Mechanisms into the development of fatty liver disease: role of free fatty acids and alcohol

Ghazali, R.

Mechanisms into the development of fatty liver disease: role of free fatty acids and alcohol

Reem Ghazali

A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of philosophy

November 2017

Background: Alcohol and Free fatty acids such as palmitate are known to promote liver injury. However less mechanistic information is available regarding omega fatty acids ratios with/out alcohol. In healthy populations omega 6/3 ratios are between 1:1 to 4:1, whereas high ratios (>15:1) are thought to correlate with the pathogenesis of fatty liver disease. This study aimed to investigate liver lipotoxicity and mitochondrial dysfunction due to imbalanced omega 6/3 ratios alone or in the presence of alcohol.

Method: Human hepatoma cell line, VL-17A cells were treated with individual fatty acids (Palmitic (PA), Stearic (SA), Linoleic (LA), alpha-Linolenic (ALA), Arachidonic (AA) and Docosahexaenoic (DHA) acids) with various concentrations ranging between 0.5 µM to 300 µM and omega 6/3 ratios (1:1, 4:1, 15:1 and 25:1) with/out alcohol (100 mM) for 24, 48 and 72 h after which lipid accumulation and cell toxicity was assessed. Subsequent studies at 24 hr examined oxidative stress, mitochondrial function and lipogenic proteins.

Results: PA treatment showed a detrimental effect on cell viability and lipid accumulation than SA after 48 h and 72 h ($P<0.05$); omega 3 (ALA and DHA) did not show any significant effect; LA exhibited a significant reduction in cell viability at 24 h only without causing any change in lipid accumulation; AA treatment significantly reduced cell viability at 48 h and 72 h ($P<0.05$) but only showed a significant lipid accumulation after 48 h. AA/DHA (omega 6/3) ratios (15:1 & 25:1) caused a significant reduction in cell viability after 24 h, 48 h and 72 h ($P<0.001$) and only (15:1) ratio inversely corresponded to an elevation in lipid level after 24 h and 48 h ($P<0.01$). At 24 hr treatment, high AA/DHA ratio of 25:1 led to increased expression of stearoyl-CoA desaturase (SCD1) and decreased peroxisome proliferator activated receptor alpha (PPARα) expression at 15:1 and 25:1 ($P<0.05$), while all ratios showed a significant decrease in the cannabinoid receptor (CB2) expression compared to control but actually increased when compared to the 1:1 ratio. Although the expression of CB1 was slightly increased ($P>0.05$), sterol regulatory element-binding protein 1 (SREBP1) did not show any change. AA/DHA ratios also showed a significant decrease in ATP production ($P<0.01$), basal respiration, maximal respiration and spare mitochondrial capacity and this effect was greater with high ratios ($P<0.001$). Reactive oxygen species (ROS) production increased significantly, particularly with high AA/DHA ratios (15:1 and 25:1) ($P<0.001$) alone and in the presence of alcohol ($P<0.01$).

Conclusion: The data suggests that lipid accumulation and toxicity occur with saturated and unsaturated fatty acids and high omega 6/3 ratios. The latter possibly due to the pro-inflammatory products of AA. This study confirms that high AA/DHA ratios with/out alcohol increase ROS production and high AA/DHA alone induce mitochondrial dysfunction and increase lipogenesis pathways by activating lipogenic factors causing steatosis and consequently promoting the development of fatty liver disease. Further work aims to elucidate the effect of fatty acid/alcohol on lipid synthetic and endocannabinoid pathways, which will further our understanding of fatty liver disease development.

Acknowledgment

Firstly, I would like to express my sincere gratitude to my Director of Studies Dr. Vinood Patel for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

Besides my supervisor, I would like to thank the rest of my thesis committee: Prof. Annie Bligh, and Dr. Ihab Tewfik, for their insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives.

My sincere thanks also go to Dr. Kosha Mehta, Dr. Meliz Sahuri Arisoylu, Dr. Zhi Song, and Rhys Mould for their help and without their precious support it would not be possible to conduct this research.

I thank my fellow labmates for the stimulating discussions, and for all the fun we have had in the last four years, especially Quratulain Khalid. In addition, I would like to express my gratitude to the staff at the University of Westminster for their corporation especially Thakor Tandel.

Last but not the least, I would like to thank my family: my parents, Fouad and Manal, my husband, AbdulKhaliq and my children, Mohammed and Lolwa for supporting me spiritually throughout writing this thesis and my life in general.

