, lack of PI uptake cannot be used in isolation to determine that cells are healthy or have not died by apoptosis. These events were observed in the lower left corners of dot plots treated with higher concentrations of ULA, namely 1mM and 2.5mM. This data corresponds to microscopic images obtained and presented in section 6.2 of this report, where a reduction in cell number and abundant particulate was observed in the media of cells treated at these concentrations. Together these data indicate that concentrations of ULA exceeding 0.5mM can induce rapid necrosis.

Cnop *et al*, (2001) suggests that excessive free fatty acids may destabilise cellular membranes and induce rapid necrosis. Linoleic acid itself can be incorporated into cellular membrane phospholipids, functioning as a structural component to contribute to and influence membrane structure and fluidity (Whelan and Fritsche, 2013). However, it has been reported that excessive fatty acids, such as linoleic acid, can disrupt cellular environments and alter cellular and organelle membrane structure and function (Hawkins *et al*, 1998, Borradaile *et al*, 2006, Suh *et al*, 2008, Malhi *et al*, 2008). Such alterations may also influence PUFA metabolic products such as lipid peroxides, prostaglandins, leukotrienes and reactive oxygen species (Hawkins *et al*, 1998). According to Innes and Calder, (2018), fatty acids can alter intracellular and

extracellular signaling pathways, through membrane modulation, resulting in altered gene expression, as well as altered physiological and metabolic responses in a number of different cell types.

According to Malhi *et al*, (2006) and Wu *et al*, (2008) free fatty acids induce c-Jun-N-terminal kinase (JNK) dependent lipoapoptosis (apoptosis associated with lipotoxicity) in hepatocytes through activation of the pro-apoptotic BCL-2 proteins BIM and BAX. These proteins trigger the mitochondrial apoptotic pathway. Hepatocytes treated with saturated fatty acids demonstrated free fatty acid induced BAX activation and lipoapoptosis that are JNK dependent (Malhi *et al*, 2008). According to Zeke *et al*, (2016), JNK activation has been reported in response to oxidative stress as well as endoplasmic reticulum stress.

According to Wu *et al*, (2008) and Feldstein *et al*, (2004), accumulation of intracellular free fatty acids resulted in lysosomal permeabilization and, as a result, lysosomal-dependent apoptosis, in rat hepatocytes and HepG2 cells respectively. Free fatty acids induced the release of cathepsin B from the lysosome into the cytosol (Feldstein *et al*, 2004, Wu *et al*, 2008). Feldstein *et al*, (2004) established that following exposure of HepG2 cells to free fatty acids, BAX, a mitochondrial pro-apoptotic BCL-2 protein, became partially colocalized with cathepsin B. This colocalization preceded cathepsin B release into the cytosol. According to Wang *et al*, (2018) cathepsins induce the proteolytic activation of BAX, resulting in the release of mitochondrial caspases. Feldstein *et al*, (2004) established that cathepsin B further promoted the production of mitochondrial reactive oxygen species and release of cytochrome C. Zhang *et al*, (2012) observed the release of cytochrome C in rat hepatoma cells treated with 0.125mM of linoleic acid, indicating that linoleic acid may induce lysosomal-dependent cell death. It may be hypothesized that over accumulation of ULA induced

lysosomal dependent cell death in HepG2 cells treated with concentrations greater than 0.25mM in this investigation.

Zhang *et al* (2012) determined that linoleic acid, at a concentration of 0.25mM, had no effect on caspase-3 activity in rat hepatoma cells, indicating that linoleic acid induced cell death was not mediated *via* the caspase-3 pathway. Cytochrome C release was observed however, in rat hepatoma cells treated with 0.125mM of linoleic acid (Zhang *et al*, 2012).

In contrast to the work carried out by Cury-Boaventura *et al*, (2004), the data obtained in the current investigation indicated that ULA, at lower concentrations, namely 0.1mM to 0.5mM did not induce significant apoptosis, when compared to higher concentrations. However, once the concentration of ULA surpasses 0.5mM rapid necrosis was observed. This hypothesis correlates with the findings of a number of authors, including Lima *et al*, (2002) and Pompeia *et al*, (2002).

While the mechanism by which free fatty acids induce apoptosis and necrosis has yet to be elucidated, Wu *et al*, (2008) proposes that more than one mechanism is involved in free fatty acid induced hepatic lipotoxicity.

6.8. Conclusion

Both humans and equines exist on a diet whereby linoleic acid is the most abundant polyunsaturated fatty acid (Hallebeek and Beynen 2002, Calder, 2012). It was also the compound of interest identified by Cooper (2005) and the IEC in the hepatic tissues of equines that had succumbed to a sudden and fatal idiopathic haemorrhagic condition, forming the origins of this research project. In addition to this, the liver

plays a key role in lipid metabolism. As such, the potential impact linoleic acid may have on hepatic inflammation and haemostasis was investigated in this chapter.

The data presented here demonstrated that ULA at concentrations surpassing 0.25mM and exposure times of more than 4 hours had a negative impact on several aspects of cellular homeostasis in HepG2 cells, including cell viability, the formation of protective intracellular lipid droplets and the production of superoxide dismutase. At higher concentrations, namely 2.5mM, ULA demonstrated induction of apoptosis and necrosis. As such it could be postulated that the deleterious effects of linoleic acid on homeostasis may be the result of persistently elevated levels.

A reoccurring postulation throughout the chapter was that the toxicity demonstrated by linoleic acid may be related to the chain length and number of double bonds (Hawkins *et al*, 1998), Lima *et al*, 2002, Iuchi *et al*, 2019).

While the mechanism of action of linoleic acid on lipotoxicity in HepG2 cells remains to be elucidated, this data supports the conclusion that linoleic acid, at supraphysiological concentrations, may elicit its effect through the disruption of cellular and organelle membrane fluidity.

Chapter 7

The *In Vitro* Effects of ULA on HUEVC Cells

Chapter 7. *In Vitro* Effects of ULA on HUVEC Cells

Once dismissed as just a passive interface between the bloodstream and the tissues, the endothelium is being recognized as an important modulator of such diverse processes as coagulation, platelet activation, vascular tone, wound healing, leukocyte migration, atherogenesis, and tumor invasion (Ramadan *et al*, 1990).

The vascular endothelium is important in many key aspects of vascular function including the release of various mediators that control vascular tone, platelet aggregation as well as several other processes (Mohazzab *et al*, 1994). As endothelial cells are constantly exposed to circulating lipids such as dietary fatty acids (Viswanathan *et al*, 2003, Young *et al*, 1998), it was decided to assess the effects of ULA on an endothelial cell line.

In the following chapter, the effects of ULA on HUVEC cells, at varying concentrations, were assessed over shorter time points ranging from 1 to 8 hours. Assays, such as MTT and Oil Red O were used to determine the effects of the fatty acid on cell viability and intracellular lipid accumulation. Further analysis, in the form of fluorescent microscopy and flow cytometry using Nile Red, as well as ELISA to test for biomarkers of inflammation were also employed to ascertain the effects of ULA on endothelial cells.

7.1. Preliminary Analysis of Cell Viability after ULA Treatment

In order to determine the effects of ULA on endothelium, preliminary analysis in the form of cell viability assays were carried out using the Human Umbilical Vein Endothelial Cell line, HUVEC. These cells were grown to 70 – 80% confluence before being treated with varying concentrations of ULA ranging from 0mM to 10mM.

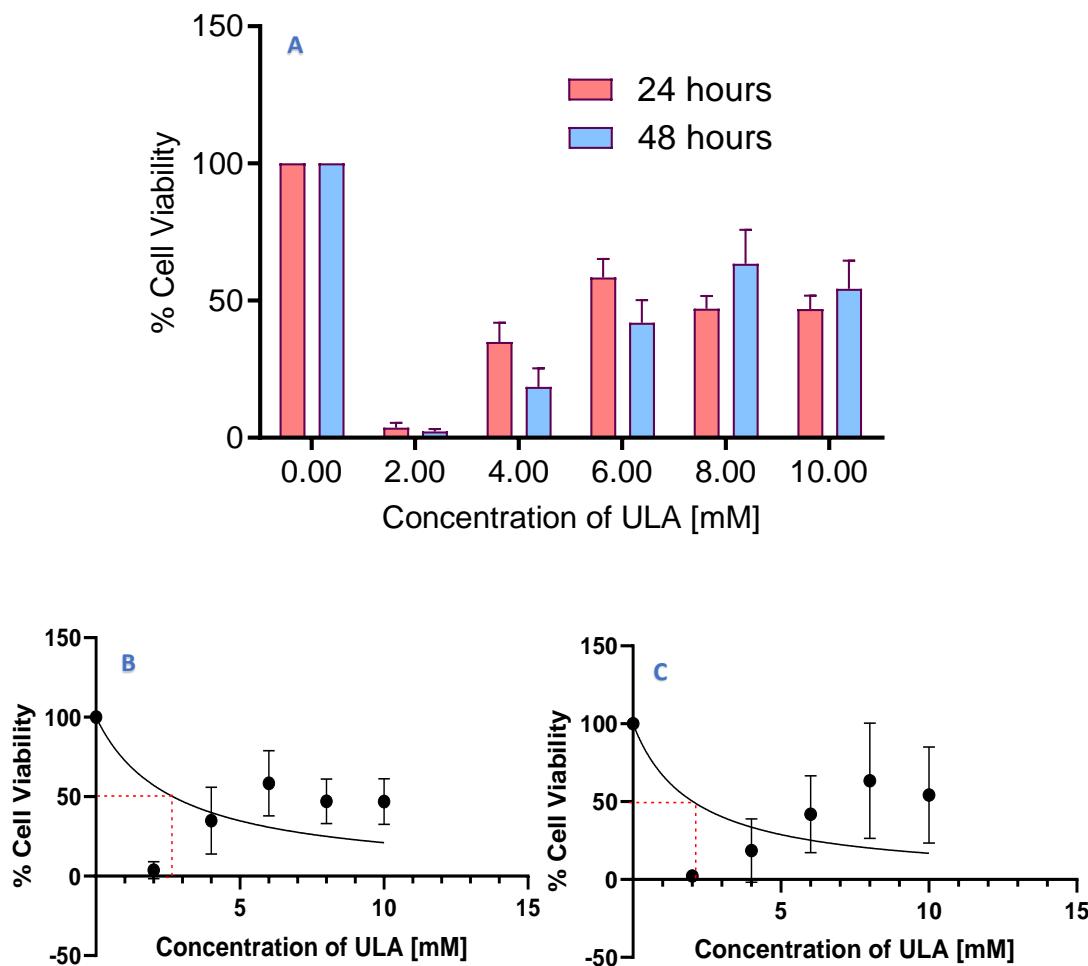


Figure 7.1. Percentage of viable HUVEC cells treated with varying concentrations of ULA over a period of 24 and 48 hours (A) compared with an untreated control ($n=3$). AlamarBlue® was used as an endpoint. Viability levels are expressed as a percent of the control (assigned as 100%). Statistical analysis was performed using mean absorbance values (\pm S.E.M.). One-way ANOVA using Dunnett's test as a post-test to compare all treatment means to the untreated control means. Concentration-response curves for 24 hours (B) and 48 hours (C) were generated to establish IC₅₀ values for ULA using GraphPad Prism software.

The results portrayed in figures 7.1 indicate that ULA significantly inhibited the ability of HUVEC cells to reduce resazurin to resorufin when compared with the untreated control ($P=0.0001$). This indicates that the fatty acid had an inhibitory effect on cell proliferation.

Table 7.1. The half-maximal inhibitory concentration (IC_{50}) of ULA in HUVEC cells treated with varying concentrations of ULA over a period or 24 and 48 hours. AlamarBlue® was used as an endpoint ($n=3$). Statistical analysis was performed using mean concentration values ($\pm S.E.M$).

Time Point	IC_{50} Concentration ($\pm S.E$)
24 hours	2.67mM ± 0.595
48 hours	2.03mM ± 0.602

Concentration-response curves were generated, from which the IC_{50} values for ULA were determined. Cells treated over 24 hours returned an IC_{50} value of 2.67mM (± 0.595), while cells treated for 48 hours presented with an IC_{50} value of 2.03mM (± 0.602).

7.2. Cell Viability in HUVEC Cells Treated with ULA over Shorter Treatment Intervals

HUVEC cells were exposed to varying concentrations of ULA over time intervals, with cell viability being assessed by MTT after 1, 2, 4, 6 and 8 hours. The following figure represents the percentage cell viability in HUVEC cells exposed to varying concentrations of ULA over 8 hours.

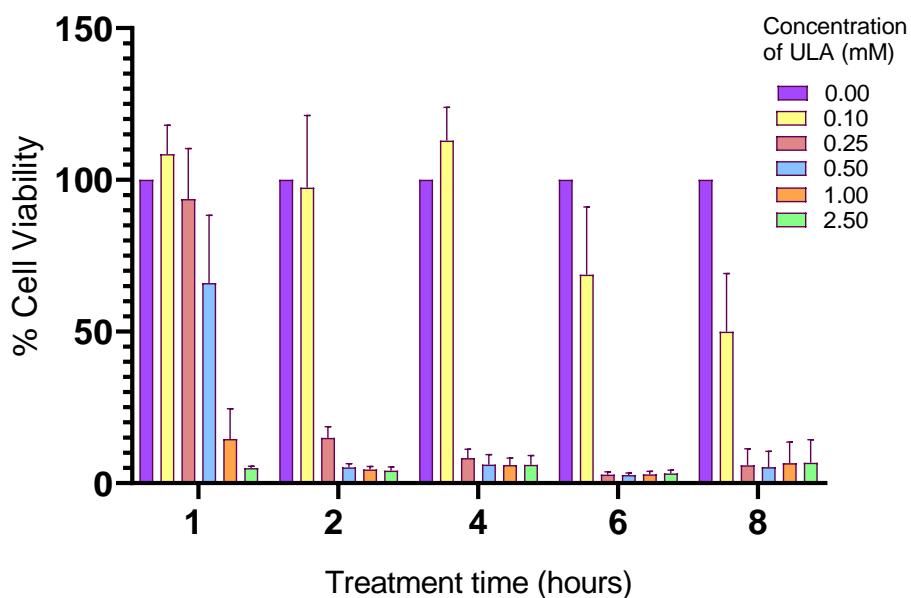


Figure 7.2. Percentage cell viability of HUVEC cells treated with varying concentrations of ULA over a period of 8 hours compared with a positive growth control. MTT was used as an endpoint ($n = 3$). Viability levels are expressed as a percent of the control (assigned as 100%). Statistical analysis was performed using mean absorbance values (\pm S.E.M). Two-way ANOVA using Bonferroni's test as a post-test to compare all means.

The results portrayed in figure 7.2 indicate that increasing concentrations, as well as increasing exposure time to ULA, initiates a significant reduction in the ability of HUVEC cells to reduce tetrazolium into formazan ($P = <0.0001$). This suggests that both the concentrations of ULA and the length of time HUVEC cells are exposed to it significantly reduce cells viability.

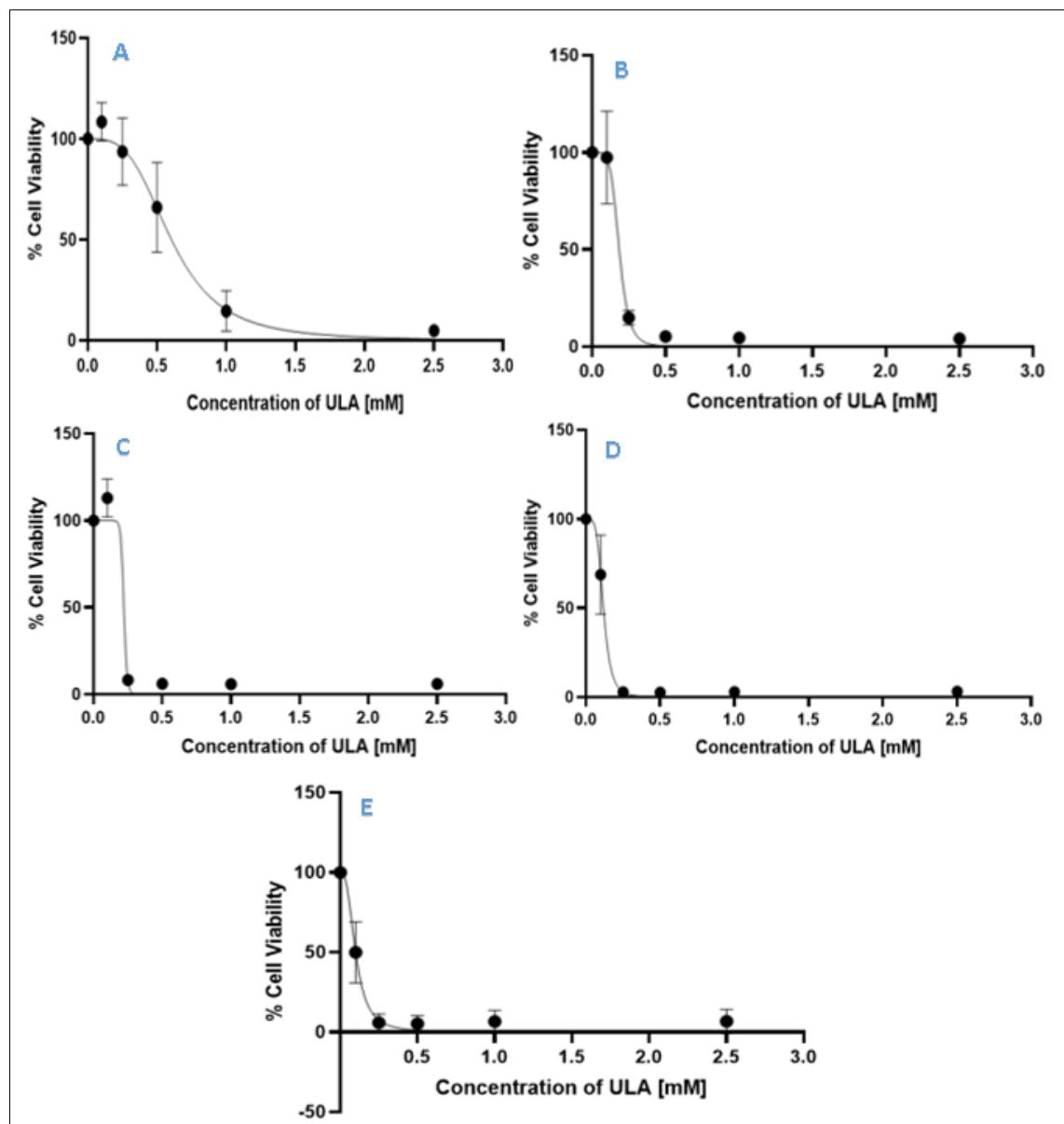


Figure 7.3. Concentration-response curves were generated from the MTT cell viability data (presented in figure 7.2) and used to determine the IC_{50} of ULA in HUVEC cells treated with varying concentrations of the fatty acid over 8 hours (A=1 hour, B=2 hours, C=4 hours, D=6 hours, E=8 hours). Statistical analysis was performed using mean concentration values (\pm S.E.M).

The IC_{50} for ULA at each time point was also determined through the use of concentration-response curves (figure 7.3) and are presented in table 7.1.

Table 7.2. The half-maximal inhibitory concentrations (IC_{50}) of ULA in HepG2 cells treated with varying concentrations of ULA over a period of 8 hours. MTT was used as an endpoint ($n = 3$). Statistical analysis was performed using mean concentration values (\pm S.E.M).

Time Point	IC50 Concentration (\pm S.E)
1 hour	0.60mM \pm 0.029
2 hours	0.18mM \pm 0.013
4 hours	0.22mM \pm 180.262
6 hours	0.12mM \pm 0.006
8 hours	0.10mM \pm 0.005

As is represented in table 7.1, as the length of exposure time increased, the IC_{50} reduced. At 1 hour, an IC_{50} of 0.60mM (± 0.029) was observed, however, as the length time that HUVEC cells were exposed to ULA, a decrease in the tolerance of the cells to ULA was observed, with an IC_{50} of 0.10mM (± 0.005) observed at 8 hours, further strengthening the indication that tolerance to ULA treatment diminishes over time.

7.3. Intracellular Lipid Accumulation in HUVEC Cells treated with ULA

7.3.1. Analysis of Intracellular Lipid Accumulation using Oil Red O

Oil Red O was used to assess the effects of increasing concentrations of ULA, over different time points, on the accumulation of intracellular lipids in HUVEC cells.

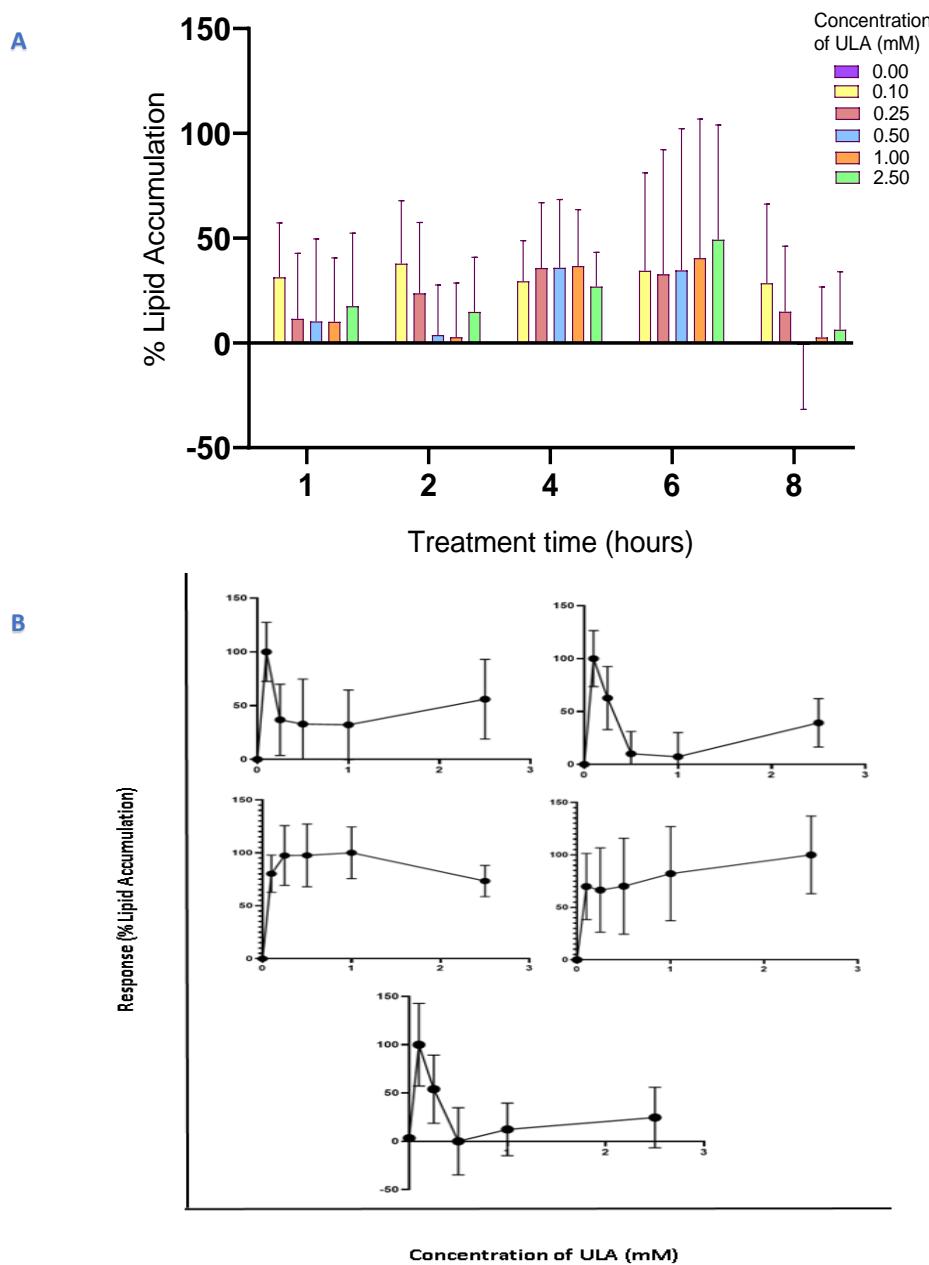
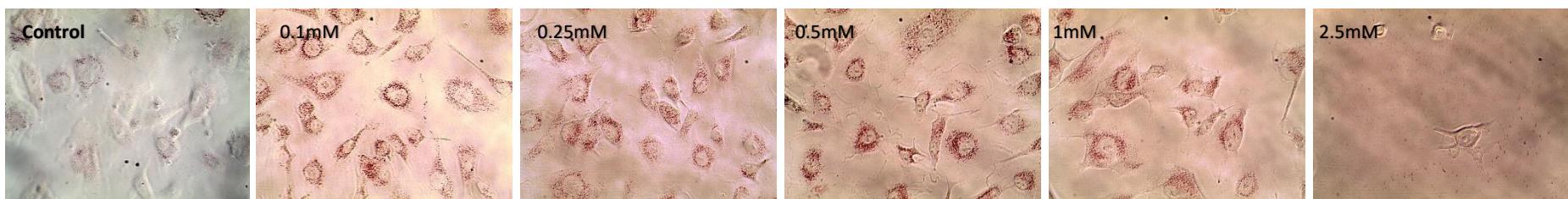


Figure 7.4. (A) Percentage lipid accumulation in HUVEC cells treated with varying concentrations of ULA assessed over a period of 8 hours. Treated cells were compared to a positive growth control in which cells were grown in fatty acid free BSA constituted media containing $\leq 1\%$ IPA ($n=3$). Lipid accumulation levels are expressed as a percent of the control (assigned as 0%). Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's test as a post-test was used to compare all means. (B) Data was also presented in the form of dot plots using GraphPad Prism Software present the relationship between each concentration.

The data presented in figure 7.4 indicated that exposure of HUVEC cells to ULA resulted in an increase in intracellular lipid accumulation compared to untreated cells. This increase was not dose dependent, however, with a non-monotonic dose response observed. Accumulation of intracellular lipids was significantly higher in cells treated with 0.1mM, 0.25mM, 0.5mM and 1mM of ULA than in the untreated control ($P \leq 0.046$). Increasing treatment times did not result in a significant increase in intracellular lipid accumulation.

Light micrographs of Oil Red O stained HUVEC cells indicate the presence of intracellular lipid accumulation. An increase in the presence of intracellular lipids can be seen (stained red), particularly in cells treated with 0.1mM of ULA between 1 hour and 8 hours of exposure. As the concentration of ULA increased, cell numbers dramatically decrease with no visible cells present after particularly at 8 hours.

1 hour



8 hours

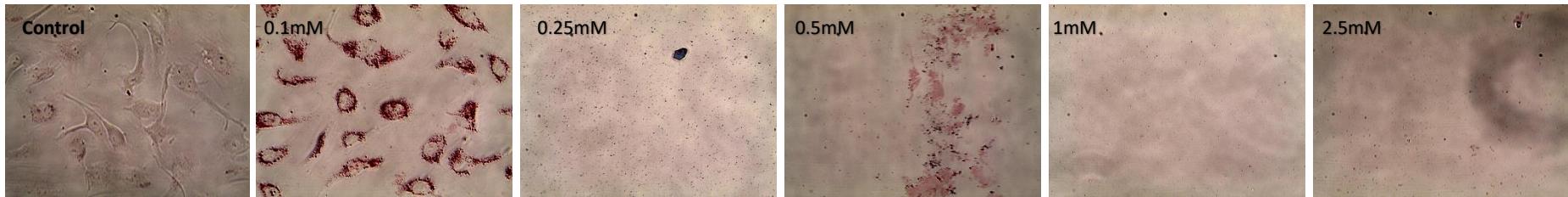


Figure 7.5. Oil Red O staining maps (x 200) of HUVEC cells treated with varying concentrations of ULA over different time intervals. Control cells were grown in media containing fatty acid free BSA and $\leq 1\%$ IPA.

7.3.2. Analysis of Intracellular Lipid Droplets in HUVEC cells treated with ULA using Nile Red Fluorescent Stain

The importance of intracellular lipid accumulation in the induction of lipotoxicity in numerous non-adipose cell types has been discussed in detail (section 2.2.5 and 6.2.1.). Endothelial cells have been reported to form lipid droplets in intact endothelial cells lining blood vessels (Kuo *et al*, 2018). According to Kuo *et al*, (2017), abnormal lipid droplet metabolism in endothelial cells may contribute to vascular disease. Simionescu, (2007) reported excessive lipid droplet accumulation in endothelial cells surrounding mammalian atheromas.

As such, intracellular lipid accumulation in HUVEC cells, as a result of increasing concentrations of ULA was further investigated using the fluorescent stain Nile Red.

Nile red was used in conjunction with fluorescent microscopy to visualise intracellular lipid droplets in HUVEC cells exposed to increasing concentrations of ULA over 8 hours. This stain was also used to determine, by flow cytometry, the effects of increasing ULA concentrations on intracellular lipid accumulation, supporting the findings in section 7.3.1.

Images obtained for ULA treated HUVEC cells. Cells fluoresced red, indicating the presence of polar lipids however, there was no gold fluorescence observed, suggesting that there were no neutral lipids present.

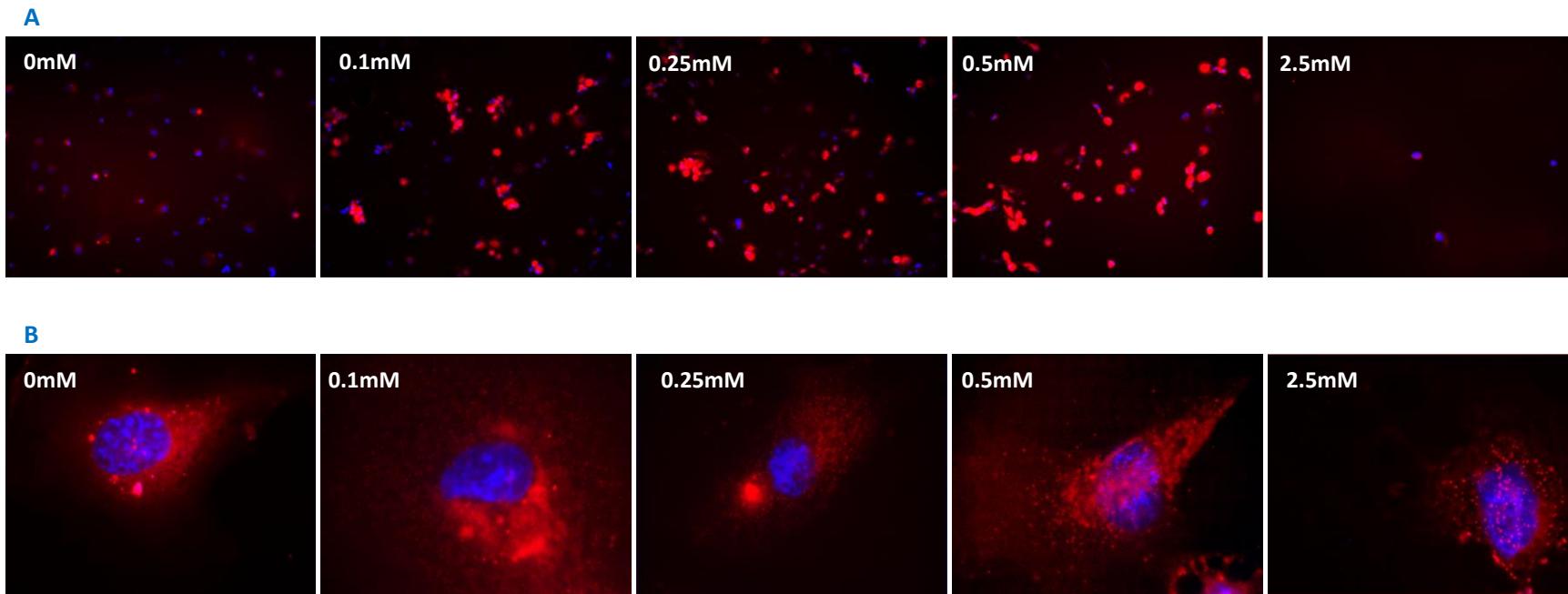


Figure 7.6.1 Qualitative determination of intracellular lipid accumulation in HUVEC cells treated with varying concentrations of ULA after 1 hour using fluorescent microscopy. Images were initially obtained at x10 (A) and at x1000 (B) using the oil immersion lens. Fluorescent images were obtained using the lipophilic stain Nile Red for intracellular lipid imaging (red) and the nuclear stain DAPI for nucleus imaging (blue). Image J software was used to enhance images for increased resolution.

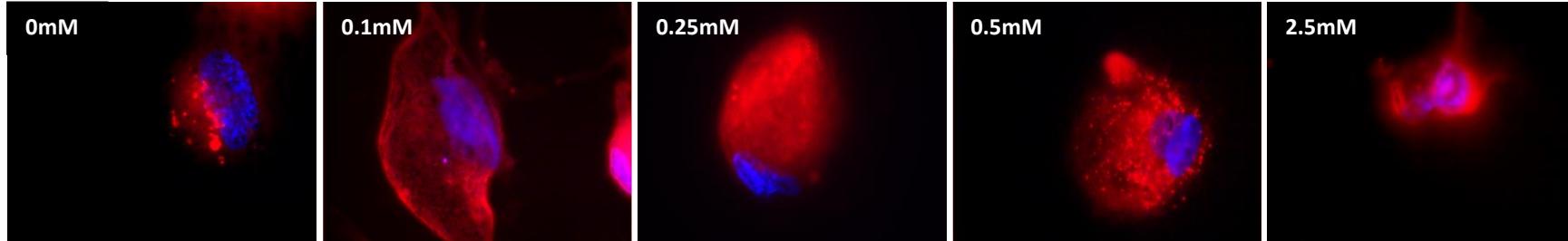


Figure 7.6.2. Intracellular lipid accumulation in HUVEC cells treated with varying concentrations of ULA after 2 hours. Intracellular lipid accumulation was qualitatively determined using fluorescent microscopy. Fluorescent images (x1000) were obtained using the lipophilic stain Nile Red for intracellular lipid imaging (red) and the nuclear stain DAPI for nucleus imaging (blue). Image J software was used to enhance images.

Fluorescent imaging was obtained from HUVEC cells treated with increasing concentrations of ULA for 1 hour and for 2 hours, as shown in figure 7.6.1 and 7.6.2. No microscopic images were obtained after 4 hours of treatment as cells could not be observed on slides, possibly due to a combination of the effects of ULA treatment and the rigorous nature of sample preparation.

Images portrayed in figure 7.6.1 (A) show HUVEC cells treated with ULA for 1 hour at x10 magnification. An increase in red fluorescence can be seen when cells treated with 0.1mM, 0.25mM and 0.5mM of ULA are compared with the untreated control (0mM), indicating an increase in cellular uptake of ULA in treatment groups. In cells treated with 2.5mM however, cell number appeared greatly diminished compared with other concentrations. In images magnified x1000 (B), small circular bodies, that may indicate lipid droplets can be seen, however, in cells treated for 2 hours (figure 7.6.2), even cells treated at 0.1mM appear irregular and amorphous.

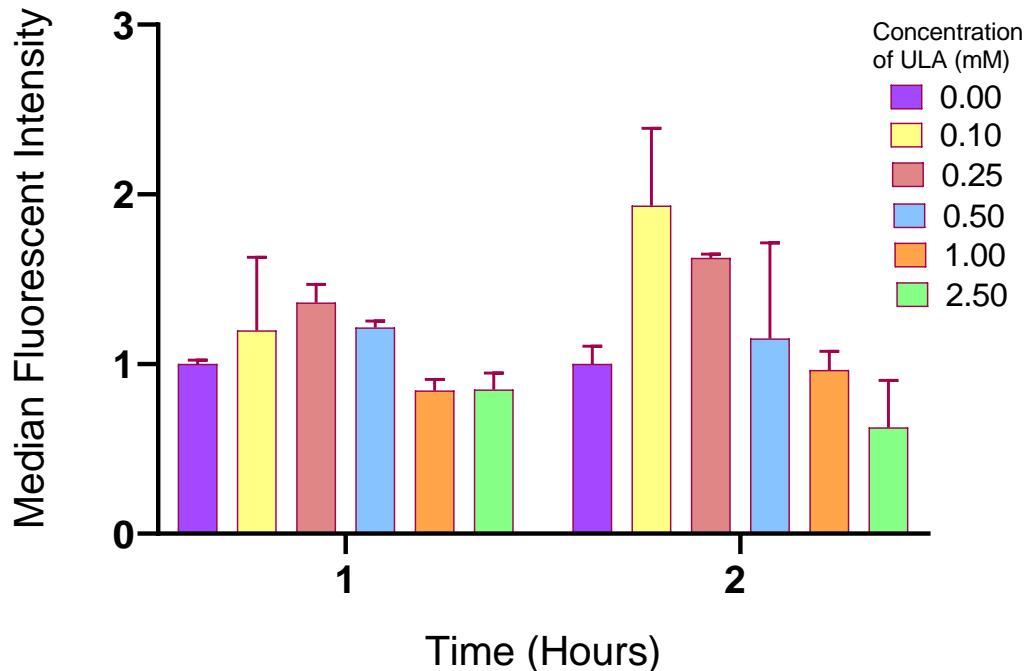


Figure 7.7. Median Fluorescence Intensity of Nile Red determined by flow cytometry in HUVEC cells treated with varying concentrations of ULA over 8 hours. Data has been normalised as described by Wolins *et al*, (2018).

Flow Cytometry was used to detect intracellular lipid accumulation through cellular uptake of the fluorescent lipophilic stain Nile Red. An increase in the median fluorescence intensity (MFI) was observed in cells treated with 0.1mM and 0.25mM of ULA after 1 hour and 2 hours of supplementation (figure 7.7). However, this increase was not considered statistically significant. Increasing concentrations did not induce a dose dependent increase in MFI.

After 4 hours of supplementation, event numbers detected by flow cytometry had reduced so much so, that no statistically relevant data could be obtained. As such, only data for 1 hour and 2 hours are portrayed here.

7.4. Assessment of the Inflammatory Effects of ULA through the Secretion of TNF- α

Linoleic acid has been associated with a pro-inflammatory response (Choque *et al*, 2014). TNF- α is a pro-inflammatory cytokine that has been widely used to study various elements ranging from cell proliferation and apoptosis to inflammation and lipid haemostasis (Chen *et al*, 2009). In order to ascertain the inflammatory effects of ULA, media from ULA treated HUVEC cells was assessed using ELISA for TNF- α secretion.

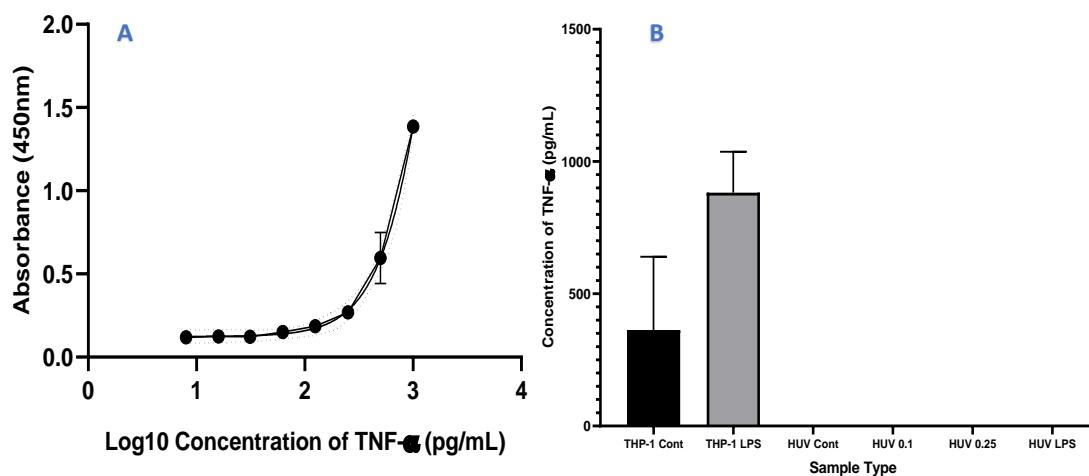


Figure 7.8. TNF- α standard curve (A). TNF- α production in HUVEC cells treated with 0.1mM and 0.25mM of ULA for 4 hours (B). LPS injured THP-1 (THP-1 LPS) cells and HUVEC cells (HUV LPS) were used as positive controls while untreated THP-1 cells (THP-1 Cont) and HUVEC cells (HUV Cont) were used as a negative control (THP-1 supernatent = 1:5 dilution).

While LPS injured THP-1 cells, successfully resulted in TNF- α secretion, TNF- α was not secreted in detectable quantities by HUVEC cells in response to ULA treatment. Absorbance values for samples were considerably lower than those obtained for the lowest standard concentration (4pg/mL TNF- α), therefore concentrations of TNF- α could not be interpolated. This indicated that TNF- α levels in the media of treated cells were so low that they were below the limit of detection produced by the standard curve, suggesting that ULA did not induce TNF- α secretion in HUVEC cells. LPS was

used to stimulate TNF- α in HUVEC cells, to serve as a positive control. However, no detectable quantities of TNF- α were produced by HUVEC cells in response to this compound.

7.5. The Effects of ULA on the activity of Superoxide Dismutase in HUVEC Cells.

The presence of superoxide dismutase in HUVEC cells was assessed as an indicator of the effects of ULA on the production of reactive oxygen species.

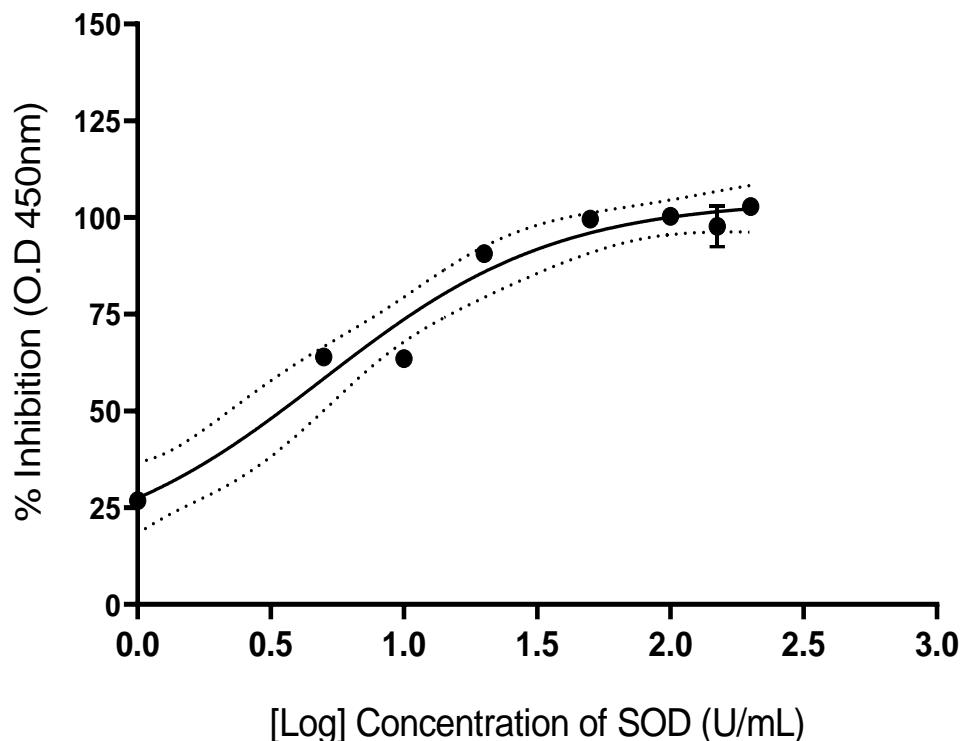


Figure 7.9. Standard curve for Superoxide Dismutase. Statistical analysis was performed using mean absorbance values \pm S.E.M ($n=2$).

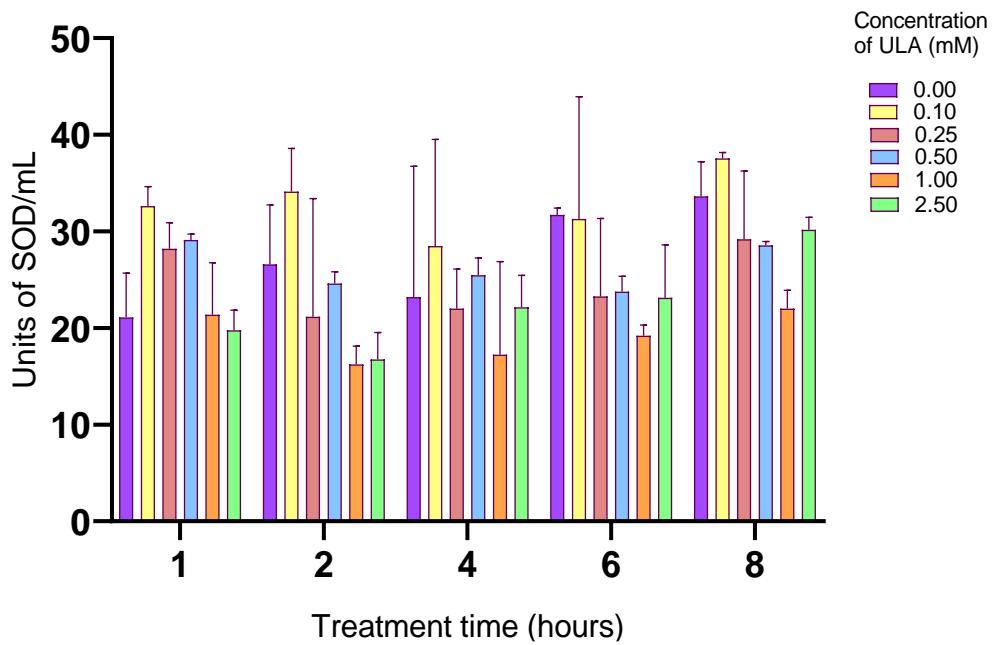


Figure 7.10. Units of superoxide dismutase (SOD) produced in HUVEC cells treated with varying concentrations of ULA over 8 hours (A). Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's test as a post-test was used to compare all means ($n=2$).

Treatment of HUVEC cells with ULA did not significantly increase or decrease in intracellular SOD content. A decrease in SOD produced by HUVEC cells was observed as the concentration of ULA surpassed 0.25mM compared with cells treated with 0.1mM of ULA, however, the trend was not dose or time dependent, nor was it statistically significant. Increasing exposure time, particularly after 4 hours of treatment appeared to induce an increase on SOD production across all treatment concentrations, however, this increase was not significant.

7.6. The Effects of ULA on Thromboxane Production in HUVEC Cells.

HUVEC cells were analysed for the production of thromboxane B₂, the stable metabolite of thromboxane A₂ (MacIntyre *et al*, 1984).

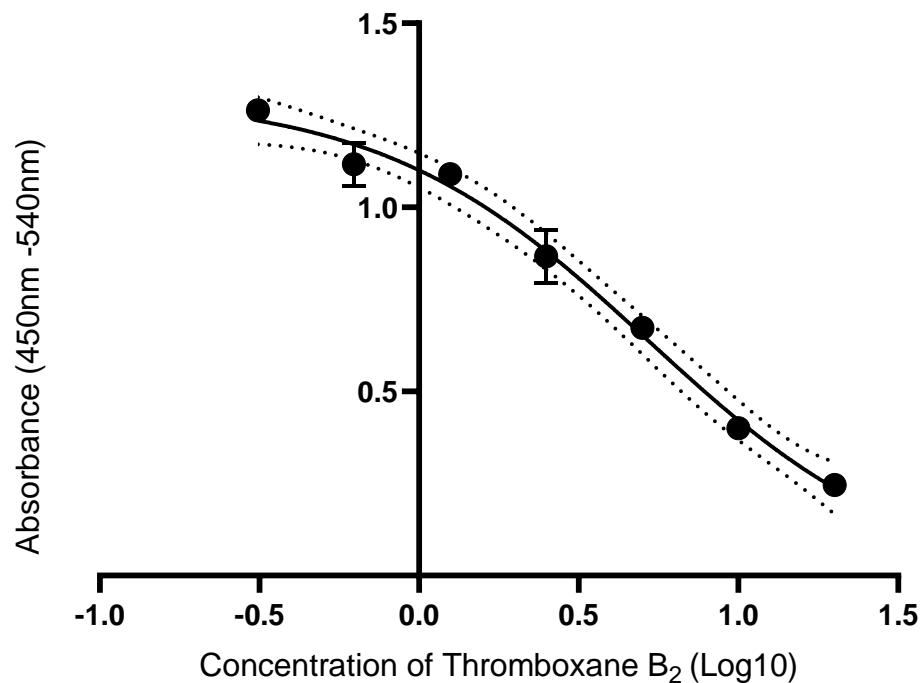


Figure 7.11. Standard curve for thromboxane B₂. Statistical analysis was performed using mean absorbance values (\pm S.E.M) ($n=2$).

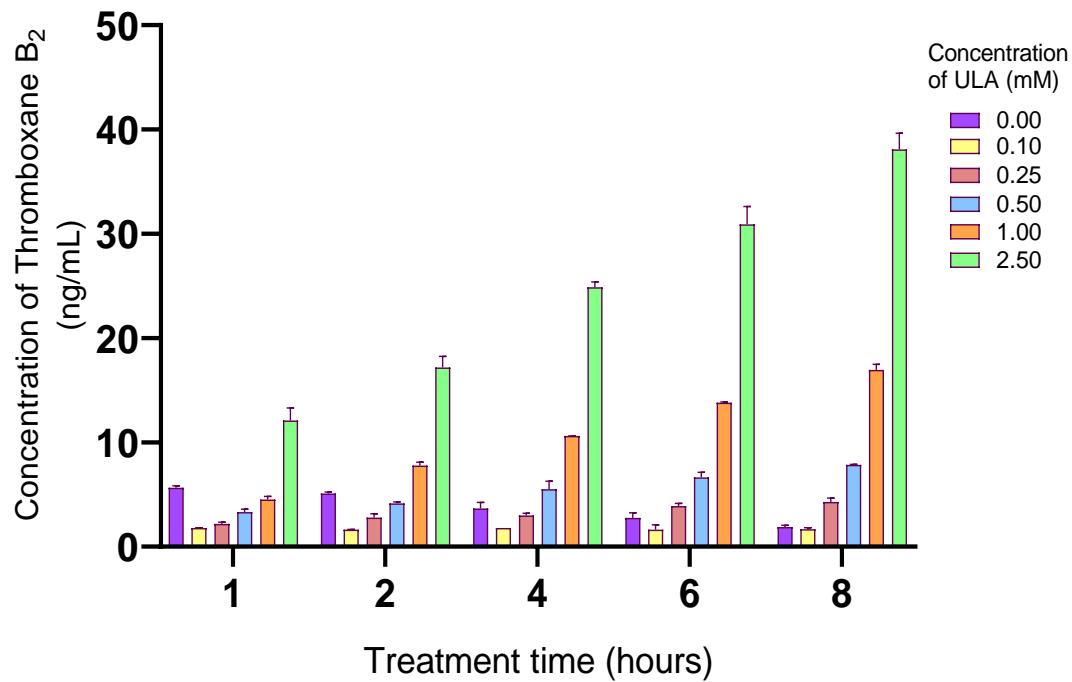


Figure 7.12. Concentration of thromboxane B₂ produced in HUVEC cells treated with varying concentrations of ULA over 8 hours. Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's test as a post-test was used to compare all means ($n=2$).

A

increased. Cells treated with 0.1mM produced the lowest concentration of thromboxane, even compared to untreated cells ($P= 0.0050$). Cells treated with 2.5mM of ULA produced the highest concentration of thromboxane, with a greater than 2-fold increase occurring compared to those treated with 1mM of ULA ($P = <0.0001$).

7.7. Discussion

The vascular endothelium, comprised of a monolayer of endothelial cells, is a tightly regulated organ that forms a vast interface between blood and neighbouring tissues (Ghosh *et al*, 2017). In order to maintain the normal function of the circulatory system, it regulates several functions. These include the balance between vasodilation and vasoconstriction and the maintenance of thrombosis and haemostasis, as well as other inflammatory responses (Ghosh *et al*, 2017).

Endothelial cells line the entire vascular system and play a crucial role maintaining the vascular homeostasis, as well as the transport of oxygen and nutrients (Kim *et al*, 2012, Kuo, *et al*, 2017). They are also involved in the release of several factors which exert antithrombogenic influences, such as tPA, or thrombogenic influences, such as PAI and vWF, (Schleger *et al*, 2004). Endothelial function is regulated by the secretion of numerous endothelium-dependent factors, such as relaxing factors, hyperpolarising factors and contracting factors (Kim *et al*, 2012).

As such, the relationship between the endothelium, blood and related tissues exposes endothelial cells to an environment comprised of leukocytes, nutrients and toxic wastes, as well as the many factors involved in haemostasis, thrombosis, and immune and inflammatory reactions (Hennig *et al*, 1994). As well as this, the endothelium can be exposed to significant levels of free fatty acids derived from lipoprotein lipase mediated hydrolysis of TAG rich lipoproteins (Young *et al*, 1998). Normal endothelial function can be hindered by harmful stimuli (Ghosh *et al*, 2017). According to Hennig *et al*, (1994), various circulating components, including nutrients, can interact with enzymes, receptors, and transport molecules located on the luminal surface of endothelial cells. These interactions may result in the production of potentially

harmful compounds such as reactive oxygen species and inflammatory proteins, leading to endothelial dysfunction (Ghosh *et al*, 2017). Endothelial dysfunction has been linked with several disorders such as obesity, diabetes and metabolic syndrome as well as cardiovascular diseases such as coronary artery disease and atherosclerosis (Edirisinghe, *et al*, 2006).

Toborek *et al*, (2002) stated that, as the lipid composition of plasma and tissue is closely related to dietary fat intake, exposure of endothelial cells to individual fatty acids can be directly influenced by the type of dietary fats consumed. Excessive consumption of linoleic acid in the western diet of both humans (Calder, 2012) and equines (Hallabeek and Beynen, 2002) has previously been highlighted (section 6.7). Free fatty acids are reported to play a role several disorders previously discussed (Edirisinghe, *et al*, 2006, Ghosh *et al*, 2017), and are known mediators of inflammation (Choque *et al*, 2012). They are also reported to facilitate apoptosis in endothelial cells (Ghosh, *et al*, 2017). Free fatty acids have also been associated with platelet dysfunction (MacIntyre, *et al* 1984, Cambien *et al*, 2003, Dhindsa, *et al*, 2015). Specifically, linoleic acid has been reported to induce marked injury to endothelial cells (Toborek *et al*, 2002). As this study involves the investigation of an idiopathic haemorrhagic condition, the HUVEC cell line was selected to determine the potential cytotoxic effects of ULA on the endothelium. According to Kocherova *et al* (2019), HUVEC cells represent a widely used source of primary endothelial cells for *in vitro* studies of the vasculature. Richardson *et al*, (2010) and Caniuguir *et al*, (2016) suggest that HUVECs appear to be the main source of human endothelial cells for *in vitro* study. HUVECs have also been reported to express many important endothelial markers and signaling molecules associated with regulation of vascular homeostasis (Caniuguir *et al*, 2016, Kocherova *et al*, 2019), including responses to pro-

inflammatory stimuli such as LPS as well as the production of several pro-inflammatory cytokines, such as interlukin 6 and interlukin 8 (Unger *et al*, 2002). They have also been used in several previous investigations to determine the effects of fatty acids, including linoleic acid, on the endothelium (Spector *et al*, 1981, Toborek *et al*, 2002, Artwohl *et al*, 2003) As such, they were considered to be a suitable *in vitro* model for this investigation.

7.7.1. Preliminary Assessment of Cell Viability in HUVEC Cells

Initially, HUVEC cells were treated with varying concentrations of ULA over two time points, 24 hours and 48 hours. Data obtained from treated HUVEC, using alamarBlue®, detailed in figure 7.1 indicated that lower concentrations of ULA exerted the most significant effect on the HUVEC cells, with a non-monotonic dose response observed. Cells treated with 2mM ULA displayed the lowest cell viability with a mean percentage cell viability of 3.7% (± 1.80) and 2.3% (± 0.96) for 24 hours treatment and 48 hours of treatment respectively, while cells treated with 8mM reported with a mean percentage cell viability of 47.1% (± 4.65)and 63.5% (± 12.33) after 24 and 48 hours respectively. However, these concentrations were considered too high to reflect realistic physiological parameters, or even, what would be considered supraphysiological. As such, the experimental parameters were adjusted to assess more physiologically appropriate concentrations. The identification of IC₅₀ values for ULA of of 2.67mM (± 0.595) (24 hours) and 2.03mM (± 0.602) (48 hours) further strengthened this conclusion.

7.7.2. Cell Viability in HUVEC Cells Treated with ULA over Shorter Treatment Intervals

Hennig *et al.* (1994) hypothesised that endothelial dysfunction may be influenced by the oxidation products of cholesterol and unsaturated fatty acids as well as the pure unsaturated fatty acids themselves. In the current investigation, HUVEC cells were treated with varying concentrations of ULA over five time points, ranging from 1 to 8 hours (figure 7.2). Cell viability assays, in the form of MTT assays, were used to identify the effects of concentration and the effects of differing periods of exposure of HUVEC cells to ULA while concentration-response curves identifying IC₅₀ values are displayed in figure 7.3 and table 7.2.

7.7.2.1. Effects of ULA Concentration on the Cell Viability of HUVEC cells

A decrease in cell viability, as the concentration of ULA increased, was apparent (figure 7.2). While an IC₅₀ of ULA in HUVEC cells (treated for 1 hour) was calculated to be 0.60mM (± 0.029), all concentrations of ULA induced a significant reduction in cell viability when compared to the untreated control ($P = 0.0001$). Cells treated with 0.25mM of ULA and higher also demonstrated a significant reduction in cell viability when compared with cells treated with 0.1mM of ULA ($P = 0.0001$).

7.7.2.2. Effects of ULA Treatment Time on the Cell Viability of HUVEC cells

Increasing treatment times also resulted in a marked decrease in cell viability (figure 7.2). In cells treated with 0.1mM of ULA, a significant reduction in cell viability was observed when exposure time surpassed 6 hours ($P = 0.0001$). Once the concentration reached and surpassed 0.25mM, a significant decrease in cell viability was observed after 1 hour of exposure ($P = 0.0001$). When IC₅₀ values were compared over the 5

time points, a decrease in these values was observed, with an IC₅₀ value of 0.60mM (± 0.029) obtained after 1 hour of treatment, decreasing to 0.10mM (± 0.005) after 8 hours.

Endothelial cell dysfunction, as a result of lipotoxicity, is mediated through a number of diverse mechanisms such as increased oxidative stress and pro-inflammatory responses (Kim *et al*, 2012). A causative factor in endothelial cell dysfunction is reduced nitric oxide bioavailability (Symons and Abel, 2013). According to Symons and Abel (2013), endothelial cell derived nitric oxide contributes to several protective mechanisms, such as vasodilation, as well as having anti-coagulant, anti-inflammatory and anti-proliferative properties.

Free fatty acid metabolism by endothelial cells is thought to reduce nitric oxide bioavailability. Kim *et al* (2005) determined that palmitic, oleic and linoleic acids reduced basal and insulin mediated nitric oxide in bovine aortic endothelial cells. Kim *et al*, (2012) indicated that the production of superoxide, potentially produced as a result of fatty acid oxidation (Kuratko and Constante, 1998, Edirisinghe *et al*, 2006), is converted into hydrogen peroxide and reacts with nitric oxide. This results in a reduction in the bioavailability of nitric oxide, reducing endothelial cell function (Kim *et al*, 2012).

More specifically, Storniolo *et al*, (2014) determined that linoleic acid, at plasma physiological concentrations (~ 0.12 mM) reduced NO synthesis by endothelial cells. Davda, *et al*, (1995) determined that linoleic acid, at concentrations of 0.1mM, reduced nitric oxide synthase activity. Torborek *et al* (1996) determined that similar concentrations of linoleic acid increased oxidative stress in porcine pulmonary endothelial cells after 6 hours of treatment. Torborek *et al*, (1996) suggested that a

unique mechanism of linoleic acid metabolism in endothelial cells may be responsible for this effect.

Artwohl *et al*, (2003) observed free fatty acid induced apoptosis in the human endothelial cell line, HUVEC, when cells were treated with 0.3mM of various free fatty acids, with varying degrees of unsaturation, including linoleic acid. Artwohl *et al*, (2009) also observed apoptosis in vascular smooth muscle cells treated with 0.3mM of linoleic acid after 24 hours of exposure. Apoptosis was observed in these cells at linoleic acid treatment concentrations of 0.1mM but only after they had been exposed to the fatty acid for 72 hours (Artwohl *et al*, 2009). In the current investigation, 0.1mM (± 0.005) was calculated to be the IC₅₀ after 8 hours of treatment with a decrease in cell viability observed at this concentration (table 7.1 and figure 7.2). This concentration was also observed in the hepatic tissues in a small number of equines, as outlined in Chapter 4. Chen *et al*, (2018) also determined a significant reduction in the cell viability as well as increased apoptosis in HUVEC cells treated with 0.3mM of palmitic acid over 24 and 48 hours. These results correlate with the findings in this investigation, whereby cells treated with 0.25mM of ULA and higher, showed a marked decrease in cell viability. Artwohl *et al*, (2003) hypothesised that the pro-apoptotic activity of PUFA's in endothelial cells may reflect the oxidisability of the double bonds present in the free fatty acid, a theory similarly presented by Hawkins *et al*, (1998). Iuchi *et al*, (2019) determined that oxidised polyunsaturated fatty acids, such as docosahexaenoic acid, eicosapentaenoic acid and linoleic acid, exhibited an anti-proliferative effect on cultured human lymphocytes. Docosahexaenoic acid, a PUFA with 6 double bonds, produced the most significant anti-proliferative effect (Iuchi *et al*, 2019). Iuchi *et al*, (2019) suggested that more double bonds provided more possible positions for hydrogen abstraction, making docosahexaenoic acid highly

oxidizable. According to Iuchi *et al*, (2019), these chemical properties may have resulted in the production of increased amounts of toxic products such as aldehydes and hydrogen peroxides, exacerbating the anti-proliferative effect of the fatty acid (Iuchi *et al*, 2019).

Artwhol *et al*, (2003) hypothesised that free fatty acids induced a direct effect on endothelial apoptosis. Chen *et al*, (2018) determined that palmitic acid treatment at concentrations ranging from 0.1mM to 0.3mM, induced apoptosis in HUVEC. Artwohl, *et al*, (2003) determined a correlation between the pro-apoptotic properties of free fatty acids and the expression of BAK, a BCL-2 apoptotic protein, involved in the release of the death promoting factor, cytochrome C, into the cytosol. Feldstein *et al*, (2004) previously observed free fatty acid induced activity of the BCL-2 pro-apoptotic protein BAX, while Zhang *et al*, (2012) observed cytochrome C release in linoleic acid treated rat hepatoma cells. Artwohl *et al*, (2003) also determined that endothelial apoptosis survival factors, such as endothelial nitric oxide synthase, endothelin-1 and clusterin, were all reduced in free fatty acid treated HUVEC cells. Diminished intracellular levels of these factors may decrease cellular defence mechanisms promoting endothelial cell dysfunction and death (Artwohl *et al*, 2003).

7.7.3. Analysis of Intracellular Lipid Accumulation in ULA treated HUVEC Cells.

In order to further assess the effects of ULA on HUVEC, its impact on intracellular lipid accumulation were investigated using Oil Red O and Nile Red in conjunction with fluorescent microscopy and flow cytometry.

7.7.3.1. Analysis of Intracellular Lipid Accumulation using Oil Red O.

While adipocytes are considered to be the most active cell type in the storage and metabolism of lipid droplets, lipid droplet formation has been reported in all eukaryote cell types, especially under pathological conditions of fatty acid excess, including endothelial cells (Kuo, *et al*, 2017).

The Oil Red O assay was used to assess intracellular lipid accumulation in HUVEC cells exposed to increasing concentrations of ULA over a period of 8 hours (figure 7.4). The untreated control was considered to have an intracellular lipid accumulation value of 0% as these cells were grown in fatty acid free BSA constituted media. For the determination of the percentage of intracellular lipid accumulation, all other treatment groups were compared to the untreated control.

Light micrographs, presented in figure 7.5, show an increase in the presence of intracellular lipids stained with Oil Red O, when treated cells were compared with untreated cells, particularly in those treated with 0.1mM and 0.25mM of ULA for 1 hour. However, as the concentration of ULA increased, cell morphology appeared to be impacted. This is particularly apparent in cells treated with 2.5mM after 1 hour.

The effects of ULA on intracellular lipid accumulation in HUVEC cells was assessed. Increasing concentrations of ULA did not induce a dose or time dependent increase in intracellular lipid accumulation (figure 7.4).

7.7.3.1.1. The Effects of ULA Concentration on Intracellular Lipid Accumulation in HUVEC

The data presented in figure 7.4 indicated that exposure of HUVEC cells to ULA resulted in an increase in intracellular lipid accumulation compared to untreated cells. This increase was not dose dependent, however, with a non-monotonic dose response observed. Statistically, accumulation of intracellular lipids was significantly higher in cells treated with 0.1mM, 0.25mM, 0.5mM and 1mM of ULA than in the untreated control with a significance value (P) of 0.046, 0.0372, 0.0368 and 0.0293 being observed respectively

7.7.3.1.2. The Effects of Time on Intracellular Lipid Accumulation in HUVEC

While intracellular lipid accumulation appeared to increase as treatment time increased, particularly after 4 and 6 hours of supplementation, the increase was not statistically significant.

Light micrographs (figure 7.5) indicated that ULA at 0.1mM induced visible Oil Red O stained lipid droplets, even after 8 hours of supplementation. Cells treated with 0.25mM and higher also demonstrated the presence of stained intracellular lipid droplets after 1 hour. However, cells treated with 2.5mM for 1 hour show depleted cell numbers in the microscopic visual field, and any cells present appear amorphous. At 8 hours cells, there were no viable cells present in wells treated with 0.25mM and higher.

Prior to work carried out Kuo *et al*, (2017), the biogenesis and degradation of lipid droplets within endothelial cells was poorly understood. Kuo *et al* (2017) determined that intact endothelial cells can form lipid droplets both *in vivo* and *in vitro*. Lipid

droplet formation in endothelial cells serves a number of functions including the prevention lipotoxicity and the provision of fatty acids for adjacent cells and tissues (Kuo *et al*, 2017). As such, further investigation into the effects of ULA on intracellular lipid accumulation in HUVEC cells was carried out in the form of fluorescent microscopy and flow cytometry using the lipophilic stain, Nile Red.

7.7.3.2. Analysis of Intracellular Lipid Accumulation using Nile Red

Nile Red was used as a lipophilic stain for the visualization of intracellular lipid accumulation in HUVEC cells, as well as the analysis of intracellular lipid accumulation using flow cytometry. According to Greenspan *et al*, (1985), Nile Red is intensely fluorescent and can serve as a sensitive vital stain for the detection of intracellular lipids. In the presence of neutral lipids, Nile Red fluoresces yellow/gold, and in the presence of polar lipid, it fluoresces red (Durandt *et al*, 2016). According to Kuo *et al*, (2017), endothelial cells are capable of forming intracellular lipid droplets and linoleic acid is readily taken up and incorporated into endothelial lipids (Spector *et al*, 1992).

7.7.3.2.1. Fluorescent Microscopy

Fluorescent microscopy was used in conjunction with Nile Red to visualise the effects of ULA on the formation of intracellular lipid droplets in HUVEC cells (figure 7.6.1 and 7.6.2. In figure 7.6.1. (A) HUVEC cells treated with ULA for 1 hour, viewed at x10 magnification, an increase in red fluorescence can be seen when cells treated with 0.1mM, 0.25mM and 0.5mM of ULA are compared with the untreated control (0mM), indicating an increase in cellular uptake of ULA in treatment groups. When cells are viewed at x1000, red staining around the blue DAPI stained nucleus appears to increase and the presence of fluorescent red circular bodies was observed, indicating

an increase in intracellular lipids. This is particularly apparent in cells treated for 1 hour (figure 7.6.1 (B)). When cells treated with increasing concentrations of ULA over 2 hours were viewed at x1000 magnification, changes in cell morphology were apparent, even in cells treated with 0.1mM of ULA.

Images were not obtained for cells treated for 4 hours and onwards due to lack of cells present on microscopic slides.

7.7.3.2.2. Flow Cytometry

Flow cytometry was used in conjunction with Nile Red to quantify intracellular lipid accumulation in HUVEC cells treated with increasing concentrations of ULA over a period of 8 hours. While data was obtained for cells treated with ULA over 1 hour and 2 hours as presented in figure 7.4, no data was obtained for cells treated for 4 hours and onwards due to low event numbers.

Cells treated for 1 hour and 2 hours showed an increase in MFI in cells treated with 0.1mM and 0.25mM when compared with the untreated control, as can be seen in figure 7.6. Statistically, however, this increase was not considered significant. Cells treated with higher concentrations of ULA (0.5mM to 2.5mM) produced MFI values comparable to untreated cells, indicating that once ULA surpassed 0.25mM cells were unable to take up ULA.

It may be postulated that, due to the lack of cells available for imaging during fluorescent microscopy experiments and the reduction in flow cytometry detectable events in cells treated with ULA for 4 hours or more, that ULA induced lipotoxicity in HUVEC cells after this time point, even in cells treated with 0.1mM of ULA.

The current data presented in section 7.4 indicates that ULA at concentrations of 0.1mM and 0.25mM induced an increase in intracellular lipid accumulation in HUVEC cells treated for 1 and 2 hours, with a decrease occurring as ULA concentrations surpassed 0.5mM. Contrary to the findings of this study, Kuo *et al*, (2017) reported that mouse lung endothelial cells treated with oleic acid, an unsaturated fatty acid, showed a dose and time dependent (0 to 16 hours) increase in intracellular lipid droplets with maximal accumulation occurring after supplementation with 1mM of the fatty acid.

However, when the same cells were exposed to palmitic and steric acid, two saturated fatty acids, lipid droplet formation was reduced and endoplasmic reticulum stress was observed (Kuo *et al*, 2017). This may suggest that fatty acid type or structure plays an important part in cellular lipid uptake and intracellular lipid accumulation in endothelial cells. Artwhol *et al*, (2003) determined different PUFAs, including linoleic acid, induced apoptosis in HUVEC at concentrations of 0.3mM after 24 hours. However, Artwhol *et al*, (2003) observed that the percentage of apoptosis induced was dependent upon the number of double bonds present in the fatty acids, with more double bonds exacting a higher rate of apoptosis. Artwhol *et al*, (2003) postulated that saturation-related proapoptotic activity of PUFAs in endothelial cells may reflect the oxidizability of double bonds in free fatty acids leading to a rise in ROS. Increased plasma lipids, ectopic lipid accumulation and intracellular lipid droplets are reported to induce oxidative stress (Kim *et al*, 2012). According to Kim *et al*, (2012), increased ROS are known to trigger endothelial cell dysfunction.

Specifically, linoleic acid has been reported to increase cellular oxidation and disrupt endothelial barrier function (Torborek, *et al*, 1996). According to Toborek *et al*,

(1996), due to low basal activity of endothelial cell elongases and Δ5 and Δ9 desaturases, arachidonic acid is not produced from linoleic acid significantly. As such, linoleic acid accumulates in HUVEC cells. While Toborek *et al*, (1994) states that linoleic acid mediated endothelial cell injury has yet to be elucidated, oxidative stress may be an important contributor to cell dysfunction. Toborek *et al*, (1996) observed a significant increase in endothelial cell oxidation in cells treated with 0.12mM of linoleic acid for 6 hours. It may be postulated that the higher concentrations of ULA used in the current experiment induced oxidative stress in HUVEC cells at 4 hours of exposure leading to endothelial barrier dysfunction and death. Linoleic acid-mediated disruption of endothelial barrier integrity may be caused by the cells inability to adequately metabolise or store large quantities of linoleic acid and the fatty acids ability to induce intracellular oxidative stress (Toborek and Hennig, 1994, Toborek *et al* 1996).

7.7.4. Assessment of the Inflammatory Effects of ULA though the Secretion of TNF- α

Free fatty acids are potent promotores of inflammation (Choque *et al*, 2012, Young *et al*, 1998). In the cardiovascular system, they promote macrophage infiltration and activation, stimulating the release of high levels of proinflammatory cytokines such as TNF- α (Chen *et al*, 2018), a potent agonist for endothelial cell activation (Imaizumi *et al*, 2000). Toborek *et al*, (2002) suggested that linoleic acid, specifically, can stimulate a pro-inflammatory environment within the vascular endothelium. Young *et al*, (1998) hypothesised that inflammatory cytokines and adhesion molecules, induced by linoleic acid in endothelial cells, contributed to inflammatory mediated endothelial

cell dysfunction. As such, HUVEC cells were assessed for ULA stimulated production of TNF- α .

In this investigation, while TNF- α was detected in LPS injured THP-1 cells (figure 7.8), there was no detectable secretion of TNF- α in either the untreated control cells, or cells treated with ULA. As previously discussed, (section 6.7.4), several cell types have been reported to secrete TNF- α in response to free fatty acid treatment (Holbrook *et al*, 2015, Suagee *et al*, 2013, Ajuwon and Spurlock, 2005). However, the literature regarding TNF- α production by HUVEC in response to free fatty acids is sparse. Li *et al*, (2018) reported that HUVEC cells treated with free fatty acids did not produce cytokines unless the cells had been activated *via* co-incubation with LPS or TNF- α , a factor that should be considered should the investigation of the effects of ULA on the cellular mechanisms within HUVEC be continued.

LPS, a potent activator of cytokine production, according to Gutierrez-Ruiz *et al*, (1999), also failed to induce TNF- α secretion in HUVEC cells. TNF- α secretion was observed in LPS injured macrophages (differentiated THP-1 cells). This was expected as macrophages are considered to be potent producers of the cytokine (Imaizumi *et al*, 2000). These results correspond with the findings of Imaizumi *et al*, (2000) who concluded that, while LPS induced TNF- α expression in HUVEC cells, there was no clear effect on its secretion. Imaizumi *et al*, (2000) reported that 10 μ g/mL of LPS stimulated HUVEC cells to express TNF- α mRNA, reaching maximal levels after 1 hour of treatment, with expression levels decreasing significantly after 8 hours. However, the cytokine was not clearly detected in the media of LPS treated cells. Imaizumi *et al*, (2000), however, did observe TNF- α secretion in media when HUVEC cells were stimulated with IL-1 α , another pro-inflammatory mediator, indicating that

the cell can secrete the cytokine, potentially *via* differential pathways. Grenon *et al*, (2012) determined that exposure of endothelial cells to arachidonic acid resulted in increased gene expression of several inflammatory biomarkers including Il-6, TNF- α and intracellular adhesion molecule-1 (ICAM-1), an intracellular protein that transports leukocytes from blood to neighbouring tissues.

As TNF- α expression was observed to increase in HUVEC cells treated with different stimulants, such as LPS (Imaizumi *et al*, 2000) or arachidonic acid (Grenon *et al*, 2012), but the cytokine was not, Imaizumi *et al*, (2000), proposed the observation of a transcriptional product, that was not readily translated to the mature cytokine by HUVEC cells. According to Greenbaum *et al*, (2003) as mRNA is eventually translated into a protein, a correlation between mRNA expression and protein production is usually expected. However, Greenbaum *et al*, (2003) postulates post-transcriptional mechanisms involved in turning mRNA into protein that are not yet sufficiently defined, may result in inverse correlations such as those observed by Imaizumi *et al*, (2000) and Grenon *et al*, (2012).

As previously discussed, the production of other cytokines in response to free fatty acid stimulation, such as ULA, may induce a negative autoregulatory effect on TNF- α (Gutierrez-Ruiz *et al*, 1999, Ajuwon and Spurlock, 2005). According to Miles *et al*, (2003) and Innes and Calder (2018) prostaglandin E₂, a pro-inflammatory eicosanoid derived from arachidonic acid, a metabolite of linoleic acid, acts as an inhibitor of TNF- α . This may explain why TNF- α was not observed in the media of ULA treated HUVEC in this investigation. While the TNF- α cytokine was not produced in detectable amounts in this study, Young *et al*, (1998) determined that HUVEC cells treated with linoleic acid produced other cytokines such as Il-8 and intracellular

adhesion molecule-1 (ICAM-1), both of which are involved in the inflammatory process. IL-8 has previously been reported to increase cell barrier permeability in HUVEC cells (Biffi *et al*, 1995). As such, Young *et al*, (1998) proposed that linoleic acid mediated IL-8 may have induced endothelial damage and dysfunction.

TNF- α is a pro-inflammatory cytokine that has been extensively studied and has been reported to play a role in endothelial dysfunction (Toborek *et al*, 1996, Young *et al*, 1998). Both linoleic acid and TNF- α is responsible for the activation of nuclear factor- κ B (NF- κ B) a protein complex, responsible for the regulation of several other inflammatory and apoptotic genes (Young *et al*, 1998, Da Silva *et al* 2017). As such, it was chosen as an inflammatory biomarker of interest in this investigation. Future work may involve the effects of ULA on TNF- α gene expression in HUVEC cells as well as the production of other inflammatory cytokines such as interleukins and adhesion molecules. TNF- α activation of HUVEC cells prior to, or in conjunction, with increasing concentrations of ULA may also yield invaluable information regarding the effects of this fatty acid in an inflamed environment.

HUVEC are considered to be an excellent model for the study of vascular endothelium properties and the main biological pathways involved in endothelium function (Baudin *et al*, 2007, Medina-Leyte *et al*, 2020). It should also be regarded however, that HUVECs may not be a suitable model for the determination of the inflammatory impact of linoleic acid on the endothelium. While TNF- α secretion was not observed in the linoleic acid treated HUVECs used in the current investigation, it does not negate the possibility that high concentrations of plasma linoleic acid may have an effect on the secretion of the cytokine *in vivo*. The phenotype of endothelial cells such as HUVECs, according to Luu *et al* (2010), are highly pliable, with environmental influences such as growth factors and shear stress impacting their responses. *In vivo*, HUVECs exist in an environment where they are exposed to constant

blood flow and the resultant shear stress (Luu *et al* 2010, Chiu and Chien, 2011, Franzoni *et al*, 2016), an environment that can be difficult to replicate *in vivo*. Luu *et al*, (2010) demonstrated that endothelial cell behaviour is powerfully modulated by culture conditions. Considering this, future studies into the impact of fatty acids on endothelial inflammation, through the use of endothelial cells such as HUVEC, may benefit from the analysis of the expression of relevant functional receptors and their continued integrity within a specific culture environment.

7.7.5. The Effects of ULA on the activity of Superoxide Dismutase in HUVEC Cells

ROS are defined as partially reduced metabolites of oxygen that possess strong oxidizing capabilities (Mittal *et al*, 2014). ROS are produced by tightly regulated enzymes such as NADPH oxidase. At physiological concentrations they serve as second messengers, transducing complex intracellular signals involved in several biological functions (Dröge, 2001, Incalza *et al*, 2018). In the endothelium, such functions include the regulation of vascular tone and enhancement of signal transduction from various membrane receptors as well as oxidative stress responses that ensure the maintenance of redox homeostasis (Dröge, 2001).

Paradoxically, at pathological concentrations, ROS can have deleterious effects (Mittal *et al*, 2014). Excessive production of ROS may occur as a result of over-stimulation of NADPH oxidases or from other sources, such as the mitochondrial electron-transport chain (Dröge, 2001), or the cytochrome P450, an enzyme family responsible for the oxidation of steroids, fatty acids, and xenobiotics (Mittal *et al*, 2014). When an aberrant production of ROS surpasses the buffering capacity of the

antioxidant defence systems or when antioxidant enzymes are defective, oxidative stress can occur. Oxidative stress has been reported to play a pivotal role in mediating cytokine production and secretion, leading to inflammation and endothelial activation and dysfunction (Incalza *et al*, 2018). According to Toborek and Hennig (1994), endothelial barrier dysfunction may be mediated by disturbances in antioxidant protection.

Specifically, in the equine, a limited number of conditions have been investigated regarding the effects of oxidative stress, particularly in the endothelium. Both Soffler, (2007) and Kirschvink *et al*, (2008) have documented several conditions detailing the specific impact of ROS and oxidative stress. Some of the more comprehensively documented conditions include injuries pertaining to ischemia and re-oxygenation or perfusion, such as laminitis and colic (Soffler, 2007), two major conditions that can contribute to multiple organ dysfunction, often leading to the euthanasia of the animal (Pouyade *et al*, 2011). According to Pouyade *et al* (2011), the endothelium plays a pivotal role in conditions involving ischemia-re-oxygenation. However, specific data regarding the production of ROS or antioxidants such as SOD by the equine endothelium is very limited. While Pouyade *et al* (2011) determined that cycles of anoxia followed by reoxygenation increased ROS production in an equine carotid endothelial cell line, the specific effects of fatty acids on the production of ROS or antioxidant enzymes in the equine endothelium, at the time of this research, have not been investigated.

As previously discussed, (section 2.4), the superoxide anion is one of the first reactive species to be generated (Incalza *et al*, 2018). Nitric oxide, an endothelial derived compound responsible for the maintenance of vascular homeostasis, reacts with the

superoxide anion (Thannickal and Fanberg, 2000, Incalza *et al*, 2018). Such reactions reduce the bioavailability of nitric oxide, leading to the production of further reactive oxygen species such as peroxynitrite, a particularly potent ROS that induces protein nitration and contributes to mitochondrial dysfunction, endothelial cell dysfunction and death (Incalza *et al*, 2018).

Cellular response to oxidative stress is the activation of antioxidant enzymes such as superoxide dismutase (SOD) (Thannickal and Fanberg, 2000, Incalza *et al*, 2018). SOD is the first detoxification enzyme and is considered to be one of the most powerful antioxidants in the cell (Ighodaro and Akinloye, 2018). In the endothelial cell, cytosolic SOD is responsible for the dismutation of superoxide anions produced by endothelial nitric oxide synthase in the synthesis of nitric oxide (Morikawa *et al*, 2003). Morikawa *et al*, (2003) have also reported that cytosolic SOD plays a pivotal role in the production factors responsible for relaxation of the endothelium.

Free fatty acids have been reported to induce oxidative stress in several cell types including HUVEC cells. Both Toborek *et al*, (1996) and Young *et al*, (1998) determined that linoleic acid markedly increased oxidative stress in HUVEC cells. Toborek *et al* (1996) reported that linoleic acid supplementation induced oxidative stress after 6 hours of treatment but not after 24 hours. Young *et al*, (1998) postulated that linoleic acid induced oxidative stress was related to cellular levels of antioxidants. Viswanathan *et al*, (2003) also observed an increase in oxidative stress in endothelial cells, including HUVEC, treated with linoleic acid, through the cytochrome P450 isomer CYP 2C9 enzymatic pathway. Viswanathan *et al*, (2003) hypothesised that metabolism of linoleic acid *via* this pathway played a major role in the generation of

ROS. Viswanathan *et al*, (2003) also determined that the superoxide anion was the most abundant ROS produced *via* linoleic acid metabolism.

While the effects of fatty acid on the production of ROS, including superoxide, in endothelial cells have been explored, data on the effects of free fatty acids on the production of ROS scavengers such as superoxide dismutase is limited. It was proposed, that as fatty acids have been shown to increase ROS production in several cell types, including endothelial cell, that intracellular enzymatic antioxidants, such as SOD, would also increase in response to the oxidative challenge (Vincent *et al*, 1999). In this investigation, the effects of ULA treatment on SOD in the endothelial cell, HUVEC, was assessed.

7.7.5.1. The Effects of ULA Concentration on SOD in HUVEC Cells

ULA treatment, at any concentration, did not induce a significant increase or decrease in SOD in HUVEC cells (figure 7.10). Cells treated with 0.1mM of ULA induced the most substantial increase in SOD when compared to the untreated control and all other treatment groups, however, this increase was not considered statistically significant. Cells treated with 0.25mM and 0.5mM also induced an initial increase in SOD when compared to the untreated control and cells treated with 1mM and 2.5mM of ULA, however, this increase was not statistically significant.

7.7.5.2. The Effects of Time on SOD in HUVEC Cells

The length of time that HUVEC cells were exposed to ULA did not induce any significant increase or decrease in SOD. Cells treated with 0.1mM of ULA showed an increase in SOD as treatment time increased, however, this increase was not statistically significant. While cells treated with 0.25mM and 0.5mM also induced an

initial increase in SOD when compared to the untreated control, the amount of SOD produced by cells treated with these concentrations did not differ significantly over the 8-hour treatment period. SOD in cells treated with 1mM and 2.5mM of ULA appeared to increase as the length of exposure time increased, however, this increase was not significant.

Neither concentration nor time induced a significant change in the production of cytosolic SOD in ULA treated HUVEC cells. While studies specifically regarding the effects of free fatty acids on endothelial cytosolic superoxide dismutase are limited, a number of studies have shown the impact of fatty acids on SOD activities in different cells types. Comparatively similar findings were reported by Phylactos *et al*, (1994) who observed no significant increase in either cytosolic or mitochondrial SOD in the cardiomyocytes of rats fed on a linoleic acid rich diet. Phylactos *et al*, (1994), however, did observe an increase in mitochondrial SOD in the cardiomyocytes of rats fed a diet rich in both linoleic acid and γ -linolenic acid. This increase was also observed in the cardiomyocytes of rats fed a diet low in linoleic acid but high in γ -linolenic acid, suggesting that n - 6 and n-3 fatty acids may have differential effects on mitochondrial SOD activity (Phylactos *et al*, 1994), possibly due to double bond position and number. Similar results were reported by De Silva *et al*, (2016) who determined that eicosapentaenoic acid had no effect on SOD activity. However, De Silva *et al*, (2016) did observe a decrease in SOD in rat skeletal muscle cells exposed to docosahexaenoic acid. While both of these fatty acids are ω -3 PUFAs, the number of double bonds differs by one, strengthening the hypothesis that the number of double bonds as well as the position may affect SOD activity

Work carried out by Vincent *et al*, (1999) also determined no significant difference in cytosolic SOD in the homogenates of the left ventricles of lean or obese mice. However, in consonance with Phylactos *et al*, (1994), Vincent *et al*, (1999) also observed an increase in mitochondrial SOD. Vincent *et al*, (1999) hypothesised that one source of ROS in obesity is the formation of superoxide radicals during mitochondrial respiration. These postulations may explain why no significant difference was observed in cytosolic SOD in HUVEC cells treated with ULA in this investigation.

While studies regarding the effects of free fatty acids on endothelial cytosolic superoxide dismutase are limited, a number of investigations have shown that cellular exposure to free fatty acids have affected other antioxidant mechanisms, such as the glutathione redox cycle, in these cell types. Toborek and Hennig, (1994) determined that exposure of porcine endothelial cells to several different free fatty acids reduced glutathione levels, with a maximal decrease occurring after 6 hours of exposure. Toborek and Hennig (1994) reported that linoleic acid induced the most significant reduction in glutathione when compared to untreated cells or cells treated with other fatty acids. As exposure times increased, Toborek and Hennig (1994) observed an increase in glutathione concentrations after 12 and 24 hours. This increase was attributed to the initial depletion of the antioxidant that was observed after 6 hours of exposure to the fatty acids (Toborek and Hennig, 1994). In liver tissue, depletion of glutathione induces its production (Reed and Fariss, 1984) and Toborek and Hennig (1994) postulated that a similar stimulation also takes place in the endothelium. Toborek *et al*, (1996) reiterated their findings, demonstrating that linoleic acid reduced glutathione levels in linoleic acid treated porcine pulmonary endothelial cells after 6

hours of exposure. Viswanathan *et al*, (2003) observed similar results, with glutathione depletion observed after 6 hours in linoleic acid treated HUVEC cells.

These data, including the results portrayed in the current investigation (figure 7.8) indicate that exposure of endothelial cells to free fatty acids, such as linoleic acid, may affect mitochondrial production of superoxide, thereby inducing mitochondrial SOD, more so than cytosolic SOD. The depletion, and subsequent renewal of glutathione, observed in studies carried out by Toborek and Hennig, (1994) and Viswanathan *et al*, (2003) suggest that other antioxidants, such as glutathione, may play a more pivotal role in the protection of endothelial cells from oxidative stress.

7.7.6. Assessment of Thromboxane B₂ Production in ULA treated HUVEC Cells

Free fatty acids, such as linoleic acid, are potent promoters of inflammation, stimulating the release of pro-inflammatory mediators, such as cytokines and eicosanoids, in several cell types (Choque *et al*, 2012, Chen *et al*, 2018). Urquhart *et al*, (2002), determined that linoleic acid stimulated the production of several eicosanoids including prostacyclin, thromboxane B₂, prostaglandin E₂ prostaglandin F_{2a} and prostaglandin D₂ in human saphenous vein endothelial cells.

Eicosanoids are essential regulators of vascular tone and haemostasis (Torres-Duarte and Vanderhoek, 2003, Araujo *et al*, 2019). They are typically produced through the enzymatic conversion of fatty acids, more specifically, arachidonic acid, into lipid signalling molecules (Araujo *et al*, 2019). Eicosanoid biosynthesis is initiated as a result of the release of arachidonic acid from membrane phospholipids, through the action of phospholipase A₂. Arachidonic acid, a metabolite of linoleic acid, is converted by cyclooxygenases and lipoxygenases, into eicosanoids such as prostaglandins, leukotrienes or thromboxane, (Khanapure *et al*, 2007).

In this study, the potential for ULA to impact haemostasis was hypothesised. As such, the effects that ULA may have on the endothelial production of thromboxane was of particular interest. In endothelial cells, Nakahata, (2008) states that thromboxane A₂ accelerates the surface expression of adhesion molecules and is also involved in angiogenesis and endothelial cell migration. Thromboxane A₂ is also proposed to work as a negative feedback regulator in the process of platelet aggregation. According to Hunt *et al*, (1992), thromboxane A₂ induces prostacyclin production in endothelial cells, which attenuates platelet aggregation and vascular smooth muscle contraction.

In order to ascertain the effects of linoleic acid on thromboxane A₂ production in HUVEC cells, cells were treated with increasing concentrations of ULA over 8 hours. Thromboxane B₂ production by HUVEC cells increased as the concentration of ULA increased. Cells treated with 0.1mM of ULA produced a mean concentration of 1.7(± 0.10)ng/mL of thromboxane B₂ after 8 hours of treatment compared to 38.1(± 1.10)ng/mL of thromboxane produced by cells treated with 2.5mM of ULA over the same treatment time.

7.7.6.1. Effects of ULA Concentration on Thromboxane Production

Thromboxane B₂ increased as the concentration of ULA increased. Statistically, untreated cells initially produced significantly more thromboxane B₂ than cells treated with 0.1mM or 0.25mM of ULA, with *P* values of 0.005 and 0.009 respectively. However, cells treated with 0.5mM, 1mM and 2.5mM of ULA all produced significantly more thromboxane B₂ than untreated cells (*P* = <0.0001).

Cells treated with 0.5mM and 1mM of ULA produced significantly more thromboxane than either untreated cells or those treated with 0.1mM or 0.25mM of ULA. However, the most significant increase in thromboxane production occurred in cells treated with

2.5mM of ULA, when compared with all other treatment groups and the untreated control ($P = <0.0001$). When this treatment group was compared with cells treated with 1mM, the next highest producer of thromboxane, there was a more than 2-fold increase in thromboxane B₂ secretion into media.

7.7.6.2. The Effects of Treatment Time on Thromboxane production

Untreated cells showed a significant reduction in thromboxane B₂ production when cells analysed after 1 hour were compared with those analysed after 8 hours ($P = 0.0014$). While cells treated with 0.1mM of ULA showed no significant difference in thromboxane B₂ across all 8 hours, cells treated with 0.25mM of ULA showed a significant increase in thromboxane B₂ production between 1 and 8 hours of treatment ($P = 0.0067$). Similarly, cells treated with 0.5mM and 1mM of ULA also showed a significant increase in thromboxane B₂ production between 1 and 8 hours with P values of 0.0013 and <0.0001 respectively. Cells treated with 2.5mM of ULA also showed that the most significant increase in thromboxane B₂ production occurred between cells treated for 1 hour and those treated for 8 hours ($P = <0.0001$). These data indicate that increasing concentrations of ULA induced thromboxane secretion by treated HUVEC cells in a time and dose dependent manner.

In the current investigation thromboxane secretion by HUVEC was increased by ULA treatment. Thromboxane production by platelets is well documented in the literature. The effects of free fatty acids such as linoleic acid on the production and action of thromboxane in platelets has been investigated by several authors. Studies carried out by Srivastava *et al* (1895), determined that treatment of human platelets with various concentrations of linoleic acid, ranging from 0.0125 to 0.5mM, reduced the production of thromboxane B₂. Needleman *et al*, (1982) also observed a 50% reduction in

thromboxane A₂ production in platelets supplemented with 0.2mM linoleic acid. Similar results were observed by MacIntyre *et al*, (1984), when they reported partial inhibition of thromboxane B₂ in human platelets treated with oleic acid and linoleic acid (0.020mM). However, Zhang *et al*, (1997) determined no alteration in thromboxane B₂ production by porcine platelets treated with linoleic acid.

The effects of free fatty acids on the production of eicosanoids, and in particular, thromboxane, by endothelial cells have also been investigated, albeit to a lesser degree. While endothelial release of thromboxane has previously been reported (Griesmacher *et al*, 1989), investigations regarding the effects of fatty acids on thromboxane production by endothelial cells is sparse.

Studies carried out by Urquhart *et al*, (2002), determined that 0.05mM of linoleic acid stimulated the production of several eicosanoids including thromboxane B₂, in human saphenous vein endothelial cells after 72 hours of treatment. Similar studies carried out by Brox and Nordøy (1983), determined that treatment of HUVEC cells with 0.05mM and 0.1mM of different PUFAs, including linoleic acid, for 3 hours failed to stimulate significant thromboxane production. However, Brox and Nordøy (1983) observed significant thromboxane B₂ production in endothelial cells treated with arachidonic acid for 24 hours, while treatment with other fatty acids, including linoleic acid failed to induce production of the eicosanoid. The findings of Brox and Nordøy (1983) could be considered comparable to those obtained in the current investigation, as cells treated with 0.1mM of ULA failed to secrete any significant amount of thromboxane B₂ compared with untreated cells, even after 8 hours of treatment.

In the current investigation, HUVEC cells treated with ULA at concentrations of 0.5mM and higher did induce significantly more thromboxane B₂ than the untreated control.

It has previously been reported that the biological effects of PUFAs are believed to be related to their incorporation into cell membrane phospholipids (Grenon *et al*, 2012). According to Araujo *et al*, (2019), PUFAs are actively incorporated into endothelial cells and are converted to both pro and anti-inflammatory mediators such as eicosanoids (Araujo *et al*, 2019, Torres-Duarte and Vanderhoek, 2003, Calder, 2009 and Innes and Calder, 2018). Ander *et al*, (2003) postulates that eicosanoid production is altered by the fatty acid composition of the cellular membrane and that this alteration may affect inflammation.

It may be hypothesised that, in the current study, linoleic acid was enzymatically converted into arachidonic acid within HUVEC cells, increasing intracellular arachidonic acid. This, in turn, was metabolised *via* COX to produce thromboxane. Gu *et al*, (2013) determined that incubating prostate cancer cells with linoleic acid resulted in a significant increase in both cellular linoleic acid and arachidonic acid, indicating that there is conversion from linoleic acid to arachidonic acid within this cell type. Ramadan *et al*, (1990) observed a 3.85-fold increase in thromboxane production in rabbit aortic endothelial cells treated with 10mM of arachidonic acid for 48 hours. However, while a number of cells are able to convert linoleic acid to arachidonic acid, according to Toborek *et al*, (1992), in endothelial cells, the low basal activity of endothelial elongases and Δ5 and Δ6 desaturases, arachidonic acid is not produced in significant quantities in endothelial cells.