Table of Contents

List of Abbreviation

2-AG	Arachidonoylglycerol
AA	Arachidonic acid
ACO	Acyl-CoA oxidase
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ALA	α-linolenic acid
ALD	Alcoholic liver disease
ALDH	Aldehyde dehydrogenase
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
AST	Aspartate transaminase
ATP	Adenosine triphosphate
CB1	Cannabinoid receptors 1
CB2	Cannabinoid receptors 2
CBD	Cannabidiol
CO2	Carbon dioxide
COX	Cyclo-oxygenase
CPT-1	Carnitine palmitoyl transferase-1
CYP	Cytochrome P450
DCFDA	2′,7′-dichlorofluorescin diacetate
DHA	Docosahexaenoic acid
DHET	Dihydroxyeicosatrienoic acid

DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EETs	Epoxyeicosatrienoic acids
ELOVL5	Elongation of very long chain fatty acids 5
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
FAEE	Fatty acid ethyl esters
FAS	Fatty acid synthase
FCS	Foetal calf serum
GGT	Gamma Glutamyltransaminase
GPAT1	Glycerol-3-phosphate acyl transferase
H2O2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HepG2	Human hepatoma cell line
HETE	Hydroxyeicosatetraenoic acids
HMG-CoA	3-hydroxy-methylglutaryl-CoA
HODE	Hydroxyoctadecaenoic acids
HpET	Hydroperoxyeicosate-traenoic acids
IL-6	Interleukin-6
LA	Linoleic acid
LDL	Low density lipoprotein

LFT	Liver function test
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotrienes
LX	Lipoxins
LXR	Liver X receptor
MEOS	Microsomal ethanol oxidising system
MPT	Mitochondrial permeability transition opening
MTT	Thiazolyl blue tetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NO	Nitric oxide
OCR	Oxygen consumption rate
P	Phosphate
PBS	Phosphate buffered saline
PG	Prostaglandins
PPAR	Peroxisome proliferator-activated receptor
PPREs	Peroxisome proliferator hormone response elements
PXR	Pregnane X receptor
ROS	Reactive oxygen species
SCD1	Stearoyl-CoA desaturase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel

sHE	Soluble epoxide hydrolase
SNP	Single nucleotide polymorphisms
SREBP1c	Sterol regulatory element-binding protein 1c
TCA	Tricarboxylic acid cycle
TG	Triglyceride
THC	Tetrahydrocannabinol
TLR4	Toll-like receptor 4
TNFα	Tumour necrosis factor-alpha
UPR	Unfolded protein response

List of Figures

List of Tables

Chapter 1

Introduction

1. 1 Global burden of non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is a chronic disease with a high prevalence rate worldwide. NAFLD is considered the second major cause of mortality in the world with a serious economic load for numerous countries due to its passive pathological consequences (Yao et al., 2016). The prevalence of NAFLD has doubled since 1980. It occurs in approximately 20-40% of Western and Asian populations and affects almost one-third of the world's population of different ages and races (Gentile and Pagliassotti, 2008; Wree et al., 2010; Williams et al., 2011; Takahashi et al., 2015; Yao et al., 2016) (Table 1.1). In addition, up to a third of the United Kingdom's population being affected by NAFLD whereas in the United States, about 80 to 100 million individuals are afflicted by NAFLD, in whom around 30% may suffer from non-alcoholic steatohepatitis (NASH), the progressive stage of NAFLD (Dyson et al., 2014; Patel et al., 2016). NASH also affects around 12% of middle-aged people and is now the third-leading cause of liver transplantation in the United States and in the United Kingdom (McCullough, 2006; Charlton et al., 2011; Takahashi et al., 2015).

Table 1.1: Prevalence of NAFLD in general population across regions (WGO, 2012).

Region	Population category	NAFLD prevalence (%)
USA	Children General population	13-14 27-34
Europe	Children General population	2.6-10 20-30
Western countries	General population	20-40
Middle East	General population	20-30
Far East	General population	15
Pakistan	General population	18

The high correlation between NAFLD and type II diabetes mellitus has significantly increased the prevalence of NAFLD worldwide to approximately 21%-78% (Olusanya et al., 2016). Moreover, the expansion of sedentary lifestyles as well as the increased consumption of unhealthy and imbalanced diets has led to an increase in the number of obese individuals (Figure 1.1) and, consequently, the prevalence of NAFLD in places such as South Africa, where about 45% of obese individuals were found to be affected by NAFLD (Olusanya et al., 2016; Severson et al., 2016). In addition, one-third of America's population suffer from obesity, with 30% being affected by NAFLD (Vernon et al., 2011).