Spector *et al*, (1981) also states that endothelial cells have a limited capacity to convert linoleic acid to arachidonic acid. According to Spector *et al*, (1981) linoleic acid also reduced arachidonic acid incorporation into cell membrane phospholipids in HUVEC cells treated with 0.15mM of linoleic acid, presumably through competitive inhibition (Spector *et al*, 1981). It could be hypothesised that incorporation of linoleic acid into the phospholipid membrane increased cytosolic arachidonic acid in ULA treated HUVEC cells in the current investigation, leading to its conversion to thromboxane A₂. According to Spector *et al*, (1981), increasing intracellular linoleic acid lead to more of it being esterified into membrane phospholipids, with arachidonic acid being excluded.

It may also be postulated that increasing concentrations of ULA passed 0.5mM induced intracellular stress that resulted in the activation of phospholipase A₂, resulting in the release of membrane arachidonic acid, leading to its subsequent conversion to thromboxane A₂. According to Ramadan *et al*, (1990), eicosanoid metabolism starts with the release of arachidonic acid from membrane phospholipids after the activation of phospholipases. The membrane phospholipid, phosphatidylcholine, which contains arachidonic acid in the *sn*-2 position, is preferentially hydrolysed by cytosolic phospholipase A₂, meaning arachidonic acid is more likely to be released (Urquhart *et al*, 2000, Hanel *et al*, 1993).

While the treatment of HUVEC cells with increasing concentrations of ULA lead to increased production of thromboxane A₂, measured as thromboxane B₂ in the current investigation, the mechanism by which this production occurred remains to be elucidated.

Hunt *et al*, (1992) observed an increase in PGI₂ in bovine aortic endothelial cells in response to the thromboxane A₂ mimetic U46619, *via* an interaction with the TP receptor, indicating that thromboxane induces PGI₂ in endothelial cells. PGI₂ is responsible for vasodilation and inhibition of platelet aggregation (Kawabe *et al*, 2010). Hunt *et al*, (1992) postulated that this release may represent a feedback mechanism whereby the vasoconstrictor and pro-aggregatory effects of thromboxane A₂ are reduced in a normal haemostatic environment. However, overstimulation of PGI₂ as a result of aberrant lipid signalling due excessive levels of ULA may initiate an imbalance in haemostasis leading to an anticoagulatory state. Taking into consideration the data presented by Needleman *et al*, (1982) and MacIntyre *et al*, (1984) regarding ULA inhibition of platelet aggregation and platelet thromboxane A₂ production, it may be hypothesised that an aberrant inflammatory response, induced by excess ULA contributes towards coagulopathy.

7.8. Conclusion

The endothelium is responsible for several functions, including the maintenance of thrombosis and haemostasis as well as other inflammatory responses involved in the regulation of circulatory homeostasis (Ghosh *et al* 2017). As the fatal idiopathic phenomenon observed by Cooper (2015) and the IEC involved hemorrhaging, the potential effects of ULA on the endothelium warranted investigation.

Endothelial cells may be exposed *in vivo* to high concentrations of free fatty acids (Toborek *et al*, 1996). As such, the potential impact that supraphysiologic concentrations of linoleic acid may have on endothelial inflammation and haemostasis was investigated in this chapter.

The data presented here demonstrated that ULA at concentrations surpassing 0.25mM and exposure times of more than 2 hours had a negative impact on several aspects of cellular homeostasis in HUVEC cells, including cell viability and the ability of HUVEC cells to form protective intracellular lipid droplets. At concentrations of 1mM and 2.5mM, ULA induced a significant and time-dependent increase on the production of thromboxane A₂.

Several authors postulate that fatty acid mediated endothelial dysfunction occurs as a result of increased oxidative stress, induced by endothelial fatty acid metabolism (Toborek and Hennig, 1994, Toborek *et al*, 1996, Kuratko and Constante, 1998, Kim *et al*, 2005, Edirisinghe *et al*, 2006, Kim et al, 2012). While the mechanism of action of linoleic acid on lipotoxicity in HUVEC cells remains to be elucidated, the current data indicates that linoleic acid, at supraphysiological concentrations, at exposure times surpassing 2 hours may mediate endothelial dysfunction.

Chapter 8

Summary Discussion and Conclusion

Chapter 8. Summary Discussion and Conclusion

This investigation originated from observations made by Cooper (2015) and the IEC that determined a link between linoleic acid in its unconjugated form and the development of a fatal haemorrhagic condition in equines. The identification of the link between linoleic acid and the haemorrhagic condition led to the postulation that an aberrant inflammatory response may be contributing towards a coagulopathy.

Linoleic acid, as a precursor for arachidonic acid, has previously been associated with a pro-inflammatory response (Simopoulos, 2008). Excessive, unresolved inflammation can lead to uncontrolled tissue damage, pathology and disease (Innes and Calder. 2018). Linoleic acid has also been associated with platelet inhibition and with a reduction in platelet thromboxane A₂ production (McIntyre *et al*, 1984).

Plasma free fatty acids are increased in several disease states including insulin resistance and diabetes (Artwhol *et al*, 2009), as well as during physiologically stressful conditions such as fasting or strenuous exercise (Høstmark, 1995, Toborek *et al*, 1996, Young *et al*, 1998). As such, this investigation aimed to elucidate the potential mechanisms by which linoleic acid at supraphysiological concentrations may impact cell health in the liver and the vasculature.

A summary of the most significant results is presented and briefly discussed in this chapter.

8.1. The Effects of ULA on HepG2

In HepG2 cells, treatment with 0.1mM of ULA had no effect on cell viability compared with an untreated control across any time point. HepG2 cells treated with this concentration also presented with a time dependent increase in intracellular lipid

accumulation, with fluorescent imaging indicating the presence of intracellular lipid droplets. This suggests that hepatic cells can sequester linoleic acid successfully at this concentration. There were also no effects on TNF- α , SOD or thromboxane A2 observed in HepG2 cells treated with 0.1mM of ULA at any time point. When HepG2 cells treated with 0.1mM of ULA were assessed for cells death, there was no significant differences observed when this treatment group was compared with an untreated control. These data indicate that the hepatic cell line HepG2 can metabolically manage ULA at this concentration. This is consistent with reports by a number of authors that physiological concentrations of fatty acids fall between 0.09mM and 1mM (Toborek *et al* 1996, Artwohl *et al*, 2003, Artwhol *et al*, 2009, Storniolo *et al*, 2013).

In the current investigation, the highest concentrations of ULA, namely 1mM and 2.5mM consistently impacted the indicators of cellular health analysed in HepG2 cells. Cells treated with these concentrations showed a significantly reduced cell viability, even after 1 hour of supplementation. Intracellular lipid accumulation also reduced in cells treated with these concentrations of ULA.

In HepG2 cells, the presence of the ROS scavenging enzyme SOD was also significantly reduced in cells treated with 1mM and 2.5mM. This reduction was consistently observed after 4 hours of exposure to these concentrations. Free fatty acids are known to increase the presence of ROS (Olusi, 2002, Videla *et al*, 2004, Haung *et al*, 2015), however, this data indicates that, at supraphysiological concentrations, they may also impact the enzymes responsible for ROS management, a postulation hypothesised and investigated by a number of researchers (Videla *et al*, 2004, Kim *et al*, 2005, Choi *et al*, 2010, Appolinário *et al*, 2015).

Linoleic acid at concentrations of 1mM and 2.5mM also significantly induced cell death, with a considerable increase in apoptosis and necrosis compared to than any other treatment concentration. The most notable observation made was the decrease in live cells and increase in events deemed to be cell debris. Several authors have reported that linoleic acid can induce apoptotic cell death (Artwohl *et al*, 2003, Cury-Boaventura *et al*, 2004, Zhang *et al*, 2012), while necrosis has been observed in cells treated with higher concentrations of fatty acids (Pompeia *et al*, 2002).

8.2. The Effects of ULA on HUVEC

Initially ULA at concentrations of 0.1mM demonstrated no impact on cell viability in HUVEC cells, however, after 8 hours of exposure to the fatty acid these vascular cells presented with a statistically significant reduction in cell viability. This data suggests that increasing the length of time that vascular cells are exposed to linoleic acid, even at concentrations considered to be within the physiological range, may impact vascular health. However, these data are in contrast to the findings of others, who observed apoptosis in similar cell types at this concentration, but only after treatment periods of 24 to 72 hours (Artwohl *et al*, 2009, Chen *et al*, 2018).

HUVEC cells treated with ULA concentrations surpassing 0.25mM, consistently presented with indicators of cellular dysfunction. Cell viability and intracellular lipid accumulation reduced after 2 hours of exposure to this and increasing concentrations of ULA, indicating that the vascular cells was particularly susceptible to the effects of the fatty acid. According to Toborek *et al*. (1996) endothelial cells are particularly sensitive to oxidative imbalances. TNF- α and SOD activity, however, were not significantly affected in HUVEC cells by ULA exposure.

The most notable result observed in HUVEC cells exposed to ULA occurred in cells treated with 1mM and 2.5mM. ULA at these concentrations induced a significant increase in thromboxane A₂ production. A number of postulations were proposed for this response, including the conversion of linoleic acid to arachidonic acid, a COX substrate for the production of eicosanoids such as thromboxane A₂ (Gu *et al*, 2013). However, the mechanism by which this production occurred has yet to be elucidated. Investigations carried out by Hunt *et al*, (1992) demonstrated that thromboxane A₂ induced PGI2 release in endothelial cells. PGI2 is responsible for vasodilation and inhibition of platelet aggregation (Kawabe *et al*, 2010). This release may represent a feedback mechanism whereby the vasoconstrictor and pro-aggregatory effects of thromboxane A₂ may be reduced. However, overstimulation of PGI2 as a result of aberrant lipid signalling due ULA overload may initiate an imbalance in haemostasis leading to an anticoagulatory state. This, in combination with data presented by Needleman *et al*, (1982) and MacIntyre *et al*, (1984) indicating that ULA inhibited platelet aggregation and reduced platelet thromboxane A₂ production, may support the postulation that an aberrant inflammatory response, induced by excess ULA contributes towards coagulopathy.

8.3. Concluding Remarks

The data portrayed in this body of research indicate that linoleic acid, at concentrations that would be considered supraphysiological, impacted cell viability and intracellular lipid sequestration, affected antioxidant activity and eicosanoid production, and induced cell death. While the literature available on the inflammatory effects of linoleic acid are conflicting, one reoccurring theme is presented throughout the discussion of the data obtained in this investigation; Linoleic acid impacts the cellular

oxidative balance (Toborek *et al*, 1994, Toborek *et al*, 1996, Cury-Boaventura *et al*, 2004, Artwohl *et al*, 2004 Cury-Boaventura *et al*, 2005). According to Masarone *et al*, (2018), free fatty acids can increase ROS leading to apoptosis.

The concentrations of linoleic acid analysed in this investigation were higher than what would be considered physiologically normal. Concentrations of fatty acids reaching 2.5mM have only been reported in aberrant physiological states such as disease, fasting or strenuous exercise (Høstmark, 1995, Toborek *et al*, 1996, Young *et al*, 1998, Artwhol *et al*, 2009).

However, in a system compromised by disease or physiological stress, increasing plasma concentrations of linoleic acid, leading to increasing ROS and diminished antioxidant capabilities may initiate or further exacerbate pathophysiology. Zaman *et al* (2010) states that in pre-existing conditions of altered inflammatory regulation, exposure to increased linoleic acid may potentiate an already dysregulated inflammatory response.

Free fatty acids and their potential impact on a plethora of disease states ranging from cardiovascular disease, non-alcoholic fatty liver disease and platelet dysfunction to amyotrophic lateral sclerosis have been investigated by several authors, as presented here. The data presented in this report, while not specific to any one pathology, indicates that excessive cellular exposure to linoleic acid can compromise cell function in a number of ways, and this compromise in conjunction with other specific dysfunctions may induce particular pathologies.

It could be postulated that, an equine under physiological duress due to an extreme exercise regime or a difficult pregnancy, on a diet incorporating high concentrations of lipids may succumb to any number of pathologies. These collective systemic

compromises, in conjunction with the equines natural tendency towards slower clotting parameters, as indicated by Bell *et al* (1955) and Byars *et al*, (2003) may manifest in the form of an idiopathic and fatal haemorrhagic condition.

Chapter 9

Future Work

Chapter 9. Future Work

9.1. ULA induced Reactive Oxygen Species Production

Long-chain fatty acids, such as linoleic acid, as well as their derivatives and metabolites, namely arachidonic acid, can modify intracellular production of ROS. According to Masarone *et al*, (2018), free fatty acids disrupt endoplasmic reticulum calcium flux leading to mitochondrial membrane permeabilization and the release of cytochrome C and increased ROS resulting in apoptosis. Free fatty acids, such as γ -linolenic, linoleic and oleic acids have all been reported to increase production of extracellular superoxide anion, with this increase being positively correlated to the number of double bonds present on each fatty acid molecule (Hatanaka *et al*, 2013). The effects of supraphysiological concentrations of ULA on the production of the superoxide anion may warrant investigation

9.2. The Effects of ULA on the Hepatic Production of Inflammatory Metabolites

Linoleic acid has several metabolic fates, depending on cellular and tissue needs. It is a precursor for several metabolites involved in many physiological and pathophysiological functions including pro-inflammatory processes such as the production of eicosanoids. In this investigation, production of the pro-inflammatory eicosanoid, thromboxane A₂, was investigated.

However, linoleic acid can also be enzymatically oxidised *via* the actions of lipoxygenases and cyclooxygenases as well as by free radical-mediated oxidation in response to oxidative stress (Reinaud *et al*, 1989, Kirpich *et al*, 2016, Schuster *et al*, 2018). These linoleic acid metabolites are referred to as OXLAMs and include 9- and 13-HODE and 9- and 13-oxoHODE (previously discussed in section 2.2.3) (Ramsden *et al*, 2012, Choque *et al*, 2014, Ramsden *et al*, 2018). According to Schuster *et al*,

(2018), excessive production of OXLAMs can increase oxidative stress and induce mitochondrial dysfunction and apoptosis. Schuster *et al*, (2018) states that these metabolites are abundant in the liver. Future work on the inflammatory effects of ULA may involve the analysis of the oxidative conversion of excessive concentrations of this fatty acid into OXLAMs by hepatocytes.

9.3. The Effects of ULA on the Induction of Hepatic Inflammation

In order to determine the true effects of supraphysiologic concentrations of ULA on hepatic inflammation, the production of pro- and anti-inflammatory eicosanoids and cytokines in response to the fatty acid by Kupffer cells warrants investigation. Kupffer cells are the resident macrophages of the liver and are responsible for the production of eicosanoids such as thromboxane A₂ and cytokines such as TNF- α (Yokoyama *et al*, 2005). Schlager *et al*, (2017) has previously reported that macrophages also accumulate neutral lipid droplets in the form of cholesterol esters, enriched with linoleic acid as well as arachidonic acid, indicating that this cell type may be a suitable *in vitro* model for the study of intracellular lipid accumulation, its impact on inflammation and lipotoxicity. As immune cells, macrophage cells are capable of altering the functional properties of the proximal parenchymal cells within their respective organs. Several eicosanoids such as leukotriene B₄ and thromboxane A₂ promote sequestration of leucocytes in tissues during inflammation and injury (Palombo *et al*, 1996). According to Palombo *et al*, (1996), persistent and uncontrolled release of these inflammatory mediators may exacerbate a predisposition to diseases of the liver. As such, Kupffer cells may be a more suitable *in vitro* model for the analyses of the inflammatory effects of ULA in the liver.

9.4. The Effects of ULA on Endothelium

While the effects of increasing concentrations of ULA on an endothelial cell, namely HUVEC, were explored in the current study, through the analysis of cytokines such as TNF- α and eicosanoids such as thromboxane A₂, analysis of more endothelial specific mediators of the inflammatory response require investigation. Prostacyclin (PGI₂) is one of the most important prostanoids involved in the regulation of cardiovascular homeostasis. PGI₂ is a potent vasodilator and an inhibitor of platelet aggregation, leukocyte adhesion, and vascular smooth muscle cell proliferation. It is also antimitogenic and inhibits DNA synthesis in vascular smooth muscle cells (Ricciotti and Fitzgerald 2010). Several types of vascular cells, including endothelial cells such as HUVEC, vascular smooth muscle cells and endothelial progenitor cells are all major source of PGI₂ (Ricciotti and Fitzgerald, 2010). In the current investigation, an increase in thromboxane A₂ production was observed in HUVEC cells treated with ULA, particularly at the higher concentrations of 1mM and 2.5mM. According to Hunt *et al.* (1992) thromboxane A₂ induces PGI₂ release in endothelial cells. Hunt *et al.* (1992) demonstrated that the thromboxane A₂ mimetic U46619 stimulated PGI₂ release from bovine aortic endothelial cells *via* an interaction with TP receptors. While this release presumably represents a feedback mechanism whereby the vasoconstrictor and pro-aggregatory effects of thromboxane A₂ may be reduced, overstimulation of this eicosanoid as a result of aberrant lipid signalling due to ULA overload may warrant investigation.

The effects of ULA on production of other eicosanoids such a prostaglandin E₂ (PGE₂), may also be worth exploring. PGE₂ is a lipid mediator, produced as a result of the cyclooxygenase mediated conversion of arachidonic acid. It regulates the

activation, maturation, migration, and cytokine secretion of several immune cells, particularly those involved in innate immunity such as macrophages and neutrophils (Agard *et al* 2013). Miles *et al*, (2002), demonstrated that PGE₂ inhibited the production of LPS mediated TNF- α in human whole blood cultures. ULA induced production of this eicosanoid may have attenuated ULA stimulated TNF- α production in HepG2 cells and HUVEC cells in the current investigation.

9.5. The Effects of ULA on Equine Platelet Aggregation and Thromboxane Production

The origin of the current investigation traces back to observations made by Cooper, (2015) and the IEC, whereby ULA was identified as a compound of interest in the hepatic tissues of equines reported to have succumbed to an idiopathic and fatal haemorrhagic condition. While the effects of ULA on platelet aggregation and platelet thromboxane inhibition have been studied in human platelets by Needleman *et al*, (1982) and MacIntyre *et al*, (1984), and later in porcine platelets by Zhang *et al*, (1997), the effects of this fatty acid on equine platelet aggregation and thromboxane production have yet to be investigated. According to Byars *et al*, (2003) equines harbour a comparatively lethargic coagulation system and have a tendency to produce clots more slowly than other domestic mammals. Studies carried out by Bell *et al*, (1955), also revealed poor clot reaction times in horses with relatively normal platelet counts by human standards. Both Needleman *et al*, (1982) and MacIntyre *et al*, (1984), observed inhibition of platelet aggregation and reduced thromboxane production in human platelets supplemented with ULA, while Zhang *et al*, (1997) determined the inhibition of cyclooxygenase by other fatty acids in porcine platelets. As linoleic acid was the major compound identified in the hepatic tissues of equines reported to have succumbed to fatal equine haemorrhage by Cooper (2015) and the IEC, the effects of

this fatty acid at supraphysiologic concentrations on platelet aggregation, thromboxane production and cyclooxygenase inhibition in equines is worth investigating.

Linoleic acid is involved in several mechanisms relating to the induction of inflammation. While several studies, many referenced within this body of work, have demonstrated associations between linoleic acid and one or more inflammatory biomarker exacerbating the risk of various chronic diseases, much more remains to be elucidated.

Chapter 10

Limitations

10.1. Project Limitations

Acknowledgement and discussion of the limitations of scientific research are essential for genuine scientific progress. The identification of weaknesses within a body of research is crucial in the development and improvement of future work as well as formulating new research questions (Ioannidis, 2007). The following are a summary of the major limitations encountered in the current investigation.

10.2. Standardisation of the Experimental Design of Lipid Studies

The current study involved the investigation of the impact of high concentrations of linoleic acid on lipotoxicity and inflammation in human *in vitro* models. The simulation of the physiology of a high plasma fatty acid state in an *in vitro* environment was a key part of this research. During extensive reviews of the current literature, several different experimental designs, outlined in peer-reviewed investigations, were observed. While the methods outlined in section 3.1.4 of this report, adapted from Di Nunzio *et al*, (2011), were identified as the most appropriate for the current research, this led to a number of limitations, including limitations in study comparability.

According to Brenna *et al*, (2018), while, fatty acids are among the most studied nutrients in human health, standardisation of the many parameters, and considerations, common to fatty acid studies has yet to be carried out. Oliveira *et al*, (2015) highlighted that the low solubility of long-chain free fatty acids, such as linoleic acid, in aqueous solutions represents one of the major limitations for *in vitro* studies involving lipids. Regardless of recent widespread interest in the impact of fatty acids on mammalian metabolism, Brenna *et al*, (2018) stated that attempts to summarize the results of multiple similar fatty acid-related studies *via* meta-analyses and systematic reviews have fallen short. This is largely because of heterogeneity in study design,

sampling, and laboratory and data analyses (Brenna *et al*, 2018). Brenna *et al*, (2018) identified several key areas, including the use of sample preparation methods known to be fit-for-purpose *via* published validation studies. As of now, the availability of standard protocols, describing validated experimental designs for the replication of *in vitro* environments that can accurately and reproducibly mimic the physiology of a high plasma fatty acid state have yet to be defined. However, such standardisation of the experimental design of fatty acid studies would allow for accurate comparisons between similar studies, leading to the translation of acquired data into future reliable recommendations.

10.3. Limitations of Cell Types used in this Research

Initial observations made by Cooper (2015) on lipid accumulation in the hepatic tissues of equines affected by fatal and idiopathic haemorrhagic disorder led this researcher to look at the impact of linoleic acid on the liver. Specifically, the human hepatoma - derived cell line, HepG2, was chosen for this study. HepG2 cells have been used previously to investigate the correlation between fatty acids and several diseases associated with the liver (Yao *et al*, 2011, Asrih *et al*, 2015, Zeng *et al*, 2020). These cells have been reported to retain the function of fully differentiated primary hepatocytes, including normal hepatic metabolic functions and are therefore widely used in hepatotoxicity studies (Lima *et al*, 2006, Asrih *et al*, 2015). For these reasons, they were considered to be a suitable *in vitro* model for this investigation.

Similarly, as the condition that was observed by Cooper (2015) was haemorrhagic in nature, the impact of linoleic acid on the vasculature and the endothelium was of particular interest. According to Kocherova *et al* (2019), HUVEC cells represent a widely used source of primary endothelial cells for *in vitro* studies of the vasculature.

Richardson *et al*, (2010) and Caniuguir *et al*, (2016) suggest that HUVECs appear to be the main source of human endothelial cells for *in vitro* study. They have also been used in several previous investigations to determine the effects of fatty acids, including linoleic acid, on the endothelium (Spector *et al*, 1981, Toborek *et al*, 2002, Artwohl *et al*, 2003) As such, they were considered to be a suitable *in vitro* model for this investigation.

However, based on the findings in this study, these cell models may be limited in terms of analysis of the inflammatory response of the liver, or the endothelium, to toxicants. While Stonans *et al*, (1999) demonstrated that HepG2 cells expressed a variety of cytokine genes, including TNF- α , indicating that cells of hepatocellular lineages may be potential producers of inflammatory markers, secretion of TNF- α was not observed during this investigation. Neuman *et al*, (1998), reported TNF- α secretion in HepG2 cells treated with 80mM of ethanol, however, in the current investigation, HepG2 cells, whether treated with ethanol, LPS or ULA, did not secrete any detectable quantities of TNF- α .

Likewise, while HUVECs have also been reported to express many important endothelial markers and signalling molecules associated with regulation of vascular homeostasis (Caniuguir *et al*, 2016, Kocherova *et al*, 2019), TNF- α secretion was not observed during the current investigation. Reports of responses to pro-inflammatory stimuli such as LPS as well as the production of several pro-inflammatory cytokines, such as IL-6 and IL-8 exist (Unger *et al*, 2002), however, literature regarding TNF- α production by HUVECs in response to free fatty acids is sparse. According to Li *et al* (2018) HUVEC cells treated with free fatty acids did not produce cytokines unless the cells had been activated *via* co-incubation with LPS or TNF- α , a further limitation of using this cell line to assess the inflammatory response.

As discussed in section 6.7.4 and section 7.7.4, with further elaboration provided in Chapter 9, these limitations may be somewhat curtailed by the selection of more suitable markers of inflammation, whether pro- or anti-inflammatory, such as IL-2, IL-8 and IL-10, whose secretion has been reported in HepG2 cells by Paul and Mukherjee (2019). IL 10, an anti-inflammatory cytokine, has also been reported to suppress pro-inflammatory cytokine responses in Hepg2 cells (Paul and Mukherjee, 2019).

Fritch (2015) states that there is no consensus regarding which inflammatory biomarker will provide the best results and the selection of multiple biomarkers does not guarantee that treatment effects will be observed. However, where a more accurate reflection of the inflammatory response is required, such limitations can be further curtailed through careful selection of a suitable cell model. As discussed Chapter 9, a more appropriate approach may be to analyse the potential inflammatory effects of linoleic acid on liver macrophages such as Kupffer cells, as well as platelets and cells involved in innate immunity, such as macrophages and neutrophils (Agard *et al* 2013).

10.4. Limitations of Oil Red for Analysis of Intracellular Lipid Droplets

Oil Red O, a hydrophobic lipid soluble diazo dye, with a maximum absorption of 518nm, was chosen as it has been reported by Mehlem *et al*, (2013) to be an accurate method for detecting and quantifying hepatic steatosis both in mouse and human liver biopsies. This stain is often used in conjunction with brightfield microscopy to visualise and quantify intracellular lipid stores (Daemen *et al* 2015, Mehlem *et al*, 2013). Oil Red O stains neutral lipids and cholesteryl esters but not biological membranes. The principle for staining is that Oil Red O is minimally soluble in IPA, with solubility further decreasing upon dilution in water. When the stain is applied to

lipid containing cells, the stain will move from the solvent to associate with the lipids (Mehlem *et al*, 2013).

According to Kraus *et al* (2016) it has been used to discern between preadipocytes and adipocytes through quantitative analysis for several years. To enable quantitative measurements, the dye is commonly eluted from the cells using IPA, and absorbance is photometrically determined at or near the absorbance maximum of Oil Red O (Kraus *et al* 2016). This method was employed by this researcher in the current investigation.

While the method used in this particular piece of research was adapted from peer-reviewed and published methods, there are a number of limitations associated with the assay. Aldrich *et al* (2013) refers to the Oil Red O assay as semi-quantitative, while Varinli *et al* (2015) deems it to be more qualitative than quantitative. Spangenburg *et al*, (2011) has indicated that, while the Oil Red O assay is a powerful tool for the measurement of lipid droplets in muscle tissue, the tissues of interest requires fixation, preventing its use on live cells. The preparation of the stain also requires that it is dissolved in isopropanol, requires heating to encourage the process, and must be filtered afterwards, which often reduces the final concentration of the solution (Spangenburg *et al*, 2011) and introduces further variability in the experimental design. Additionally, lipophilic dyes, such as Oil Red O, have high affinity toward hydrophobic surfaces, resulting in background staining when plastic culture dishes are used (Varinli *et al*, 2015).

According to Kraus *el al* (2016), the validity of this method has been called into question through a lack of reported data on the performance of the Oil Red O assay, with several published protocols differing in the detail of the experimental design.

Masone *et al* (2017) also states that intrinsic information such as lipid droplet number and size is lost using the Oil Red O method.

These are limitations that need to be considered carefully during the future experimental designs that include the use of this assay. In order to reduce these limitations, Kraus *et al*, (2016) has presented an optimised quantitative staining protocol that is reported to be validated, however the assay appears to be specifically for the quantitative assessment of adipocyte differentiation. The limitations of this assay may also be further controlled through the use of optimised methods such as an automatic procedure for digital image processing that allows for the efficient quantification and characterisation of lipid droplets as described by Masone *et al*, (2017)

10.5. Thromboxane Control

Linoleic acid has been reported to effect eicosanoid production in a number of cells types, including human breast cancer cells and the hepatic carcinoma cell line, HepG2 cells (Eder, et al, 2002). Linoleic acid was also reported to have antithrombotic properties (MacIntyre *et al*, 1984, Needleman *et al*, 1982). As such, the production of thromboxane B₂ (an inactive stable metabolite of thromboxane A₂) by ULA treated HepG2 cells and HUVECs was analysed. While successful standard curves were obtained and thromboxane production was reported in both cell lines, the omission of a positive thromboxane control introduced an assay limitation. Best practice dictates that a sample of known concentration that falls within the linear section of the standard curve should be used as a positive control sample. The use of stimulated platelets as a positive control for thromboxane production, or the use of a quantitative positive control specific to the assay kit (outlined in section 3.1.14) would have strengthened the findings and added further validity to the final assay conclusion.

10.6. Conclusion.

Ioannidis (2007) states that the identification of weaknesses within a body of research is essential in improving future research methodology and the validity of extrapolation of results. While there were a number of limitations observed throughout the course of this investigation, the key areas have been highlighted in this chapter so that careful considerations can be made during future experimental design.

Chapter 11

Dissemination of Research

11.1. Dissemination of Research

Plans are in progress to disseminate this piece of research in the following research journals:

Publication 1: Journal of Lipid Research

Research Paper: Working Title – In Vitro Effects of Unconjugated Linoleic Acid on the Liver and its Potential Impact on Haemostasis (see Appendix 5 for draft).

Publication 2: Journal of Lipid Research/ Prostaglandins, Leukotrienes & Essential Fatty Acids

Review Paper: Working Title – Linoleic acid and its involvement in Inflammation and Coagulation.

Publication 3: Journal of Veterinary Diagnostic Investigation/Equine Veterinary Education.

Review Paper: Working Title –The Potential impact of a High Fatty Acid Diets on Coagulation and Inflammation in the Equine

Publication 4: Journal of Lipid Research

Research Paper: Working Title – In Vitro Effects of Unconjugated Linoleic Acid on the Endothelium and its Potential Impact on Haemostasis

Chapter 12

Bibliography

12.1. Bibliography

Abdule-Ghani, M. a., Muller F. L., Liu, Y., Chavez, A. O., Balas, B., Zuo, P., Chang, Z., Tripathy, D., Jani, R., Molina-Carrion, M., Amonroy, A., Folli, F., Van Remmen, H. and DeFronzo, R. (2008), Deleterious action of FA metabolites on ATP synthesis: possible link between lipotoxicity, mitochondrial dysfunction, and insulin resistance, *American Journal of Physiology, Endocrinology and Metabolism*, vol. 295, pp. E678 – E685.

Achneck, H. E., Sileshi, B., Parikh, A., Milano, C. A., Welsby, I. J. and Lawson, J. H. (2010), Pathophysiology of bleeding and clotting in the cardiac surgery patient from vascular endothelium to circulatory assist device surface, *Circulation*, vol. 22, pp. 2068-2077.

AFSSA (Agence française de sécurité Sanitaire des aliments), 2007. Avis de l'Agence française de sécurité des aliments sur l'innocuité de la glycérine utilisée en tant que matière première en alimentation animale pour toutes espèces. Saisine n° 2007-SA-0013, 1-4. In: European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM), (2010), Scientific Opinion on the abiotic risks for public and animal health of glycerine as co-product from the biodiesel production from Category 1 animal by-products (ABP) and vegetable oils. *EFSA Journal*, vol. 8, pp. 1 – 22.

Agard, M., Asakrah, S. and Morici, L. A. (2013) PGE₂ suppression of innate immunity during mucosal bacterial infection, *Frontiers in Cellular and Infection Microbiology*, vol. 3, pp. 1 – 11.

Ahern, K. and Rajagopal, I., (2012), *Biochemistry, Free and Easy*, Available at: <http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy> [Accessed on the 1st March 2016].

Ahern, K. and Rajagopal, I., (2019) Fatty Acid Oxidation, *Biochemistry, Free and Easy*, Available at: [https://bio.libretexts.org/Bookshelves/Biochemistry/Book%3A_Biochemistry_Free_and_Easy_\(Ahern_and_Rajagopal\)/06%3A_Metabolism_I/6.11%3A_Fatty_Acid_Oxidation](https://bio.libretexts.org/Bookshelves/Biochemistry/Book%3A_Biochemistry_Free_and_Easy_(Ahern_and_Rajagopal)/06%3A_Metabolism_I/6.11%3A_Fatty_Acid_Oxidation) [Accessed on the 21st November 2019].

Ajuwon, K. M. and Spurlock, M. E. (2005) Palmitate activates the NF-κB transcription factor and induces IL-6 and TNF-α expression in 3T3-L1 adipocytes, *Biochemical and Molecular Actions of Nutrients*, vol. 135, pp.1841 – 1846.

Aldai, N., Murray, B. E., Najera, A. I., Troy, D. J. and Oroso, K. (2005) Derivatisation of fatty acids and its application for conjugated linoleic acid studies in ruminant meat lipids, *Journal of the Science of Food and Agriculture*, vol. 85, pp. 1073 – 1083.

Aldridge, A., Kouroupis, D., Churchman, S., English, A., Ingham, E. and Jones, E. (2013) Assay validation for the assessment of adipogenesis of multipotential stromal cells—a direct comparison of four different methods, *Cytotherapy*, vol. 15, pp. 89 – 101.

- Alsabeeh, N., Chausse, B. Kakimoto, P. A., Kowaltowski, A. J. and Shirihai, O. (2018) Cell culture models of fatty acid overload: problems and solutions, *Biochimica et Biophysica Acta*, vol. 1863, pp. 143 – 151.
- Ambrojo, K. S., Gardon Poggi, J. C. and Muñoz Juzado, A. (2013), Use of laboratory testing to diagnose liver and biliary dysfunction in the horse, *Journal of Gastroenterology and Hepatology*, vol. 2, pp. 807 – 813.
- Ander, B. P., Dupasquier, C. M. C., Prociuk, M. A. and Pierce, G. N. (2003), Polyunsaturated fatty acids and their effects on cardiovascular disease, *Experimental and Clinical Cardiology*, vol. 8, pp. 164 – 172.
- Andreyev, A.Y., Fahy, E., Guan, Z., Kelly, S., Li, X., McDonald, J. G., Milne, S., Myers, D., Park, H., Ryan, A., Thompson, B. M., Wang, W., Zhao Y., Brown, H. A., Merrill, A. H., Raetz, C. R. H., Russell, D. W., Subramaniam, S. and Dennis, E. A. (2010), Subcellular organelle lipidomics in TLR -4-activated macrophages, *Journal of Lipid Research*, vol. 10, pp. 2785 – 2797.
- Aon, M. A., Bhatt, N. and Cortassa, S. C. (2014) Mitochondrial and cellular mechanisms of managing lipid excess, *Frontiers in Physiology*, vol. 5, pp. 1 – 13.
- Appolinário, P. P., Medinas, D. B., Chaves-Filho, A. B., Genaro-Mattos, T. C., Cussiol, J. R. R., Soares Netto, E. L., Augusto, O. and Miyamoto, S. (2015) Oligomerization of cu,zn-superoxide dismutase (sod1) by docosahexaenoic acid and its hydroperoxides in vitro: aggregation dependence on fatty acid unsaturation and thiols, *OLoS ONE*, vol. 10, pp. 1 – 15.
- Araujo, P., Belghit, I., Aarsæther, N., Espe, M. Lucena, E. and Holen, E. (2019) The effect of omega-3 and omega-6 polyunsaturated fatty acids on the production of cyclooxygenase and lipoxygenase metabolites by human umbilical vein endothelial cells, *Nutrients*, vol. 11, pp. 1 – 18.
- Artwohl, M., Lindenmair, A., Roden, M., Waldhausl, W., Freudenthaler, A., Klosner, G., Ilhan, A., Luger, A. and Baumgartner-Parzer, S. M. (2009) Fatty acids induce apoptosis in human smooth muscle cells depending on chain length, saturation, and duration of exposure, *Atherosclerosis*, vol. 202, pp. 351–362.
- Artwohl, M., Roden, M., Waldhausl, W., Freudenthaler, A. and Baumgartner-Parzer, S. M. (2003), Free fatty acids trigger apoptosis and inhibit cell cycle progression in human vascular endothelial cells, *The Federation of American Societies for Experimental Biology*, vol. 18, pp. 146 – 148.
- Asrih, M., Montessuit, C., Philippe, J., and Jornayvaz, F. (2015) Free Fatty Acids Impair FGF21 Action in HepG2 Cells, *Cellular Physiology and Biochemistry*, pp. 1767 – 1778.
- Austin, S. K. (2017), Haemostasis, *Medicine*, vol. 45, pp. 204 – 208.
- Ayora-Canada, M. J., Dominguez-Vidal, A. and Lendl, (2010), Monitoring oxidation of lipids in edible oils and complex food systems by vibrational spectroscopy, In: Chalmers, J and Griffiths, P. (ed.) *The handbook of Vibrational Spectroscopy*, vol. 1, pp. 1 – 20.

Baraona, E. & Lieber, C. S., (1979), Effects of ethanol on lipid metabolism, *Journal of Lipid Research*, vol. 20, pp. 289-315.

Barton, B. M., Morris, D. D., Reed, S. M. & Bayly, W. M., (1998), *Equine Internal Medicine*. Philadelphia: W. B. Saunders.

Barrera, G. (2012) Oxidative stress and lipid peroxidation products in cancer progression and therapy, *International Scholarly Research Network Oncology*, vol. 2012, pp. 1 – 21.

Barroso, J. M. (2010), Commission Regulation No 892/2010 on the status of certain products with regards to feed additives within the scope regulations (EC) No 1831/2003 of the European Parliament and of the Council, *Official Journal of the European Union*, The European Commission.

Basinska, K., Maryc, K., Śmieszek, A. and Nicpoń, J. (2014) The production and distribution of IL-6 and TNF- α in subcutaneous adipose tissue and their correlation with serum concentrations in Welsh ponies with equine metabolic syndrome, *Journal of Veterinary Science*, vol. 16, pp. 113 – 120.

Baudin, B.; Bruneel, A.; Bosselut, N.; Vaubourdolle, M. (2007), A protocol for isolation and culture of human umbilical vein endothelial cells, *Nature Protocols*, vol. 2, pp. 481–485.

Beards, G. (2012), Coagulation in vivo.png, available at https://commons.wikimedia.org/wiki/File:Coagulation_in_vivo.png [accessed on the 30th June 17].

Bell, W. N., Tomlin, S. C. and Archer, R. K., (1955), The coagulation mechanism of the blood of the horse with particular reference to its “haemophiloid” status, *Journal of Comparative Pathology*, vol. 65, pp. 255 – 261.

Belury, M. A., Cole, R. M., Snoke D. B., Banh, T. and Angelotti, A. (2018) Linoleic acid, glycemic control and type 2 diabetes, *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 132, pp. 30 – 33.

Biffl, W. L., Moore, E. E., Moore F. A., Carl, R. S., Franciose, R. J., Banerjee, A. (1995) Interleukin-8 increases endothelial permeability independent of neutrophils, *Journal of Trauma: Injury, Infection, and Critical Care*, vol. 39 pp. 98-103

Blaser, H., Dostert, C., Mak, T. W. and Brenner, D. (2016) TNF and ROS crosstalk in inflammation, *Trends in Cell Biology*, vol. 26, pp. 249 – 261.

Bogatchev, N. V., Sergeev, M. G., Dudek, S. M. and Verin, A. D. (2005) Arachidonic acid cascade in endothelial pathobiology, *Microvascular Research*, vol. 69, pp. 107 – 127.

Bollheimer, L. C., Skelly, R. H., Chester, M. W., McGarry, J. D. and Rhodes, C. J. (1998), Chronic exposure to free fatty acid reduces pancreatic β cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation, *Journal of Clinical Investigation*, vol. 101, pp. 1094 – 1101.

- Borgstahl, G. E. O. and Oberley-Deegan, R. E. (2018) Superoxide dismutases (SODs) and SOD Mimetics, *Antioxidants*, vol. 7, pp. 1 – 3.
- Borradaile, N. M., Han, X., Harp, J. D., Gale, S. E., Ory, D. S., and Schaffer, J. E. (2006) Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death, *Journal of Lipid Research*, vol. 47, pp. 2726 – 2737.
- Bradley, R. M., Stark, K. D. and Duncan, R. E. (2016) Influence of tissue, diet, and enzymatic remodelling on cardiolipin fatty acyl profile, *Molecular Nutrition and Food Research*, pp. 1 – 15.
- Bradley, R. L., Fisher, F. M. and Flier, E. M. (2008) Dietary fatty acids differentially regulate production of TNF- α and IL-10 by murine 3T3-L1 adipocytes, *Obesity*, vol. 16, pp. 938 – 944.
- Brand, M. D. and Nicholls, D. G. (2011), Assessing mitochondrial dysfunction in cells, *Biochemical Journal*, vol. 435, pp. 297 – 312.
- Brands, M., Hoeks, J., Sauerwine, H. P., Ackermans, M. T., Ouwendijk, M., Lammers, N. M., van der Plas, M. N., Schrauwen, P., Groen, A. K. and Serlie, M. J. (2011) Short-term increase of plasma free fatty acids does not interfere with intrinsic mitochondrial function in healthy young men, *Metabolism*, vol. 60, pp. 1398 – 1405.
- Brayer, R. M., Bagdassarian, C. K., Myers, S. A. and Breyer, M. D. (2001) Prostanoid receptors: subtypes and signalling, *Annual Review of Pharmacology and Toxicology*, vol. 41, pp. 661–690.
- Brehm, A., Krššák, M., Schmid, A. I., Waldhäusl, W. and Roden, M. (2010) Acute elevation of plasma lipids does not affect ATP synthesis in human skeletal muscle, *American Journal of Physiology – Endocrinology and Metabolism*, vol. 299, pp. E33 – E38.
- Brenna, T. J., Plourde, M., Stark, K. D., Jones, P. J. and Lin, Y. H. (2016) Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids, *The American Journal of Clinical Nutrition*, vol. 108, pp. 211 – 227.
- Brennan, F. M., Maini, R. N. and Feldmann, M. (1995) Cytokine expression in chronic inflammatory disease, *British Medical Bulletin*, vol. 51, pp. 368 – 384.
- Bullock, C., (2010), The Biochemistry of Alcohol Metabolism, *Biochemical Education*, vol. 18(2), pp. 62-66.
- Brox, J. H. and Nordøy, A. (1983) The effect of polyunsaturated fatty acids on endothelial cells and their production of prostacyclin, thromboxane and platelet inhibitor activity, *Thrombosis and Haemostasis*, vol. 4, pp. 762 – 767.
- Bulua, A. C., Simon, A., Maddipati, R., Pelletier, M., Park, H., Kim, K. Y., Sack, M. N., Kastner, D. L. and Siegel, R. M. (2011) Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS)*The Journal of Experimental Medicine*, vol. 208, pp. 519-533.

- Burke, J. E. and Dennis, E. A. (2009) Phospholipase A2 structure/function, mechanism, and signalling, *Journal of Lipid Research*, vol. 50, pp. S237 - S242.
- Byars, T. D., David, D. and Divers, T. J. (2003), Coagulation in the equine intensive care patient, *Clinical Techniques in Equine Practice*, vol. 2, pp. 178 – 187.
- Calder, P. C., Ahluwali, N., Albers, R., Bosco, N., Bourdet-Sicard, R., Haller, D., Holgate, S. T., Jonsson, L. S., Latulippe, M. E., Marcos A., Moreines, J., Rini, C. M., Muller, M., Pawelec, G., van Neerven, R. J. J., Watz, B. and Zhao, J. (2013) A consideration of biomarkers to be used for evaluation of inflammation in human nutritional studies, *British Journal of Nutrition*, vol. 109, pp. S1 – S34.
- Calder, P. C. (2012), Omega-3 polyunsaturated fatty acids and inflammation processes: nutrition or pharmacology? *British Journal of Clinical Pharmacology*, vol. 75, pp. 645 – 662.
- Calder, P. C. (2009a) Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale, *Biochimi*, vol. 91, pp. 791–795.
- Calder, P. C., Albers, R., Antoine, J. M., Blum, S., Bourdet-Sicard, R., Ferns, G. A., Folkerts, G., Friedmann, P. S., Frost, G. S., Gaurner, F., Lovik, M., Macfarlan, S., Meyer, P. D, Rabet, L. M., Serafini, M., van Eden, W., van Loo, J., Vas Dias, W., Vidry, S., Winklhofer-Roob, B. M, and Zhao, J. (2009b) Inflammatory disease processes and interactions with nutrition, *British Journal of Nutrition*, vol. 101, pp. S1 – S42.
- Calder, P. C. (2005) Polyunsaturated fatty acids and inflammation, *Biochemical Society Transactions*, vol. 33, pp. 423 – 427.
- Cambien, B., Bergmeier, W., Saffaripour, S., Mitchell, H. A. and Wagner, D. D. (2003), Antithrombotic activity of TNF- α , *The Journal of Clinical Investigation*, vol. 112, pp. 1589 – 1596.
- Cameron, A. J., Boyko, E. J., Sicree, R. A., Zimmett, P. Z., Soderberg, S., Alberti, K. G. M. M., Tuomilehto, J. Chitson, P. and Shaw, J. E. (2008), Central obesity as a precursor to the metabolic syndrome in the AusDiab study and Mauritius, *Obesity*, vol. 16, pp. 2707 – 2716.
- Caniuguir, A., Krause, B. J., Hernandez, C., Uauy, R. and Casanello (2016), Markers of early endothelial dysfunction in intrauterine growth restriction-derived human umbilical vein endothelial cells revealed by 2D-DIGE and mass spectrometry analyses, *Placenta*, vol. 41, pp. 14 – 26.
- Canty, T. G., Boyle, E. M., Farr, A., Morgan, E. N., Verrier, E. D. and Pohlman, T. H. (1999) Oxidative stress induces nf-kb nuclear translocation without degradation of I κ B α , *Circulation*, vol. 100, pp. II-361-II-364.
- Cao, P., Huang, G., Yang, Q., Guo, J., Su, Z. (2016), The effect of chitooligosaccharides on oleic acid-induced lipid accumulation in HepG2 cells, *Saudi Pharmaceutical Journal*, vol. 24, pp. 292 – 298.

Capozzi, M. E., McCollum, G. W., Cousins, D. B. and Penn, J. S. (2016) Linoleic Acid is a Diabetes-relevant Stimulator of Retinal Inflammation in Human Retinal Muller Cells and Microvascular Endothelial Cells, *Journal of Diabetes and Metabolism*, vol. 7, pp. 1 – 3.

Carina Paola Van Nieuwenhove, Victoria Terán and Silvia Nelina González, (2012), Conjugated Linoleic and Linolenic Acid Production by Bacteria: Development of Functional Foods, INTECH Open Access Publisher.

Carlier, H., Bernard, A. and Caselli, C. (1991) Digestion and absorption of polyunsaturated fatty acids, *Reproduction Nutrition Development*, vol. 31, pp. 475–500.

Carro, M., Buschiazzo, J., Ríos, G. L., Oresti, G. M. and Alberio, R. H. (2013) Linoleic acid stimulates neutral lipid accumulation in lipid droplets of maturing bovine oocytes, *Theriogenology*, vol. 79, pp. 687 – 694.

Casella, S., Giannetto, C., Fazio, F., Giudiec, E., and Piccione, G. (2009) Assessment of prothrombin time, activated partial thromboplastin time, and fibrinogen concentration on equine plasma samples following different storage conditions, *Journal of Veterinary Diagnostic Investigation*, vol. 21, pp. 674 – 678.

Cassar, K., Bachoo, P. and Brittenden, J. (2003), The role of platelets in peripheral vascular disease, *European Journal of Vascular and Endovascular Surgery*, vol. 25, pp. 6 – 15.

Ceballos-Picot, I., Nicole, A., Clement, M., Bourre, J. M. and Sinet, P. M. (1992) Age related changes in antioxidant enzymes and lipid peroxidation in brains of control and transgenic mice overexpressing copper-zinc superoxide dismutase, *Mutation Research*, vol. 275, pp. 281 – 293.

Chambaz, J., Guillouzo, A., Cardot, P., Pepin D. and Bereziat, G. (1986), Essential fatty acid uptake and esterification in primary culture of rat hepatocytes, *Biochimica et Biophysica Acta*, vol. 878, pp. 310 – 319.