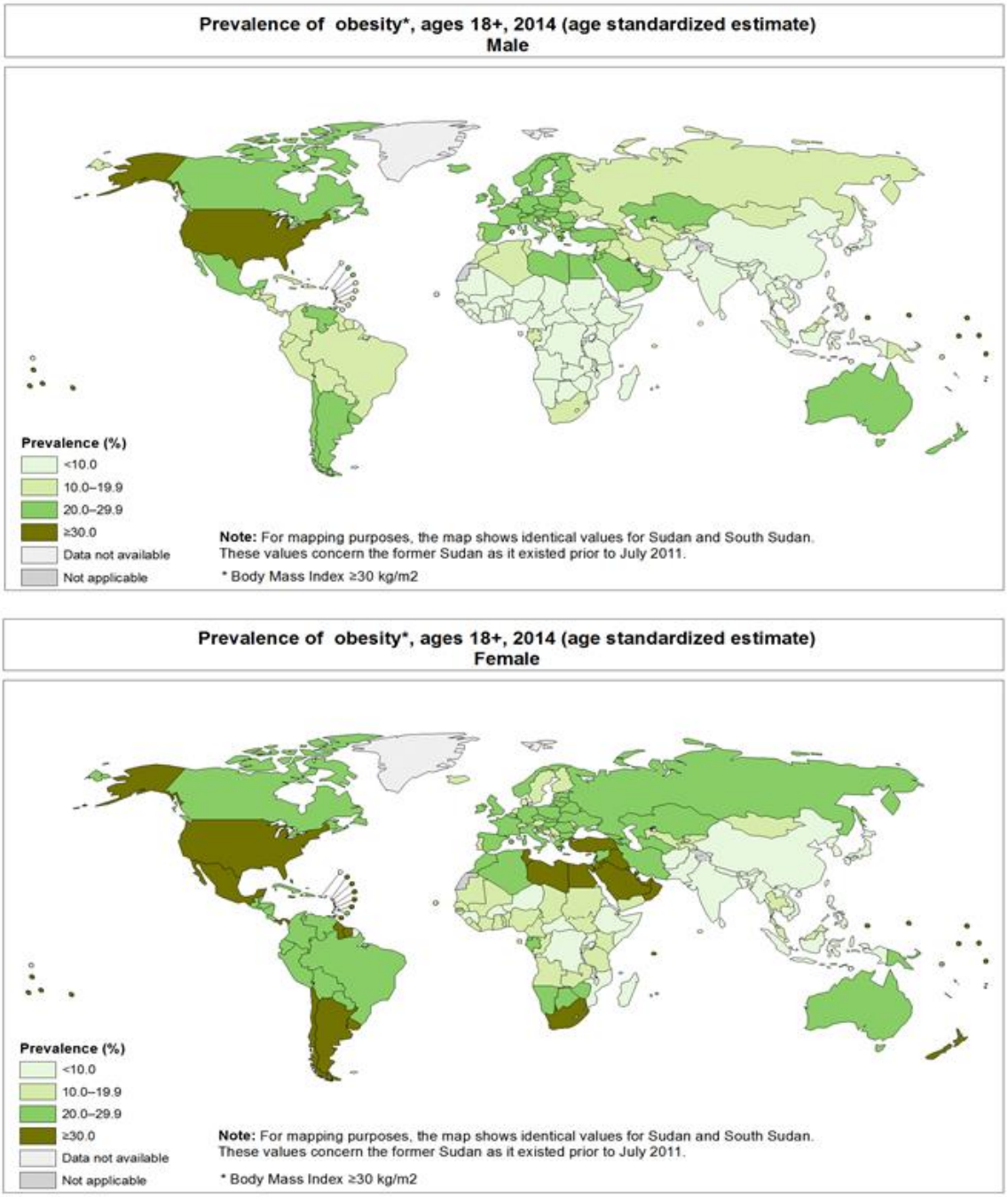

Figure 1.1: Global prevalence of obesity in both sexes, ages 18 +. Source: For more on the WHO Global Health Observatory Map Gallery on non communicable diseases see http://gamapserver.who.int/mapLibrary

1.2 Pathogenesis of NAFLD

NAFLD is caused by high dietary fat or carbohydrate consumption and is not related to excessive alcohol use. As previously mentioned, NAFLD is also highly related to the incidence of some metabolic syndromes, such as obesity, type II diabetes and insulin resistance (Wree et al., 2010; Mota et al., 2016). The latter is believed to be one of the vital factors involved in the development of NAFLD, as it increases the breakdown of adipose tissue causing an increase in the level of free fatty acids in circulation, which, in turn, gives rise to liver steatosis (Papandreou and Andreou, 2015). Simple hepatic steatosis is the first manifestation of NAFLD. This disease is known to have a broad spectrum and develops into NASH; this condition is characterised by hepatic ballooning, inflammation and fibrosis, which may then progress rapidly into cirrhosis and in some cases, ultimately lead to hepatocellular carcinoma (HCC) (Gusdon et al., 2014).

1.2.1 Multi-hit hypothesis

The multi-hit theory is a global hypothesis for the pathogenesis of NAFLD, which comprises three theories. Gentile and Pagliassotti (2008) described the ‘first hit’ as the stage where triglycerides (TG) accumulate in the hepatocyte (steatosis) and make the liver more susceptible to injury by many mediators. These include elevated levels of the leptin hormone and some cytokines, such as tumour necrosis factor (TNF-α) and interleukin-6 (IL-6), in the serum as well as mitochondrial dysfunction, oxidative stress and endoplasmic reticulum stress (ER), which, in turn, lead to the development of NASH. This stage is known as the ‘second hit’ (Dowman et al., 2010). The second-hit theory was proposed in

1998 by Day et al. and it is believed that this theory plays a crucial role in understanding the pathogenesis of NAFLD, which may in some cases be accompanied by the occurrence of fibrosis and then develop to cirrhosis ‘third hit’ (Figure 1.2) (Gentile and Pagliassotti, 2008).

Indeed, despite the existing literature on this topic, the pathogenesis of fatty liver disease is still largely unclear. In addition, most patients with NAFLD are asymptomatic until the developed stages of the disease, and currently, the only reliable diagnostic method in use is liver biopsy, which provides precise data on the grading of fibrosis (Wang and Liu, 2003; Adams et al., 2005; Gyamfi and Wan, 2010; Papandreou and Andreou, 2015).

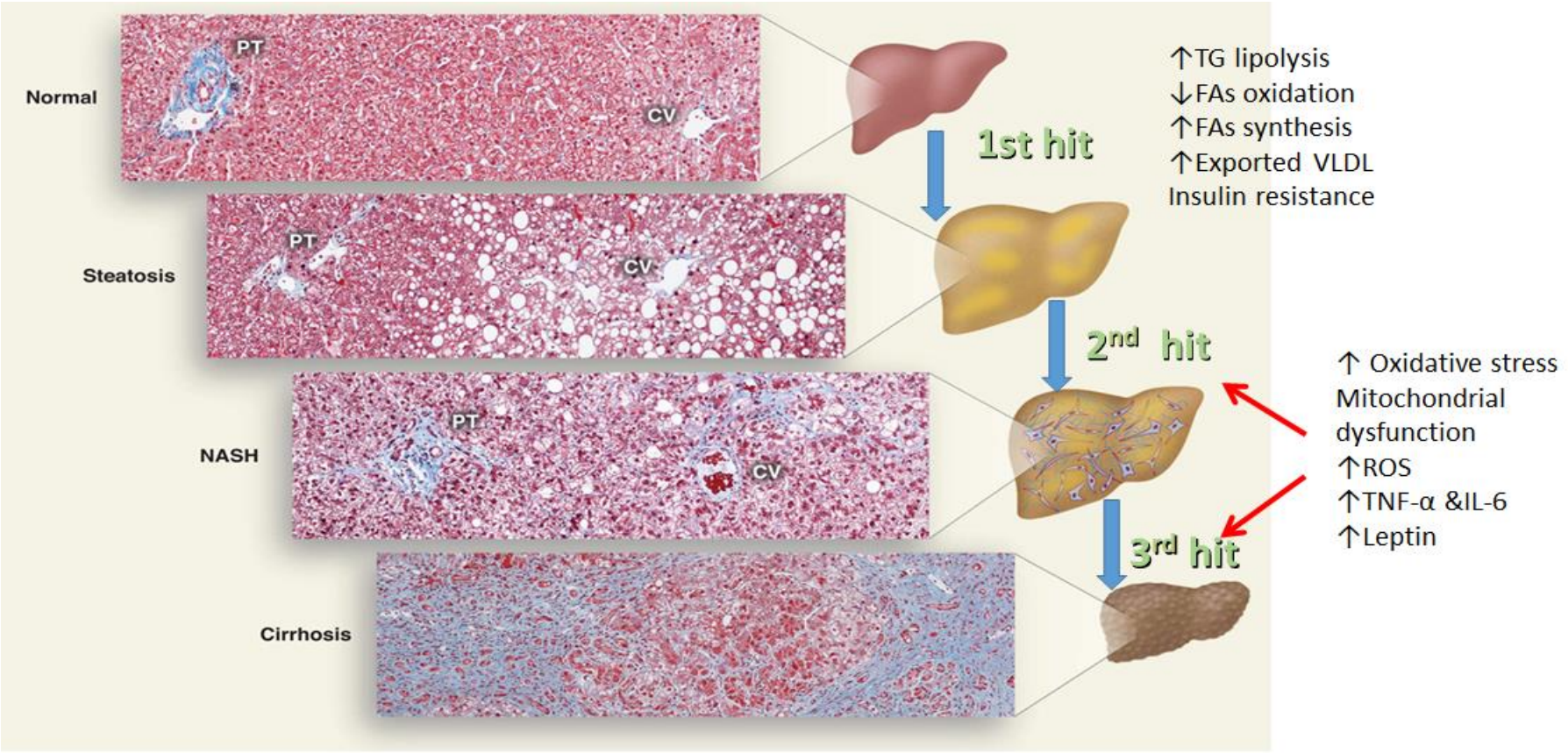

Figure 1.2: Pathogenesis of NAFLD (multi-hit theory). The onset of fatty liver occurs as a result of elevated fat import, decreased fat oxidation and insulin resistance. Liver then will be susceptible to injuries because of the high production of ROS which then increases the oxidative stress and consequently causes mitochondrial dysfunction. This dysfunction will then activates the production of TNF-α and IL-6 and leads to inflammation with fibrosis which then can develop to cirrhosis. ROS, reactive oxygen species; CV, central vein; PT, portal triad, which comprises the portal vein, hepatic artery and bile duct. Source: Adapted from Cohen *et al.*, (2011).