Chavez, A. O., Kamath, S., Jani, R., Sharma, L. K., Monroy, A., Abdul-Ghani, M. A., Centonze, V. E., Sathyaranayana, P., Coletta, D. K., Jenkinson, C. P., Bai, Y., Folli, F., DeFronzo, R. A. and Tripathy, D. (2010) Effect of short-term free fatty acids elevation on mitochondrial function in skeletal muscle of healthy individuals, *Journal of Clinical Endocrinology and Metabolism*, vol. 95, pp. 422 – 429.

Chelombitko, M. A. (2018) Role of reactive oxygen species in inflammation: a minireview, *Moscow University Biological Sciences Bulletin*, vol. 73, pp. 199 – 202.

Chen, L., Yu, C. X., Song, B., Cai, W. Liu, C. and Guan, Q. B. (2018) Free fatty acids mediates human umbilical vein endothelial cells inflammation through toll-like receptor-4, *European Review for Medical and Pharmacological Sciences*, vol. 22, pp. 2421 – 2431.

Chen, X., Xun, K., Chen, L. and Wang, Y. (2009), TNF- α , a potent lipid metabolism regulator, *Cell Biochemistry and Function*, vol. 27, pp. 407-416.

Chen, X., Andresen, B. T., Hill, M., Zhang, J., Booth, F. and Zhang, C. (2008) Role of reactive oxygen species in tumor necrosis factor-alpha induced endothelial dysfunction, *Current Hypertension Reviews*, vol. 4, pp., 245 – 255.

Chen, Z., Bertin, R. and Froldi, G. (2013), EC₅₀ estimation of antioxidant activity in DPPH radical assay using several statistical programs, *Food Chemistry*, vol. 138, pp. 414 – 420.

Chin, S. F., Liu, W., Storkson, J. M., Ha, Y. L. and Pariza, M. W. (1992) Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens, *Journal of Food Composition and Analysis*, vol. 5, pp. 185- 197.

Chiu, J. J. and Chien, S. (2011), Effects of Disturbed Flow on Vascular Endothelium: Pathophysiological Basis and Clinical Perspectives, *Physiological Reviews*, vil. 91, pp. 1 – 106.

Choi, I., Yang, Y. I., Song, H. D., Lee, J. S., Kang, T., Sung, J. J., Yi, J. (2011) Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide dismutase with structurally disordered regions, *Biochimica et Biophysica Acta*, vol. 1812, pp. 41 – 48

Choque, B., Catheline, D., Rioux, V. and Legrand, P., (2014), Linoleic Acid: Between doubts and certainties, *Biochimie*, vol. 96, pp. 14 – 21.

Christopherson, P. W., Insalaco, T. A., van Santen, V. L., Livesey, L., Bourne, C., and Boudreaux, M. K., (2006) Characterization of the cDNA encoding α IIb and β 3 in normal horses and two horses with Glanzmann thrombasthenia, *Veterinary Pathology*, vol. 43, pp. 78 – 82.

Christy, W. W. (1998) Gas chromatography-mass spectrometry methods for structural analysis of fatty acids, *Lipids*, vol. 33, pp. 343 – 353

Circu, M. L. and Aw, T. Y. (2010) Reactive oxygen species, cellular redox systems, and apoptosis, *Free Radical Biology & Medicine*, vol, 48, pp. 749–762

Clauss, M., Grum, C. and Hatt, J. M. (2008) Polyunsaturated fatty acid content in adipose tissue in foregut and hindgut fermenting mammalian herbivores: A literature survey, *Mammalian Biology*, vol. 74, pp. 153 – 158.

Clay, H. B., Parl, A. K., MitchellS. L., Singh, L., Bell, L. N. and Murdock, D. G. (2016) Altering the mitochondrial fatty acid synthesis (mtfasii) pathway modulates cellular metabolic states and bioactive lipid profiles as revealed by metabolomic profiling, *PLoS One*, vol. 11, pp. 1 – 23.

Clayden, J., Greeves, N. and Warren, S. (2012), Organic Chemistry, 2nd Ed. Oxford, Oxford University Press.

Cnop, M., Hannaert, J. C., Hoorens, A., Eizirik, D. L. and Pipeleers, D. G. (2001) Inverse Relationship Between Cytotoxicity of Free Fatty Acids in Pancreatic Islet Cells and Cellular Triglyceride Accumulation, *Diabetes*, vol. 50, pp. 1771–1777.

Coates, J. (2000), Interpretation of Infrared Spectra, a practical approach, In: Mayer, R. A., (ed.) *Encyclopaedia of Analytical Chemistry*, Chichester, Wiley & Sons Ltd., pp. 1 – 23.

Cohen, T. (2012), Bounding out to work: Farmer ditches modern technology and turns to horses, *Mail online*, Available at: <http://www.dailymail.co.uk/news/article-2131846/Farmer-Robert-Sampson-Ringwood-farms-using-horses-traditional-way.html> [Accessed on the 4th June 17].

Collin, F. (2019) Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases, *International Journal of Molecular Sciences*, vol. 20, pp. 1 – 17.

Collins, J., Hanlon, A., More, S., Wall, P. and Duggan, V., (2010), Challenges and Solutions to Support Good Equine Welfare Practice in Ireland, University College Dublin Publishers, Available at: <http://www.ucd.ie/t4cms/UCD%20Equine%20Welfare-web%20secure.pdf> [Accessed on the 5th November 2015].

Cooper, B., (2015), *The Identification and Investigation of a Putative Anticoagulant associated with Fatal Equine Haemorrhaging*, Ph.D, Athlone Institute of Technology.

Cooper, A. d. (1997) Hepatic uptake of chylomicron remnants, *Journal of Lipid research*, vol. 38, pp. 2173 – 2192.

Corbally, A. and Quinn, K. (2012), Economic contribution of the sport horse industry to the irish economy, school of agriculture and food science, university college Dublin Available at <http://www.horseportireland.ie/wp-content/uploads/2014/01/Economic-Contributions-of-the-Sport-Horse-Industry-to-the-Irish-Economy4.pdf> [Accessed on the 27th October 2015].

Cox, D., Salvato, M. S. and Zapata, J. C. (2013) The Role of Platelets in Viral Hemorrhagic Fevers, *Journal of Bioterrorism & Biodefense*, s. 12, pp. 1 – 10.

Crowley, L. C., Marfell, B. J., Scott, A. P., Boughaba, J. A., Chojnowski, G., Christensen, M. E. and Waterhouse, N. J. (2016) Dead cert: measuring cell death, *Cold Spring Harbour Protocols*, vol. 12, pp. 1064 – 1072.

Cryer, A. and Bartlet, W. (1973), Studies on the adaption of rats to a diet high in glycerol, *International Journal of Biochemistry*, vol. 4, PP. 293 – 308.

Cury-Boventura, M. F., Pompeia, C. and Curi, R. (2005), Comparative toxicity of oleic acid and linoleic acid on Raji cells, *Nutrition* vol. 21, pp. 395-405.

Cury-Boventura, M. F., Pompeia, C. and Curi, R. (2004), Comparative toxicity of oleic acid and linoleic acid on Jurkat cells, *Clinical Nutrition*, vol. 23, pp. 721 – 732.

Czekanska, E. M., (2011), Assessment of cell proliferation with resazurin-based fluorescent dye, In: Stoddart, M. J., ed. *Mammalian Cell Viability, Methods and Protocols*, New York, USA, Springer Science + Business Media LLC.

Da Silva, M. S., Julien, P., Bilodeau, J. F., Barbier, O. and Rudkowska, I. (2017) Trans fatty acids suppress TNF- α -induced inflammatory gene expression in endothelial (HUVEC) and hepatocellular carcinoma (Hepg2) cells, *Lipids*, vol. 52, pp. 315 – 325.

Da Silva, Jr. E. P., Nachbar, R. T., Levada-Pires, A. C. Hirabara, S. M. and Lambertucci, R. H. (2016) Omega-3 fatty acids differentially modulate enzymatic anti-oxidant systems in skeletal muscle cells, *Cell Stress and Chaperones*, vol. 21, pp. 87–95.

Daemen S. Van Zandvoort, M. A. M.J., Parekh, S. H., and Hesselink, M. K. C. (2015), Microscopy tools for the investigation of intracellular lipid storage and dynamics, *Molecular Metabolism*, vol. 5, pp. 153 – 163.

Das, U. (2011) Essential fatty acids enhance free radical generation and lipid peroxidation to induce apoptosis of tumor cells, *Clinical Lipidology*, vol. 6, pp. 463-489.

Davda, R. K., Stepniakowski, K. T., Lu, G., Ullian, M. E., Goodfriend, T. L. and Egan, B., M. (1995) Oleic acid inhibits endothelial nitric oxide synthase by a protein kinase c-independent mechanism, *Hypertension*, vol. 26, pp. 764 – 770.

Dawson, P. (2016), The demise of the Irish Sport Horse, *Horse Network*, available at: <http://horsenetwork.com/2016/06/demise-irish-sport-horse/> [Accessed on the 6th June 17].

De Hoffmann, E. and Stroobent, V. (2007) *Mass Spectrometry, principles and applications*, 3rd Ed., John Wiley & Sons Ltd, Chichester, UK.

De Sousa Araujo, S., Casimiro Fernandes, T. C., Cardona, Y. T., De Almeide, P. M., Marin-Morales, M. A., Dos Santos, A. V., Perrelli Randau, K., Benko-Iseppon, A. M. and Brasileiro-Vidal, A. C., (2015), Cytotoxic and genotoxic effects of ethanolic extract of *Euphorbia hyssopifolia L.* on HepG2 cells, *Journal of Ethnopharmacology*, vol. 170, pp. 16 – 19.

Di Nunzio, M., Valli, V and Bordoni, A., (2011), Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells, *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 85, pp. 121 – 127.

Di Pasquale, M. G. (2009) The essentials of essential fatty acids, *Journal of Dietary Supplements*, vol. 6, pp. 143 – 161.

Diaz, G., Melis, M., Batetta, B., Angius, F. and Falchi, A. M. (2008) Hydrophobic characterization of intracellular lipids in situ by Nile Red red/yellow emission ratio, *Micron*, vol. 39, pp. 819-824.

Divers, T. J., (2015), The equine liver in health and disease, *American Association of Equine Practitioners Proceedings*, vol. 61, pp. 66-103.

Dixon, J. L. and Ginsberg, H. N. (1993) Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells, *Journal of Lipid Research*, vol. 34, pp. 167-178.

Dhindsa, S., Ghanim, H. and Dandona, P. (2015) Nonesterified fatty acids, albumin, and platelet aggregation, *Diabetes*, vol. 64, pp. 703 – 705.

Dokko, R. C., Cho, H. B. S. and Chung, B. H. (1998), Cellular uptake of stearic, oleic, linoleic, and linolenic acid and their effects on synthesis and secretion of lipids in HepG2 cells, *The international Journal of Biochemistry and Cell Biology*, vo. 30, pp. 65 – 76.

Donkin, S. S., (2008), Glycerol from biodiesel production: the new corn for dairy cattle, *Revista Brasileira de Zootecnia*, vol. 37, pp. 280-286.

Dröge, W. (2002) Free radicals in the physiological control of cell function, *Physiological Review*, vol. 82, pp. 47–95.

Dunuwila, D. D. and Berglund, K. A. (1997), ATR-FTIR spectroscopy for in situ measurement of supersaturation, *Journal of Crystal Growth*, vol. 179, pp. 185 – 193.

Durandt, C., van Vollenstee, F. A., Dessels, C., Kallmeyer, K., de Villiers, D., Murdoch, C., Potgieter, M. and Pepper, M. S. (2016) Novel flow cytometric approach for the detection of adipocyte sub-populations during adipogenesis, *Journal of Lipid Research*, vol. 57, pp. 729 – 742.

Eaton, D. L. and Gilbert, S. G. (2008) ‘Principles of Toxicology’, in Klaassen, C. D., *Cararett and Doulls Toxicology, The Basic Science of Poisons*, 7th ed., McGraw-Hill, USA, pp 11 - 43.

Eaton, R. P., Berman, M. and Steinberg, (1969) Kinetic studies of plasma free fatty acid and triglyceride metabolism in man, *The Journal of Clinical Investigation*, vol. 48, pp. 1560 – 1579.

Eckel, R. H., Alberti, K. G. M. M., Grundy, S. M. and Zimmet, P. Z. (2010) The metabolic syndrome, *The Lancet*, vol. 375, pp. 181 – 183.

Eder, K., Slomma, N. and Becker, K. (2002) *Trans-10,cis-12 Conjugated linoleic acid suppresses the desaturation of linoleic and α-linolenic acids in Hepg2 cells*, *American Journal of Nutritional Sciences*, vol. 132, pp. 1115 – 1121.

Edirisinghe, I., McCormack-Hallam, K. and Kappagoda, C. T. (2006), Effects of fatty acids on endothelium-dependent relaxation in the rabbit aorta, *Clinical Science*, vol. 111, pp. 145 – 151.

Epstein, K.L. (2014), Coagulopathies in horses, *Veterinary Clinics: Equine Practice*, vol. 30, pp. 437 - 452.

Escobar, M. L., Echeverría, O. M. and Vázquez-Nin, G. H. (2015x` Necrosis as programmed cell death, In; Ntuli, T. *Cell Death, Autophagy, Apoptosis and Necrosis*, IntechOpen, pp. 419 – 434.

Esmon, C. T. (1999) Possible involvement of cytokines in diffuse intravascular coagulation and thrombosis, *Bailliere's Clinical Haematology*, vol. 12, pp. 343 -359.

Esmon, C. T. (2000) Does inflammation contribute to thrombotic events? *Haemostasis*, vol. 30, pp. 34 – 40.

European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM), (2010), Scientific Opinion on the abiotic risks for public and animal health of glycerine as co-product from the biodiesel production from Category 1 animal by-products (ABP) and vegetable oils. *EFSA Journal*, vol. 8, pp. 1 – 22.

Fahy, E., Subramaniam, S., Brown, A. H., Glass, C. K., Merrill, A. H., Murphy, R. C., Raetz, C. R. H., Russell, D. W., Seyama, Y., Shaw, W., Shimizu, T., Spence, F., Van Meer, G., VanNieuwenhze, M. S., White, S. H., Witztum, J. L. and Dennis, E. A., (2005), A comprehensive classification system for lipids, *Journal of Lipid Research*, vol. 46, pp. 839 – 862.

Fajardo, V. A., Mikhaeil, J. S., Leveille, C. F., Saint, C. and LeBlanc, P. J. (2017) Cardiolipin content, linoleic acid composition, and tafazzin expression in response to skeletal muscle overload and unload stimuli, *Nature Scientific Reports*, vol. 7, pp. 1 – 9.

Feige, K. (2009), Coagulopathies, In: Robinson, N. E. and Sprayberry, K. A., *Current Therapy in Equine Medicine*, 6th ed., Missouri USA, Saunders Elsevier, pp. 227 – 231.

Feldstein, A. E., Werneburg, N. W., Canbay, A., Guicciardi, M. Bronk, S. F., Rydzewski, R., Burgart, L J. and Gores, G. J., (2004), Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway, *Hepatology*, vol. 40, pp. 185 – 194.

Felter, S. P., Vassallo, J. D., Carlton, B. D. and Daston, G. P. (2006), A safety assessment of coumarin taking into account species-specificity of toxicokinetics, *Food and Chemical Toxicology*, vol. 44, pp. 462 – 475.

Fischer, L. J., Erfle, J. D., Lodge, G. A. and Sauer, F. D., (1973), Effects of propylene glycol or glycerol supplementation on the diet of dairy cows on feed intake, milk yield and composition, and incidence of ketosis, *Canadian Journal of Animal Science*, vol. 53, pp. 289 – 296.

Fisher, R. A., Robertson, S. M., and Olson, M. S. (1987), Stimulation of glycogenolysis and vasoconstriction in the perfused rat liver by the thromboxane A₂ analogue U-46619, *Journal of Biological Chemistry*, vol. 262, pp. 4631-4638.

Food and Agriculture Organisation of the United Nations Statistics Division, (2015), FAOSTAT, Available at: <http://faostat3.fao.org/download/Q/QA/E> [Accessed on the 27th October 2015].

- Forfang, K., Zimmermann, B., Kosa, G., Kohler, A., Shapaval, V. (2017), FTIR spectroscopy for evaluation and monitoring of lipid extraction efficiency for *Oleaginous* fungi, *Public Library of Science*, vol. 12, pp. 1 – 17.
- Forrester, S. J., Kikuchi, D. S., Hernandes, M. S., Xu, Q., Griendling, K. K. (2018) Reactive oxygen species in metabolic and inflammatory signalling, *Circulation Research*, vol. 122, pp. 877-902.
- Frankel, E. N., (2005), *Lipid Oxidation*, 2nd ed., Cambridge UK, Woodhead Publishing Ltd, pp. 1.
- Franzoni, M., Cattaneo, I., Ene-Iordache, B., Oldani, A., Righettini, P. and Remuzz, A. (2016), Design of a cone-and-plate device for controlled realistic shear stress stimulation on endothelial cell monolayers, *Cytotechnology*, vol. 68, 1885 – 1896.
- Frayn, K. N., Williams, C. M. and Arner, P. (1996) Are increased plasma non-esterified fatty acid concentrations a risk marker for coronary heart disease and other chronic diseases?, *Clinical Science*, vol. 90, pp. 243 – 253.
- Frezza, C. Cipolat, S and Scorrano, L. (2006), Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts, *Nature Methods*, vo. 2, pp. 287 – 295.
- Fritzsche, K. L. (2015), The science of fatty acids and inflammation, *American Society for Nutrition, Advances in Nutrition*, vol. 6, pp. 293S – 301S.
- Fritzsche, K. L. (2008), Too much linoleic acid promotes inflammation—doesn't it? *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 79, pp. 173 – 175.
- Fry, M. M., Walker, N. J., Blevins, G. M., Magdesian, K. G. and Tablin, F. (2005), Platelet function defect in Thoroughbred filly, *Journal of Veterinary Internal Medicine*, vol. 19, pp. 359 – 362.
- Furuhashi, M. and Hotamisligil, G. S. (2008) Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets, *Nature Reviews Drug Discovery*, vol. 7, pp. 2 – 30.
- Gallo, M. A. (2008) 'History and Scope of Toxicology ', in Klaassen, C. D., *Cararett and Doulls Toxicology ,The Basic Science of Poisons*, 7th ed., McGraw-Hill, USA, pp 3 - 10.
- Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M., Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M. J. M., Bianchi, K., Blagosklonny, M. V., Blomgren, K., Borner, C., Boya, P., Brenner, C., Campanella, M., Candi, E., Carmona-Gutierrez, D., Ceconni, F., Chan, F. K., Chandel, N. S., Cheng, E. H., Chipuk, J. E., Cidlowski, J. A., Ciechanover, A., Cohen, G. M., Conrad, M., Cubillos-Ruiz, J. R., Czabotar, P. E., D'Angiolella, V., Dawson, T. M., Dawson, V. L., De Laurenzi, V., De Maria, R., Debatin, K. M., DeBerardinis, R. J., Deshmukh, M., Di Daniele, N., Di Virgilio, F., Dixit, V. M., Dixon, S. J., Duckett, C. S., Dynlacht, B. D., El-Deiry, W. S., Elrod, J. W., Fimia, G. M., Fulda, S., García-Sáez, A. J., Garg, A. D., Garrido, C., Gavathiotis,

E., Golstein, P., Gottlieb, E., Green, D. R., Greene, L. A., Gronemeyer, H., Gross, A., Hajnoczky, G., Hardwick, J. M., Harris, I. S., Hengartner, M. O., Hetz, C., Ichijo, H., Jäättelä, M., Joseph, B., Jost, P. J., Juin, P. P., Kaiser, W. J., Karin, M., Kaufmann, T., Keep, O., Kimchi, A., Kitsis, R. N., Klionsky, D. J., Knight, R. A., Kumar, S., Lee, S. W., Lemasters, J. J., Levine, B., Linkermann, A., Lipton, S. A., Lockshin, R. A., López-Otín, C., Lowe, S. W., Luedde, T., Lugli, E., MacFarlane, M., Madeo, F., Malewicz, M., Malorni, W., Manic, G., Marine, J. C., Martin, S. J., Martinou, J. C., Medema, J. P., Mehlen, P., Meier, P., Melino, S., Miao, E. A., Molkentin, J. D., Moll, U. M., Muñoz-Pinedo, C., Nagata, S., Nuñez, G., Oberst, A., Oren, M., Overholtzer, M., Pagano, M., Panaretakis, T., Pasparakis, M., Penninger, J. M., Pereira, D. M., Pervaiz, S., Peter, M. E., Piacentini, M., Pinton, P., Prehn, J. H. M., Puthalakath, H., Rabinovich, G. A., Rehm, M., Rizzuto, R., Rodrigues, C. M. P., Rubinsztein, D. C., Rudel, T., Ryan, K. M., Sayan, E., Scorrano, L., Shao, F., Shi, Y., Silke, J., Simon, H. U., Sistigu, A., Stockwell, B. R., Strasser, A., Szabadkai, G., Tait, S. W. G., Tang, D., Tavernarakis, N., Thorburn, A., Tsujimoto, Y., Turk, B., Vanden Berghe, T., Vandebaele, P., Vander Heiden, M. G., Villunger, A., Virgin, H. W., Vousden, K. H., Vucic, D., Wagner, E. F., Walczak, H., Wallach, D., Wang, Y., Wells, J. A., Wood, W., Yuan, J., Zakeri, Z., Zhivotovsky, B., Zitvogel, L., Melino, G., Kroemer, G. (2018) Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018, *Cell Death & Differentiation*, vol. 25, pp. 486–541.

Ghosh, A. Gao, L., Thakur, A., Siu, P. M., Lai, C. W. (2017) Role of free fatty acids in endothelial dysfunction, *Journal of Biomedical Science*, vol. 24, pp. 1 – 15.

Gibbons, A., (2014), Racing for Disaster: Breeding thoroughbreds for speed may harm their health, *Science*, vol. 344, pp. 1213-1214.

Gloire, G., Legrand-Poels, S. and Piette, J. (2006) NF-κB activation by reactive oxygen species: Fifteen years later, *Biochemical Pharmacology*, vol. 72, pp. 1493 – 1501.

Golan, D. E., Tashjian, A. H. & Armstrong, E. J., (2012) Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy. Third ed. s.l.:Lippincott, Williams and Wilkins.

Gomaa, N., Koeller, G. and Schusser, G. F. (2009) Triglycerides, free fatty acids and total bilirubin, in horses with left ventral colon impaction, *Pferdheilkunde Equine Medicine*, vol. 2, pp 137-140.

Gong, J., Sun, Z., Wu, L., Xu, W., Schieber, N., Xu, D., Shui, G., Yang, H., Parton, R. G. and Li, P. (2011) Fsp27 promotes lipid droplet growth by lipid exchange and transfer at lipid droplet contact sites, *The Journal of Cell Biology*, vol. 195, pp. 953 – 963.

Goré, J., Hoinard, C., Couet, C. (1994) Linoleic Acid Uptake by Isolated Enterocytes: Influence of a-Linolenic Acid on Absorption, *Lipids*, vol. 29, pp. 701-706.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. (2003) Comparing protein abundance and mRNA expression levels on a genomic scale, *Genome Biology*, vol. 4, pp. 1 – 9.

- Greenspan, P., Mayer, E. P., and Fowler, S. D. (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets, *The Journal of Cell Biology*, vol. 100, pp. 965 – 973.
- Geffre, A., Grollier, S., Hanot, C., Vergez, F., Trumel, C. and Braum, J. P. (2010) Canine reference intervals for coagulation markers using the STA Satellite and the STA-R Evolution analyzers, *Journal of Veterinary Diagnostic Investigation*, vol. 22, pp. 690 – 695.
- Grenon, S. M., Aguado-Zuniga, J., Hatton, J. P., Owens, C. D., Conte, M. S., Hughes-Fulford, M. (2012) Effects of fatty acids on endothelial cells: inflammation and monocyte adhesion, *Journal of Surgical Research*, vol. 177, pp. 1 – 16.
- Griesmacher, A., Weigel, G., Schriener, W. and Muller, M. M. (1989) Thromboxane A₂ generation by human umbilical endothelial cells, *Thrombosis Research*, vol. 6, pp. 611 – 623.
- Gu, Z., Wu, J., Wang, S., Suburu, J., Chen, H., Thomas, M. J., Shi, L., Edwards, I. J., Berquin, I., M. and Chen, Y. Q. (2013), Polyunsaturated fatty acids affect the localization and signalling of PIP3/AKT in prostate cancer cells, *Carcinogenesis*, vol. 34, pp. 1968 – 1975.
- Guillen M. D. and Cabo, N. (1997), Characterisation of edible oils and lard by Fourier Transform Infrared Spectroscopy. Relationships between composition and frequency of concrete bands in the fingerprinting region, *Journal of the American Oil Chemists Society*, vol. 74, pp. 1281 – 1286.
- Guo, Y., Cordes K. R., Farese, Jr, R. V. and Walther, T. C. (2009) Lipid droplets at a glance, *Journal of Cell Science*, vol. 122, pp. 749 – 742.
- Gutierrez-Ruiz, M. C., Quiroz, S. C., Souza, V., Bucio, L., Hernandez, E., Olivares, I. P., Llorente, L., Vargas-Vorackova, F., and Kershenobich, D. (1998) Cytokines, growth factors, and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde, and LPS, *Toxicology*, vol. 134, pp. 197 – 207.
- Haberkant, P . and Holthuis, J. C. M. (2014) Fat & fabulous: Bifunctional lipids in the spotlight, *Biochimica et Biophysica Acta*, VOL. 1841, PP. 1022 – 1030.
- Hallebeek, J. M. & Beynen, A. C., (2002), Dietary fats and lipid metabolism in relation to equine health, performance and disease, s.l.:Department of Nutrition, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
- Halim, I., (2008) *Essential Revision Notes in Surgery for Medical Students*, Cheshire: Cambridge University Press.
- Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides, *Proceedings of the National Academy of Sciences*, vol. 72, pp. 2994-2998.
- Hanel, A. M., Schuttel, S., Gelb, M. H. (1993) Processive interfacial catalysis by mammalian 85-kilodalton phospholipase A₂ enzymes on product-containing vesicles:

application to the determination of substrate preferences, *Biochemistry*, vol. 32, pp. 5949-5958.

Hartnell, W., Hodgett, A. and O'Keeffe, N., (2013), Analysis of the Economic Impact of the Irish Thoroughbred Industry , The Irish Thoroughbred Breeders Association, Available at <http://www.itba.info/wp-content/uploads/2013/07/Dukes-Report-II-October-2013-Update.pdf> [Accessed on the 27th October 2015].

Hashemi, H. F. and Goodman, J. M., (2015), The life cycle of the lipid droplet, *Current Opinion in Cell Biology*, vol. 33, pp. 119-124.

Hata, A. N. and Breyer, R. M. (2004) Pharmacology and signalling of prostaglandin receptors: multiple roles in inflammation and immune modulation, *Pharmacology & Therapeutics*, vol. 103, pp. 147– 166.

Hatanaka, E., Dermargos, A., Hirat, A. E., Ramirez Vinolo, M. A., Carpinelli, A. R., Newsholme, P., Armelin, H. A. and Curi, R. (2013) Oleic, linoleic and linolenic acids increase ROS production by fibroblasts via NADPH oxidase activation, *PLoS ONE*, vol. 8, pp. 1 – 8.

Hatanaka, E., Levada-Pires, A. C., Pithon-Curi, T. C. and Curi, R. (2006) Systematic study on ROS production induced by oleic, linoleic, and γ -linolenic acids in human and rat neutrophils, *Free Radical Biology & Medicine*, vol. 41, pp. 1124–1132.

Hausberger, M., Roche, H., Henry, S. and Visser, E. K., (2008), A review of the human-horse relationship, *Applied Animal Behaviour Science*, vol. 109 (1), pp. 1-24.

Hawkins, R. A., Sangster, K. and Arends, M. J., (1998), Apoptotic death of pancreatic cancer cells induced by polyunsaturated fatty acids varies with double bond number and involves an oxidative mechanism, *Journal of Pathology*, vol. 185, pp. 61 – 70.

Hennessey, A. A., Ross, P. R., Fitzgerald, G. F. and Stanton, C. (2016), Sources and Bioactive Properties of Conjugated Dietary Fatty Acids, *Lipids*, pp. 1 – 21.

Hennig, B., Lei, W., Arzuaga, X., Das Ghosh, D., Saraswathi, V., Toborek, M. (2006) *The Journal of Nutritional Biochemistry*, vol. 17, pp.766 – 772.

Hennig, B., Torborek, M., Cader, A. A. and Decker, E. A. (1994) Nutrition, endothelial cell metabolism, and atherosclerosis, *Critical Reviews in Food Science and Nutrition*, vol. 34, pp. 253 – 282.

Henson, P. M. (2005) Dampening inflammation, *Nature Immunology*, vol. 6, pp. 1179 – 1181.

Hesse, D., Jaschke, A., Chung, B. and Schurmann, A. (2013) Trans-Golgi proteins participate in the control of lipid droplet and chylomicron formation, *Bioscience Reports*, vol. 33, pp. 1 – 9.

Heuwieser, W., Biesel, M. and Grunert, E. (1989) Physiological coagulation profile of dairy cattle, *Journal of Veterinary Medicine*, vol. 36, pp. 24 – 31.

- Higgs, E. A., Moncada, S. and Vane, J. R. (1986) Prostaglandins and thromboxanes from fatty acids, *Progress in Lipid Research*, vol. 25, pp. 5 – 11.
- Hill, C. E., Myers, J. P. and Vandenberg, L. N. (2018) Non-monotonic dose–response curves occur in dose ranges that are relevant to regulatory decision-making, *Dose-Response: An International Journal*, pp. 1 – 4.
- Hiltunen, J. K., Schonauer, M. S., Autio, K. J., Mittelmeier, T. M., Kastaniotis, A. J. and Dieckmann, C. L., (2009), Mitochondrial fatty acid synthesis type II: more than just fatty acids, *Journal of Biological Chemistry*, vol. 284, pp. 9011 – 9015.
- Hinchcliff, K. W., Goer, R. J. and Kaneps, A. J., (2008), *Equine Exercise Physiology, the Science of Exercise in the Athletic Horse*, Edinburgh, Saunders/Elsevier.
- Hodgson, D. R., McKeever, K. H. and McGowan, C. M. (2014), *The Athletic Horse, Principles and Practice of Equine Sports Medicine*, 2nd ed., St. Louis, Missouri USA, Elsevier, pp. 24.
- Holbrook, T. C., Tipton, T. and McFarlane, D. (2015) Neutrophil and cytokine dysregulation in hyperinsulinemic obese horses, *Veterinary Immunology and Immunopathology*, vol. 145, pp. 283 – 289.
- Homma, T. and Fujii, J. (2019) Oxidative stress and dysfunction of the intracellular proteolytic machinery: a pathological hallmark of nonalcoholic fatty liver disease: In: eds: Watson, R. R. and Preedy, V. R. *Dietary Interventions in Liver Disease, Foods, Nutrients, and Dietary Supplements*, Cambridge, USA, Academic Press, pp. 59-70.
- Horrobin, D. F. (1993) Fatty acid metabolism in health and disease: the role of Δ6 desaturase, *American Journal of Clinical Nutrition*, vol. 57, pp. 732S – 737S.
- Horvath, S. I. and Daum, G. (2013), Lipids of Mitochondria, *Research in Lipid Research*, issue 52, pp. 590-614.
- Høstmark, A.T (1995) Serum fatty acid/ albumin molar ratio and the risk of diseases, *Medical Hypotheses* vol. 44, pp. 539 – 541.
- Hu, S., Luo, X., Wan, C. and Li, Y. (2012), Characterisation of Crude Glycerol from Biodiesel Plants, *Journal of Agriculture and Food Chemistry*, vol. 60, pp. 5915 – 5921.
- Huang, C. J., McAllister, M. J., Slusher, A. L., Webb, H. E., Mock, T. J. and Acevedo, E. O. (2015) Obesity-related oxidative stress: the impact of physical activity and diet manipulation, *Sports Medicine*, vol. 1, pp. 1 – 12.
- Hulsman, M., Van Dooren, E. and Holvoet, P. (2012), Mitochondrial reactive oxygen species and risk of atherosclerosis, *Current Atherosclerosis Reports*, vol. 14, pp. 264 – 276.
- Hunt, J. A., Merritt, J. E., MacDermot, J. and Keen, M. (1992) Characterization of the thromboxane receptor mediating prostacyclin release from cultured endothelial cells, *Biochemical Pharmacology*, vol. 43, pp. 1747-1752.

Hwang, L. C., Tsai, C. H. and Chen, T. H. (2005), Overweight and Obesity-related Metabolic Disorders in Hospital Employees, *Journal of the Formosan Medical Association*, vol. 105, pp. 56 – 63.

Ibarguren, M., Lopez, D. J. and Escriba, P. V., (2014) The effect of natural and synthetic fatty acids on membrane structure, microdomain organization, cellular functions and human health, *Biochimica et Biophysica Acta*, vol. 1838, pp. 1518– 1528.

Ighodaro, O. M. and Akinloye, O. A. (2018), First line defence antioxidants-superoxide dismutase (SOD), catalase(CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid, *Alexandria Journal of Medicine*, vol. 54, pp. 287 – 293.

Imaizumi, T., Itaya, H., Fujita, K., Kudoh, D., Kudoh, S., Mori , K., Fujimoto, K., Matsumiya, T., Yoshida, H. and Satoh, K. (2000) Expression of tumor necrosis factor-a in cultured human endothelial cells stimulated with lipopolysaccharide or interleukin-1 α , *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, pp. 410 – 415.

Incalza, M. A., D'Oria, R., Natalicchio, A., Perrini, S., Laviola, L. and Giorgino, F. (2018) Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases, *Vascular Pharmacology*, vol. 100, pp. 1 – 19.

Innes, J. K. and Calder P. C., (2018) Omega-6 fatty acids and inflammation, *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 132, pp. 41 – 48.

Inoguchi, T., Li, P., Umeda, F., Yu, H. Y., Kakimoto, M., Imamura, M., Aoki, T., Etoh, T., Hashimoto, T., Naruse, M., Sano, H., Utsumi, H. and Nawata, (2000), High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase c-dependent activation of NAD(P)H oxidase in cultured vascular cells, *Diabetes*, vol. 49, pp. 1939 – 1945.

Ioannidis, J. P. A. (2007) Limitations are not properly acknowledged in the scientific literature, *Journal of Clinical Epidemiology*, vol. 60, pp. 324 – 329.

Ipsen, D. H., Lykkesfeldt. and Tveden-Nyborg, P. (2018) Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease, *Cellular and Molecular Life Sciences*, vol. 75, pp. 3313 – 3327.

Iqbal, J. and Hussain, M. M., (2009), Intestinal lipid absorption, *The American Journal of Physiology – Endocrinology and Metabolism*, vol. 296, pp. 1183 – 1194.

Irish Equine Centre, (2015), Second OIE Reference Laboratory, Irish Equine Centre, Available at:

<http://www.irishequinecentre.ie/NewsAndResearch/NewOrResearchArticle?title=OIERefLab> [Downloaded on the 5th October 2015].

Ishizuka, T., Kawakami, M., Hidaka, T., Matsuki, Y., Takamizawa, M., Suzuki, K., Kurita, A. and Nakamura, H. (1998) Stimulation with thromboxane A₂ (TXA₂) receptor agonist enhances ICAM-1, VCAM-1 or ELAM-1 expression by human

vascular endothelial cells, *Clinical and Experimental Immunology*, vol. 112, pp. 464 – 470.

Iuchi, K., Ema, M., Suzuki, M., Yokoyama, C. and Hisatomi, H. (2019) Oxidized unsaturated fatty acids induce apoptotic cell death in cultured cells, *Molecular Medicine Reports*, vol. 19, pp. 2767 – 2773.

Jaishy, B. and Abel, E. D. (2016) Lipid, lysosomes and autophagy, *Journal of Lipid Research*, vol. 57, pp. 1619 – 1635.

Jambunatha, S., Yin, J., Kahn, W., Tamori, Y. and Puri, V (2011) FSP27 promotes lipid droplet clustering and then fusion to regulate triglyceride accumulation, *PLoS ONE*, vol. 6, pp. 1 – 12.

James, M. J., Gibson, R. A. and Cleland, I. g. (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production, *American Journal of Clinical Nutrition*, vol. 71, pp. 343S – 348S.

Johnson, G. H. and Fritzsche, K. (2012) Effect of dietary linoleic acid on markers of inflammation in healthy persons: a systematic review of randomized controlled trials, *Journal of the Academy of Nutrition and Dietetics*, vol. 112, pp. 1029-1041.

Josephs, S. F., Ichim, T. E., Prince, S. M., Kesari, S., Marincola, F. M., Escobedo, A. R. and Jafri, A. (2018) Unleashing endogenous TNF-alpha as a cancer immunotherapeutic, *Journal of Translational Medicine*, vol. 16, pp. 1 – 8.

Kabashima, K., Murata, T., Tanaka, H., Matsuoka, T., Sakata, D., Yoshida, N., Katagiri, K., Kinashi, T., Tanaka, T., Miyasaka, M., Nagai, H., Ushikubi, F. & Narumiya, S. (2003) Thromboxane A2 modulates interaction of dendritic cells and T cells and regulates acquired immunity, *Nature Immunology*, vol. 4., pp. 694 – 701.

Kale, J., Osterlund, E. J. and Andrews, D. W. (2018) BCL-2 family proteins: changing partners in the dance towards death, *Cell Death and Differentiation*, vol. 25, pp. 65– 80.

Kao, L. S. and Green, C. E. (2008) Analysis of Variance: is there a difference in means and what does it mean? *Journal of Surgical Research*, vol. 144. Pp. 158 – 170.

Karim, S., Habib, A., Le'vy-Toledano, S. and Maclouf, J. (1996) Cyclooxygenases-1 and -2 of Endothelial Cells Utilize Exogenous or Endogenous Arachidonic Acid for Transcellular Production of Thromboxane, *The Journal of Biological Chemistry*, vol. 271, pp. 12042–12048.

Karpe, F. Dickmann, J. R., and Frayn, K. N. (2011), Fatty Acids, Obesity, and Insulin Resistance: Time for a Re-evaluation, *Diabetes*, vol. 60, pp. 2441 – 2449.

Kastaniotis, A. J., Autio, K. J., Kerätär, J. M., Monteuijs, G., Mäkelä, A. M., Nair, R. R., Pietikäinen, L. P., Shvetsova, A., Chen, Z. and Hiltunen, J. K. (2017) Mitochondrial fatty acid synthesis, fatty acids and mitochondrial physiology, *Biochimica et Biophysica Acta*, vol. 1862, pp. 39–48.

- Katagiri, H., Ito, Y., Ishii, K., Hayashi, I., Suematsu, M., Yamashina, S., Murata, T., Narumiya, S., Kakita, A., Majima, M. (2004) Role of thromboxane derived from cox-1 and -2 in hepatic microcirculatory dysfunction during endotoxemia in mice, *Hepatology*, vol. 39, pp. 139–150.
- Kawabe, J., Ushikubi, F. and Hasebe, N. (2010) Prostacyclin in Vascular Diseases, Recent Insights and Future Perspectives, *Circulation Journal*, vol. 74, pp. 836 – 843.
- Kern, P. A., Saghizadeh, M., Ong, J. M., Bosch, R. J., Deem, R. and Simsolo, R. B. (1995) The expression of tumor necrosis factor in human adipose tissue, *Journal of Clinical Investigation*, vol. 95, pp. 2111 – 2119.
- Kerr, B. J., Shurson, G. C., Johnston, L. J. and Dozier, W. A. (2011), Utilisation of Crude Glycerin in Nonruminants, *Biodiesel- Quality, Emissions and By-Products*, Dr. Gisela Montero (Ed.), ISBN: 978- 953-307-784-0, InTech, Available from: <http://www.intechopen.com/books/biodiesel-quality-emissions-and-byproducts/utilization-of-crude-glycerin-in-nonruminants>. [Accessed on the 10th November 2016].
- Kerr, B. J., Webber, T. E., Dozier, W. A. and Kidd, M. T. (2009), Digestible and metabolisable energy content of crude glycerine originating from different sources in nursery pigs, *American Society of Animal Science*, vol. 87, pp. 4042 – 4049.
- Khanapure, S. P., Garvey, D. S., Janero, D. R. and Letts, L. G. (2007) Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers, *Current Topics in Medicinal Chemistry*, Vol. 7, PP. 311-340.
- Kim, J. H., Kang, S. I., Shin, H. S., Yoon, S. A., Kang, S. W., Ko, H. C., and Kim, S. J. (2013), Sasa quelpaertensis and p-Coumaric Acid Attenuate Oleic Acid-Induced Lipid Accumulation in HepG2 Cells, *Bioscience, Biotechnology and Biochemistry*, vol. 77, pp. 1595 – 1598.
- Kim, J. Montagnani, M., Chandrasekran, S. and Quon, M. J. (2012), Role of lipotoxicity in endothelial cell dysfunction, *Heart Failure Clinics*, vol. 8, pp. 589 – 607.
- Kim, C., Kim, J. Y. and Kim, J. H. (2008) Cytosolic phospholipase A2, lipoxygenase metabolites, and reactive oxygen species, *BMB reports*, vol. 41, pp. 555-559.
- Kim, Y. J. Nakatomi, R., Akagi, T., Hashikawa, T. and Takahashi, R. (2005) Unsaturated fatty acids induce cytotoxic aggregate formation of amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutants, *The Journal of Biological Chemistry*, vol. 280, pp. 21515–21521.
- Kinnunen, S., Atalay, M., Hyppä, S., Lehmuskero, A., Hänninen, O. and Oksala, N. (2005) Effects of prolonged exercise on oxidative stress and antioxidant defense in endurance horse, *Journal of Sports Science and Medicine*, vol. 4, pp. 415 – 421.
- Kirpitch, I. A., Miller, M. E., Cave, M. C., Joshi-Barve, S. and McClain, C. J. (2016) Alcoholic liver disease: update on the role of dietary fat, *Biomolecules*, vol. 6, pp. 1 – 17.

- Kirschvink, N., Moffarts, B. and Lekeux, P. (2008) The oxidant/antioxidant equilibrium in horses, *The Veterinary Journal*, vol. 177, pp. 178–191.
- Kocherova, I., Bryja, A., Mozdziak, P., Volponi, A. A., Dyszkiewicz-Konwinska, M., Piotrowska-Kempisty, H., Antosil, P., Bukowska, D., Bruska, M., Izyczki, D., Zabel, M., Nowicki, M. and Kempisty, B. (2019), Human Umbilical Vein Endothelial Cells (HUVECs) Co-Culture with Osteogenic Cells: From Molecular Communication to Engineering Prevascularised Bone Grafts, *Journal of Clinical Medicine*, vol. 8, pp. 1 – 21.
- Kohjima, M., Enjoji, M., Higuchi, N., Kato, M., Kotoh, K., Nakashima, M. and Nakamura, M. (2009) The effects of unsaturated fatty acids on lipid metabolism in HepG2 cells, *In Vitro Cellular & Developmental Biology – Animal*, vol., 45, pp. 6 – 9.
- Kondratskyi, A., Kondratskyi, K., Skryma, R., Prevarskaia, N. (2015) Ion channels in the regulation of apoptosis, *Biochimica et Biophysica Acta*, vol. 1848, pp. 2532–2546.
- Kounakis, K., Chaniotakis, M., Markaki, M. and Tavernarakis, N. (2019) Emerging roles of lipophagy in health and disease, *Frontiers in Cell and Developmental Biology*, vol. 7, pp. pp. 1 – 8.
- Koutsari, C., Basu, R., Rizza, R. A., Nair, K. S., Khosla, S. and Jensen, M. D. (2011) Nonoxidative free fatty acid disposal is greater in young women than men, *Journal of Clinical Endocrinology and Metabolism*, vol. 92, pp. 541 – 547.
- Kraus, N. A., Ehebauer, F., Zapp, B., Rudolphi, B., Kraus, B. J. and Kraus, D. (2016) Quantitative assessment of adipocyte differentiation in cell culture, *Adipocyte*, vol. 5, pp. 351 – 358.
- Kuo, A., Lee, M. Y., and Sessa, W. C., (2017) Lipid droplet biogenesis and function in the endothelium, *Circulation Research*, vol. 120, pp. 1289-1297.
- Kuratko, C. N. and Constante, B. J., (1998), Linoleic acid and tumour necrosis factor- α increase manganese superoxide dismutase activity in intestinal cells, *Cancer Letters*, vol. 130, pp. 191-196.
- Kyrou, I., Randeva, H. S., Tsigos, C., Kaltsas, G. and Weickert, M. O., (2018) Clinical problems caused by obesity, in: Feingold, K. R., Anawalt, B., Boyce, A., Chrousos, G., Dungan, K., Grossman, A., Hershman, J. M., Kaltsas, G., Koch, C., Kopp, P., Korbonits, M., McLachlan, R., Morley, J. E., New, M., Perreault, L., Purnell, J., Rebar, R., Singer, F., Treince, D. L., Vinik, A. and Wilson, D. P. (eds.) *Endotext*, available at: <https://www.ncbi.nlm.nih.gov/books/NBK278973/> (downloaded: 28th June 2019).
- Lagarde, F., Beausoleil, C., Belcher, S. M., Belzunce, L. P., Emound, C., Guerbet, M. and Rousselle, C., (2015), Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment, *Environmental Health*, vol. 14, pp. 1 – 15.
- Lammi, C., Zanoni, C. and Arnoldi, A, (2015), A simple and high-throughput in-cell Western assay using HepG2 cell line for investigating the potential

hypcholesterolemic effects of food components and nutraceutics, *Food Chemistry*, vol. 169, pp. 59 – 64.

Lassen, E. D. and Swardson (1995), Haematology and haemostasis in the horse: normal functions and common abnormalities, *Clinical Pathology*, vol. 11, pp. 351 – 389.

Laug, W. E. (1983), Ethyl alcohol enhances plasminogen activator secretion by endothelial cells, *Journal of American Medical Association*, vol. 250, pp. 772 – 776.

Li, M., Esch, B, C. A. M., Henricks, P. A J., Garssen, J. and Folkerts, G. (2018) Time and concentration dependent effects of short chain fatty acids on lipopolysaccharide- or Tumor Necrosis Factor- α -induced endothelial activation, *Frontiers in Pharmacology*, vol. 9, pp. 1 – 12.

Li, X., Xu, M., Liu, M., Ji, Y. and Li, Z. (2015) TNF-alpha and IL-6 inhibit apolipoprotein A-IV production induced by linoleic acid in human intestinal Caco2 cells, *Journal of Inflammation*, vol. 12, pp. 1 – 8.

Lichtenstein, A. H., (2005), Fats and Oils, In: Engelking, L. R. (2014), *Textbook of Veterinary Physiological Chemistry*, Neatherlands, Elsiver, pp. 177 – 186,

Lin, C. L., Huang, H. C. and Lin, J. K. (2007) Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells, *Journal of Lipid Research*, vol. 48, pp. 2334 – 2343.

Lima, C. F., Fernandes-Ferreire, M. and Pereira-Wilson, C. (2006), Phenolic compounds protect HepG2 cells from oxidative damage: relevance of glutathione levels, *Life Sciences*, vol. 79, pp. 2056 – 2068.

Lima, T. M., Kanunfre, C. C., Pompeia, C. and Curi, R. V. R. (2002) Ranking the toxicity of fatty acids on Jurkat and Raji cells by flow cytometric analysis, *Toxicology in Vitro*, vol. 16, pp. 741–747.

Lopez L. R., Guyer, K. E., De La Torre, I. G., Pitts, K. R., Matsuura, E. and Ames P. R. J. (2014) Platelet thromboxane (11-dehydro-Thromboxane B2) and aspirin response in patients with diabetes and coronary artery disease, *World Journal of Diabetes*, vol. 5, pp. 115-127.

Lu, Y., A., J., Wang, G., Hao, H., Huang, Q., Yan, B., Zha, W., Gu, S., Ren, H., Zhang, Y., Fan, X., Zhang, M. and Hao, K., (2008) Gas chromatography/time-of-flight mass spectrometry based metabonomic approach to differentiating hypertension- and age-related metabolic variation in spontaneously hypertensive rats, *Rapid Communications In Mass Spectrometry*, vol. 22, pp. 2882 – 2888.