1.3 Role of free fatty acids and some molecular mediators in the development of NAFLD

The accumulation of free fatty acids in the hepatocytes due to hyperinsulinemia or hyperglycemia is regulated by the transcriptional factor sterol regulatory element-binding protein 1c (SREBP1c), peroxisome proliferator-activated receptor (PPAR-α) and high dietary fat (Mantena et al., 2009; Tsochatzis et al., 2009). Also, how stearoyl-CoA desaturase (SCD1) and cannabinoid receptors intervene in the development of this disease is to be discussed in this chapter.

1.3.1 Role of SREBP1c

SREBP family is involved in controlling some genes that are coded for some proteins in lipid metabolism, such as 3-hydroxy-methylglutaryl-CoA (HMG-Co A) reductase, Acetyl-CoA carboxylase, fatty acid synthase (FAS) and low density lipoprotein (LDL) receptor. These proteins are employed in the regulation of lipid and carbohydrate metabolism by controlling the lipogenesis and gluconeogenesis pathways (Chakravarty et al., 2004; Mullen et al., 2004; Quan et al., 2013; Peng et al., 2016). Mammals have two SREBP genes, SREBP1 and SREBP2. SREBP1a and c isoforms are responsible for regulating the metabolism of fatty acids and are encoded by a single gene known as SREBF-1. On the other hand, SREBP2 is the master regulator of cholesterol metabolism but has been found as weak regulator of fatty acids biosynthesis and is encoded by a different gene, SREBF-2. SREBP1a, is a potent transcriptional activator of genes encoded for enzymes involved in the biosynthesis of fatty acids and cholesterol, while SREBP1c activates transcription of enzymes contributing in the synthesis of unsaturated fatty acids (DeBose-Boyd et al., 2001). SREBP1c is a lipogenic

transcriptional factor that is predominantly found in the liver, skeletal muscles and adipose tissue. It is also believed that SREBP1c is involved in controlling the enzymatic synthesis of TG and fatty acids in the liver. (Chakravarty et al., 2004; Mullen et al., 2004; Quan et al., 2013; Peng et al., 2016). Insulin resistance and diabetes are highly associated with NAFLD in which the latter is a consequence of SREBP1c overexpression (Figure 1.3) (Vitto et al., 2012).

1.3.2 Role of SCD1

SCD1 is a delta 9 desaturase enzyme involved in the biosynthesis of unsaturated fatty acids. It is highly expressed in the liver and this rate-limiting enzyme converts saturated fatty acids into monounsaturated fatty acids by adding a cis double bond to the delta 9^{th} carbon of fatty acyl CoA (Gutiérrez-Juárez et al., 2006; Matsui et al., 2012; Caputo et al., 2014). Thus, stearoyl-CoA and palmitoyl-CoA, which are saturated fatty acids, are transformed by SCD1 into oleoyl-CoA and palmitoleoyl-CoA, respectively. SCD1 plays a vital role in hepatic lipid oxidation and lipogenesis. The produced monounsaturated fatty acids act as structural components of the cellular membrane phospholipid and are considered to be preferred substrates in the synthesis of complex lipids, such as TG and cholesterol ester. These complex lipids intervene in apoptosis, membrane fluidity, transduction signalling and energy metabolism (Matsui et al., 2012; Zhang et al., 2013; Caputo et al., 2014). Various factors, such as insulin, polyunsaturated fatty acids, fructose and glucose, can regulate SCD1 expression (Ntambi et al., 2004). Moreover, liver X receptor (LXR) activation, positively regulates the expression of SCD1 and promotes lipogenesis. This regulation can occur either (a) directly by the binding of LXR to the SCD1 gene promoter via an LXR response element or (b) indirectly by the activation of SREBP1-c transcription via LXR (Kim et al.,

2002; Liang et al., 2002; Chu et al., 2006). Therefore, as mentioned previously, the activation of SREBP1c will activate some lipogenic enzymes involved in the synthesis of TG and consequently induce hepatic steatosis.

.