Luu, N. T., Rahman, M., Stone, P. C., Rainger, G. E. and Nash G. B. (2010), Responses of Endothelial Cells from Different Vessels to Inflammatory Cytokines and Shear Stress: Evidence for the Pliability of Endothelial Phenotype, *Journal of Vascular Research*, vol. 47, pp. 451 – 461.

MacFarlane, R. G., (1964), An enzyme cascadein blood clotting mechanism, and its function as a biochemical amplifier, *Nature*, vol. 202, pp. 498 – 499.

MacIntyre, D. E., Hoover, R. L., Smith, M., Steer, M., Lynch, M., Karnovsky, M. J. and SalzmanE. W. (1984) Inhibition of platelet function by *cis*-unsaturated fatty acids, *Blood*, vol. 63, pp. 848 – 875.

Magtanong, L. Ko, P. J. and Dixon, S. J. (2016) Emerging roles for lipids in non-apoptotic cell death, *Cell Death and Differentiation*, vol. 23, pp. 1099 – 1109.

Malhi, H and Gores, J. (2008) Molecular mechanisms of lipotoxicity in non-alcoholic fatty liver disease, *Seminars in Liver Disease*, vol. 28, pp. 360 – 369.

Malhi, H., Bronk, S. F., Werneburg, N. W. and Gores, G. J. (2006) Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis, *The Journal of Biological Chemistry*, vol. 281, pp. 12093–12101.

Mannucci, P. M. & Tripodi, A., (2013), Liver disease, coagulopathies and transfusion therapy, *Journal of Blood Transfusion*, vol.11, pp. 32-36.

Mansbach II, C. M. and Gorelick, F., (2007), Development and Physiological Regulation of Intestinal Lipid Absorption. II. Dietary lipid absorption, complex lipid synthesis, and the intracellular packaging and secretion of chylomicrons, *The American Journal of Physiology – Gastrointestinal and Liver Physiology*, vol. 293, pp. 645 – 650.

Marchesini, G., Moscatiello, S., Di Domizio, S. and Forlani, G. (2008) Obesity-Associated Liver Disease, *Journal of Clinical Endocrinology and Metabolism*, vol. 93, pp. S74 – S80.

Marchix, J., Choque, B., Kouba, M., Fautrel, A., Catheline, D. and Legrand, P. (2015) Excessive dietary linoleic acid induces proinflammatory markers in rats, *Journal of Nutritional Biochemistry*, vol. 26, pp. 1434–1441

Mari, M., Caballero, F., Colell, A., Morales, A., Caballeria, J., Fernandez, A., Enrich, C., Fernandez-Checa, J. C. and Garcia-Rui, c. (2006) Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis, *Cell Metabolism*, vol. 4, pp. 185 – 198.

Masarone, M., Rosato, V., Dallio, M., Gerarda Gravina, A., Aglitti, A., Loguercio, C., Federico, A. and Persico, M. (2018) Role of oxidative stress in pathophysiology of nonalcoholic fatty liver disease, *Oxidative Medicine and Cellular Longevity*, vol. 2018, pp. 1 – 14.

Mashek, D. G., Khan, S. A., Sathyaranarayan, A., Ploeger, J. M. and Franklin, M. P. (2015) Hepatic lipid droplet biology: getting to the root of fatty liver, *Hepatology*, vol. 62, pp. 964 – 967.

Masone, D., Gojanovich, A. D., Frontini-Lopez, Y. R., DEL Veliz, S., Uhart, M. and Bustos, D. M. (2017), Freely Available Tool (FAT) for automated quantification of lipid droplets in stained cell, *Biocell*, vol. 41, pp. 55 – 58.

Mayr, J. A. (2014), Lipid metabolism in mitochondrial membranes, *Journal of Inherited Metabolic Disease*, vol. 1, pp. 137 – 144.

McCord, J. M. (2008) Superoxide dismutase, lipid peroxidation, and bell-shaped dose response curves, *Dose Response*, vol. 6, pp. 223 – 238.

McMillian, M. K., Li, L., Parker, J. B., Patel, L., Zhong, Z., Gunnett, J. W., Powers, W. J. and Johnson, M. D., (2002), An improved resazurin-based cytotoxicity assay for hepatic cells, *Cell Biology and Toxicology*, vol. 18, pp. 157 – 173.

Medina-Leyte, D. J., Domínguez-Pérez, M., Mercado, I., Villarreal-Molina, M. T. and Jacobo-Albavera, L. (2020), Use of Human Umbilical Vein Endothelial Cells (HUVEC) as a Model to Study Cardiovascular Disease: A Review, *Applied Sciences*, vol. 10, pp. 1 – 25.

Mehlem, A., Hagberg, C. E., Muhl, L., Eriksson, U. and Falkevall, A. (2013) Imaging of neutral lipids by oil red o for analysing the metabolic status in health and disease, *Nature Protocols*, vol. 8, pp. 1149 – 1154.

Miles, E. A., Allen, E. and Calder, P. C. (2002) In vitro effects of eicosanoids derived from different 20-carbon fatty acids on production of monocyte-derived cytokines in human whole blood cultures, *Cytokine*, vol. 20, pp. 215 – 223.

Mills, C. E., Hall, W. L. and Berry, S. E. E. (2017) What are interesterified fats and should we be worried about them in our diet?, *Nutrition Bulletin*, vol. 42, pp. 153 – 158.

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., and Malik, A. B. (2014) Reactive oxygen species in inflammation and tissue injury, *Antioxidants & Redox Signalling*, vol. 20, pp. 1126 – 1166.

Mondola, P., Damiano, S., Sasso, A. and Santillo, M. (2016) The Cu,ZnSuperoxide dismutase: not only a dismutase enzyme, *Frontiers in Physiology*, vol. 7, pp. 1 – 8.

Morgan, D., Oliveira-Emilio, H. R., Keane, D., Hirata, A. E., Santos da Rocha, M., Curi, R., Newsholme, P. and Carpinelli, A. R. (2007) Glucose, palmitate and proinflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line, *Diabetologia*, vol. 50, pp. 359–369.

Mohd, M. A. (2012). Advanced Gas Chromatography - *Progress in Agricultural, Biomedical and Industrial Applications*. InTech Open.

Morikawa, K., Shimokawa, H., Matoba, T., Kubota, H., Akaike, T., Talukder, M. A., Hatanaka, M., Fujiki, T., Maeda, H., Takahashi, S. and Takeshita, A. (2003) Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization, *Journal of Clinical Investigation*, vol. 112, pp. 1871-1879.

Morita, I. (2002) Distinct functions of COX-1 and COX-2, *Prostaglandins & other Lipid Mediators*, vol. 68, pp. 165 – 175.

Mu, H. and Høy, C. E. (2004), The digestion of dietary triacylglycerols, *Progress in Lipid Research*, vol. 43, pp. 105 – 133.

Mu, H. and Porsgaard, T. (2005), The metabolism of structured triacylglycerols, *Progress in Lipid Research*, vol. 44, pp. 430 – 448.

Munoz, C., Carlet, J., Fitting, C., Misset, B., Bieriot, J. P., and Cavaillon, J. M. (1991) Dysregulation of In Vitro Cytokine Production by Monocytes during Sepsis, *Journal of Clinical Investigation*, vol. 88 pp. 1747-1754.

Murphy, S., Martin, S. and Parton, R. G. (2010) Quantitative analysis of lipid droplet fusion: inefficient steady state fusion but rapid stimulation by chemical fusogens, *PLoS ONE*, vol. 5, pp. 1 – 12.

Murray, G., Munstermann, S. and Lam, K., (2013), *Benefits and challenges posed by the worldwide expansion of equestrian events – new standards for the population of competition horses and equine disease free zones in countries*, Paris: World Organisation for Animal Health.

Na, H. G., Kim, D. G., Kin, Y. H., Han, J. H. and Jung, E. S. (2014) Effects of glucose concentration in the medium on rat hepatocyte culture, *Annals of Surgical Treatment and Research*, vol. 87, pp. 53 – 60.

Nagao, K. and Yanagita T. (2005) Conjugated Fatty Acids in Food and Their Health Benefits, *Journal of Bioscience And Bioengineering*, vol. 100, pp. 152 – 157.

Nagata, S. (2018) Apoptosis and clearance of apoptotic cells, *Annual Review of Immunology*, vol. 36, pp. 489–517

Nakahata, N. (2008) Thromboxane A₂: Physiology/pathophysiology, cellular signal transduction and pharmacology, *Pharmacology & Therapeutics*, VOL. 118, PP. 18 – 35.

Nanji, A. A., Khettry, U., Sadrzadeh,S. M. H. and Yamanaka, T, (1993) Severity of liver injury in experimental alcoholic liver disease, correlation with plasma endotoxin, Prostaglandin E₂, Leukotriene B₄, and Thromboxane B₂, *American journal of Pathology*, vol. 142, pp. 367 – 373.

National Centre for Biotechnology Information, (2017), PubChem Compound Database; CID=753, Available at: <https://pubchem.ncbi.nlm.nih.gov/compound/753>, [accessed 4th August 2017].

Nassir, F. and Ibdah, J. A. (2014), Role of mitochondria in alcoholic liver disease, *World Journal of Gastroenterology*, vol. 20, pp. 2136 – 2142.

Nedjadi, T., Moran, A. W., Al-Rammahi, M. A. and Shirazi-Beechey, S. P. (2014) Characterization of butyrate transport across the luminal membranes of equine large intestine, *Experimental Physiology*, vol. 99, pp. 1335 – 1347.

Needleman, S. W., Spector, A. A. and Hoak, J. C. (1982), Enrichment of human platelet phospholipids with linoleic acid diminishes thromboxane release, *Prostaglandins*, vol. 24, pp. 607 - 6.22.

Nelson, D. L. and Cox, M. M., (2017), *Lehninger Principles of Biochemistry*, 7th ed., New York, USA, Worth Publishers, pp. 363 – 388.

Ness, S. A. (2014), Blood coagulation disorders, In: Orsini, J. A. and Divers, T. J., ed. *Equine Emergencies, treatment and procedures*, 4th ed., Missouri, USA, Elsiver Saunders, pp. 118 – 123.

Neuman, M. G., Shear, N. H., Bellentani, S. and Tiribelli, C. (1998) Role of cytokines in ethanol-induced cytotoxicity in vitro in HepG2 cells, *Gastroenterology*, vol. 115, pp. 157 – 166.

Niedzwiedz, A. and Jaworski, Z. (2014) Oxidant-antioxidant status in the blood of horses with symptomatic recurrent airway obstruction (RAO), *Journal of Veterinary Internal Medicine*, vol. 28, pp. 1845 – 1852.

Nitayavardhana, S. and Khanal, S. K. (2011), Biodiesel derived crude glycerol bioconversion to animal feed: A sustainable option for a biodiesel refinery, *Bioresource Technology*, vol. 102, pp. 5808 – 5814.

Norris, J. W., PrattS. M., Auh, J. H., Wilson, S. J., ClutterD., Magdesian, M. g., Farraro, G. L. and Tablin, F. (2006), Investigation of a novel, heritable bleeding diathesis of Thoroughbred horses and development of a screening assay, *Journal of Veterinary Internal Medicine*, vol. 20, pp. 1450 – 1456.

Nourse, B. M., Rolle, M. W., Pabon, L. M. and Murray, C. E., (2007), Selective control of endothelial cell proliferation with a synthetic dimerizer of FGF receptor-1, *Laboratory Investigation*, vol. 87, pp. 828 -835.

Oh, Y. S., Bae, G. D., Baek, D. J., Park, E. Y. and Jun, H. S. (2018) Fatty acid-induced lipotoxicity in pancreatic beta-cells during development of type 2 diabetes, *Frontiers in Endocrinology*, vol. 9, pp. 1 – 10.

Oliveira A.F., Cunha, D. A., Ladriere, L., Igoillo-Esteve, M., Bugiani, M., MarchettiP, and Cnop, M., (2015) *In Vitro* use of free fatty acids bound to albumin: A comparison of protocols, *Biotechniques*, vol, 58, pp 228 – 233.

Olusi, S. O. (2002) Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotective enzymes in humans, *International Journal of Obesity*, col. 26, pp. 1159–1164

Olzmann, J. A., and Carvalho, P. (2018) Dynamics and functions of lipid droplets, *Nature Reviews*, vol. 20, pp. 137 – 155.

O’Quinn, P. R., Nelssen, J. L., Goodband, R. D. and Tokach, M. D. (2000), Conjugated linoleic acid, *Animal Health Research reviews*, vol. 1, pp. 35 – 46.

O’Sullivan, L. (2016), Horse Sense: Managing exercised induced pulmonary haemorrhage, *The Irish Field*, available at: <http://www.theirishfield.ie/horse-sense-managing-exercise-induced-pulmonary-haemorrhage-211046/> [Downloaded on the 4th June 2017].

Orata, F. (2012) ‘Derivatisation reactions and reagents for gas chromatography analysis’ in Mohd, M. A. (Ed.) *Advanced Chromatography – Progress in Agricultural, Biomedical and Industrial Applications*, Intech, pp. 83 – 109.

Ore, A. and Akinloye, O. A. (2019), oxidative stress and antioxidant biomarkers in clinical and experimental models of non-alcoholic fatty liver disease, *Medicina*, vol. 55, pp. 1 – 13.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., Bao, J. K. (2012) Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis, *Cell Proliferation*, vol. 45, pp. 487 – 498.

Page, M. J., Bester, J. and Pretorius, E. (2018), The inflammatory effects of TNF- α and complement component 3 on coagulation, *Nature Scientific Reports*, vol. 8, pp. 1 – 9.

Palombo, J. D., DeMichele, S. J., Lydon, E. E., Gregory, T. J., Banks, P. L., Forse, R. A. and Bistrian, B. R. (1996) Rapid modulation of lung and liver macrophage phospholipid fatty acids in endotoxemic rats by continuous enteral feeding with n-3 and gamma-linolenic fatty acids, *American Journal of Clinical Nutrition*, vol. 63, pp. 208 – 219.

Palta, S., Saroa, R. and Palta, A. (2014), Overview of the coagulation system, *Indian Journal of Anaesthesia*, vol. 58, pp. 515 – 523.

Parameswaran, N. and Patial, S. (2010) Tumor necrosis factor- α signaling in macrophages, *Critical Reviews in Eukaryotic Gene Expression*, vol. 20, pp. 87–103.

Park, J. and Koh, J. W. (2018) Era of bloodless surgery: spotlights on haemostatic materials and techniques, *Hanyang Medical Reviews*, vol. 38, pp. 3 – 15.

Parker, R. (2013), *Equine Science*, 4th ed., New York, USA, Dalmar Cengage Learning, pp. 1.

Paul, T. and Mukherjee, S. K. (2019) Induction of inflammatory response in human cell lines by arsenic-contaminated soil-isolated bacterium *Micrococcus* sp. KUMAs15, *Egyptian Journal of Medical Human Genetics*, vol. 20, pp. 1 – 7.

Pelley, J W. (2007), Fatty Acids, *Elseviers Integrated Biochemistry*, pp. 13.

Peskin, A. V. and Winterbourn, C. C. (2017) Assay of superoxide dismutase activity in a plate assay using WST-1, *Free Radicle Biology and Medicine*, vol. 103, pp. 188 – 191.

Phylactos, A. C., Harbige, L. S. and Crawford, M. A. (1994) Essential fatty acids alter the activity of manganese-superoxide dismutase in rat heart, *Lipids*, vol. 29, pp. 111 – 115.

Pol, A., Gross, S. P. and Parton, R. G., (2014), Biogenesis of multifunctional lipid drople: lipids, proteins and sites, *The Journal of Cell Biology*, vol. 204, issue no. 5, pp. 635 – 646.

Pompeia, C., Freitas, J. J. S., Kim, J. S., Zyngier, S. B. and Curi, R. (2002) Arachidonic acid cytotoxicity in leukocytes: implications of oxidative stress and eicosanoid synthesis, vol. 94, pp. 251–265.

Pouyade, G. R., Salcicci, A., Ceusters, J., Deby-Dupon, G., Serteyn, G. and Mouithys-Mickalad, A. (2011) Production of free radicals and oxygen consumption by primary equine endothelial cells during anoxia-reoxygenation, *The Open Biochemistry Journal*, vol. 5, pp. 52-59.

Puddey, I. B., Zilkens, R. R. Croft K. D. and Beilin, L. J. (2001), Alcohol and endothelial function: a brief overview, *Clinical and experimental Pharmacology and Physiology*, vol. 28, pp. 1020 – 1024.

Präbst, K., Engelhardt, H., Ringgeler, S., Hübner, H. (2017), ‘Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin’, in Gilbert, D. F. and Friedrich, O., (eds.) *Cell Viability Assays: Methods and Protocols*, Methods in Molecular biology, vol. 1601, Springer Science+Business Media, Germany.

Price, S. R., Olivecrona, T. and Pekala, P. H. (1986), Regulation of lipoprotein lipase synthesis by recombinant tumour necrosis factor – the primary regulatory role hormone in 3T3-L1 adipocytes, *Archives of Biochemistry and Biophysics*, vol. 251, pp. 738 – 746.

Prinz, W. A. (2010), Lipid trafficking sans vesicles: where, why, how? *Cell*, issue 143, pp. 870- 874.

Quehenberger, O., Armando, A. M. and Dennis, E. A. (2011) High Sensitivity Quantitative Lipidomics Analysis of Fatty Acids in Biological Samples by Gas Chromatography-Mass Spectrometry, *Biochim Biophys Acta*, vol. 1811, pp 648 – 656.

Radzikowska, U. Rinaldi, A. O., Sözener, Z. C., Karaguzel, D., Wojcik, M., Cypryk, K., Akdis, M., Akdis, C. A. and Sokolowska, M. (2019) The Influence of Dietary Fatty Acids on Immune Responses, *Nutrients*, vol. 11, pp. 1 – 52.

Ramadan, F. M., Upchurch, G. R., Keagy, B. A. and Johnson, G. (1990) Endothelial cell thromboxane production and its inhibition by a calcium-channel blocker, *The Annals of Thoracic Surgery*, vol. 49, pp. 916 – 919.

Ramadori, G., Van Damme, J., Rieder, H. and Meyer, K. H. (1988) Interleukin 6, the third mediator of acute-phase reaction, modulates hepatic protein synthesis in human and mouse. Comparison with interleukin 1 β and tumor necrosis factor- α , *European Journal of Immunology*, vol. 18, pp. 1259-1264.

- Rambold, S. A., Cohen, S., Lippincott-Schwartz, J. (2015), Fatty acid trafficking in starved cells: Regulation by lipid droplet lipolysis, autophagy and mitochondrial fusion dynamics, *Developmental Cell*, vol. 23, pp. 678–692.
- Ramirez-Zacarias, J. L., Castro-Mufiozledo, F. and Kuri-Harcuch, W. (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O, *Histochemistry*, vol. 97, pp. 493 – 497.
- Ramsden, C. E., Hennebelle, M., Schuster, S., Keyes, G., Johnson, C. D., Kirpich, I. A., Dahlen, J. E., Horowitz, M. S., Zamora, D., Feldstein, A. E., McClain, C. J., Muhlhausler, B. S., Makrides, M., Gibson, R. A. and Taha, A. Y. (2018) Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice, *BBA - Molecular and Cell Biology of Lipids*, vol. 1863, pp. 1206–1213
- Ramsden, C. E., Ringel, A., Feldstein, A. E., Taha, A. Y., MacIntosh, B. A., Hibbeln., J. R., Majchrzak-Hong, S. F., Faurot, K. R., Rapoport, S. I., Cheon, Y., Chung, Y. M., Berk, M. and Mann, J. D. (2012), Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans, *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 87, pp. 135–141.
- Ray, P. D., Huang, B. W. and Tsuji, Y. (2012) Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signalling, *Cellular Signalling*, vol. 24, pp. 981 – 990.
- Reardon, A. J. F., Elliot, J. A. W. and McGann, L. E. (2014) Fluorescence as an alternative to light-scatter gating strategies to identify frozen–thawed cells with flow cytometry, *Cryobiology*, vol. 69, pp. 91 – 99.
- Reccai, I., Kumar, J., Akladios, C., Virdis, F., Pai, M., Habib, N. and Spalding, D. (2017), Non-alcoholic fatty liver disease: A Sign of systemic disease, *Metabolism*, vol. 72, pp. 94 – 108.
- Reed, D. J., and Fariss, M. W., (1984) Glutathione depletion and susceptibility, *Pharmacological Review*, vol. 36, pp. 25 – 33.
- Reinaud, O., Delaforge, M., Boucher, J. L., Ricchicioli, F. and Mansuy, D. (1989) Oxidative metabolism of linoleic acid, by human leukocytes, *Biochemical and Biophysical Research Communications*, vol. pp. 883 – 891.
- Rett, B. S. and Whelan, J. (2011) Increasing dietary linoleic acid does not increase, pp. tissue arachidonic acid content in adults consuming Western-type diets: a systematic review, *Nutrition & Metabolism*, vol. 8, pp. 1 – 15.
- Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., Fantoni, L. I., Marra, F., Bertolotti, M., Banni, S., Lonardo, A., Carulli, N., and Loria, P. (2009) Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes, *Journal of Gastroenterology and Hepatology*, vol. 84, pp. 830 – 840.
- Ricciotti, E. and FitzGerald, G. A. (2010) Prostaglandins and inflammation, *Arteriosclerosis, Thrombosis and Vascular Biology*, vol. 31, pp. 986 – 1000.

Richardson, D. K., Kashyap, S., Bajaj, M. Cusi, K., Mandarino, S. J., Finlayson, J., DeFronzo, R. A., Jenkinson, C. P. and Mandarino, L. J. (2005) Lipid infusion decreases the expression of nuclear encoded mitochondrial genes and increases the expression of extracellular matrix genes in human skeletal muscle, *Journal of Biological Chemistry*, vol. 280, pp. 10290 – 10297.

Richardson, M. R., Lai, X., Witzmann, F. A. and Yoder, M. C. (2010) Venous and arterial endothelial proteomics: mining for markers and mechanisms of endothelial diversity, *Expert Review of Proteomics*, vol. 7, pp. 823 – 831.

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J. and Minor, L. (2016), Cell Viability Assays, *Assay Guidance Manual*, Eli Lilly & Company and the National Center for Advancing Translational Sciences Bethesda (MD), pp. 2 – 31.

Robb, J., Harper, R. B., Hintz, H. F., Reid, J. T., Lowe, J. E., Schryver, H. F. and Rhee, M. S. S. (1972), Chemical composition and energy value of the body, fatty acid composition of adipose tissue, and liver and kidney size in the horse, *Animal Production*, vol. 14, pp 25-34.

Robson, N. (2016), Rule the World is Grand National hero at Aintree, *The Irish News*, Available at: <http://www.irishnews.com/sport/racing/2016/04/09/news/rule-the-world-is-grand-national-hero-at-aintree-480765/> [Accessed on the 4th June 17].

Rocha, V. Z. and Libby, P. (2009) Obesity, inflammation, and atherosclerosis, *Nature Reviews*, vol. 6, pp. 399 – 409.

Rohman, A. and Che Mann, Y. B., (2010), Fourier Transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil, *Food Research International*, vol. 43, pp. 886 – 892.

Romano, G. G., Menten, J.F.M., Freitas, L. W., Lima, M.B., Pereira, R., Zavarize, K. C. and Dias, C. T. S. (2014), Effects of glycerol on the metabolism of broilers fed increasing glycerine levels, *Brazilian Journal of Poultry Science*, vol. 16, pp. 97 – 106.

Rosenberg, M., Azevedo, N. F. and Ivask, A. (2019) Propidium iodide staining underestimates viability of adherent bacterial cells, *Nature Scientific Reports*, vol. 9, pp. 1 – 12.

Rui, L. (2014), Energy metabolism in the liver, *Comprehensive Physiology*, vol. 4 pp. 1 – 42.

Saad, B., Frei, K., Scholl, F. A., Fontana, A. and Maier, P. (1995), Hepatocyte-derived interleukin-6 and tumor-necrosis factor- α mediate the lipopolysaccharide-induced acute-phase response and nitric oxide release by cultured rat hepatocytes, *European Journal of Biochemistry*, vol. 229, pp. 349 – 355.

Safar, M., Bertrand, D., Robert, P., Devaux, M. F. and Genot, C. (1994), Characterisation of edible oils, butters and margerines by Fourier Transform Infrared with Attenuated Total Reflectance, *Journal of the American Oil Chemists Society*, vol. 71, pp. 371 – 377.

Saha, P. and Smith, A. (2018), TNF- α (Tumor Necrosis Factor- α) A Paradox in Thrombosis, *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 38, pp. 25442 – 2543.

Sako, Y. and Grill, V. E. (1990) A 48-hour Lipid Infusion in the Rat Time-Dependently Inhibits Glucose-Induced Insulin Secretion and B Cell Oxidation Through a Process Likely Coupled to Fatty Acid Oxidation, *Endocrinology*, vol. 127, pp. 1580 – 1589.

Salehpour, S. and Dube, M. A. (2012), Reaction monitoring of glycerol step-growth polymerisation using ATR-FTIR spectroscopy, *Macromolecular Reaction Engineering*, vol. 6, pp. 85-92.

Salimon, J., Omar, T. A. and Salih, N. (2017) An accurate and reliable method for the identification and quantification of fatty acids and transfatty acids in food fats samples using gas chromatography, *Arabian Journal of Chemistry*, vol. 10, pp. S1875 – S1882.

Satue, K. Gardon, J. C. and Munoz, A. (2017) Interpretation of platelets in the horse, *Journal of Haematology Research*, vol. 4, pp. 19 – 25.

Savonen, R., Hiden, M., Hultin, M., Zechner, R., Levak-Frank, S., Olivecrona, G. and Olivecrona, T. (2015) The tissue distribution of lipoprotein lipase determines where chylomicrons bind, *Journal of Lipid Research*, vol. 56, pp. 588 – 598.

Schie, I. W., Wu, J., Zern, M., Rutledge, J. C. and Huser, T (2011) Label-free characterization of rapid lipid accumulation in living primary hepatocytes after exposure to lipoprotein lipolysis products, *Journal of Biophotonics*, vol. 4, pp. 425 – 434.

Schlager, S., Vujic, N., Korbelius, M., Duta-Mare. M., Dorow, J., Leopold, C., Rainer, S., Wegscheider, M., Reicher, H., Ceglarek, U., Sattler, W., Radovic, B. and Kratky, D. (2017) Lysosomal lipid hydrolysis provides substrates for lipid mediator synthesis in murine macrophages, *Immunology*, vol. 8, pp. 40037-40051.

Schleger, C., Platz, S. J. and Deschl, U. (2004), Development of an *in vitro* model for vascular injury with human endothelial cells, *Alternatives to Animal Experimentation*, vol. 21, pp. 12 – 19.

Schönenfeld, P. and Wojtczak, L. (2008) Fatty acids as modulators of the cellular production of reactive oxygen species, *Free Radical Biology & Medicine*, vol. 45, pp. 231 – 241.

Schuster, S., Johnson, C. D., Hennebelle, M., Holtmann, T., Taha, A. T., Kirpich, I. A., Eguchi, A., Ramsden, C. E., Papouchado, B. G., McClain, C. J., and Feldstein, A. E. (2018) Oxidized linoleic acid metabolites induce liver mitochondrial dysfunction, apoptosis and NLRP3 activation in mice, *Journal of Lipid Research*, vol. 59, pp. 1597-1609.

Schweiger, M., Schreiber, R., Haemmerle, G., Lass, A., Fledelius, C., Jacobsen, P., Tornqvist, H., Zechner, R. and Zimmermann, R. (2006), Adipose Triglyceride Lipase and Hormone-sensitive Lipase Are the Major Enzymes in Adipose Tissue

Triacylglycerol Catabolism, *The Journal of Biological Chemistry*, vol. 218, no. 52, pp. 40236-40241.

Shen, H. M. and Pervaiz, S. (2006) TNF receptor superfamily-induced cell death: redox dependent execution, *Federation of American Societies for Experimental Biology*, vol. 20 pp. 1589 – 1598.

Shen, R. F. and Tai, H. H., (1998) Thromboxanes: synthase and receptors, *Journal of Biomedical Science*, vol. 5, pp. 153 – 172.

Shultz, T. D. (1991) physiological free fatty acid concentrations do not increase free estradiol in plasma, *Journal of Clinical Endocrinology and Metabolism*, vol. 72, pp. 65 – 68.

Simionescu, M. (2007) Implications of early structural-functional changes in the endothelium for vascular disease, Arteriosclerosis, *Thrombosis and Vascular Biology*, vol. 27, pp. 266 – 247.

Simopoulos, A. P. (2016) An increase in the omega-6/omega-3 fatty acid ratio increases the risk for obesity, *Nutrient*, vol. 8, pp. 1 – 17.

Simopoulos, A. P. (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases, *Experimental Biology and Medicine*, vol. 233, pp. 674-688.

Singh, R., Letai, A. and Sarosiek, K. (2019) Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins, *Nature Reviews: Molecular Cell Biology*, vol. 20, pp. 175-193.

Siqueira, R. F., Weigel, R. A., Nunes, G. R., Mori, C. S. and Fernandes, W. R. (2014) Oxidative profiles of endurance horses racing different distances, *Arq. Bras. Med. Vet. Zootec*, vol. 66, pp. 455-461.

Sira J. and Eyre, L. (2016), Physiology of haemostasis, *Anaesthesia and Intensive Care Medicine*, vol. 17, pp. 79 – 82.

Skrzypski, J., Bellenger, S., Bellenger, J., Sinclair, A., Poisson, J. P., Tessier, C., Rialland, M. and Narce, M. (2009) Revisiting delta-6 desaturase regulation by C18 unsaturated fatty acids, depending on the nutritional status, *Biochimie*, vol. 91 pp. 1443–1449.

Smith, J. B. (1981), Prostaglandins and platelet aggregation, *Acta Medica Scandinavica Supplement*, vol. 651, pp. 91 – 99.

Soffler, C. (2007) Oxidative stress, *Veterinary Clinics Equine Practice*, vol. 23, pp. 135 – 157.

Spangenburg, E. E., Pratt, S. J. P., Wohlers, L. M. and Lovering, R. M. (2011) Use of BODIPY (493/503) to visualize intramuscular lipid droplets in skeletal muscle, *Journal of Biomedicine and Biotechnology*, vol. 2011, pp. 1 – 8.

Sparks, L. M., Xie, H., Koza, R. A., Mynatt, R., Hulver, M. W., Bray, G. A. and Smith, S. R. (2005) A high-fat diet co-ordinately downregulates genes required for

mitochondrial oxidative phosphorylation in skeletal muscle, *Diabetes*, vol. 54, pp. 1926 – 1933.

Spolarics, Z., Tanacs, B., Garzo, T., Mandl, J., Mucha, I., Antoni, F., Machovich, R. and Horvath, I., (1984) Prostaglandin and thromboxane synthesizing activity in isolated murine hepatocytes and nonparenchymal liver cells, *Prostaglandins, Leukotrienes and Medicine*, pp. 379 – 388.

Sprayberry, K. A. and Robinson, N. E., (2015), *Robinsons Current Therapy in Equine Medicine*, 7th Ed., Missouri, Saunders/Elsevier.

Srivastava, K., C. (1895), Docosahexaenoic acid (C22:6 ω3) and linoleic acid are anti-aggregatory, and alter arachidonic acid metabolism in human platelets, *Prostaglandins Leukotrienes and Medicine*, vol. 17, pp. 319-327.

Staiculescu, M. C., Foote, C., Meininger, G. A. and Martinez-Lemus, L. A. (2014) The role of reactive oxygen species in microvascular remodelling, *International Journal of Molecular Sciences*, vol. 15, pp. 23792 – 23835.

Stonans, I., Stonane, E., Rußwurm, S., Deigner, h. p., Böhm, K. J., Wiederhold, M., Jäger, L. and Reinhart, K., (1999) HepG2 Human hepatoma cells express multiple cytokine genes, *Cytokine*, vol. 11, pp. 151 – 156.

Storniolo, C. E., Roselló-Catafau, J., Pintó, X., Mitjavila, M. T. and Moreno, J. J. (2014) Polyphenol fraction of extra virgin olive oil protects against endothelial dysfunction induced by high glucose and free fatty acids through modulation of nitric oxide and endothelin-1, *Redox Biology*, vol. 2., pp. 971 – 977.

Suagee, J. K., Corl, B. A., Crisman, M. V., Pleasant, R.S., Thatcher, C. D. and Geor, R. J. (2013), Relationships between body condition score and plasma inflammatory cytokines, insulin, and lipids in a mixed population of light-breed horses, *Journal of Veterinary Internal Medicine*, vol. 27, pp. 157 – 163.

Suh, H. N., Huong, H. T., Song, C. H., Lee, J. H. and Han, H. J. (2008) Linoleic acid stimulates gluconeogenesis via Ca²⁺/PLC, cPLA₂, and PPAR pathways through GPR40 in primary cultured chicken hepatocytes, *American Journal of Physiology – Cell Physiology*, vol. 295, pp. C1518–C1527.

Sumantran, V. N. (2011) Cellular Chemosensitivity Assays: an overview, In: Cree I. (ed.) *Cancer Cell Culture. Methods in Molecular Biology* (Methods and Protocols), vol 731. New York, Humana Press.

Sun, Y., Oberly, L. W. and Li, Y. (1988) A simple method for clinical assay of superoxide dismutase, *Clinical Chemistry*, vol. 34, pp. 497 – 500.

Sutherland, R. J., Cambridge, H. and Bolton, J. R. (1989), Functional and morphological studies on blood platelets in a thrombasthenic horse, *Australian Veterinary Journal*, vol. 66, pp.366 – 370.

Sweeting, J. N., Siu, M., McCallum, G. P., Miller, L. and Wells, P. G. (2010), Species differences in methanol and formic acid pharmacokinetics in mice, rabbits and primates, *Toxicology and Applied Pharmacology*, vol. 247, pp. 28 – 35.

- Symons, J. D. and Abel, E. D. (2013), Lipotoxicity contributes to endothelial dysfunction: a focus on the contribution of ceramide, *Reviews in Endocrine and Metabolic disorders*, vol. 14, pp. 59 – 68.
- Swystun, L. L. and Liaw, P. C. (2016), The role of leukocytes in thrombosis, *Blood*, vol. 128, pp. 753 – 762.
- Tait, S. W. G. and Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond, *Nature Reviews Molecular Cell Biology*, vol. 11, pp. 621–632.
- Thannickal, V. J. and Fanburg, B. L. (2000) Reactive oxygen species in cell signalling, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, vol. 279, pp. L1005–L1028.
- Thompson, J. C. (2006), Characterisation of Crude Glycerol from Biodiesel Production from Multiple Feedstocks, *American Society of Agriculture and Biological Engineers*, vol. 22, pp. 261 – 265.
- Tikanoja, S. H., Joutti, A., and Liewendahl, B. K., (1989) Association between increased concentrations of free thyroxine and unsaturated free fatty acids, *Clinica Chimica Acta*, vol. 179, PP. 33 – 44.
- Toborek, M., Lee, Y. W., Kaiser, S. and Hennig, B. (2002) Measurement of Inflammatory Properties of Fatty Acids in Human Endothelial Cells, *Methods In Enzymology*, vol. 352, pp. 198 – 219.
- Toborek, M., Barger, S. W., Mattson, M. P., Barve, S., McClain, C. J. and Hennig, B. (1996), Linoleic acid and TNF- α cross amplify oxidative injury and dysfunction of endothelial cells, *Journal of Lipid Research*, vol. 37, pp. 123 – 135.
- Toborek, M. and Hennig, B. (1994) Fatty acid-mediated effects on the glutathione redox cycle in cultured endothelial cells, *The American Journal of Clinical Nutrition*, vol. 59, pp. 60 -65.
- Toda N. and Ayajiki, K. (2010), Pharmacology and cell metabolism, vascular actions of nitric oxide as affected by exposure to alcohol, *Alcohol and Alcoholism*, vol. 3, pp. 347 – 355.
- Togo, M., Konari, N., Tsukamoto, M., Yamaguchi1, T., Takeda, H. and Kambayashi, I. (2018) Effects of a high-fat diet on superoxide anion generation and membrane fluidity in liver mitochondria in rats, *Journal of the International Society of Sports Nutrition*, vol. 15, pp. 1 – 8.
- Torres-Duarte, A. P. and Vanderhoek, J. Y. (2003) Conjugated linoleic acid exhibits stimulatory and inhibitory effects on prostanoid production in human endothelial cells and platelets, *Biochimica et Biophysica Acta*, vol. 1640, pp. 69 – 76.
- Tripathy, D., Mohanty, P., Dhindsa, S., Syed, T., Ghanim, H., Aljada, A. and Dandona, P. (2003) Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects, *Diabetes*, vol. 52, pp. 2882–2887.

Turner, M. D., Nedjai, B., Hurst, T. and Pennington, D. J. (2014) Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease, *Biochimica et Biophysica Acta*, vol. 1843, pp. 2563 – 2582.

Unger, R. E., Krump-Konvalinkova, V., Peters, K. and Kirkpatrick, C. J. (2002) In Vitro Expression of the Endothelial Phenotype: Comparative Study of Primary Isolated Cells and Cell Lines, Including the Novel Cell Line HPMEC-ST1.6R, *Microvascular Research*, vol. 64, pp. 384 – 397.

Unger, R. H., Clark, G. O. Scherer, P. E. and Orci, L., (2010), Lipid homeostasis, lipotoxicity and the metabolic syndrome, *Biochimica et Biophysica Acta*, vol. 1801, pp. 209 – 214.

Unger, R. H. (2003) Lipid overload and overflow: metabolic trauma and the metabolic syndrome, *Trends in Endocrinology and Metabolism*, vol. 14, pp.398 – 403.Unger, R. H. (2002) lipotoxic diseases, *Annual Review of Medicine*, vol. 53, pp. 319 – 336.

Unger, R.H. and Orci, L. (2002) Lipoapoptosis: its mechanism and its diseases, *Biochimica et Biophysica Acta*, vol. 1585, pp. 202 – 212.

Unger, R. H. (1995) Lipotoxicity in the pathogenesis of obesity-dependent NIDDM, genetic and clinical implications, *Perspectives in Diabetes*, vol. 44, pp. 863 – 870.

Urquhart, P., Parkin, SM., Rogers, J. S., Bosley, J., A. and Nicolaou, A. (2002) The effect of conjugated linoleic acid on arachidonic acid metabolism and eicosanoid production in human saphenous vein endothelial cells, *Biochimica et Biophysica Acta*, vol. 1580, pp. 150 – 160.

Vallee, E., Gougat, J. and Ageron, M., (1980) Inhibition of platelet phospholipase-A₂ as a mechanism for the anti-aggregating effect of linoleic acid, *Agents and Actions*, vol. 10, pp. 57 – 61.

Van Nieuwenhove, C. P., Terán V., and González S. N., (2012), Conjugated Linoleic and Linolenic Acid Production by Bacteria: Development of Functional Foods, *InTech Open Access Publisher*, pp. 55 – 80.

Vecchini, A., Ceccarelli, C., Susta, F., Caligiana, P., Orvietani, P., Binaglia, L., Nocentini,G., Riccardi, C., Calviello, G., Palozza, P., Maggiano, N. and Di Nardo, P (2004) Dietary α-linolenic acid reduces COX-2 expression and induces apoptosis of hepatoma cells, *Journal of Lipid Research*, vol. 45, pp. 308 – 316.

Videla, L. A., Rodrigo, R., Orellana, M., Fernandez, V., Tapia, G., Quinones, L., Varela, N., Contreras, J., Lazarte, R., Csendes, A., Rojas, J., Maluenda, F., Burdiles, P., Diaz, J. C., Smok, G., Thielemann, L. and Poniachik, J. (2004) Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients, *Clinical Science*, vol. 106, pp. 261 – 268.

Vincent H. K., Powers, S. K., Stewart, D. J., Shanely, R. A., Demirel, H. and Naito, H. (1999) Obesity is associated with increased myocardial oxidative stress, *International Journal of Obesity*, vol. 23, pp. 67 – 74.

Virtanen, J. K., Mursu, J., Voutilainen, S. and Tuomainen, T. P. (2017) The associations of serum n-6 polyunsaturated fatty acids with serum C-reactive protein in

men: the Kuopio Ischaemic Heart Disease Risk Factor Study, *European Journal of Clinical Nutrition*, vol. 72, pp. 342 – 348.

Viswanathan, S., Hammock, B. D., Newman, J. W., Meerarani, P., Toborek, M. and Hennig, B. (2003) Involvement of CYP 2C9 in mediating the proinflammatory effects of linoleic acid in vascular endothelial cells, *Journal of the American College of Nutrition*, vol. 22, pp. 502–510.

Voet, D. and Voet, J. G. (2011), *Biochemistry*, 4th Ed, New Jersey, USA, John Wiley & Sons, Inc., pp – 386.

Walch, L., Copic, A. and Jackson, C. L., (2015), Fatty acid metabolism meets organelle dynamics, *Developmental Cell*, issue 32, pp. 657-658.

Wallage R. H. and Watterson, J. H. (2008), Formic acid and methanol concentrations in death investigations, *Journal of Analytic Toxicology*, vol. 32, pp. 241 – 247.

Wallberg, F., Tenev, T. and Meier, P. (2016) Analysis of Apoptosis and Necroptosis by Fluorescence-Activated Cell Sorting, *Cold Spring Harbour Protocols*, pp. 347 – 353.

Walther, T. C. and Farese Jr., R. V. (2012) Lipid droplets and cellular lipid metabolism, *Lipid Droplets and Cellular Lipid Metabolism*, vol. 81, pp. 687 – 714.

Wang, H., Cheng, H., Wang, F., Wei, D. and Wang, X., (2010), An improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of *Escherichia coli* cells, *Journal of Microbiological methods*, vol. 82, pp. 330 – 333.

Watkins, P. A. (2013), Fatty acyl-CoA Synthetases, *Encyclopaedia of Biological Chemistry*, pp. 290-295.

Weber, K., Roelandt, R., Bruggeman, I., Estornes, Y., Vandenabeele, P. (2018) Nuclear RIPK3 and MLKL contribute to cytosolic necosome formation and necroptosis, *Nature Communications Biology*, vol. 1, pp. 1 – 13.

Wei, Y, Wang, D. and Pagliassotti, M. J. (2007) Saturated fatty acid-mediated endoplasmic reticulum stress and apoptosis are augmented by trans-10, cis-12-conjugated linoleic acid in liver cells, *Molecular and Cellular Biochemistry*, vol. 303, pp. 105 – 113.

Weinberg, J. M. (2006) Lipotoxicity, *Kidney International*, vol. 70, pp. 1560 – 1566.

Weller, P. F., Ackerman, S. J., Nicholson-Weller, A. and Dvorak, A. M (1989) Cytoplasmic lipid bodies of human neutrophilic leukocytes, *American Journal of Pathology*, vol. 135, pp. 947 – 959.

Welte, M. A. (2015), Expanding roles for lipid droplets, *Current Biology*, vol. 25, pp. R470 – R481.

Weltman, M. D., Farrell, G. C. and Liddle C. (1996) Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation, *Gastroenterology*, vol. 111, pp. 1645–1653.

- West, H. J. (1996), Clinical and pathological studies in horses with hepatic disease, *Equine Veterinary Journal*, vol. 28, pp. 146 – 156.
- Whelan, J. and Fritsche, K. (2013) Linoleic acid, *Advances in Nutrition*, vol. 4, pp. 311–312.
- White, S. W., Zheng, J., Zhang, Y. M. and Rock, C. O. (2005), The structural biology of type II fatty acid biosynthesis, *Annual Review of Biochemistry*, vol. 74, pp. 791 – 831.
- Whitehead, R. H., (1909), A note on the absorption of fat, *American Journal of Physiology*, vol. 24, pp. 294 – 296.
- Wilfling, F., Wang, H., Hass, J. T., Krahmer, N., Gould, T. J., Uchida, A., Cheng, J. X., Graham, M., Christiano, R., Frohlich, F., Lui, X., Buhman, K. K., Coleman, R. A., Bewersdorf, J., Farese, Jr., R. V. and Walther, T. C. (2013) Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocating from the ER to lipid droplets, *Developmental Cell*, vol. 24, pp. 384 – 399.
- Williams, C. A., Kronfeld, D. S., Hess, T. M., Saker, K. E., Waldron, J. N., Crandell, K. M., Hoffman, R. M., and Harris, P. A. (2004) Antioxidant supplementation and subsequent oxidative stress of horses during an 80-km endurance race, *Journal of Animal Science*, vol. 82, pp. 588–594.
- Wolins, N. E., DeHaan, K. N., Cifarelli, V. and Stoeckman, A. K. (2018) Normalized Neutral Lipid Quantitation by Flow Cytometry, *Journal of Lipid Research*, vol. 59, pp. 1294 – 1300.
- Wong, D. A., Bassilian, S., Lim, S. and Lee, W. P., (2004), Coordination of Peroxisomal β -Oxidation and Fatty Acid Elongation in HepG2 Cells, *Journal of Biological Chemistry*, vol. 279 pp. 41302 – 41309.
- Woo, Y. R., Cho, D. H. and Park, H. J. (2017) Molecular mechanisms and management of a cutaneous inflammatory disorder: psoriasis, *International Journal of Molecular Sciences*, vol. 18, pp. 1 – 26.
- Wu, Y. and Zhou, B. P. (2010) TNF-a/NF- κ B/Snail pathway in cancer cell migration and invasion, *British Journal of Cancer*, vol. 102, pp. 639 – 644.
- Wu, X., Zhang, L., Gurley, E., Struder, E., Shang, J., Wang, T., Wang, C., Yan, M., Jiang, Z., Hylemon, P. B., Sanyal, A. J., Pandak, W. M. and Zhou, H. (2008), Prevention of free fatty acid-induced hepatic lipotoxicity by 18 β -Glycyrrhetic acid through lysosomal and mitochondrial pathways, *Hepatology*, vol. 47, pp. 1905 – 1915.
- Yan, Q., Song, Y., Zhang, L., Chen, Z., Yang, C., Liu, S., Yuan, X., Gao, H., Ding, G., and Wnag, H. (2018) Autophagy activation contributes to lipid accumulation in tubular epithelial cells during kidney fibrosis, *Cell Death Discovery*, vol. 4, pp. 1 – 14.
- Yang, Y. Jiang, G., Zhang, P. and Fan, J. (2015) Programmed cell death and its role in inflammation, *Military Medical Research*, vol. 2, pp. 1 – 12.

Yang, F., Hanna, M. A. and Sun, R. (2012), Value-added uses for crude glycerol – a by-product of biodiesel production, *Biotechnology for biofuels*, vol. 5, pp. 1 – 10.

Yao, R., H., Liu, J., Plumeri, D., Cao, Y., B., He, T., Lin, L., Li, Y., Jiang, Y. Y., Li, J., and Shang, J., (2011), Lipotoxicity in HepG2 cells triggered by free fatty acids, *American Journal of Translational Research*, vol. 3 pp. 284 – 291.

Yamashina, S., Sato, N., Kon, K., Ikejima, K. and Watanabe, S., (2009), Role of mitochondria in liver pathophysiology, *Drug Discovery Today: Disease Mechanisms/Mitochondrial Mechanisms*, vol. 6, pp. 25 – 30.

Yoboue, E. D., Sitia, R., Simmen, T. (2018) Redox crosstalk at endoplasmic reticulum (ER) membrane contact sites (MCS) uses toxic waste to deliver messages, *Cell Death and Disease*, vol. 9, pp. 1 – 14.

Yokoyama, Y., Nimura, Y., Nagino, M., Bland, K. I. and Chaudry, I. H. (2005) Role of thromboxane in producing hepatic injury during hepatic stress, *Archives of Surgery*, vol. 140, pp. 801 – 807.

Yoon, B. K., Jackman, J. A., Valle-González, E. R. and Cho, N. J. (2018) Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications, *International Journal of Molecular Sciences*, vol. 19, pp. 1 – 40.

Yoshizumi, M., Perrella, M A., Burnett, J. C., Lee, M. E., (1993), Tumour necrosis factor down regulates endothelial nitric oxide synthase mRNA by shortening its half-life, *Circulation Research*, vol. 73, pp. 205 – 209.

Young, V. M., Toborek, M., Yang, F., McClain, C. J. and Hennig, B. (1998) Effect of linoleic acid on endothelial cell inflammatory mediators, *Metabolism*, vol. 47, pp. 566 – 572.

Younus, H. (2018) Therapeutic potentials of superoxide dismutase, *International Journal of Health Sciences*, vol. 13, pp. 88 – 93.

Zaar, M., Fedyk, C. G., Pidcock, H. F., Scherer, M. R., Ryan, K. L., Rickards, C. A., Hinojosa-Laborde, C., Convertino, V. A. and Cap, A. P. (2014), Platelet activation after presyncope by lower body negative pressure in humans, *PLoS One*, vol. 9, pp. 1 – 12.

Zaman, M. M., Martin, C. R., Andersson, C., Bhutta, A. Q., Cluette-Brown, J. E., Laposata, M. and Freedman, S. D. (2010) Linoleic acid supplementation results in increased arachidonic acid and eicosanoid production in CF airway cells and in cftr^{-/-} transgenic mice, *American Journal Physiology Lung Cell Molecular Physiology*, vol. 299, pp. L599 – L606.

Zechner, R., Zimmermann, R., Eichmann, T. O., Kohlwein, S. D., Haemmerle, G., Lass, A. and Madeo, F., (2012), Fat Signals – Lipases and lipolysis in lipid metabolism and signalling, *Cell Metabolism*, vol. 15, pp. 279 – 291.

Zeke, A., Misheva, M. Reményi, A., and Bogoyevitch, M. A. (2016) JNK signalling: regulation and functions based on complex protein-protein partnerships, *Microbiology and Molecular Biology Reviews*, vol. 80, pp. 793 – 821.

Zeng, X., Zhu, M., Liu, X., Chen, X., Yuan, Y., Li, L., Liu, J., Lu, Y., Cheng, J. and Chen, Y. (2020), Oleic acid ameliorates palmitic acid induced hepatocellular lipotoxicity by inhibition of ER stress and pyroptosis, *Nutrition and Metabolism*, vol. 17, pp. 1 - 14.

Zhang, J. Y., Kothapalli, K. S. D. and Brenna J. T. (2016) Desaturase and elongase limiting endogenous long chain polyunsaturated fatty acid biosynthesis, *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 19, pp. 103–110.

Zhang, Y., Xue, R., Zhang, Z., Yang, X. and Shi, H. (2012) Palmitic and linoleic acids induce ER stress and apoptosis in hepatoma cells, *Lipids in Health and Disease*, vol. 11, pp. 1 – 8.

Zhang, L., Keung, W., Samokhyalov, V., Wang, W. and Lopaschuk, G. D. (2010), Role of fatty acid uptake and fatty acid β -oxidation in mediating insulin resistance in heart and skeletal muscle, *Biochimica et Biophysica Acta –Molecular and Cell Biology of Lipids*, vol. 1801, no. 1, pp. 1-22.

Zhang, T., Murray, M., Klock, K., Miles, J. M. and Holman, R. T. (1997), Effects of incubation of long chain polyunsaturated fatty acids on platelet lipids and thromboxane release, *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 56, pp. 259 – 264.

Zhao, Y. Y., Miao, H., Cheng, X. L. and Wei, F., (2015), Lipidomics: Novel insight into the biochemical mechanism of lipid metabolism and dysregulation-associated diseases, *Chemico-Biological Interactions*, vol. 240, pp. 220-238.

Zhou, Y. P. and Grill, V. E. (1994) Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle, *The Journal of Clinical Investigation*, vol. 93, pp. 870 – 876.

Appendices

Appendix 1 – Analysis of the Hepatic Extract.

HPLC Standard Curve

Using a commercial standard of ULA, a five-point calibration curve was established by plotting the area under the curve of the standard peak verses the concentration of the fatty acid. A linear relationship was determined over five different concentrations ($20\mu\text{g}/\mu\text{L}$ to $100\mu\text{g}/\mu\text{L}$) of ULA with a correlation coefficient (R^2) of 0.9892 indicating a strong linear response.

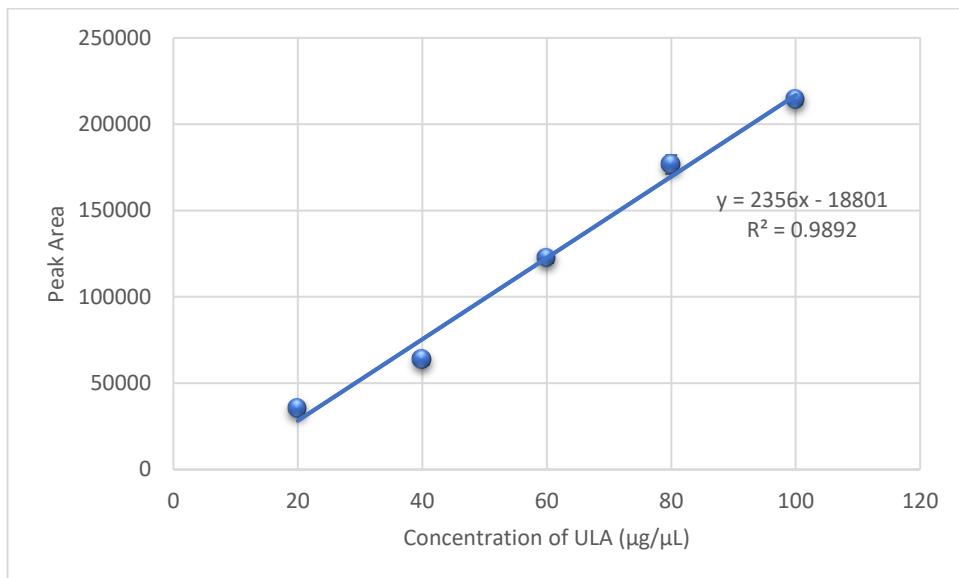
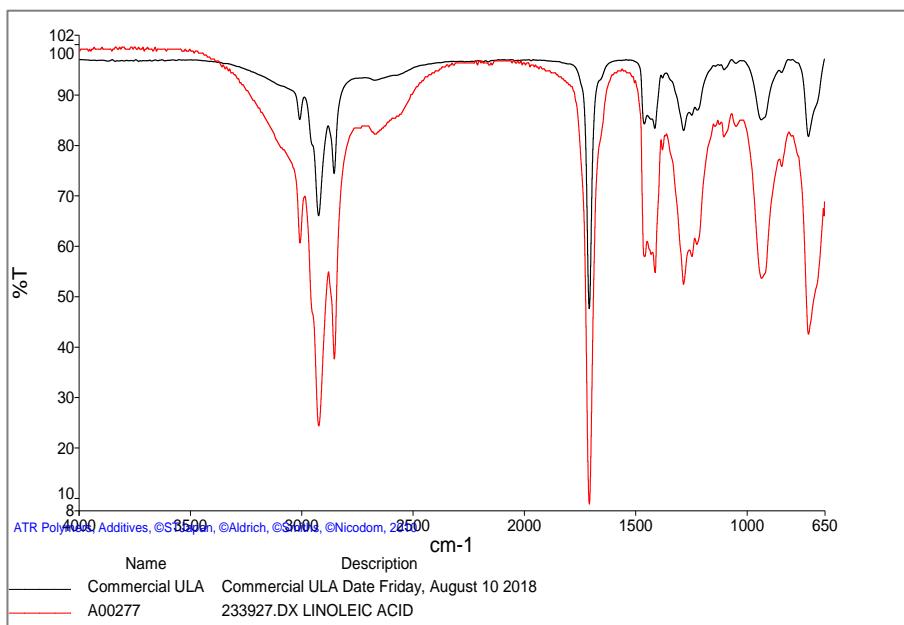
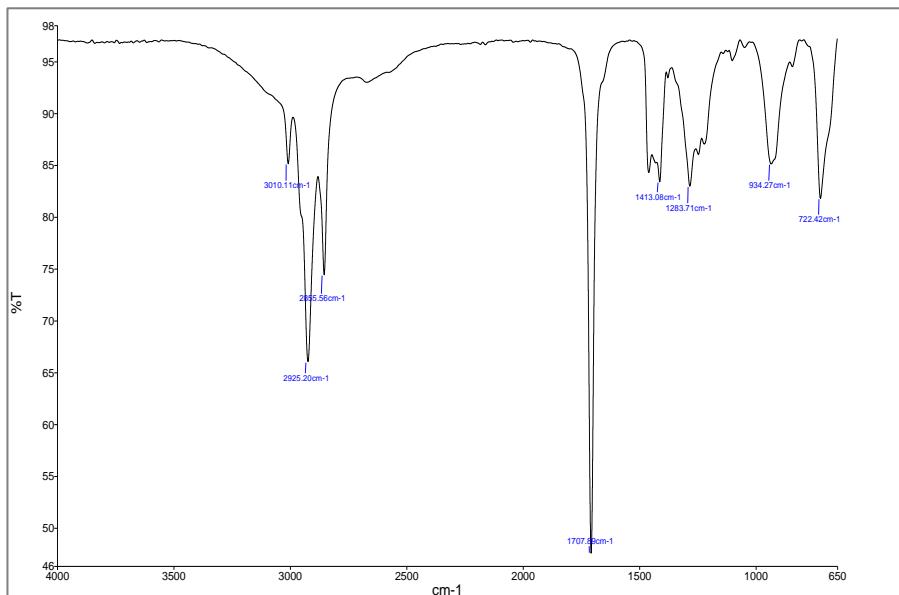


Figure A1.1. Calibration curve for commercial unconjugated linoleic acid obtained using a $250 \times 4.6\text{mm}$ Excil C18 $5\mu\text{m}$ reverse phase analytical column.

FTIR Analysis of the Hepatic Extract

Commercial Linoleic Acid



Search Score Search Reference Spectrum Description Search Library

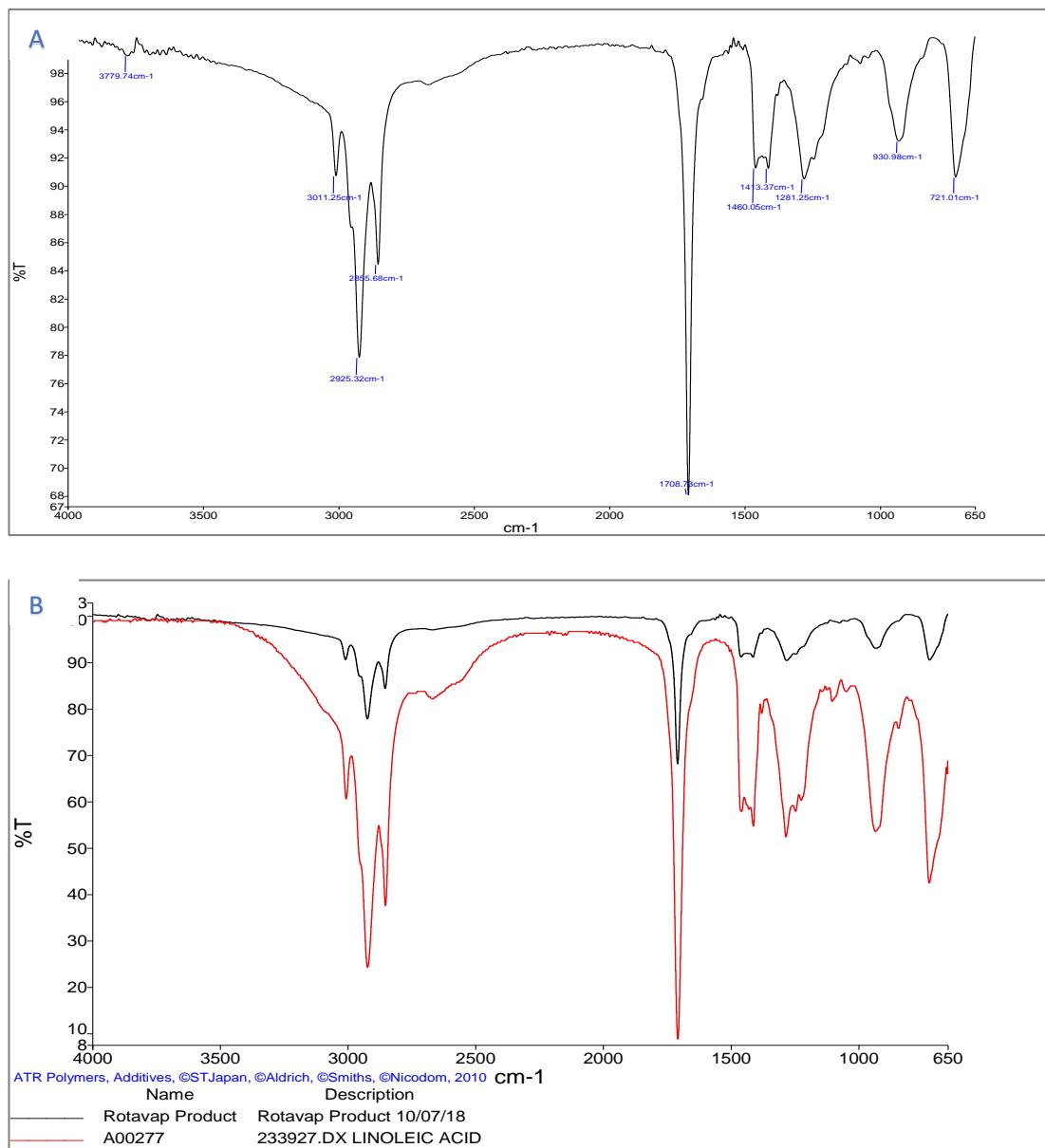
0.996878

233927.DX LINOLEIC ACID

POLYAM40

Figure A1.2. Calibration curve for commercial unconjugated linoleic acid obtained using a 250 x 4.6mm Excil C18 5 μ m reverse phase analytical column.

Hepatic Extract



Search Reference Spectrum Description

233927.DX LINOLEIC ACID

Search Score

0.988069

Search Reference Spectrum Description

233927.DX LINOLEIC ACID

Figure A1.2. Raw FTIR data obtained for the Hepatic extract (A). FTIR data for hepatic extract overlaid against the library spectrum for linoleic acid (B).

GC-MS analysis of underivatised ULA and the Hepatic Extract

In order to further confirm that the fraction collected during HPLC analysis of the hepatic extract was unconjugated linoleic acid, a crude, underivatized sample of both commercial ULA and the hepatic extract was initially analysed using GC-MS. According to Quehenberger *et al* (2011), GC has become widely adopted as a reliable tool for the analysis of complex mixtures of fatty acids.

Gas Chromatography-Time of Flight-Mass Spectrometry (GC-TOF-MS) was used initially to tentatively identify the presence of ULA in the hepatic extract collected in the HPLC fraction. Comparisons were made between an underivatised standard of commercial ULA and the underivatised hepatic extract.

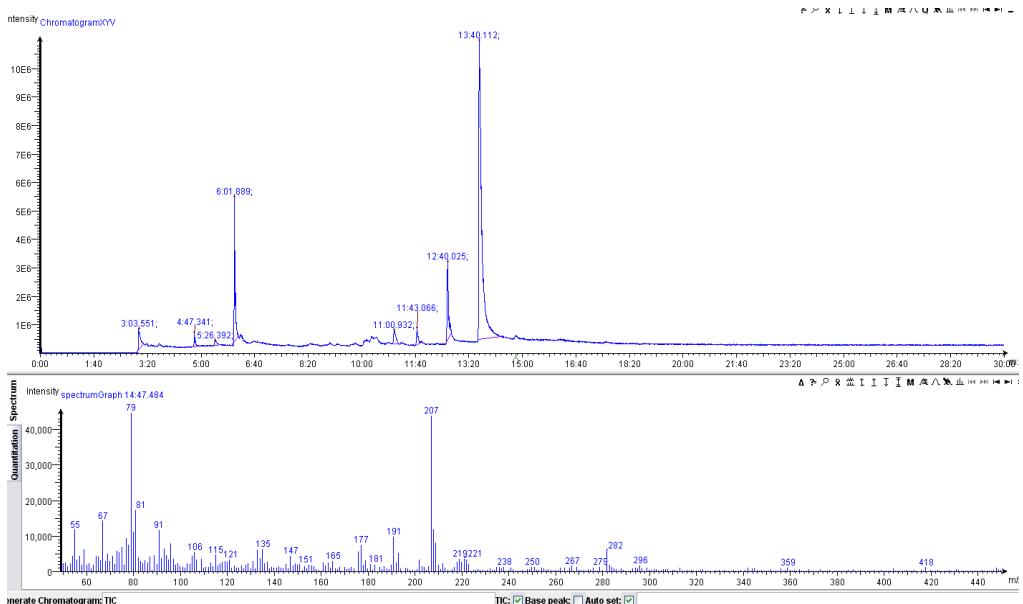


Figure A1.3. GC-MS Chromatogram of the derivatised hepatic extract. Peaks are labelled with retention times

Table A1.1.. Identification of the other peaks observed in the underivatised sample of hepatic extract.

Retention (minutes)	time	MS library match
20.14		9-Hexadecenoic Acid (Palmitoleic Acid)
20.25		9-Octadecenoic Acid (Oleic Acid)
21.32		8, 11-Octadecenoic Acid, methyl-ester
22.12		9, 12-Octadecanoic Acid (Z, Z) (Linoleic Acid)
23.21		5,8,11,14-Eicosatetraenoic Acid, methyl-ester (Arachidonic Acid, methyl-ester)
24.11		5,8,11,14-Eicosatetraenoic Acid, methyl-ester (Arachidonic Acid, methyl-ester)

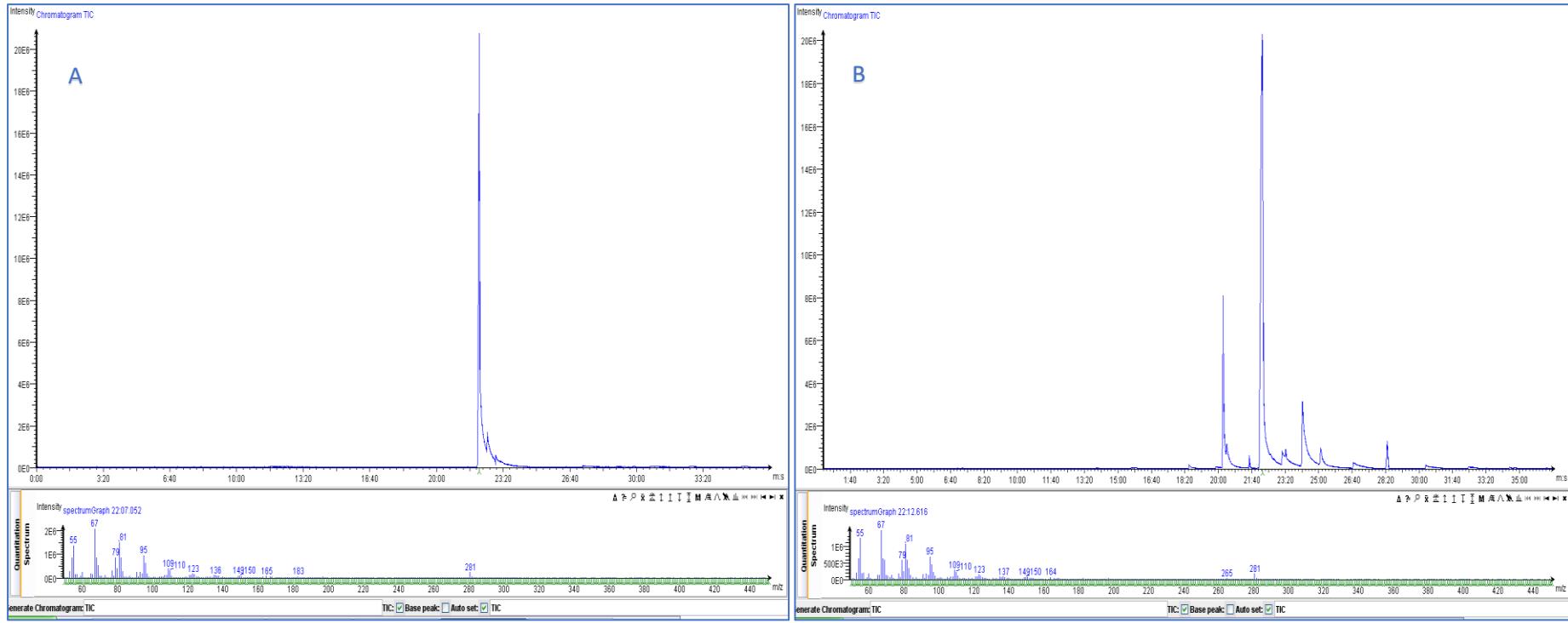


Figure A1.4. GC-MS Chromatogram of an underivatised standard of commercial linoleic acid (A) and an underivatised sample of the hepatic extract fraction (B). The main peak in each chromatogram, appearing at 22:07 and 22:12 respectively, have been identified, based on the NIST spectral library, as linoleic acid in its *cis* 9, *cis* 12 formation. The smaller peaks that appear to be tailing the main peak in chromatogram A occur as a result of the polar carbonyl groups associated with the underivatised fatty acid. In chromatogram B, several other peaks were identified as other fatty acids.

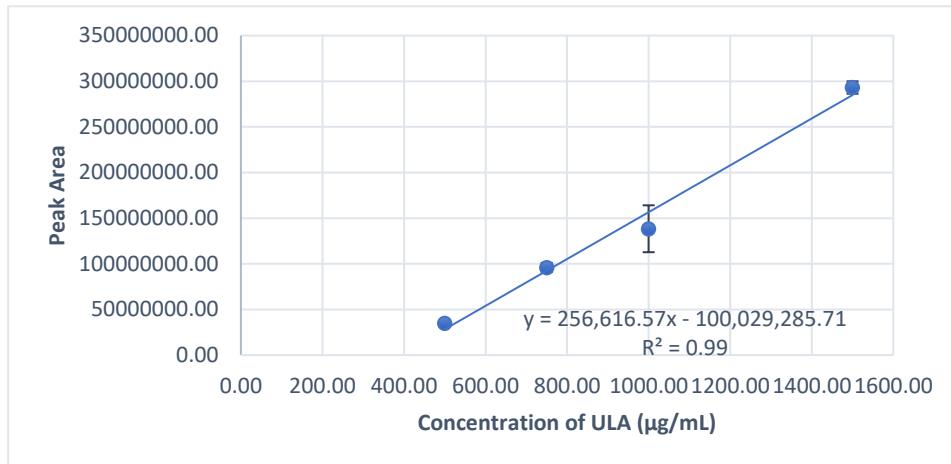


Figure A1.4. Calibration curve for commercial unconjugated linoleic acid obtained using GC-MS.

A four-point calibration curve was constructed from the analysis of the aforementioned working standard of the commercial ULA (Figure 7.6) for quantitative purposes.

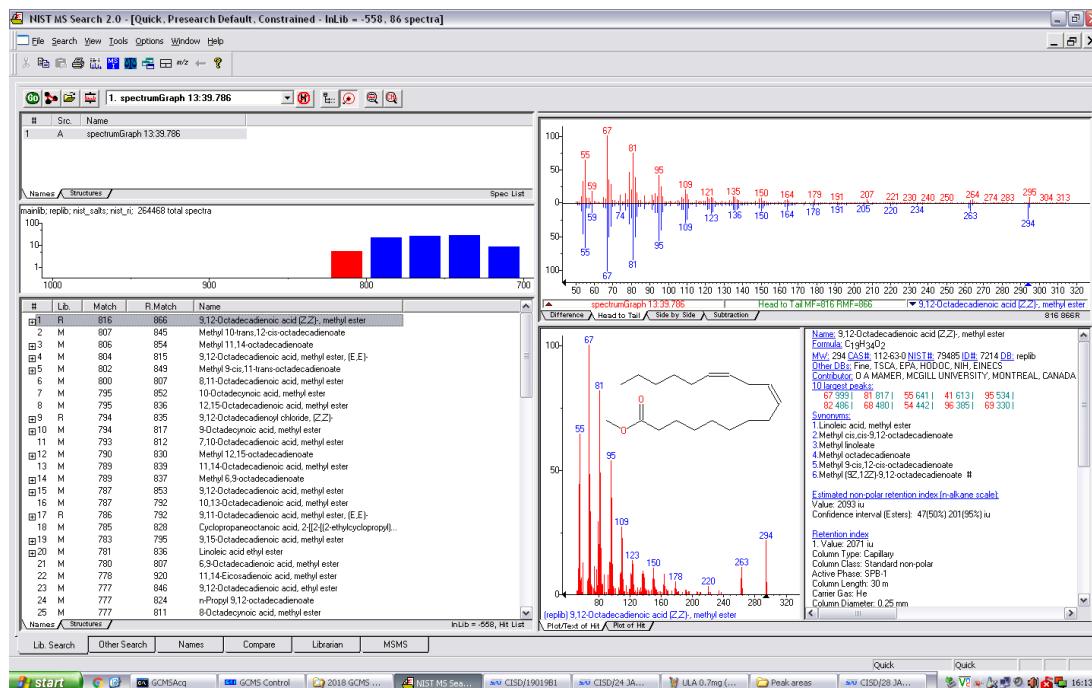


Figure A1.5. NIST Library match for the commercial standard of ULA

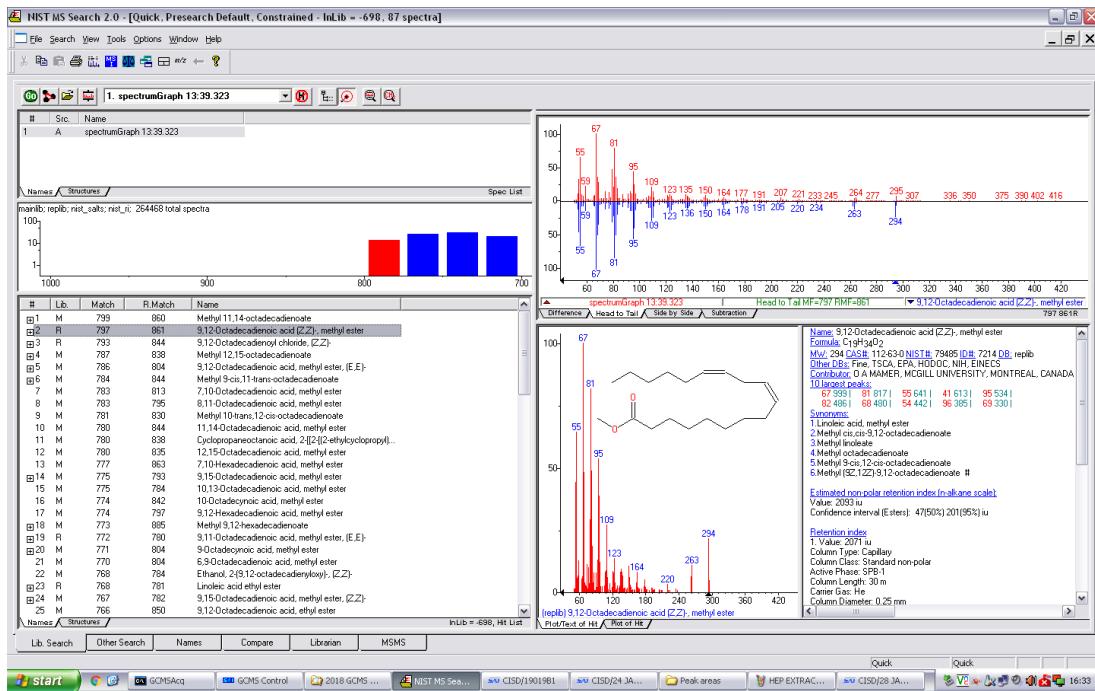


Figure A1.6. NIST Library match for the Hepatic Extract

The Effects of the Hepatic Extract on Cell Viability in HepG2 Cells

As the extract was derived from the hepatic tissue of equines that had succumbed to a fatal haemorrhagic condition, it was decided to assess the effects of the hepatic extract on the viability of HepG2 cells. Cells were treated with the extract at dilution ratios comparable to those used for the analysis of a commercial standard of ULA (figure 4.5).

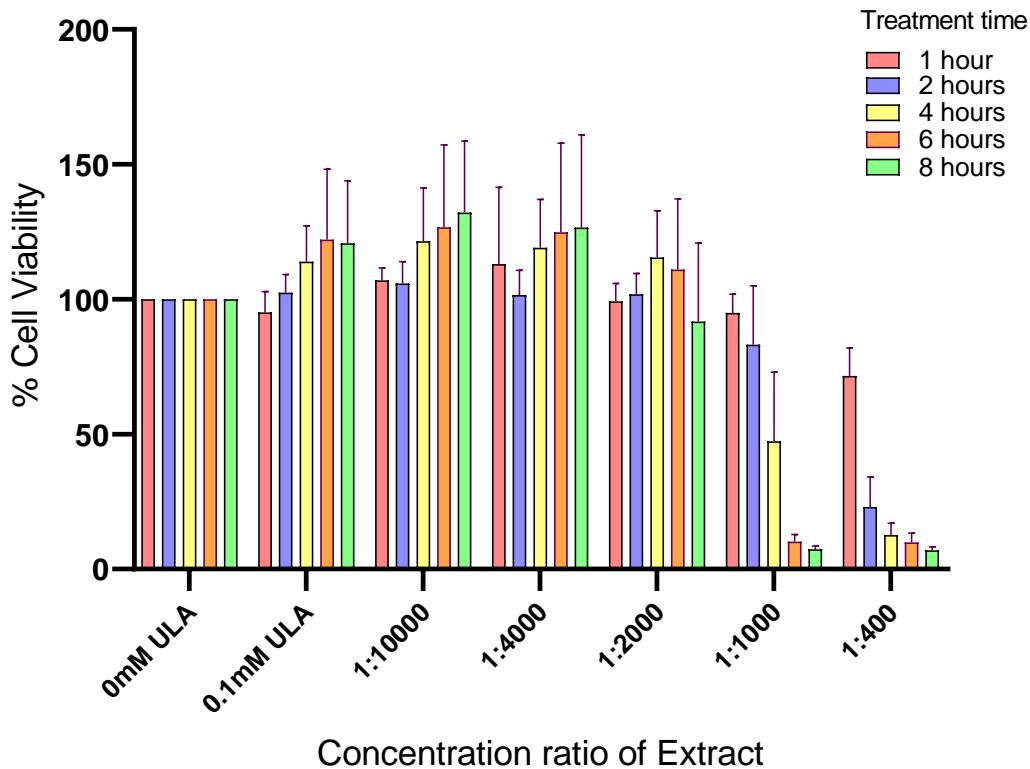
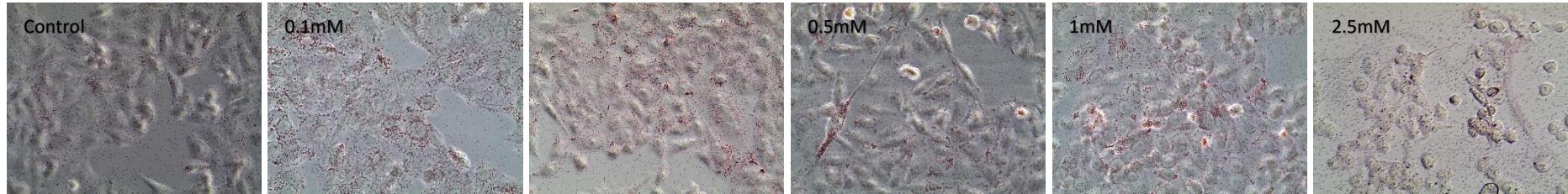


Figure 4.5. The effects of the hepatic extract on cell viability in HepG2 Cells

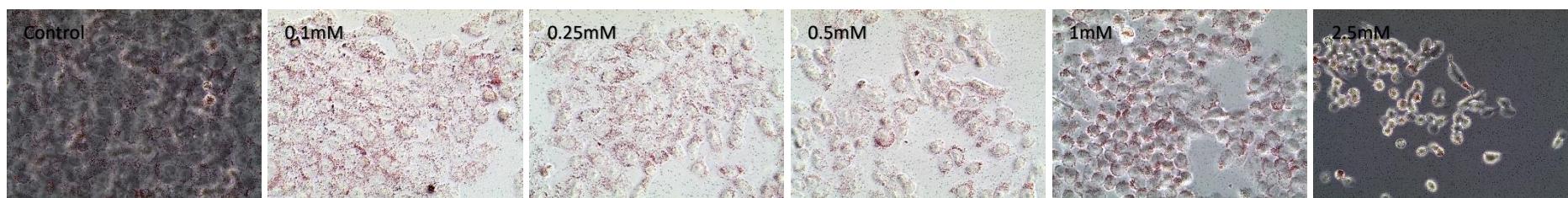
Cells treated with the hepatic extract at dilutions of 1:1000 and 1:400 induced the most significant reduction in cell viability, particularly after 6 and 8 hours of exposure. Cells treated with the extract at lower dilutions, i.e. 1:10,000, 1:4000 and 1:2000 induced a similar effect on cell viability as cells treated with 0.1mM of the ULA standard, with a slight hermetic response being observed.

Appendix 2

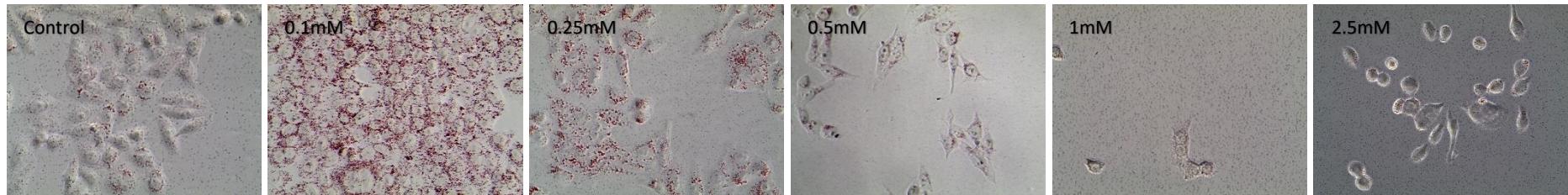
1 hour



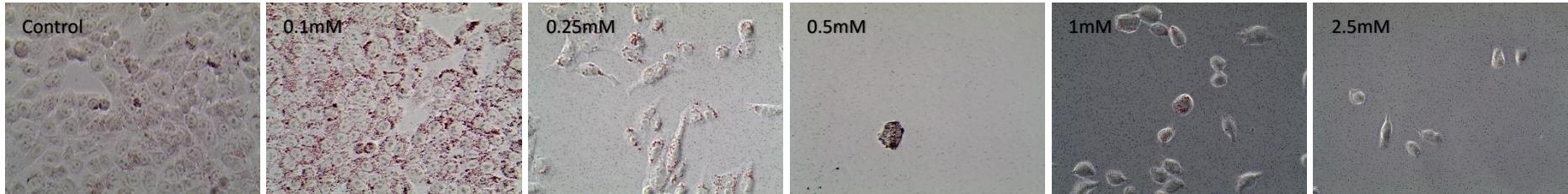
2 hours



4 hours



6 hours



8 hours

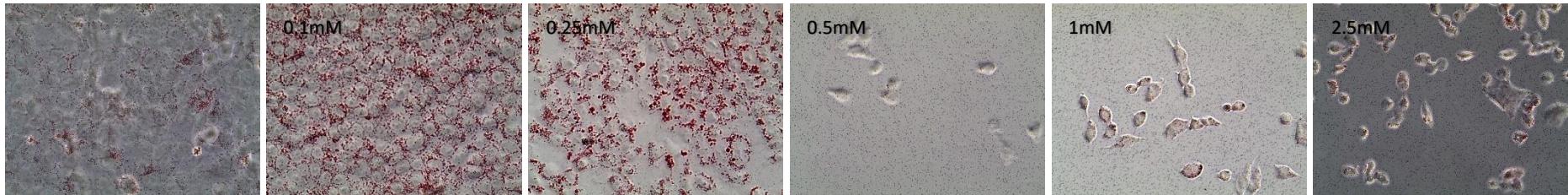
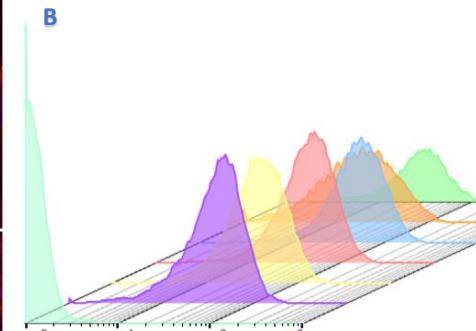
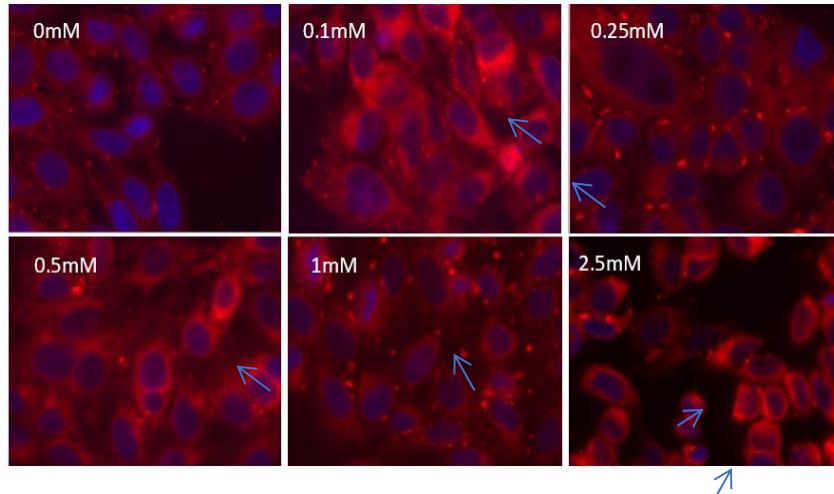


Figure A2.1 Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over different time intervals. Control cells were grown in media containing fatty acid free BSA and 1% IPA.

A – 1 hour



A – 2 hours

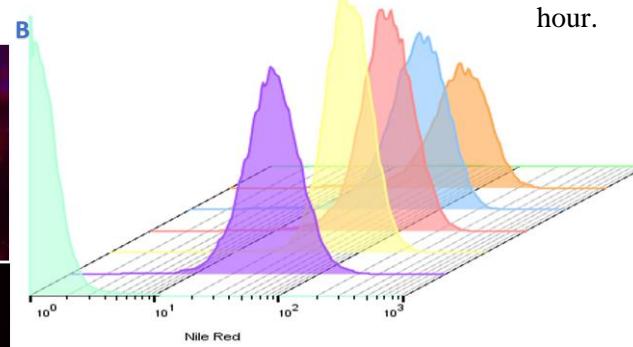
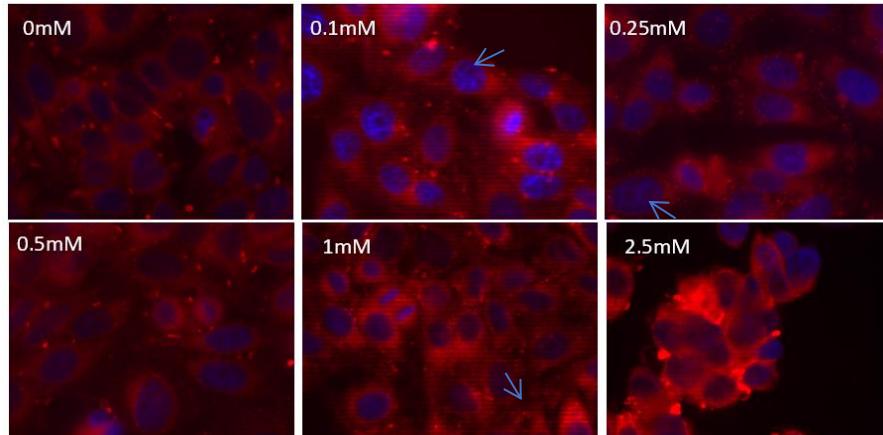
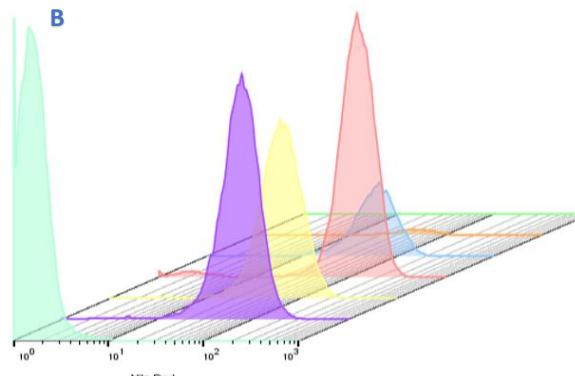
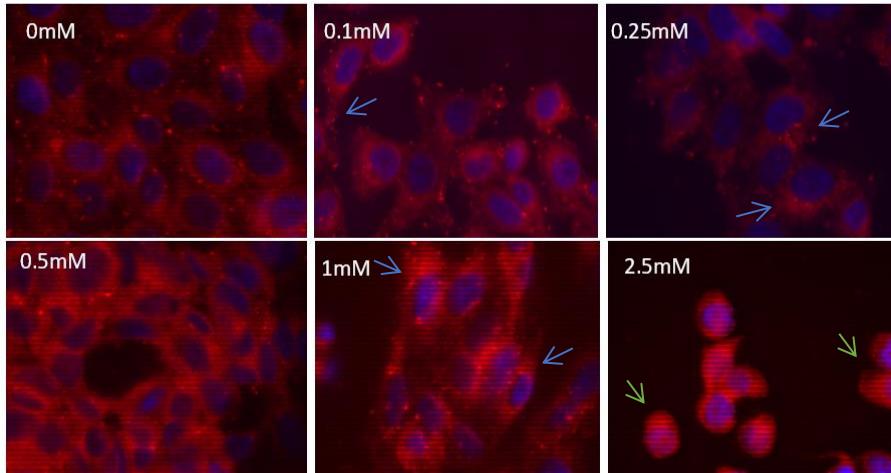


Figure A2.2.1. The following figures depict intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA. Intracellular lipid accumulation was qualitatively determined using fluorescent microscopy (A). Fluorescent images ($\times 1000$) were obtained using the lipophilic stain Nile Red for intracellular lipid imaging (red) and the nuclear stain DAPI for nucleic imaging (blue). Intracellular lipid accumulation was quantified using flow cytometry (B).

Figure A2.2.2. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 1 hour.

Figure A2.2.3. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 2 hours.

A – 4 hours



A – 6 hours

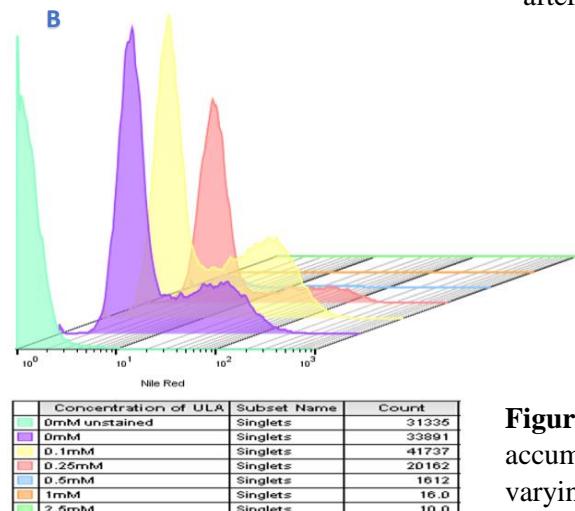
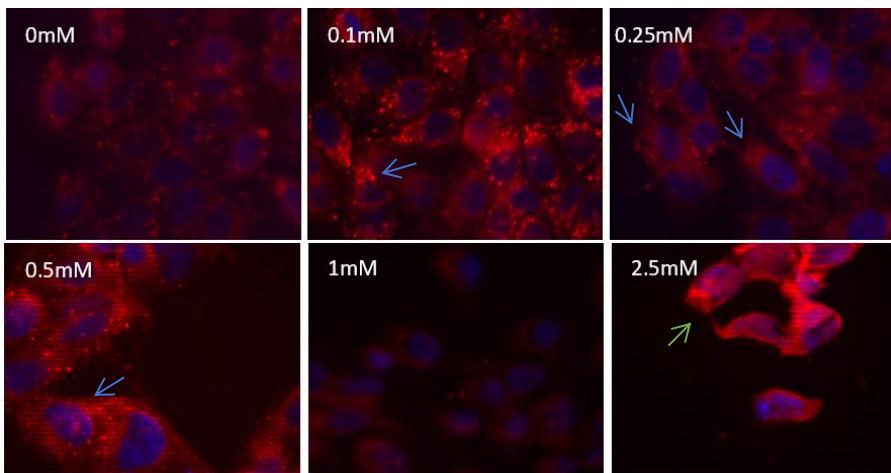


Figure A2.2.4. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 4 hours.

Figure A2.2.5. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 6 hours.

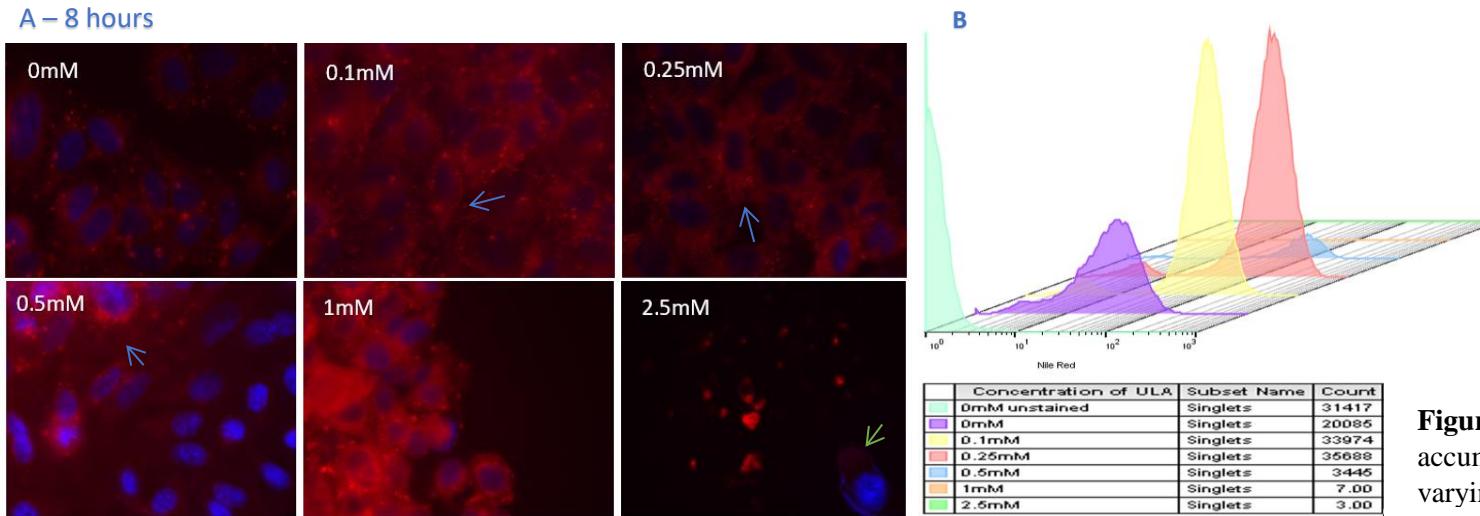


Figure A2.2.6. Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 8 hours.