1.3.3 Role of PPAR-α

PPAR is another nuclear ligand-activated transcriptional factor that acts as a hormonal receptor, which belongs to the steroid family receptors. It is involved in fatty acids metabolism and plays a vital role in fatty acids oxidation (Gyamfi and Patel, 2009; Grygiel-Górniak, 2014; Blednov et al., 2016). PPAR has two binding domains; the first domain binds to the deoxyribonucleic acid (DNA) sites of the target gene, which are called peroxisome proliferator hormone response elements (PPREs), via the DNA-binding domain located in the N-terminus of the PPAR, whereas the C-terminal has the ligand-binding second domain. Thus, after interacting with some ligands, it is translocated to the nucleus and induces gene transcription by changing its structure (Grygiel-Górniak, 2014). PPARs have three different isoforms: PPARγ, PPARβ/δ and PPAR-α (Berger and Moller, 2002). The latter is highly expressed in the brown adipose tissue, skeletal muscles, liver and heart. Therefore, when it is activated, it plays an important role in fatty acids oxidation and in lowering the level of lipids and consequently fat storage, and it prevents steatosis (Rao and Reddy, 2004; Neschen et al., 2007). However, inactivating PPAR-α reduces the oxidation of free fatty acids and energy utilisation, causing liver steatosis, which may develop into NASH (Figure 1.3) (Schwimmer et al., 2005). PPAR isoforms differ from one another in their sites of distribution and physiological effects, so they either activate or inhibit the

various genes involved in lipid and glucose haemostasis (Willson et al., 2000; Sertznig et al., 2007).

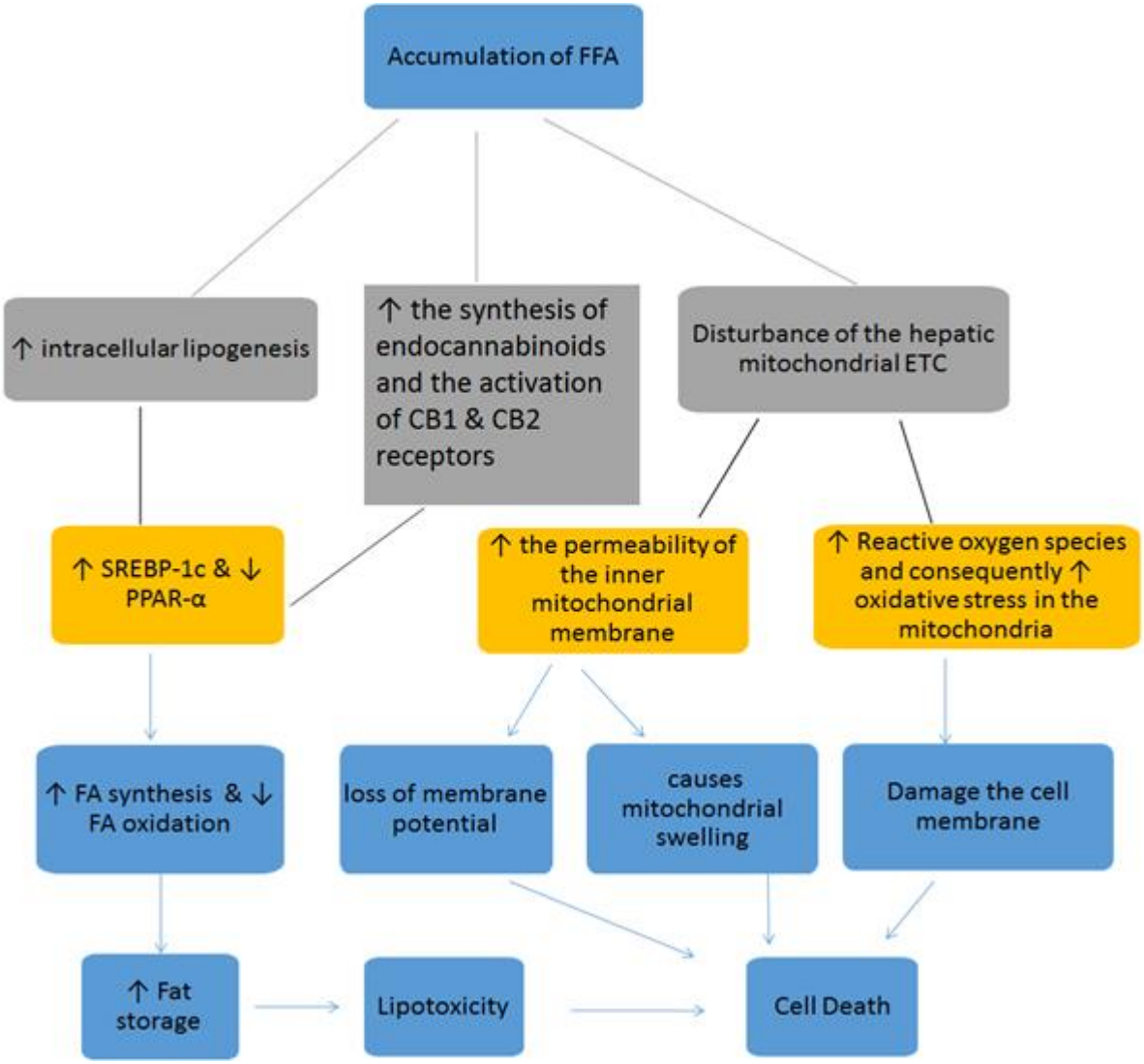

Figure 1.3: Mechanisms into fatty liver disease.