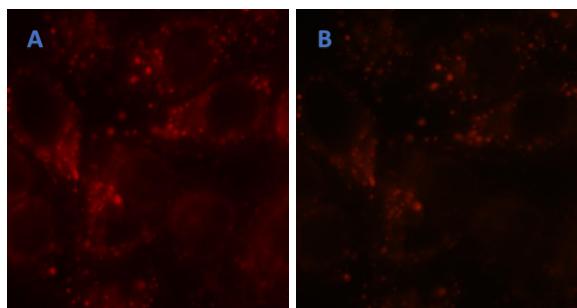
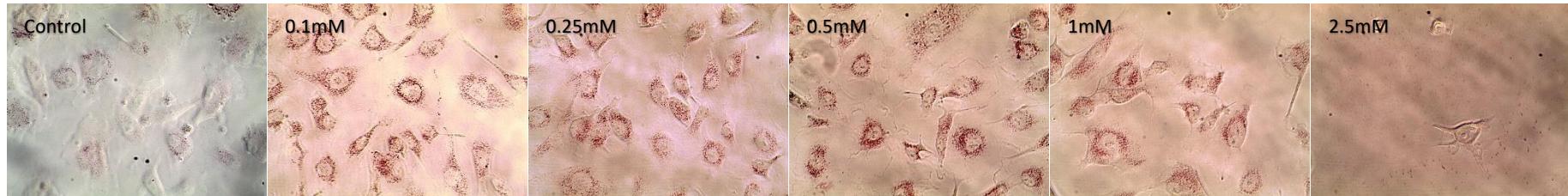


Figure A2.2.7. Examples of red fluorescence (A) and yellow/gold fluorescence (B) in the same cells treated with 0.1mM of ULA for 6 hours.

Fluorescent micrographs allowed for the visualisation of intracellular lipid accumulation in HepG2 cells. As Nile Red is reported to fluoresces yellow gold in the presence of neutral lipids and red in the presence of polar lipids (Diaz *et al*, 2008), micrographs were captured using two spectral settings and the merged to increase lipid body resolution, as described in section 3.1.10. Distinct lipid droplets can be seen in ULA treated cells, as indicated by the blue arrows, particularly at 0.1mM. As the concentration of ULA and exposure time increases, cell morphology changes dramatically, particularly in cells treated with 2.5mM, as indicated by green arrows, suggesting that higher concentrations impacted cellular integrity.

Appendix 3

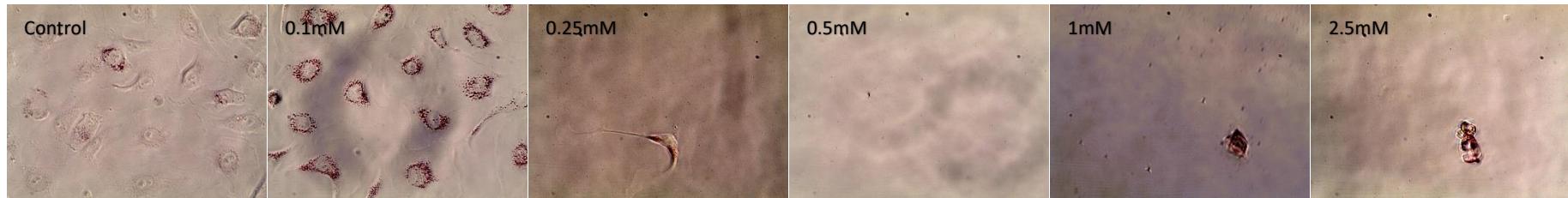
1 hour



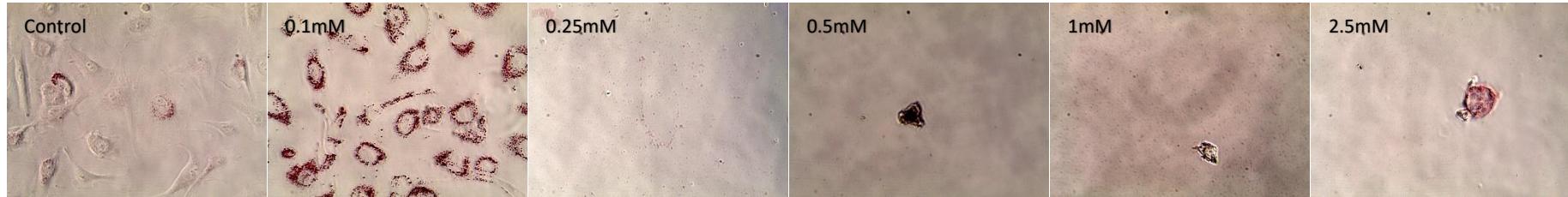
2 hours



4 hours



6 hours



8 hours

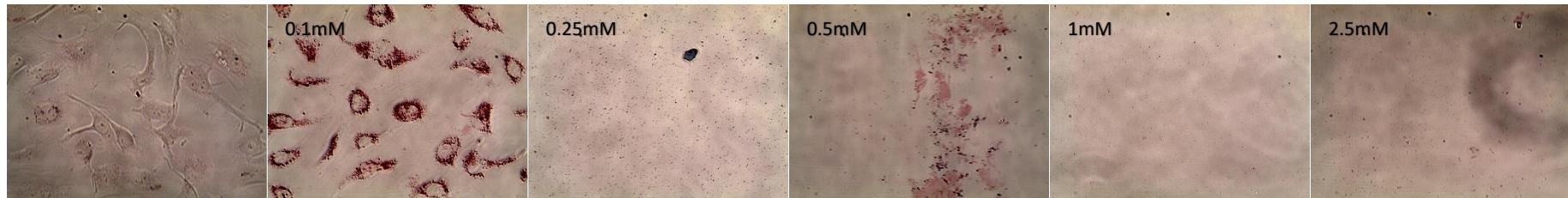
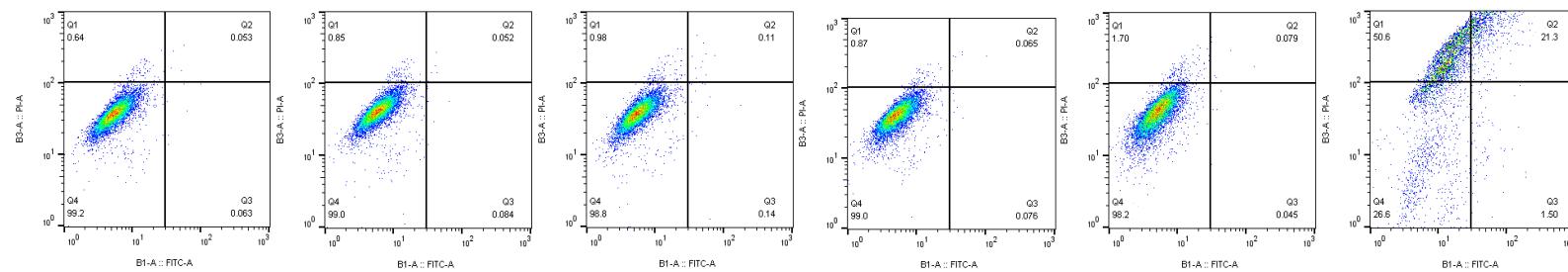


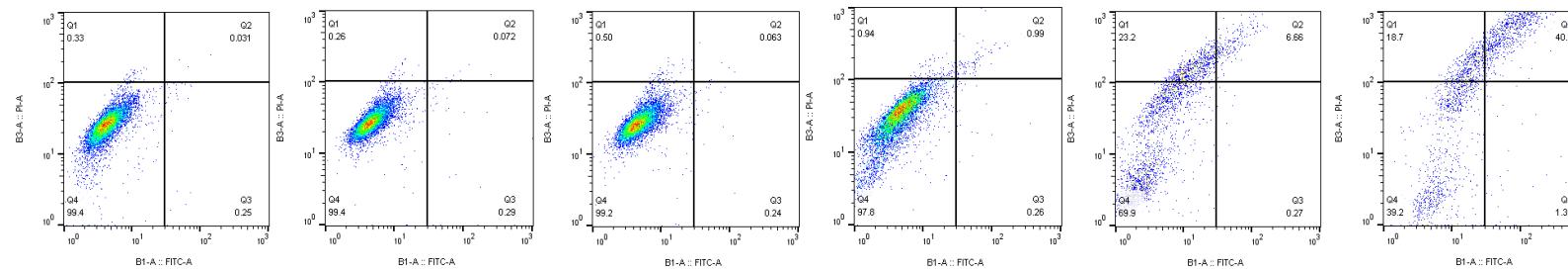
Figure A3.1. Oil Red O staining maps (x 200) of HUVEC cells treated with varying concentrations of ULA over different time intervals. Control cells were grown in media containing fatty acid free BSA and 1% IPA.

Appendix 4

2 hour



6 hour



Appendix 5 – Draft Publication 1 - Journal of Lipid Research

Working Title – *In Vitro* Effects of Unconjugated Linoleic Acid on the Liver and its Potential Impact on Haemostasis

Catriona Kielty^a, Damien B. Brady^{a, b} and Carmel Kealey^{a, b}

^a Bioscience Research Institute, Athlone Institute of Technology, Athlone, Co. Westmeath, Ireland.

^b Department of Life & Physical Science, Athlone Institute of Technology, Athlone, Co. Westmeath, Ireland.

Running Title:

Corresponding Author: Carmel Kealey

Email: ckealey@ait.ie

Athlone Institute of Technology,
Athlone,
Co. Westmeath
Ireland

Abbreviations Used:

ULA, Unconjugated linoleic acid, MFI, median fluorescence intensity, ROS, reactive oxygen species, SOD, superoxide dismutase, BCL-2, B Cell Lymphoma – 2, BAX, BCL-2-associated X protein, BIM, BCL-2-interacting mediator of cell death,

Abstract and Keywords

Key Words: Apoptosis, Cytokines, Eicosanoids, Fatty Acid, Fatty Liver, Lipotoxicity, Linoleic acid.

Introduction: Unconjugated Linoleic Acid (ULA), an essential $\omega 6$ fatty acid, has been linked with an increased inflammatory response, platelet inhibition and reduced platelet thromboxane. Excessive unresolved inflammation has been associated with several inflammatory mediated diseases. Plasma free fatty acids are known to increase in several disease states as well as during fasting and strenuous exercise. This preliminary investigation aimed to elucidate the potential mechanisms by which ULA at supraphysiological concentrations may impact hepatic cell health.

Methods: Human hepatoma cells, HepG2, were assayed for the effects of ULA on cell viability and intracellular lipid accumulation. ELISA determined the effects of ULA on thromboxane A₂ and TNF- α production. Cell lysates were assayed for superoxide dismutase (SOD) activity. Flowcytometry was used assess ULA induced apoptosis and necrosis.

Results: Supraphysiological concentrations of ULA (1mM and 2.5Mm) induced a significant reduction in cell viability and the ability of HepG2 cells to sequester lipids intracellularly. ULA treated HepG2 cells presented with a significant reduction in SOD activity and a significant increase in thromboxane production. They also presented with increased apoptosis and necrosis.

Conclusion: ULA, at supraphysiological concentrations induces cell dysfunction in several ways. While ULA may not directly induce specific disease states, in a system compromised by disease or physiological stress, increasing plasma concentrations of ULA, leading to increasing ROS and diminished antioxidant capabilities may initiate or further exacerbate pathophysiology.

Introduction

Lipids, particularly, fatty acids are among the most abundant cellular metabolites, with immense diversity in structure and function. They play an essential role in cellular membrane assembly and architecture, provide anhydrous stores of energy and serve as signalling molecules in an array of cellular processes (1).

Dietary linoleic acid (C18:2 Δ9, Δ12) (ULA), found in its unconjugated form, is an ω-6 essential fatty acid. It is an eighteen-carbon polyunsaturated fatty acid with two *cis* double bonds after the ninth and twelfth carbon from the carboxyl end. ULA is a precursor for arachidonic acid, as well as numerous molecules involved in inflammation and coagulation. It has previously been associated with a pro-inflammatory response (2), through the production of pro-inflammatory cytokines and eicosanoids (3). Excessive, unresolved inflammation has been reported to lead to uncontrolled tissue damage, pathology and disease (4). Linoleic acid has previously been associated with platelet inhibition and with a reduction in platelet thromboxane A₂ production (5).

In contrast, the ω-3 PUFAs such as α-linolenic acid, have been implicated in anti-inflammatory mechanisms (6). Both α-linolenic acid and ULA are metabolised by the same enzyme, Δ6-desaturase (6). While the preferred substrate for this enzyme is α-linolenic acid, the abundance of ULA in the western human diet means that its metabolism, according to Calder (2012) is quantitatively more important. Linoleic acid has been reported to lower the rate of metabolism of ω-3 PUFAs, resulting in a decrease in their availability and in turn their physiological functions (3).

The liver is the main metabolic organ in the body, playing a vital role in lipid metabolism. It is responsible for the uptake and esterification of free fatty acids into triacylglycerols for assembly into very low-density lipoproteins (VLDL), which in turn deliver the constituent fatty acids to various other tissues (7, 8). The liver is also the primary source of several circulating coagulation factors, and acute liver injury as well as chronic liver disease are associated with alterations in blood coagulation (9).

Disturbed hepatic lipid metabolism is associated with elevated circulating free fatty acids, such as palmitic and oleic acid (10). According to Karpe *et al.* (2011), and Eaton *et al.* (1969), free fatty acid

turn-over in plasma is extremely rapid, with a half-life of 2 to 4 minutes, however, several authors (13, 14, 15) have implied that the deleterious effects of free fatty acids on homeostasis are as a result of persistently elevated plasma free fatty acid levels.

Based on evidence linking ULA to a strong inflammatory response (3), and the involvement of the liver in important systemic functions including coagulation and immunity (7), this preliminary investigation aimed to begin the process of elucidating the potential mechanisms by which linoleic acid at supraphysiological concentrations may impact cellular health in the liver. The human hepatoma - derived cell line, HepG2, was chosen for this study. These cells have been reported to retain the function of fully differentiated primary hepatocytes, including normal hepatic metabolic functions and are therefore widely used in hepatotoxicity studies (16, 10).

Materials and Methods

Cell Culture Conditions and Fatty Acid Treatments

Human Hepatoma (HepG2) cells (Dr. Emily Crowley, Athlone Institute of Technology) were cultured in low glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and 1% L-glutamine (GibcoTM). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

ULA (Fisher Scientific) was dissolved in 100% molecular biology grade isopropyl alcohol (IPA) (Sigma Aldrich) (17) to create a 1M solution. From this stock, ULA treatment concentrations of 0.1mM, 0.25mM, 0.5mM, 1mM and 2.5mM were prepared in DMEM supplemented with fatty acid free BSA, at a concentration of 0.5% as the fatty acid carrier. The concentration of IPA was ≤1%. Cells were treated over intervals of 1, 2, 4, 6 and 8 hours. Control cells were treated with fatty acid free BSA supplemented DMEM containing molecular biology grade IPA at a concentration no higher than 1%.

Cell Viability in HepG2 Cells treated with LA

Analysis of cell viability was carried out using the Tetrazolium Bromide (MTT) (Sigma Aldrich) cell viability assay. HepG2 cells (2×10^4 cells/well) were allowed to adhere over night before being treated as described. A 0.5% stock solution of MTT was prepared in PBS after which a 1:10 working dilution was prepared in the appropriate treatment media. After each treatment interval, treatments were removed and media containing MTT was added to each well. Cells were then incubated at 37°C in a humidified atmosphere containing 5% CO₂, HepG2 cells, for 3 hours and HUVEC cells, for 4 hours. After incubation, the media was removed from all wells and 100μl dimethyl sulfoxide (DMSO) was added to each well. Absorbance was then measured at 540nm using a Biotek® multiwell plate reader.

Intracellular Lipid Accumulation in HepG2 Cells treated with ULA using Oil Red O Assay

A stock solution of Oil Red O was prepared by dissolving 360mg of Oil Red O powder in 100mL of molecular biology grade IPA (w/v), stirred at room temperature overnight, filtered using a 0.2um filter and stored at 4°C until in use. Using this stock solution, a 1.5:1 dilution was prepared using deionised

distilled water (ddH₂O), filtered again and left to stand for 20 minutes at room temperature before use. The working solution was freshly prepared for each assay.

HepG2 cells were seeded in 24 well plates at a density of 1 x 10⁵ cells/well, incubated overnight and treated as previously described. After treatment, media was removed, and cells were washed once with ice cold PBS before fixation in 10% formalin (500µL/well) for 1 hour. After fixation, cells were washed twice with ddH₂O. They were then incubated for 5 minutes with 60% IPA. The IPA was removed, and cells were dried completely before the addition of Oil Red O working solution (400µL/well). Cell were incubated for 10 minutes, after which the staining solution was removed. Cells were washed immediately 4 times with ddH₂O. After the fourth wash 500µL of ddH₂O was left on the cells and microscopic images were acquired (method adapted from Kim *et al*, 2013 and Cao *et al*, 2016). In order to quantify intracellular lipid accumulation, the ddH₂O was then removed and cells were dried completely. The stained cells were incubated at room temperature for 10 minutes in 100% IPA at a volume of 500µL/well, the absorbance of the elution from each well was measured at 500nm. Data was expressed as the percentage of lipid accumulation when compared to a positive growth control, cells that were grown in fatty acid free BSA constituted media, containing ≤1% IPA.

Fluorescent Microscopy for the Visualisation of Intracellular Lipid Droplets in HepG2 Cells treated with ULA using Nile Red

HepG2 cells were cultured on glass cell culture slides in 6 well plates at a seeding density of 1 x 10⁶ cells/well and allowed to adhere overnight prior to being treated with varying concentrations of ULA as previously described. After treatment, media was removed, and cells were washed five times with warm PBS. Cells were fixed with 500µL of cold 4% paraformaldehyde for 15 minutes. After fixation, cells were washed twice with cold PBS before being incubated in 500µL of cold permeabilization buffer (0.1% Triton X in PBS) for 5 minutes. Again, cells washed twice with cold PBS before 1mL of Nile Red (0.02µg/mL) was added to each well. The following steps were then carried out in a dark laboratory to avoid sample degradation. Cells were incubated in Nile Red for 15 minutes in the dark. After staining, Nile Red was removed, and cells were washed five times in cold PBS. Cover slips were then mounted with Fluoroshield™ mounting media containing DAPI. Slides were stored at -20°C overnight.

Fluorescent 12 bit images were obtained using a Leica DM 2000 fluorescent microscope fitted with a Leica DFC425 C digital camera and Leica Application Suite software. Fluorescence was viewed at two spectral settings. Yellow/gold fluorescence was viewed in the blue excitation range using a 450 – 490nm band pass exciter filter, a 510nm dichromatic mirror beam splitter and a 515nm long pass suppression filter. Red was viewed in the green excitation range using a 515 – 560nm band pass exciter filter, a 580nm dichromatic mirror beam splitter and a 590nm long pass suppression filter. Minimal image processing was performed using ImageJ software.

Quantification of Intracellular Lipid Droplets in HepG2 Cells treated with ULA using Nile Red and Flow Cytometry

Flow cytometry was used to quantify the accumulation of intracellular lipid droplets with both HepG2 cells and HUVEC cells. HepG2 cells were seeded in 24 well plates at a density of 1×10^5 cells/well and allowed to adhere over night before being treated as previously described. After treatment, trypsinised cells were washed with cold PBS twice before being re-suspended in 500 μ L of Nile Red and incubated in the dark at room temperature for 15 minutes. After incubation in Nile Red, cells were centrifuged as above, supernatant removed, and the cell pellet was washed twice in cold PBS. After the final wash step the cell pellet was re-suspended in 500 μ L of flow buffer and analysed using a MACSQuant® Analyse 10 flow cytometer (Miltenyl Biotec, Germany). Intracellular lipid accumulation was detected by fluorescence activated cell sorting (FACS). The fluorescence signals of both unstained cells and stained cells were obtained using a laser with an excitation of 488 nm and an emission of 585/40 nm. Unstained cells were used as an auto-fluorescence control. Data was obtained using FlowJo v10 software and was expressed using the arbitrary unit, median fluorescence intensity (MFI) (20).

Collection of Cell Lysates

HepG2 cells were seeded at a density of 4×10^5 cells/well in 6 well plates and allowed to adhere overnight before being treated as previously described. After treatment, media was collects, centrifuged and saved in individual Eppendorf tubes before being stored at -80°C. Cells were then washed with warm (37°C) PBS twice, after which 150 μ L of ice-cold lysis buffer (Sigma Aldrich), containing

protease inhibitor cocktail (Roche) was added to each well. Cells were then incubated on ice for 15 minutes and gently agitated, Cells were then scraped from each well, subjected to further agitation by pipetting to mechanically aid cell lysis, after which the lysates were transferred into a sterile Eppendorf tube. This suspension was then incubated on ice for a further 30 minutes before being centrifuged at 4000xg for 15 minutes at 4°C. The supernatant from each tube was then aliquoted into a fresh tube and stored at -80°C. The lysis pellet was resuspended in ice cold sterile PBS and stored at -80°C.

Thromboxane B₂ (TXB₂) in HepG2 Cells treated with LA

A Parameter™ Thromboxane B₂ Assay kit (R and D Systems) was used as per manufacturer's instructions.

Superoxide Dismutase (SOD) in HepG2 Cells treated with LA

A Superoxide Dismutase Assay –WST-1 kit (Sigma Aldrich) was used as per manufacturer's instructions.

Analysis of Apoptosis in HepG2 Cells treated with LA

Apoptosis was assessed using the Tonbo Biosciences® Annexin V-FITC (R & D Systems). Cells were cultured in 24 well plates at a density of 5 x 10⁵ cells/well and left to adhere overnight before being treated as previously described. Once the treatment time point was reached, cells were harvested using trypsin-EDTA (0.25%) supplemented with BSA (0.2%) (w/v) to minimise enzymatic damage to cellular membranes. Cell culture media, containing dead and dying cells, was also collected. Once all cells were fully detached and collected, they were centrifuged to remove trypsin. Cells were then washed three times with 500µL of warm (37°C) PBS and centrifuged once more to create a pellet. Annexin V-FITC staining was carried out as per manufacturer's instructions. Immediately before analysis propidium iodide (Miltenyl Biotec, Germany) was added to each sample at a rate of 5µL per 500 µL (v/v) of cell suspension. Samples were then analysed, at a rate of 10,000 events per sample by flow cytometry using a MACSQuant® Analyse 10 flow cytometer (Miltenyl Biotec, Germany).

Statistical Analysis

Data are presented as the mean \pm standard error of the mean (S.E.M). Statistical analysis was performed on scientific data using GraphPad Prism 8 software. Significance was determined by one-way-ANOVA, followed by Bonferroni's post hoc test.

Results

Cell Viability

Cell viability assays (figure 3) indicated that increasing concentrations of ULA as well as increasing exposure time to the fatty acid initiated a significant reduction in the ability of HepG2 cells to reduce tetrazolium into formazan ($P = <0.0001$). The production of concentration-response curves substantiated this observation. At 1 hour an IC_{50} of 0.44mM (± 0.021) was observed, however, as the length of time that HepG2 cells were exposed to ULA, a decrease in the tolerance of the cells to ULA was observed, with an IC_{50} of 0.16mM (± 0.005) observed at 8 hours. This suggested that both the concentrations of ULA and the length of time HepG2 cells are exposed to ULA significantly reduced cell viability.

Intracellular Lipid Accumulation

Flow Cytometry was used to determine intracellular lipid accumulation through cellular uptake of the fluorescent lipophilic stain Nile Red, with increasing median fluorescence intensity (MFI) correlating to increasing lipid accumulation. MFI can be used as an indicator of intracellular lipid accumulation (20, 21). An increase in MFI was observed in cells treated with 0.1mM and 0.25mM of ULA across all time points when compared to the untreated control (figure 4a). Cells treated with 0.25mM of ULA, induced the greatest increase in MFI, with an MFI value of 2.2 (± 0.19) compared to an MFI of 1.0 (± 0.28) for untreated cells ($P = 0.0370$). However, increasing concentrations did not induce a dose dependent increase in MFI. While lower concentrations (0.1mM and 0.25mM of ULA) induced an increase in MFI over 8 hours, a decrease in MFI was observed in cells treated with higher concentrations, namely 1mM and 2.5mM.

Light micrographs (figure 4b) of Oil Red O stained HepG2 cells indicated the occurrence of intracellular lipid accumulation through the presence of intracellular lipid droplets. An increase in the presence of intracellular lipid droplets was observed (red stained circular bodies), particularly in cells treated with 0.1mM and 0.25mM of ULA between 1 hour and 8 hours of exposure. These images correlate with

results presented in figure 4a, indicating that HepG2 cells possess the ability to tolerate these concentrations of ULA, potentially through the formation of intracellular lipid droplets.

Fluorescent micrographs of Nile Red stained ULA treated cells also demonstrated the presence of intracellular lipid droplets. Distinct lipid droplets were observed in ULA treated cells, as indicated by in figure 4b, particularly at 0.1mM in cells treated for 1 hour. As the concentration of ULA and exposure time increased, a dramatic change in cell morphology was observed, particularly in cells treated with 2.5mM (figure 4a and 4b, 8 hours). This suggested that higher concentrations impacted cellular integrity.

The Effects of ULA on Superoxide Dismutase (SOD) in HepG2 Cells.

A decrease in SOD activity in HepG2 cells was observed as the concentration of ULA increased, however, the trend was not dose or time dependent (figure 5). The decrease in SOD activity was apparent as the treatment time increased past 4 hours. SOD activity in cells treated with 2.5mM of ULA appeared to be significantly reduced when compared to SOD in cells treated with 0.1mM of ULA ($P = 0.0009$).

The effects of ULA on the Production of Thromboxane in HepG2 cells.

Figure 6 demonstrates thromboxane B₂ production in HepG2 cells treated with varying concentrations of LA. ULA did not induce a time or dose dependent release of thromboxane B₂ in HepG2 cells. Cells treated with 0.1mM and 0.25mM produced significantly less thromboxane B₂ than the untreated control ($P = 0.0195$ and 0.0078 respectively). They also produced significantly less thromboxane B₂ than cells treated with 2.5mM ($P = 0.0172$ and 0.0070 respectively). This observation occurred after 4 hours of exposure. After this time point, cells treated with 2.5mM of ULA produced the highest concentration of thromboxane B₂.

The Effects of ULA on Apoptosis in HepG2 cells

The effects of ULA on cell death was analysed using flow cytometry and are presented in figure 7a and 7b. Increasing concentrations of the fatty acid over increasing exposure times had a marked effect on

cell viability as already observed in figure 3. Figure 7 A demonstrates that as concentrations reached 1mM and 2.5mM, cell population dispersed dramatically. Two distinct populations were observed in Q4 of the dot plots for cells treated at these concentrations, particularly after 6 (dot plots not presented) and 8 hours. Figure 7b represents the effects of ULA on the percentage of cells, at each treatment time point in each state; live, early apoptotic, late apoptotic and necrotic.

Concentrations of 0.1mM and 0.25mM of ULA along with untreated cells did not have any significant impact on ULA induced cell death (figure 7b). These data correlate with observations made during analysis of cell viability and intracellular lipid accumulation.

Cells treated with 1mM and 2.5mM had the most significant impact on cell death in this investigation. Cells treated with these concentrations of ULA showed a significant decrease in the number of events presenting as live cells when compared to untreated controls (figure 7b). While cells in these two treatment groups presented with signs of early and late apoptosis, necrosis was observed to be the most prominent form of cell death ($10.75 \pm 1.8\%$ in cells treated with 1mM and $46.55 \pm 4.1\%$ in cells treated with 2.5mM after 4 hours).

Discussion

Free fatty acids are essential substrates required for numerous functions (22). However, fatty acid overload can result in the impairment of normal membrane function, endoplasmic reticular stress, mitochondrial dysfunction and eventual cell death (23).

Cell Viability

As the concentration of ULA surpassed 0.25mM of ULA (figure 2), a significant decrease in cell viability was observed. This was not anticipated as several authors describe normal plasma free fatty acid concentrations ranging from 0.3 to 0.6mM in the overnight fasting state (24, 25, 26, 27). However, the most common plasma free fatty acids are palmitic acid and oleic acid (13), which indicates that the fatty acid type, structure or degree of saturation may affect cell viability. Molecular structure may play

a role in how a particular fatty acid impacts cellular activity (28, 29). Iuchi *et al* (2019) demonstrated the anti-proliferative effects of various PUFAs, including linoleic acid, and observed a correlation between increasing number of double bonds and decreasing cell viability. Hawkins *et al*, (1998) and Iuchi *et al*, (2019) proposed that PUFAs with more double bonds are more easily oxidised into highly active and potentially cytotoxic products such as hydrogen peroxides and aldehydes. Hawkins *et al*, (1998) also postulated that the mechanism of action may be due to the disruption of cellular membrane fluidity as well as the production of by-products of lipid metabolism, such as reactive oxygen species.

Intracellular Lipid Accumulation

Intracellular lipid accumulation is an accumulation of triacylglycerol-containing lipid droplets within the cytosol (31). Lipid droplets have been recognised as organelles with key functions and are considered to work as universal storage organelles for neutral lipids in most mammalian cells (32). It is also hypothesised that, in cases of intracellular lipid overload, lipid droplets may serve as buffers for potentially toxic lipids, preventing lipotoxicity and oxidative stress (32, 33).

However, intracellular lipid overload resulting in the accumulation of high concentrations of intracellular lipid droplets may induce toxicity, resulting in cellular dysfunction and death (34, 35). HepG2 cells treated with ULA, particularly lower concentrations (0.1mM and 0.25mM of ULA) induced an increase in MFI, correlating to an increase in intracellular lipid accumulation (36). Cells treated with 0.25mM of ULA, induced the greatest increase in MFI, however, as the concentration of ULA surpassed 0.5mM, MFI decreased, even after 1 hour of treatment (figure 4a) indicating a reduced cellular capacity to generate protective lipid droplets. While hepatocytes have the greatest capacity, next to adipocytes, to store lipid droplets, excessive accumulation of intracellular lipids within the hepatocyte, referred to as hepatic steatosis (37), has been associated with cell death (38). When non-adipose cells are chronically overloaded with fatty acids, inappropriate lipid accumulation can lead to cellular dysfunction resulting in eventual cell death (23, 39, 40). Apoptosis appears to be a principal route of cell death; however, necrosis may also occur (41).

In figures 4b and 4c, microscopic imaging shows an increase in red stain was observed between treated and untreated cells, particularly those treated with 0.1mM of ULA. Small circular bodies were observed in all micrographs including the untreated controls and appeared to surround the nucleus. These circular bodies were more abundant in treated cells. As lipid droplets are thought to form off the endoplasmic reticulum (42, 43), it may be postulated that red stained circular bodies observed close to the nucleus, stained blue, in figure 4c, are lipid droplets. Cells exposed to 2.5mM also showed a distinct change in morphology, even after 1 hour of treatment.

Due to the close association of lipid droplets with the endoplasmic reticulum, lipotoxicity in hepatocytes may induce endoplasmic reticulum stress and associated apoptosis (40). Wei *et al* (2007) demonstrated that saturated free fatty acids, at physiological concentrations (~0.1mM) induced endoplasmic reticulum stress within four hours in a rat hepatoma cell line. Zhang *et al*, (2012) also observed endoplasmic reticulum stress, in the form of elevated calcium flux, induced by linoleic acid in rat hepatoma cells. In the current investigation, a decrease in HepG2 cell viability was observed in cells treated with 0.25mM of ULA after 1 hour (figure 2), however, intracellular lipid accumulation decreased, once the treatment reached this concentration after 4 hours of exposure (figure 3). Borradaile *et al*, (2006) also reported endoplasmic reticulum stress, associated with increased reactive oxygen species, and cell death in cardiomyocytes and Chinese hamster ovary cells treated with 0.5mM of saturated fatty acids.

As the concentration of ULA increased, images obtained from both light micrographs through Oil Red O staining (figure 4b) and fluorescent micrographs through Nile Red staining (figure 4c) show a distinct change in cellular morphology. These data suggested that the ectopic over-accumulation of lipids resulted in a loss of cellular integrity. Over accumulation of lipids in non-adipose cells may enter non-oxidative pathways, such as unnecessary cellular signalling pathways (45), leading to cell injury and death (46, 47), however the specificities remain to be elucidated.

Assessment of the Inflammatory Effects of ULA though the Secretion of TNF- α

TNF- α is reported to be one of the most important cytokines, exerting a series of biological effects on several cell and tissue types (48). It has been titled a master regulator in several biological process,

including apoptosis and necroapoptosis and, as such, has been extensively studied (49). Systemic administration of TNF- α has also been reported to decrease platelet activation and inhibit thrombi formation in mice (50). Linoleic acid at concentrations of 0.25mM to 1mM, have been shown to stimulate TNF- α secretion in a number of cell lines, including Caco2 intestinal cells (51). In this investigation, there was no detectable secretion of TNF- α in either the untreated control cells, or HepG2 cells treated with ULA. These results are in agreement with the findings of Gutierrez-Ruiz *et al*, (1999), who reported that, while a number of pro-inflammatory compounds, including LPS, induced TNF- α expression in HepG2 cells, there was no effect on its secretion. Two positive controls, LPS, a potent activator of cytokine production according to Gutierrez-Ruiz *et al*, (1999), and ethanol (80mM), also both failed to induce TNF- α secretion in HepG2 cells. While Saad *et al*, (1995) observed TNF- α secretion in cultured rat hepatocytes treated with LPS at a concentration of 10 μ g/mL, this was not observed in HepG2 cells treated with the same concentration of LPS in this study, even though LPS injured THP-1 cells successfully resulted in TNF- α secretion. According to Neuman *et al*, (1998), treatment of HepG2 cells with 80mM of ethanol induced TNF- α secretion (31.5pg/mL). Contrary to the findings in this investigation, Neuman *et al*, (1998) also reported a TNF- α concentration of 8pg/mL in untreated HepG2 cells. These results indicate that, while free fatty acids, such as linoleic acid may induce TNF- α expression, secretion of the cytokine may not always occur. According to Josephs *et al* (2018), TNF- α is found in a soluble and membrane bound form. The soluble plasma form of TNF-alpha is cleaved from the membrane form by TNF-alpha-converting enzyme (TACE). While TACE expression has been reported in HepG2 cells (56), the impact of linoleic acid on this converting enzyme remains unknown.

The Effects of ULA on the activity of Superoxide Dismutase in HepG2 Cells.

Reactive oxygen species (ROS) are by-products of various enzymatic reactions in several cellular compartments, such as the cell membrane, cytoplasm, endoplasmic reticulum and mitochondria (57). At physiological concentrations, they regulate cellular homeostasis and participate in normal physiological processes, serving as second messengers and transducing complex intracellular signals involved in several biological functions (57, 58, 59). However, at pathological concentrations, ROS can

contribute to maladaptive responses leading to metabolic dysfunction, inflammatory signalling and cellular damage resulting in disease pathophysiology (57). Several studies have reported a link between obesity, fatty acid metabolism and increased ROS (60, 61, 62, 63). The superoxide anion, considered to be the primary type of ROS produced in the body (64), is continually produced during normal metabolism (65). According to Incalza *et al*, (2018) it is one of the first reactive species to be generated and is constantly produced in the inner membrane of mitochondria during electron transfer in the electron transport chain (66, 67). In order to protect against the potentially damaging effects of ROS, cells possess several antioxidant enzymes such as superoxide dismutases (SOD) (68).

Based on previous reports in the literature, free fatty acids increase oxidative stress and ROS production both *in vitro* and *in vivo* (26, 69, 70, 71, 72, 73). It was postulated that an increase in ROS in response to fatty acid exposure would initiate an increase in the antioxidant enzymes responsible for their intracellular clearance, a hypothesis shared by Vincent *et al*, (1999). This hypothesis was strengthened by the findings of Kuratko and Constante (1998) who observed an increase in MnSOD in normal rat intestinal epithelial cells in response to supplementation with 0.04mM various free fatty acids, including linoleic acid.

Increasing concentrations of ULA induced a non-monotonic decrease in cytosolic SOD activity in HepG2 cells. Similarly, Videla *et al*, (2004) reported that SOD activity was decreased in the livers of human patients suffering from NAFLD. Videla *et al*, (2004) found that NAFLD patients exhibited a pro-oxidant state in the liver, however, this pro-oxidant condition occurred concomitantly with a significant decrease in hepatic SOD. Videla *et al*, (2004) postulated that the progressive functional deficiency associated with the development of liver injury, particularly protein synthesis, may contribute to the lower activity of SOD observed in patients suffering from NAFLD. Several studies have hypothesised that lipid molecules such as free fatty acids may be positive modulators for misfolded protein aggregations (76, 77, 78). Choi *et al*, (2011) hypothesised that as cytosolic SOD is frequently in contact with lipids, lipid-protein interactions could potentially promote the lipid molecule mediated abnormal assembly of SOD.

However, both Kim *et al* (2005) and Choi *et al* (2011) determined that incubation of cytosolic SOD with saturated fatty acids, did not form aggregates. This suggests SOD oligomerisation may require at least monounsaturated fatty acids, implicating the involvement of double bonds in the process (Kim *et al*, 2005) and reiterating the suppositions of Hawkins (1998) and Ricchi *et al*, (2009) regarding the involvement of molecular structure.

The Effects of ULA on the activity of Thromboxane Production in HepG2 Cells

Diseases of the liver are strongly linked to coagulopathies mainly due to its role in haemostasis (7). Linoleic acid has been reported to effect eicosanoid production in a number of cell types, including the hepatic carcinoma cell line, HepG2 (79). Yokoyama *et al*, (2005) states that eicosanoids such as thromboxane play a pivotal role in producing hepatic liver injury. According to Nanji *et al* (1993) and Yokoyama *et al*, (2005), thromboxane levels in the liver increase during hepatic stress such as alcoholic liver injury and cirrhosis. Thromboxane has also been reported to promote hepatic inflammation and induce hepatic vasoconstriction and leucocyte adhesion in sinusoids (80, 91, 92).

Previously linoleic acid was reported to have antithrombotic properties with a number of authors reporting a reduction in thromboxane production in thrombin-stimulated platelets treated with the fatty acid (93, 94).

In the current investigation, HepG2 cells produced significant amounts of thromboxane B₂ when treated with higher concentrations of ULA, namely 2.5mM, when compared to cells treated with the lower concentrations of 0.1mM and 0.25mM. Untreated cells also produced significantly more thromboxane than cells treated with the lower concentrations of ULA. The data obtained from cells treated with 0.1mM and 0.25mM of ULA are consistent with work carried out by Needleman *et al*, (1982) and MacIntyre *et al*, (1984) who observed inhibition of thromboxane B₂ in human platelets treated with 0.2mM and 0.035mM of ULA respectively, when compared to untreated control cells.

In contrast, cells treated with higher concentrations, specifically 2.5mM of ULA, showed a significantly higher production of the eicosanoid. As arachidonic acid release from the phospholipid membrane relies on the activation of phospholipase A₂, it may be hypothesized that linoleic acid induced the activation

of phospholipase A₂ in ULA treated HepG2 cells. This, in turn, initiated the release of arachidonic acid from the cell membranes, consequently leading to its conversion to thromboxane. Suh *et al*, (2008) observed the release of arachidonic acid from the phospholipid membranes of primary chicken hepatocytes as a result of linoleic acid (0.1mM) induced phosphorylation of cytosolic phospholipase A₂, after 12 hours of supplementation. While comparable concentrations of ULA did not lead to increased thromboxane production in supplemented HepG2 cells in the current study, higher concentrations may have initiated more rapid intracellular reactions in response to lipid overload, the disparity may also be attributed to cell type.

It may also be hypothesised that intracellular conversion of ULA to arachidonic acid via Δ6, elongases such as ELOVL5 and Δ5 desaturase lead to the COX induced production of thromboxane. According to Whelan and Fritzsche, (2013), linoleic acid can be elongated and desaturated to other bioactive ω6 PUFAs, such as δ-linolenic acid and arachidonic acid. Subsequently, arachidonic acid can be converted to a number of bioactive compounds including eicosanoids, such as thromboxane.

Cell Death

In the present study, cells treated with 1mM and 2.5mM of ULA had the most significant impact on all types of cell death. Cells treated at these concentrations induced statistically more apoptosis and necrosis than any other treatment concentration. According to Lima *et al*, (2002) concentrations of certain fatty acids, particularly PUFAs can induce apoptotic cell death at close to physiological concentrations (0.1mM), however as concentrations increase, necrosis may be observed.

Cury- Boaventura *et al*, (2004) demonstrated that linoleic acid at concentrations ranging from 0.025mM to 0.2mM, induced apoptosis in Jurkat cells (immortalised T-lymphocytes), with evidence of DNA fragmentation, chromatin condensation and phosphatidylserine externalization, indicating induction of apoptosis (31). However, in the current investigation, significant late apoptosis was only observed in HepG2 cells after ULA treatments reached 1mM and 2.5mM for 8 hours (figure 7b). This may be due to the hepatocyte's propensity for lipid metabolism and sequestration.

According to Malhi *et al*, (2006) and Wu *et al*, (2008) free fatty acids induce c-Jun-N-terminal kinase (JNK) dependent lipoapoptosis in hepatocytes through activation of the pro-apoptotic BCL-2 proteins BIM and BAX, proteins that trigger the mitochondrial apoptotic pathway. Previous work shows that hepatocytes treated with saturated fatty acids demonstrated free fatty acid induced BAX activation and lipoapoptosis that are JNK dependent (99). According to Zeke *et al*, (2016), JNK activation has been reported in response to oxidative stress as well as endoplasmic reticulum stress. According to Wu *et al*, (2008) and Feldstein *et al*, (2004), accumulation of intracellular free fatty acids resulted in lysosomal permeabilization and, as a result, lysosomal-dependent apoptosis, in rat hepatocytes and HepG2 cells respectively.

Significant necrosis was observed in HepG2 cells treated with 1mM and 2.5mM more rapidly, after 4 hours and 2 hours of treatment respectively (figure 7b). Cnop *et al*, (2001) suggests that excessive free fatty acids may destabilise cellular membranes and induce rapid necrosis. They can also disrupt cellular environments and alter cellular and organelle membrane structure and function (23, 28, 95, 99). This is apparent in the microscopic imaging presented in figure 4b and 4c. Linoleic acid itself can be incorporated into cellular membrane phospholipids, functioning as a structural component to contribute to and influence membrane structure and fluidity (96). Pompeia *et al*, (2002) observed the potential onset of arachidonic acid mediated necrosis in leucocytes within 15 minutes. While necrosis did not appear to occur until cells had been treated with ULA for 2 hours in the current study (figure 7b), it may be postulated that the cytotoxicity of certain fatty acids is related to the carbon chain length and number of double bonds, with increasing chain length and number of double bonds correlating to increasing cytotoxicity (16).

While the mechanism by which free fatty acids induce apoptosis and necrosis have yet to be completely elucidated, Wu *et al*, (2008) proposes that more than one mechanism is involved in free fatty acid induced hepatic lipotoxicity.

Conclusion

Humans exist on a diet whereby ULA is the most abundant polyunsaturated fatty acid (103). The liver plays a key role in lipid metabolism. As such, the potential impact ULA may have on hepatic inflammation and haemostasis was investigated. This preliminary study demonstrates that ULA, at concentrations surpassing 0.25mM and exposure times of more than 4 hours, has a negative impact on several aspects of cellular homeostasis in HepG2 cells, including cell viability, the formation of protective intracellular lipid droplets and the activity of superoxide dismutase. At higher concentrations, namely 1mM and 2.5mM, ULA demonstrated induction of apoptosis and necrosis. As such it could be postulated that the deleterious effects of ULA on homeostasis may be the result of persistently elevated levels.

The current study indicates that ULA, at concentrations that would be considered supraphysiological, impact cell viability and intracellular lipid sequestration, affect antioxidant activity and eicosanoid production, and induces cell death. While the concentrations of ULA analysed in this investigation were higher than what would be considered physiologically normal, concentrations of fatty acids reaching 2.5mM have been reported in aberrant physiological states such as disease, fasting or strenuous exercise (26, 69, 104, 105). In a system compromised by disease or physiological stress, increasing plasma concentrations of ULA, leading to increasing ROS and diminished antioxidant capabilities may initiate or further exacerbate pathophysiology. Zaman *et al* (2010) states in pre-existing conditions of altered inflammatory regulation, exposure to increased ULA may potentiate an already dysregulated inflammatory response. A reoccurring postulation throughout the investigation was that the toxicity demonstrated by ULA may be related to the chain length and number of double bonds (16, 28, 30). While the mechanism of action of ULA on lipotoxicity in HepG2 cells remains to be elucidated, this preliminary study supports the proposition that ULA, at supraphysiological concentrations, may elicit its effect through the disruption of cellular and organelle membrane fluidity, a proposition that warrants further future investigation.

References

1. Haberkant, P . and Holthuis, J. C. M. (2014) Fat & fabulous: Bifunctional lipids in the spotlight, *Biochimica et Biophysica Acta*, VOL. 1841, PP. 1022 – 1030.
2. Simopoulos, A. P. (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases, *Experimental Biology and Medicine*, vol. 233, pp. 674-688.
3. Choque, B., Catheline, D., Rioux, V. and Legrand, P., (2014), Linoleic Acid: Between doubts and certainties, *Biochimie*, vol. 96, pp. 14 – 21.
4. Innes, J. K. and Calder P. C., (2018) Omega-6 fatty acids and inflammation, *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 132, pp. 41 – 48.
5. MacIntyre, D. E., Hoover, R. L., Smith, M., Steer, M., Lynch, M., Karnovsky, M. J. and SalzmanE. W. (1984) Inhibition of platelet function by *cis*-unsaturated fatty acids, *Blood*, vol. 63, pp. 848 – 875.
6. Calder, P. C. (2012), Omega-3 polyunsaturated fatty acids and inflammation processes: nutrition or pharmacology? *British Journal of Clinical Pharmacology*, vol. 75, pp. 645 – 662.
7. Ambrojo, K. S., Gardon Poggi, J. C. and Muñoz Juzado, A. (2013), Use of laboratory testing to diagnose liver and biliary dysfunction in the horse, *Journal of Gastroenterology and Hepatology*, vol. 2, pp. 807 – 813.
8. Barton, B. M., Morris, D. D., Reed, S. M. & Bayly, W. M., (1998), Equine Internal Medicine. Philadelphia: W. B. Saunders.
9. Kopec, A. K and Luyendyk, (2014) Coagulation in liver toxicity and disease: role of hepatocyte tissue factor, *Thrombosis Research*, vol. 133, pp. S57 – S59.
10. Asrih, M., Montessuit, C., Philippe, J., and Jornayvaz, F. (2015) Free Fatty Acids Impair FGF21 Action in HepG2 Cells, *Cellular Physiology and Biochemistry*, pp. 1767 – 1778.
11. Karpe, F. Dickmann, J. R., and Frayn, K. N. (2011), Fatty Acids, Obesity, and Insulin Resistance: Time for a Re-evaluation, *Diabetes*, vol. 60, pp. 2441 – 2449.
12. Eaton, R. P., Berman, M. and Steinberg, (1969) Kinetic studies of plasma free fatty acid and triglyceride metabolism in man, *The Journal of Clinical Investigation*, vol. 48, pp. 1560 – 1579.
13. Zhou, Y. P. and Grill, V. E. (1994) Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle, *The Journal of Clinical Investigation*, vol. 93, pp. 870 – 876.
14. Unger, R. H. (1995) Lipotoxicity in the pathogenesis of obesity-dependent NIDDM, genetic and clinical implications, *Perspectives in Diabetes*, vol. 44, pp. 863 – 870
15. Bollheimer, L. C., Skelly, R. H., Chester, M. W., McGarry, J. D. and Rhodes, C. J. (1998), Chronic exposure to free fatty acid reduces pancreatic β cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation, *Journal of Clinical Investigation*, vol. 101, pp. 1094 – 1101.
16. Lima, C. F., Fernandes-Ferreire, M. and Pereira-Wilson, C. (2006), Phenolic compounds protect HepG2 cells from oxidative damage: relevance of glutathione levels, *Life Sciences*, vol. 79, pp. 2056 – 2068.

17. Di Nunzio, M., Valli, V and Bordoni, A., (2011), Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells, *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 85, pp. 121 – 127
18. Kim, J. H., Kang, S. I., Shin, H. S., Yoon, S. A., Kang, S. W., Ko, H. C., and Kim, S. J. (2013), Sasa quelpaertensis and p-Coumaric Acid Attenuate Oleic Acid-Induced Lipid Accumulation in HepG2 Cells, *Bioscience, Biotechnology and Biochemistry*, vol. 77, pp. 1595 – 1598.
19. Cao, P., Huang, G., Yang, Q., Guo, J., Su, Z. (2016), The effect of chitooligosaccharides on oleic acid-induced lipid accumulation in HepG2 cells, *Saudi Pharmaceutical Journal*, vol. 24, pp. 292 – 298.
20. Wolins, N. E., DeHaan, K. N., Cifarelli, V. and Stoeckman, A. K. (2018) Normalized Neutral Lipid Quantitation by Flow Cytometry, *Journal of Lipid Research*, vol. 59, pp. 1294 – 1300.
21. Greenspan, P., Mayer, E. P., and Fowler, S. D. (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets, *The Journal of Cell Biology*, vol. 100, pp. 965 – 973.
22. Zechner, R., Zimmermann, R., Eichmann, T. O., Kohlwein, S. D., Haemmerle, G., Lass, A. and Madeo, F., (2012), Fat Signals – Lipases and lipolysis in lipid metabolism and signalling, *Cell Metabolism*, vol. 15, pp. 279 – 291.
23. Borradaile, N. M., Han, X., Harp, J. D., Gale, S. E., Ory, D. S., and Schaffer, J. E. (2006) Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death, *Journal of Lipid Research*, vol. 47, pp. 2726 – 2737.
24. Tikanoja, S. H., Joutti, A., and Liewendahl, B. K., (1989) Association between increased concentrations of free thyroxine and unsaturated free fatty acids, *Clinica Chimica Acta*, vol. 179, PP. 33 – 44.
25. Shultz, T. D. (1991) physiological free fatty acid concentrations do not increase free estradiol in plasma, *Journal of Clinical Endocrinology and Metabolism*, vol. 72, pp. 65 – 68.
26. Toborek, M., Barger, S. W., Mattson, M. P., Barve, S., McClain, C. J. and Hennig, B. (1996), Linoleic acid and TNF- α cross amplify oxidative injury and dysfunction of endothelial cells, *Journal of Lipid Research*, vol. 37, pp. 123 – 135.
27. Artwohl, M., Roden, M., Waldhausl, W., Freudenthaler, A. and Baumgartner-Parzer, S. M. (2003), Free fatty acids trigger apoptosis and inhibit cell cycle progression in human vascular endothelial cells, *The Federation of American Societies for Experimental Biology*, vol. 18, pp. 146 – 148.
28. Hawkins, R. A., Sangster, K. and Arends, M. J., (1998), Apoptotic death of pancreatic cancer cells induced by polyunsaturated fatty acids varies with double bond number and involves an oxidative mechanism, *Journal of Pathology*, vol. 185, pp. 61 – 70.
29. Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., Fantoni, L. I., Marra, F., Bertolotti, M., Banni, S., Lonardo, A., Carulli, N., and Loria, P. (2009) Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes, *Journal of Gastroenterology and Hepatology*, vol. 84, pp. 830 – 840.
30. Iuchi, K., Ema, M., Suzuki, M., Yokoyama, C. and Hisatomi, H. (2019) Oxidized unsaturated fatty acids induce apoptotic cell death in cultured cells, *Molecular Medicine Reports*, vol. 19, pp. 2767 – 2773.
31. Cury-Boventura, M. F., Pompeia, C. and Curi, R. (2004), Comparative toxicity of oleic acid and linoleic acid on Jurkat cells, *Clinical Nutrition*, vol. 23, pp. 721 – 732.

32. Olzmann, J. A., and Carvalho, P. (2018) Dynamics and functions of lipid droplets, *Nature Reviews*, vol. 20, pp. 137 – 155.
33. Welte, M. A. (2015), Expanding roles for lipid droplets, *Current Biology*, vol. 25, pp. R470 – R481.
34. Yao, R., H., Liu, J., Plumeri, D., Cao, Y., B., He, T., Lin, L., Li, Y., Jiang, Y. Y., Li, J., and Shang, J., (2011), Lipotoxicity in HepG2 cells triggered by free fatty acids, *American Journal of Translational Research*, vol. 3 pp. 284 – 291.
35. Jaishy, B. and Abel, E. D. (2016) Lipid, lysosomes and autophagy, *Journal of Lipid Research*, vol. 57, pp. 1619 – 1635.
36. Wolins, N. E., DeHaan, K. N., Cifarelli, V. and Stoeckman, A. K. (2018) Normalized Neutral Lipid Quantitation by Flow Cytometry, *Journal of Lipid Research*, vol. 59, pp. 1294 – 1300.
37. Walther, T. C. and Farese Jr., R. V. (2012) Lipid droplets and cellular lipid metabolism, *Lipid Droplets and Cellular Lipid Metabolism*, vol. 81, pp. 687 – 714.
38. Ipsen, D. H., Lykkesfeldt. and Tveden-Nyborg, P. (2018) Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease, *Cellular and Molecular Life Sciences*, vol. 75, pp. 3313 – 3327.
39. Unger, R. H. (2003) Lipid overload and overflow: metabolic trauma and the metabolic syndrome, *Trends in Endocrinology and Metabolism*, vol. 14, pp.398 – 403.
40. Wei, Y, Wang, D. and Pagliassotti, M. J. (2007) Saturated fatty acid-mediated endoplasmic reticulum stress and apoptosis are augmented by trans-10, cis-12-conjugated linoleic acid in liver cells, *Molecular and Cellular Biochemistry*, vol. 303, pp. 105 – 113.
41. Unger, R.H. and Orci, L. (2002) Lipoapoptosis: its mechanism and its diseases, *Biochimica et Biophysica Acta*, vol. 1585, pp. 202 – 212.
42. Guo, Y., Cordes K. R., Farese, Jr, R. V. and Walther, T. C. (2009) Lipid droplets at a glance, *Journal of Cell Science*, vol. 122, pp. 749 – 742.
43. Hashemi, H. F. and Goodman, J. M., (2015), The life cycle of the lipid droplet, *Current Opinion in Cell Biology*, vol. 33, pp. 119-124.
44. Zhang, Y., Xue, R, Zhang, Z., Yang, X. and Shi, H. (2012) Palmitic and linoleic acids induce ER stress and apoptosis in hepatoma cells, *Lipids in Health and Disease*, vol. 11, pp. 1 – 8.
45. Koutsari, C., Basu, R., Rizza, R. A., Nair, K. S., Khosla, S. and Jensen, M. D. (2011) Nonoxidative free fatty acid disposal is greater in young women than men, *Journal of Clinical Endocrinology and Metabolism*, vol. 92, pp. 541 – 547.
46. Feldstein, A. E., Werneburg, N. W., Canbay, A., Guicciardi, M. Bronk, S. F., Rydzewski, R., Burgart, L J. and Gores, G. J., (2004), Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway, *Hepatology*, vol. 40, pp. 185 – 194.
47. Unger, R. H., Clark, G. O. Scherer, P. E. and Orci, L., (2010), Lipid homeostasis, lipotoxicity and the metabolic syndrome, *Biochimica et Biophysica Acta*, vol. 1801, pp. 209 – 214.
48. Chen, X., Xun, K., Chen, L. and Wang, Y. (2009), TNF- α , a potent lipid metabolism regulator, *Cell Biochemistry and Function*, vol. 27, pp. 407-416.

49. Blaser, H., Dostert, C., Mak, T. W. and Brenner, D. (2016) TNF and ROS crosstalk in inflammation, *Trends in Cell Biology*, vol. 26, pp. 249 – 261.
50. Cambien, B., Bergmeier, W., Saffaripour, S., Mitchell, H. A. and Wagner, D. D. (2003), Antithrombotic activity of TNF- α , *The Journal of Clinical Investigation*, vol. 112, pp. 1589 – 1596.
51. Li, X., Xu, M., Liu, M., Ji, Y. and Li, Z. (2015) TNF-alpha and IL-6 inhibit apolipoprotein A-IV production induced by linoleic acid in human intestinal Caco2 cells, *Journal of Inflammation*, vol. 12, pp. 1 – 8.
52. Gutierrez-Ruiz, M. C., Quiroz, S. C., Souza, V., Bucio, L., Hernandez, E., Olivares, I. P., Llorente, L., Vargas-Vorackova, F., and Kershenobich, D. (1998) Cytokines, growth factors, and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde, and LPS, *Toxicology*, vol. 134, pp. 197 – 207.
53. Saad, B., Frei, K., Scholl, F. A., Fontana, A. and Maier, P. (1995), Hepatocyte-derived interleukin-6 and tumor-necrosis factor- α mediate the lipopolysaccharide-induced acute-phase response and nitric oxide release by cultured rat hepatocytes, *European Journal of Biochemistry*, vol. 229, pp. 349 – 355.
54. Neuman, M. G., Shear, N. H., Bellentani, S. and Tiribelli, C. (1998) Role of cytokines in ethanol-induced cytotoxicity in vitro in HepG2 cells, *Gastroenterology*, vol. 115, pp. 157 – 166.
55. Josephs, S. F., Ichim, T. E., Prince, S. M., Kesari, S., Marincola, F. M., Escobedo, A. R. and Jafri, A. (2018) Unleashing endogenous TNF-alpha as a cancer immunotherapeutic, *Journal of Translational Medicine*, vol. 16, pp. 1 – 8.
56. Gao, F., Joan, L., Zafar, I. M., Du., W., Cai, Q., Shafqat, R. A. and Lu. (2015) 4-Hydroxyisoleucine improves insulin resistance in HepG2 cells by decreasing TNF- α and regulating the expression of insulin signal transduction proteins, *Molecular Medicine Reports*, vol. 12, pp. 6555 – 6560.
57. Forrester, S. J., Kikuchi, D. S., Hernandes, M. S., Xu, Q., Griendl, K. K. (2018) Reactive oxygen species in metabolic and inflammatory signalling, *Circulation Research*, vol. 122, pp. 877-902.
58. Dröge, W. (2002) Free radicals in the physiological control of cell function, *Physiological Review*, vol. 82, pp. 47–95.
59. Incalza, M. A., D'Oria, R., Natalicchio, A., Perrini, S., Laviola, L. and Giorgino, F. (2018) Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases, *Vascular Pharmacology*, vol. 100, pp. 1 – 19.
60. Olusi, S. O. (2002) Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotective enzymes in humans, *International Journal of Obesity*, vol. 26, pp. 1159– 1164
61. Videla, L. A., Rodrigo, R., Orellana, M., Fernandez, V., Tapia, G., Quinones, L., Varela, N., Contreras, J., Lazarte, R., Csendes, A., Rojas, J., Maluenda, F., Burdiles, P., Diaz, J. C., Smok, G., Thielemann, L. and Poniachik, J. (2004) Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients, *Clinical Science*, vol. 106, pp. 261 – 268.
62. Huang, C. J., McAllister, M. J., Slusher, A. L., Webb, H. E., Mock, T. J. and Acevedo, E. O. (2015) Obesity-related oxidative stress: the impact of physical activity and diet manipulation, *Sports Medicine*, vol. 1, pp. 1 – 12.
63. Masarone, M., Rosato, V., Dallio, M., Gerarda Gravina, A., Aglitti, A., Loguercio, C., Federico, A. and Persico, M. (2018) Role of oxidative stress in pathophysiology of nonalcoholic fatty liver disease, *Oxidative Medicine and Cellular Longevity*, vol. 2018, pp. 1 – 14.

64. Homma, T. and Fujii, J. (2019) Oxidative stress and dysfunction of the intracellular proteolytic machinery: a pathological hallmark of nonalcoholic fatty liver disease: In: eds: Watson, R. R. and Preedy, V. R. *Dietary Interventions in Liver Disease, Foods, Nutrients, and Dietary Supplements*, Cambridge, USA, Academic Press, pp. 59-70.
65. Ighodaro, O. M. and Akinloye, O. A. (2018), First line defence antioxidants-superoxide dismutase (SOD), catalase(CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid, *Alexandria Journal of Medicine*, vol. 54, pp. 287 – 293.
66. Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., and Malik, A. B. (2014) Reactive oxygen species in inflammation and tissue injury, *Antioxidants & Redox Signalling*, vol. 20, pp. 1126 – 1166.
67. Togo, M., Konari, N., Tsukamoto, M., Yamaguchi1, T., Takeda, H. and Kambayashi, I. (2018) Effects of a high-fat diet on superoxide anion generation and membrane fluidity in liver mitochondria in rats, *Journal of the International Society of Sports Nutrition*, vol. 15, pp. 1 – 8.
68. Thannickal, V. J. and Fanburg, B. L. (2000) Reactive oxygen species in cell signalling, *American Journal of Physiology – Lung Cellular and Molecular Physiology*, vol. 279, pp. L1005–L1028.
69. Young, V. M., Toborek, M., Yang, F., McClain, C. J. and Hennig, B. (1998) Effect of linoleic acid on endothelial cell inflammatory mediators, *Metabolism*, vol. 47, pp. 566 – 572.
70. Hatanaka, E., Levada-Pires, A. C., Pithon-Curi, T. C. and Curi, R. (2006) Systematic study on ROS production induced by oleic, linoleic, and γ -linolenic acids in human and rat neutrophils, *Free Radical Biology & Medicine*, vol. 41, pp. 1124–1132.
71. Morgan, D., Oliveira-Emilio, H. R., Keane, D., Hirata, A. E., Santos da Rocha, M., Curi, R., Newsholme, P. and Carpinelli, A. R. (2007) Glucose, palmitate and proinflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line, *Diabetologia*, vol. 50, pp. 359–369.
72. Hatanaka, E., Dermargos, A., Hirat, A. E., Ramirez Vinolo, M. A., Carpinelli, A. R., Newsholme, P., Armelin, H. A. and Curi, R. (2013) Oleic, linoleic and linolenic acids increase ROS production by fibroblasts via NADPH oxidase activation, *PLoS ONE*, vol. 8, pp. 1 – 8.
73. Siqueira, R. F., Weigel, R. A., Nunes, G. R., Mori, C. S. and Fernandes, W. R. (2014) Oxidative profiles of endurance horses racing different distances, *Arq. Bras. Med. Vet. Zootec*, vol. 66, pp. 455–461.
74. Vincent H. K., Powers, S. K., Stewart, D. J., Shanely, R. A., Demirel, H. and Naito, H. (1999) Obesity is associated with increased myocardial oxidative stress, *International Journal of Obesity*, vol. 23, pp. 67 – 74.
75. Kuratko, C. N. and Constante, B. J., (1998), Linoleic acid and tumour necrosis factor- α increase manganese superoxide dismutase activity in intestinal cells, *Cancer Letters*, vol. 130, pp. 191-196.
76. Kim, Y. J. Nakatomi, R., Akagi, T., Hashikawa, T. and Takahashi, R. (2005) Unsaturated fatty acids induce cytotoxic aggregate formation of amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutants, *The Journal of Biological Chemistry*, vol. 280, pp. 21515–21521.
77. Choi, I., Yang, Y. I., Song, H. D., Lee, J. S., Kang, T., Sung, J. J., Yi, J. (2011) Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide dismutase with structurally disordered regions, *Biochimica et Biophysica Acta*, vol. 1812, pp. 41 – 48.

78. Appolinário, P. P., Medinas, D. B., Chaves-Filho, A. B., Genaro-Mattos, T. C., Cussiol, J. R. R., Soares Netto, E. L., Augusto, O. and Miyamoto, S. (2015) Oligomerization of cu,zn-superoxide dismutase (sod1) by docosahexaenoic acid and its hydroperoxides in vitro: aggregation dependence on fatty acid unsaturation and thiols, *PLoS ONE*, vol. 10, pp. 1 – 15.
79. Eder, K., Slomma, N. and Becker, K. (2002) Trans-10,cis-12 Conjugated linoleic acid suppresses the desaturation of linoleic and α -linolenic acids in Hepg2 cells, *American Journal of Nutritional Sciences*, vol. 132, pp. 1115 – 1121.
80. Yokoyama, Y., Nimura, Y., Nagino, M., Bland, K. I. and Chaudry, I. H. (2005) Role of thromboxane in producing hepatic injury during hepatic stress, *Archives of Surgery*, vol. 140, pp. 801 – 807.
90. Nanji, A. A., Khettry, U., Sadrzadeh,S. M. H. and Yamanaka, T, (1993) Severity of liver injury in experimental alcoholic liver disease, correlation with plasma endotoxin, Prostaglandin E2, Leukotriene B4, and Thromboxane B2, *American journal of Pathology*, vol. 142, pp. 367 – 373.
91. Fisher, R. A., Robertson, S. M., and Olson, M. S. (1987), Stimulation of glycogenolysis and vasoconstriction in the perfused rat liver by the thromboxane A2 analogue U-46619, *Journal of Biological Chemistry*, vol. 262, pp. 4631-4638.
92. Katagiri, H., Ito, Y., Ishii, K., Hayashi, I., Suematsu, M., Yamashina, S., Murata, T., Narumiya, S., Kakita, A., Majima, M. (2004) Role of thromboxane derived from cox-1 and -2 in hepatic microcirculatory dysfunction during endotoxemia in mice, *Hepatology*, vol. 39, pp. 139–150.
93. Needleman, S. W., Spector, A. A. and Hoak, J. C. (1982), Enrichment of human platelet phospholipids with linoleic acid diminishes thromboxane release, *Prostaglandins*, vol. 24, pp. 607 - 6.22.
94. MacIntyre, D. E., Hoover, R. L., Smith, M., Steer, M., Lynch, M., Karnovsky, M. J. and SalzmanE. W. (1984) Inhibition of platelet function by cis-unsaturated fatty acids, *Blood*, vol. 63, pp. 848 – 875.
95. Suh, H. N., Huong, H. T., Song, C. H., Lee, J. H. and Han, H. J. (2008) Linoleic acid stimulates gluconeogenesis via Ca2/PLC, cPLA2, and PPAR pathways through GPR40 in primary cultured chicken hepatocytes, *American Journal of Physiology – Cell Physiology*, vol. 295, pp. C1518–C1527.
96. Whelan, J. and Fritsche, K. (2013) Linoleic acid, *Advances in Nutrition*, vol. 4, pp. 311–312.
97. Malhi, H., Bronk, S. F., Werneburg, N. W. and Gores, G. J. (2006) Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis, *The Journal of Biological Chemistry*, vol. 281, pp. 12093–12101.
98. Wu, X., Zhang, L., Gurley, E., Struder, E., Shang, J., Wang, T., Wang, C., Yan, M., Jiang, Z., Hylemon, P. B., Sanyal, A. J., Pandak, W. M. and Zhou, H. (2008), Prevention of free fatty acid-induced hepatic lipotoxicity by 18 β -Glycyrrhetic acid through lysosomal and mitochondrial pathways, *Hepatology*, vol. 47, pp. 1905 – 1915.
99. Malhi, H and Gores, J. (2008) Molecular mechanisms of lipotoxicity in non-alcoholic fatty liver disease, *Seminars in Liver Disease*, vol. 28, pp. 360 – 369.
100. Zeke, A., Misheva, M. Reményi, A., and Bogoyevitch, M. A. (2016) JNK signalling: regulation and functions based on complex protein-protein partnerships, *Microbiology and Molecular Biology Reviews*, vol. 80, pp. 793 – 821.

101. Cnop, M., Hannaert, J. C., Hoorens, A., Eizirik, D. L. and Pipeleers, D. G. (2001) Inverse Relationship Between Cytotoxicity of Free Fatty Acids in Pancreatic Islet Cells and Cellular Triglyceride Accumulation, *Diabetes*, vol. 50, pp. 1771–1777.
102. Pompeia, C., Freitas, J. J. S., Kim, J. S., Zyngier, S. B. and Curi, R. (2002) Arachidonic acid cytotoxicity in leukocytes: implications of oxidative stress and eicosanoid synthesis, vol. 94, pp. 251–265.
103. Hallebeek, J. M. & Beynen, A. C., (2002), Dietary fats and lipid metabolism in relation to equine health, performance and disease, *s.l.: Department of Nutrition, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.*
104. Høstmark, A.T (1995) Serum fatty acid/ albumin molar ratio and the risk of diseases, *Medical Hypotheses* vol. 44, pp. 539 – 541.
105. Artwohl, M., Lindenmair, A., Roden, M., Waldhausl, W., Freudenthaler, A., Klosner, G., Ilhan, A., Luger, A. and Baumgartner-Parzer, S. M. (2009) Fatty acids induce apoptosis in human smooth muscle cells depending on chain length, saturation, and duration of exposure, *Atherosclerosis*, vol. 202, pp. 351–362.
106. Zaman, M. M., Martin, C. R., Andersson, C., Bhutta, A. Q., Cluette-Brown, J. E., Laposata, M. and Freedman, S. D. (2010) Linoleic acid supplementation results in increased arachidonic acid and eicosanoid production in CF airway cells and in cftr^{-/-} transgenic mice, *American Journal Physiology Lung Cell Molecular Physiology*, vol. 299, pp. L599 – L606.

Figures

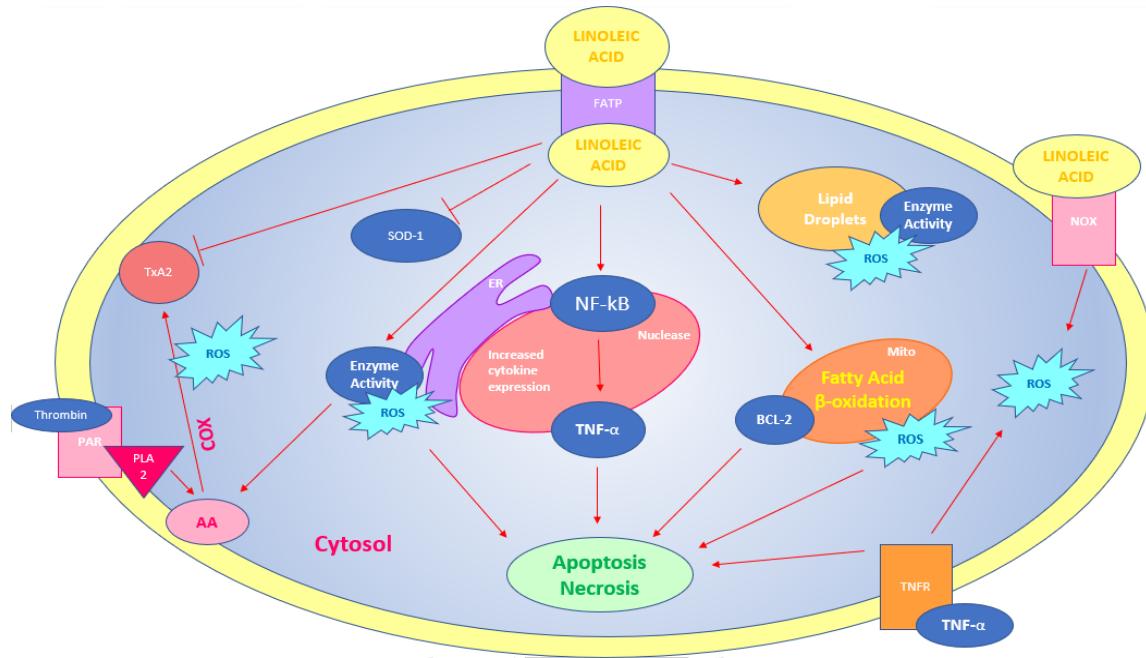


Figure. 1. Schematic of the possible intracellular fates of linoleic acid. Once inside the cell it is transported via fatty acid binding proteins where it can be packaged into lipid droplets, used for energy production in the mitochondria through β -oxidation or enzymatically transformed into derivatives such as arachidonic acid. Excessive intracellular linoleic acid may lead to increased production and secretion of proinflammatory eicosanoids such as thromboxane A₂ (Tx_A₂) as a result of excess arachidonic acid. Linoleic acid may also inhibit Tx_A₂ function. Production of excess proinflammatory cytokines such as TNF- α may occur through linoleic acid induced NF- κ B activation. Increased intracellular metabolism of linoleic acid in several organelles can lead to increased production of reactive oxygen species (ROS). Linoleic acid may also inhibit ROS scavenger enzymes such as superoxide dismutase-1 (SOD-1). These factors may induce intracellular stress, leading to apoptosis.

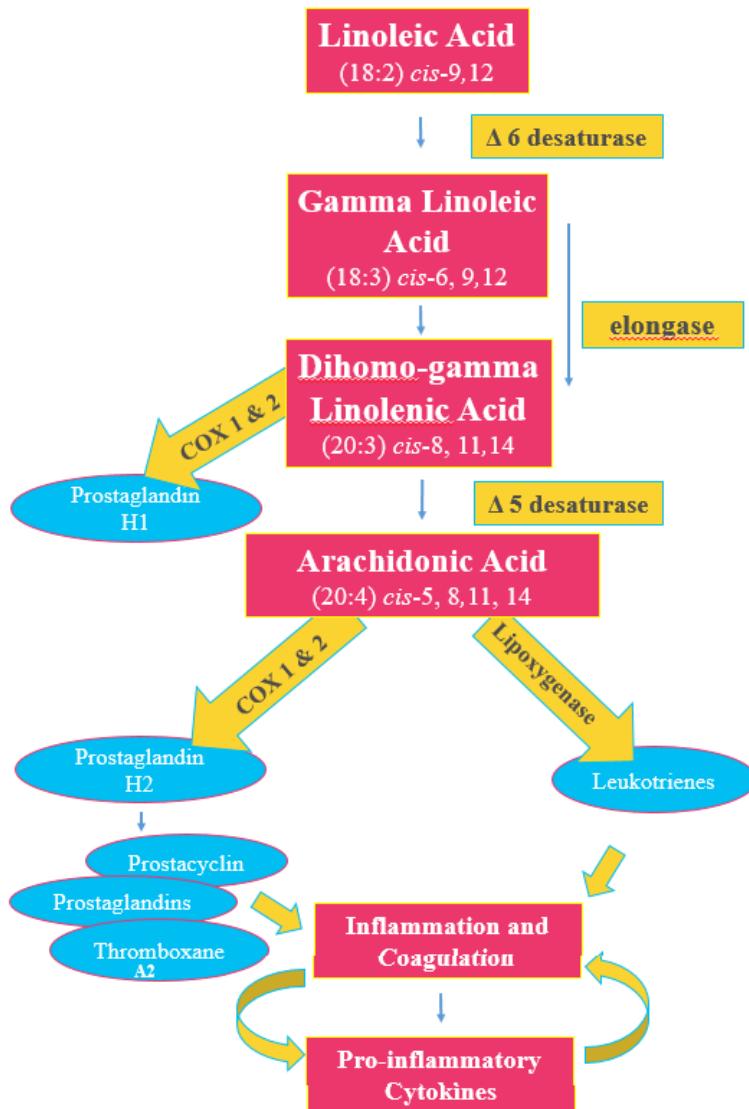


Figure 2. Schematic of the production of eicosanoids from linoleic acid *via* the cyclooxygenase (COX) metabolic pathway (authors own work).

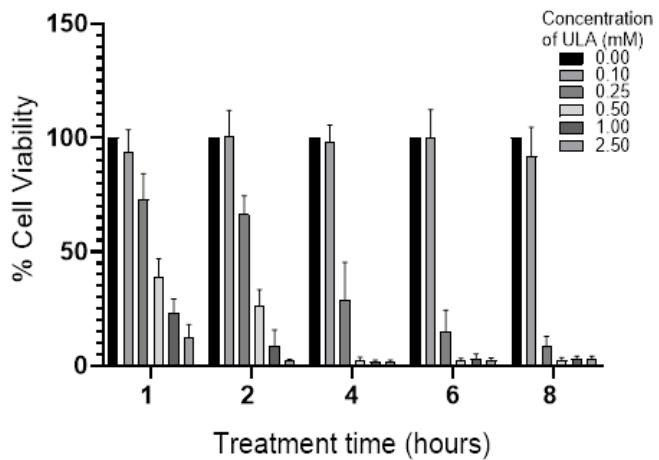


Figure 3. Percentage cell viability of HepG2 cells treated with varying concentrations of ULA over a period of 8 hours compared with a positive growth control. MTT was used as an endpoint ($n = 3$). Viability levels are expressed as a percent of the control (assigned as 100%). Statistical analysis was performed using mean absorbance values (\pm S.E.M.). One-way ANOVA using Bonferroni's test as a post-test to compare the treated cells to the untreated control.

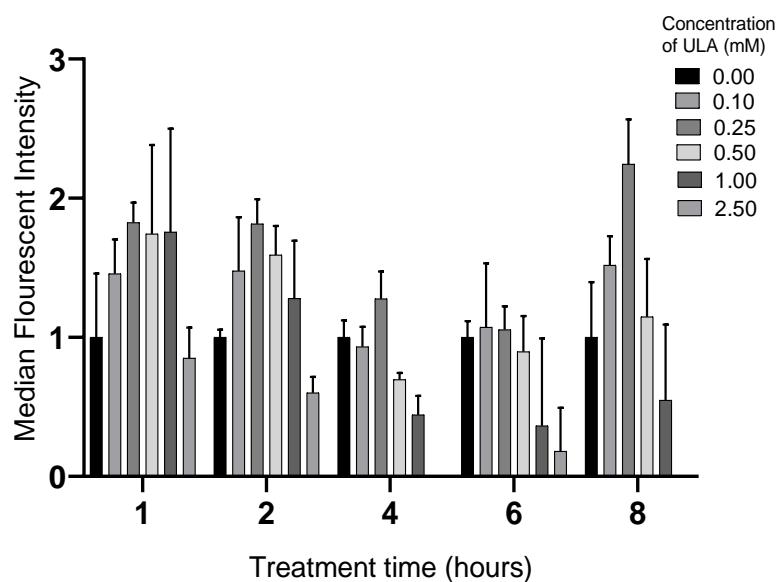
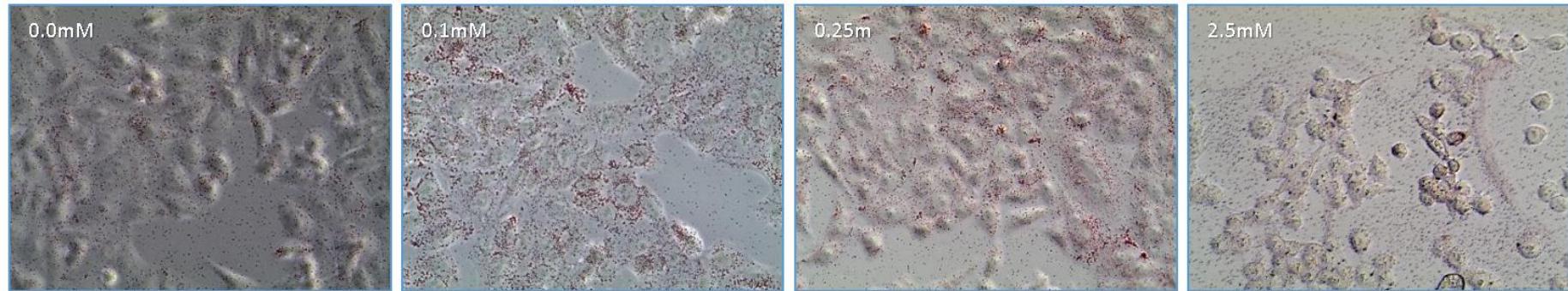


Figure 4a. Median Fluorescence Intensity of Nile Red determined by flow cytometry in HepG2 cells treated with varying concentrations of ULA over 8 hours. Data has been normalised as described by Wolins *et al*, (2018).

1 hour



8 hours

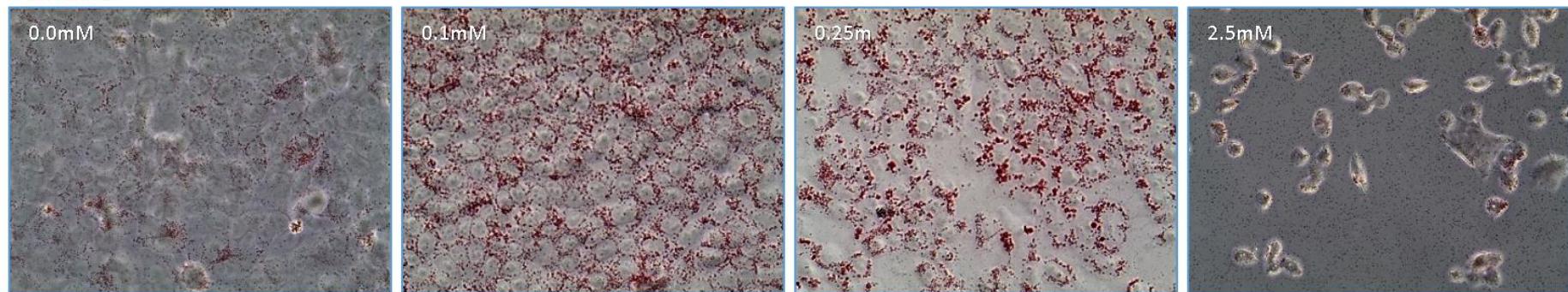


Figure 4b. Oil Red O staining maps (x 200) of HepG2 cells treated with varying concentrations of ULA over different time intervals. Control cells were grown in media containing fatty acid free BSA and $\leq 1\%$ IPA

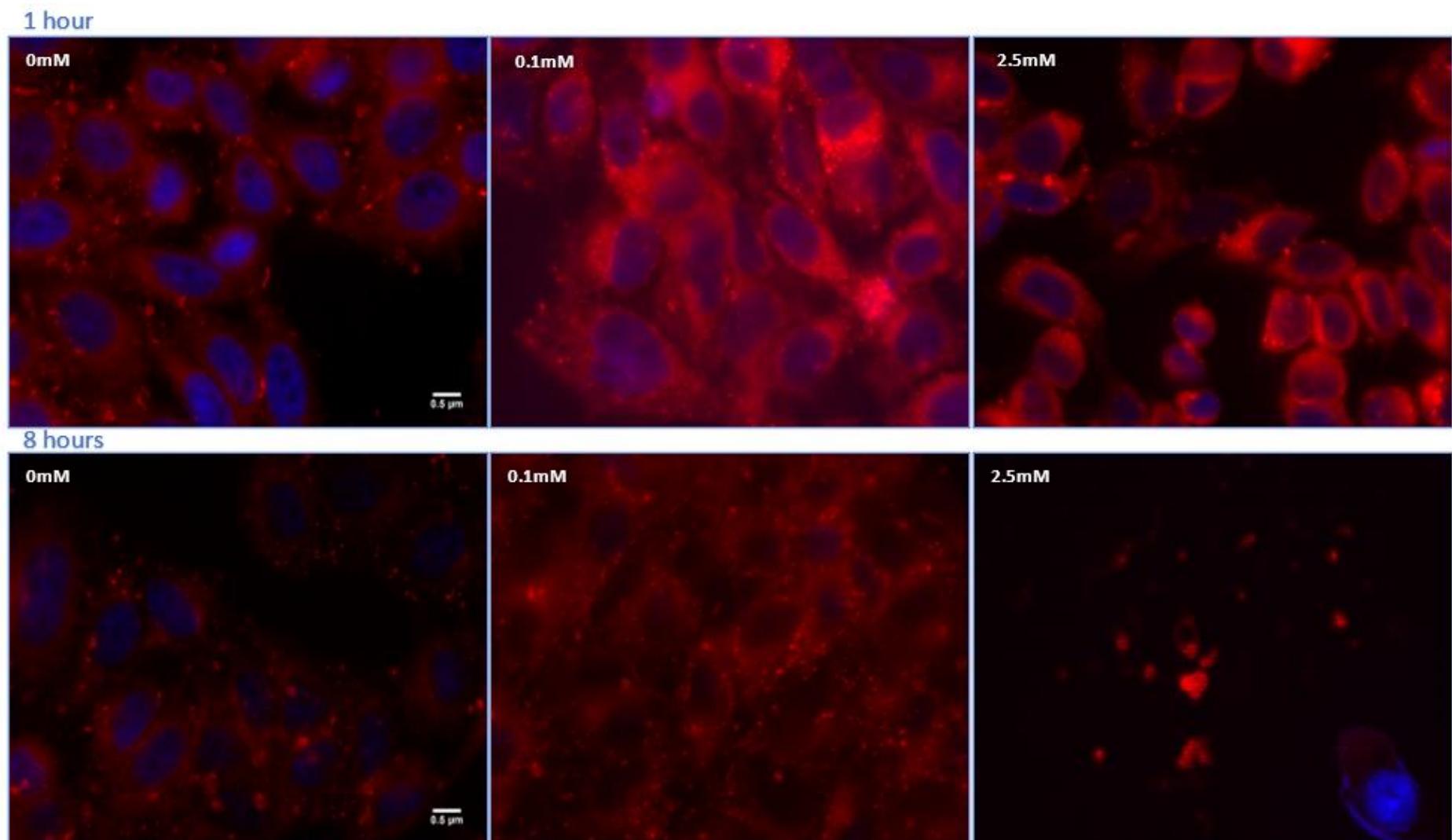


Figure 4c Intracellular lipid accumulation in HepG2 cells treated with varying concentrations of ULA after 1 and 2 hours of supplementation. Intracellular lipid accumulation was qualitatively determined using fluorescent microscopy. Fluorescent images (x1000) were obtained using the lipophilic stain Nile Red for intracellular lipid imaging (red) and the nuclear stain DAPI for nucleus imaging (blue). Scale bar represents 0.5μm across all images..

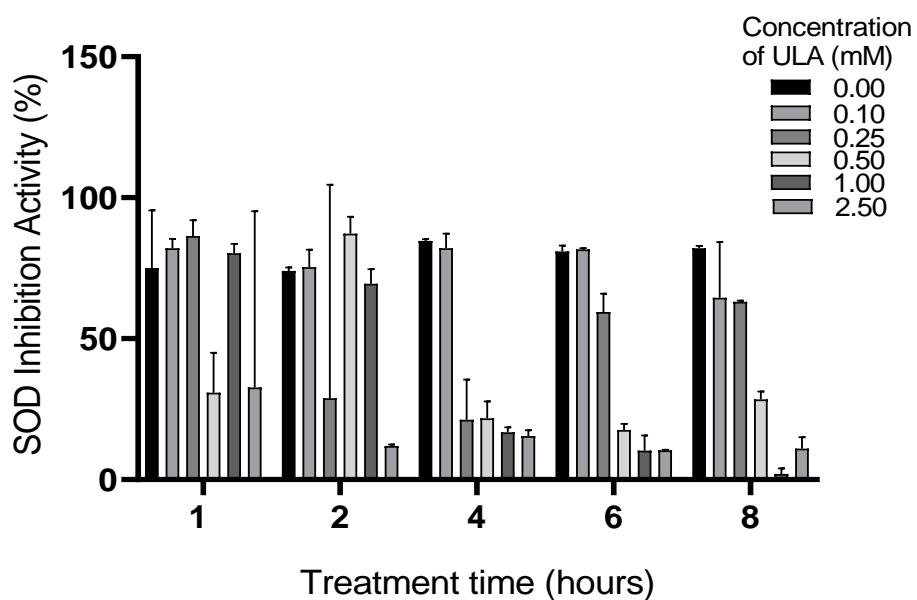


Figure 5. Superoxide dismutase (SOD) inhibition activity (%) in HepG2 cells treated with varying concentrations of ULA over 8 hours. SOD activity was determined as described in the methods. SOD inhibits the reduction of water-soluble tetrazolium salt (WST-1) by the superoxide anion to form a water-soluble formazan dye. This inhibition activity can be determined *via* colorimetric method.

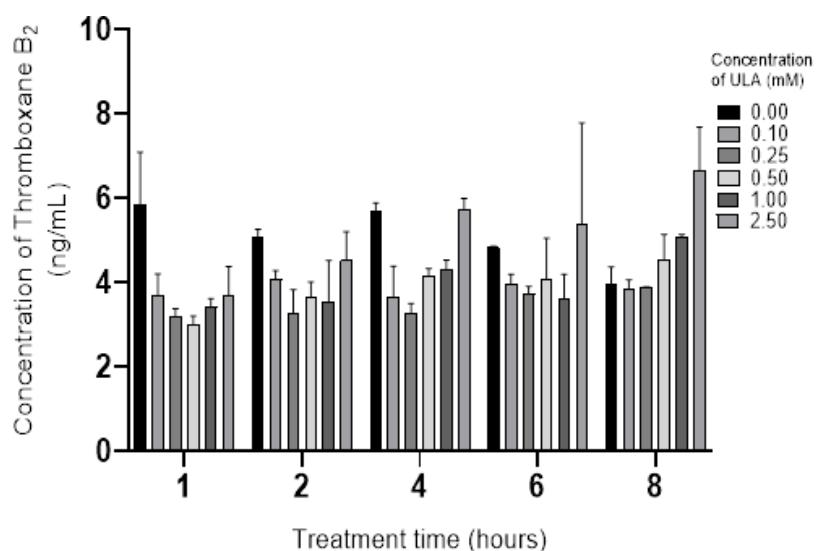


Figure 6. Concentration of thromboxane B₂ produced in HepG2 cells treated with varying concentrations of ULA over 8 hours. Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's *post-hoc* test was used to compare all means ($n=2$).

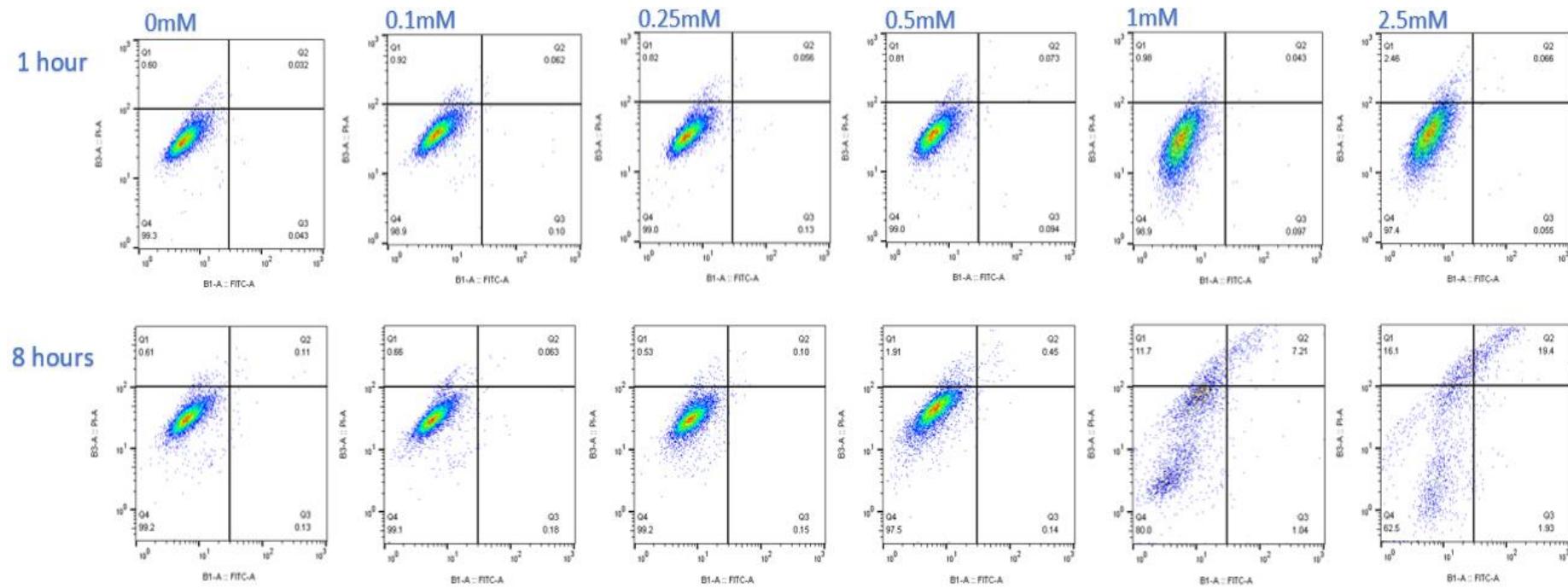


Figure 7a. Flow cytometry dot plots representing HepG2 cells treated with increasing concentrations of ULA. Viable, apoptotic and necrotic cells in the whole cell population were measured by AV-FITC/PI flow cytometry. The whole cell population was divided into different quadrants (Q); Q 1 were considered necrotic, Q2, late apoptotic, Q3, early apoptotic and Q 4 were considered live.

HepG2 cells were stained with Annexin V-FITC (AV-FITC) and propidium iodide (PI) to detect cells with disrupted membranes. According to the cell staining, cells were categorized as; live cells, negative for both AV-FITC and PI (Q4) (bottom left quarter); early apoptotic cells, positive for AV-FITC and negative for PI (Q3)(bottom right quarter); late apoptotic cells, positive both for AV-FITC and PI (Q2) (top right quarter) while cells positive only for PI were considered as necrotic (Q1) (top left quarter).

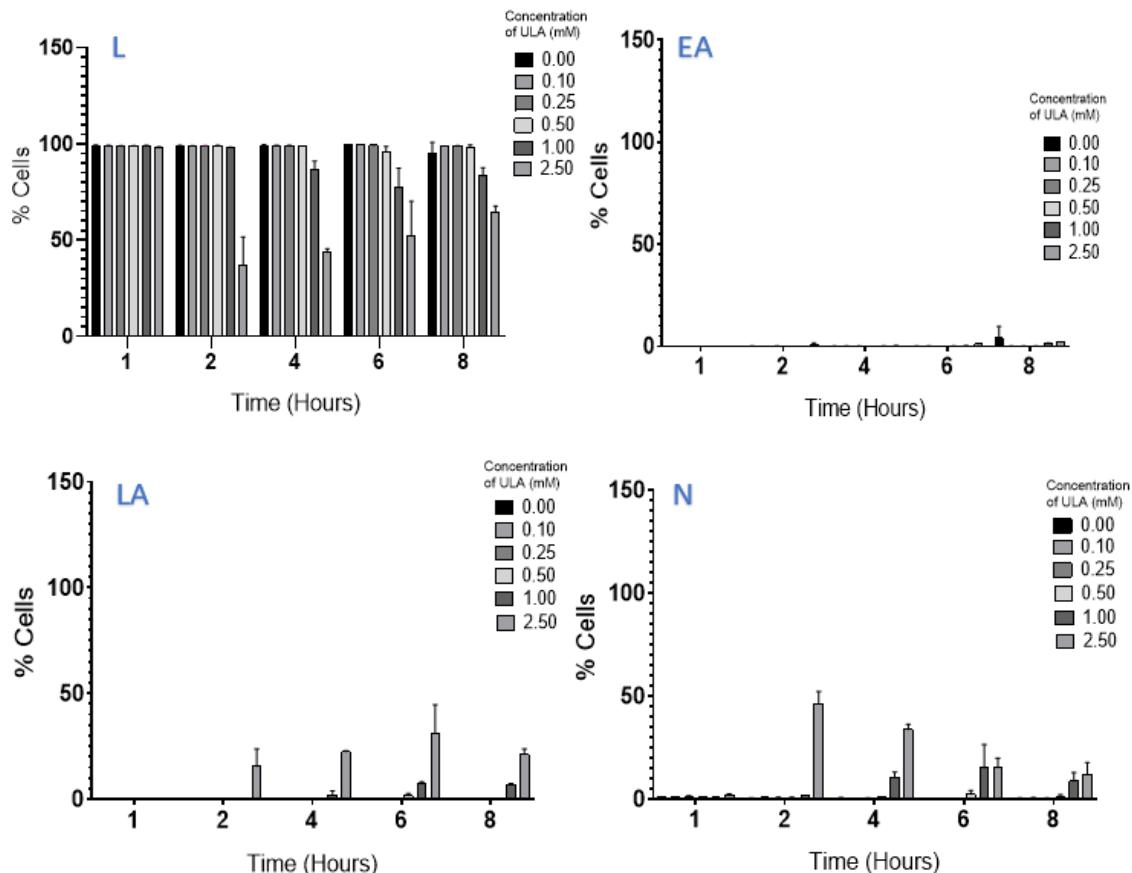


Figure 7b. Induction of ULA mediated cell death in HepG2 cells treated with ULA from 1 to 8 hours. Data is presented as the effects of increasing concentrations of ULA on the percentage of cells in each cellular state, live, early apoptotic, late apoptotic, and necrotic cells for each time point. Statistical analysis was performed using mean absorbance values (\pm S.E.M). One-way ANOVA using Bonferroni's *post-hoc* test was used to compare all means ($n=2$). L; live. EA; early apoptosis. LA; late apoptosis. N; necrosis.