The accumulation of FFA through the ingestion of high fat diet leads to an increase in the intracellular lipogenesis and the synthesis of endocannabinoids. The latter, stimulates the activation of CB1 and CB2 receptors. Both mechanisms result in activating and deactivating some lipogenic genes SREBP1-c and PPARα, respectively. This effect consequently, increases FA synthesis and reduces FA oxidation causing cell death due to lipotoxicity caused by an increase of fat. Another crucial mechanism that can also lead to cell death is the disturbance of the mitochondrial ETC by destabilising the inner mitochondrial membrane. This in turn, causes loss in the membrane potential and mitochondrial swelling as a result of electron leakage and oxidative phosphorylation uncoupling of the respiratory chain, which then reduces the production of ATP. ETC disturbance also increases the production of ROS and consequently increases oxidative stress which has the ability to damage the complexes of the ETC and causes cell death. FFA, free fatty acid; FA, fatty acid; SREBP1-c, sterol regulatory element binding protein 1-c; PPAR-α, Peroxisome proliferator-activated receptors; ETC, electron transport chain; ROS, reactive oxygen species.

1.3.4 Role of Free Fatty acids

Free fatty acids play a crucial role in developing NAFLD by causing liver mitochondrial dysfunction through disturbing the electron transport chain (ETC) in the liver mitochondria and consequently reducing the production of the adenosine triphosphate (ATP) (Figure. 1.3) (Mantena et al., 2009; Tsochatzis et al., 2009).

Electron transport chain (ETC)

To understand how the ETC is involved in the development of NAFLD, it is necessary to describe this briefly in this chapter. In fact, the main function of ETC is energy production in the form of ATP, which is generated mainly via fat and carbohydrate oxidation under aerobic condition in the inner mitochondrial membrane and the cytoplasm, respectively. This process is recognised as oxidation phosphorylation and comprises (a) a respiratory chain that has four complexes and (b) the stage of ATP synthesis (Schon et al., 2010).

Five multiprotein complexes (I-V) exist in the ETC, along with two electron carriers (coenzyme Q and cytochrome C). Normally, electrons are released from reduced Flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH), which are generated from the oxidation of Acetyl-coA in the Krebs cycle, and subsequently, they pass through the respiratory chain and reduce the oxygen atom in complex IV to form water. The movement of electrons across the ETC will produce energy because of the redox reactions, where the electron transfers from an electron donor to an electron acceptor. This energy will then be used in generating an electrochemical proton gradient across the inner

membrane of the mitochondria by pumping protons (H^+) from the matrix of the mitochondria to the intermembrane space, across the respiratory chain (Acın-Perez et al., 2008; Schon et al., 2010; Nassir and Ibdah, 2014). As a result, ETC will be coupled with oxidative phosphorylation via an electrochemical gradient, and therefore, the energy that this gradient generates will be used to produce ATP from inorganic phosphate (P) and adenosine diphosphate (ADP) in complex V (ATP synthase) via oxidative phosphorylation (Figure. 1.4) (Acın-Perez et al., 2008).

Reactive oxygen species

Complexes (I & III) are considered the main sites of electron leakage in the inner mitochondrial membrane. Normally, reactive oxygen species (ROS) are produced in the mitochondria by complex I and III via the reduction of oxygen to the superoxide anion (Nassir and Ibdah, 2014). However, the excessive flow of electrons to the respiratory chain will cause the accumulation of electrons and results in electron leakage, leading to oxygen-forming ROS (Murphy, 2009). Indeed, electron leakage prevents complex IV from reducing a molecular oxygen atom to water, allowing the formation of a free radical, such as the superoxide anion (Mantena et al., 2008; Schönfeld and Wojtczak, 2008). Under physiological conditions, about 1%-2% of the oxygen atoms in the mitochondria are consumed to produce ROS, where some of them act as strong oxidising factors and play major roles in signalling pathways (Boveris and Chance., 1973; Leamy et al., 2013). Mitochondria plays a prime role in the homeostasis of ROS as the mitochondrial production of ROS accounts for 90% of the cellular production (Balaban et al., 2005; Nassir and Ibdah, 2014).

The increased production of ROS, particularly the superoxide anion in the mitochondria, results in the elevation of oxidative stress, such as in NASH, where the oxidation of free fatty acids increases (Begriche et al., 2006; Nassir and Ibdah, 2014). Accordingly, a high level of oxidative stress in the mitochondria can damage complexes (I-IV) of the ETC leading to cell death. ROS molecules, such as hydrogen peroxide, hydroxyl radicals and superoxide radicals, have the oxidative ability to damage DNA as well as the membrane of the cell. The latter is induced through the production of the lipid diffusible molecules known as reactive aldehydes, such as malondialdehyde and 4-hydroxynonenal. These two molecules also activate hepatic stellate cells, leading to collagen synthesis and the development of liver fibrosis (Bailey and Cunningham, 2002; Fromenty, et al., 2004; Mantena et al., 2008; Schönfeld and Wojtczak, 2008; Leamy et al., 2013; Papandreou and Andreou, 2015).

A high ROS level usually occurs in response to some factors, such as depletion in the antioxidant defence, cellular stress and mitochondrial dysfunction (Leamy et al*.,* 2013). The latter is also caused by electron leakage that causes mitochondrial membrane potential destabilisation, which, in turn, impairs the flow of electrons in the ETC and consequently increases the permeability of the inner mitochondrial membrane. This leads to mitochondrial swelling, the loss of the mitochondrial membrane potential via oxidative phosphorylation uncoupling and, subsequently, ATP synthesis reduction (Mantena et al., 2009; Tsochatzis et al., 2009).

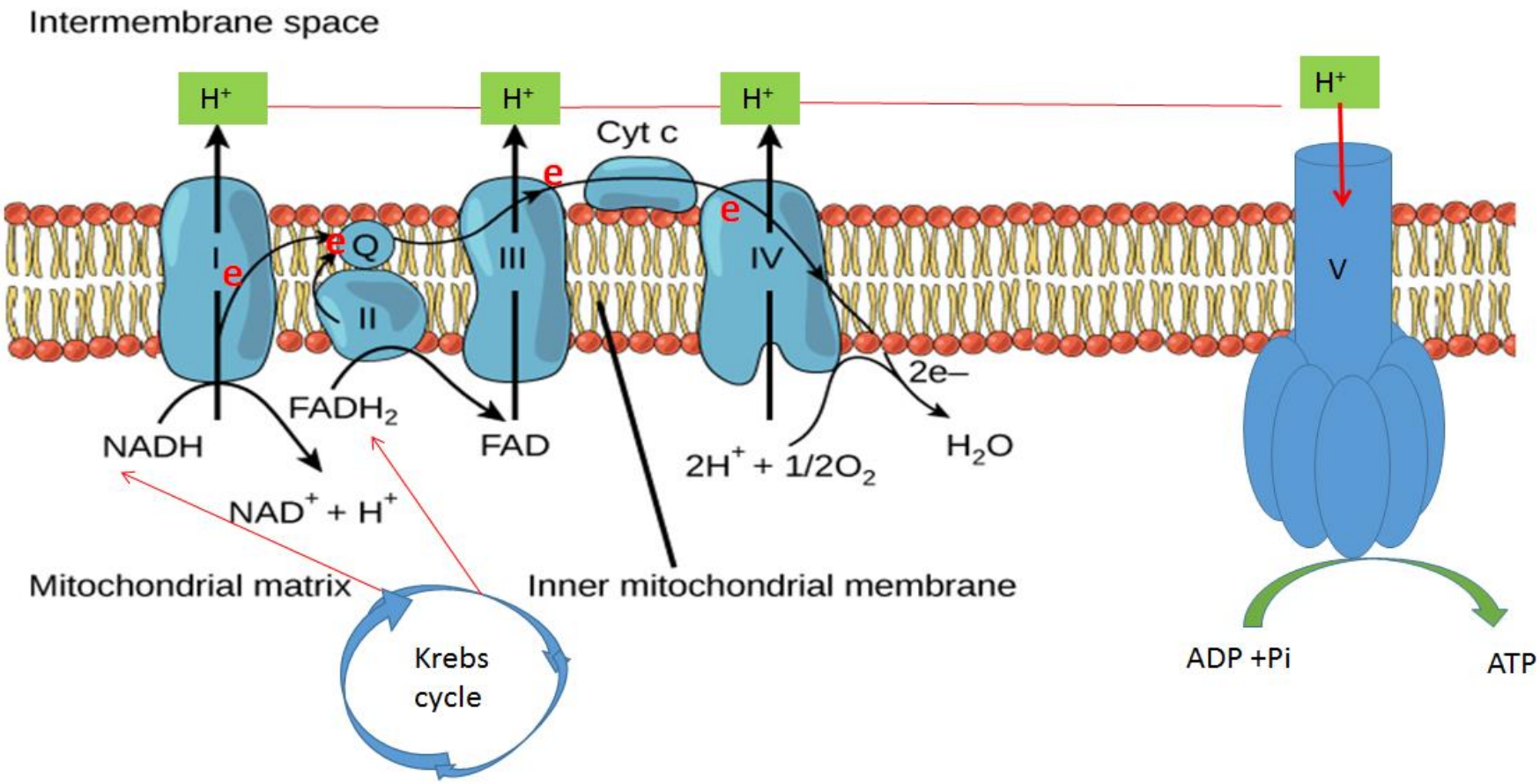

Figure 1.4: Electron transport chain in the mitochondria. It comprises four complexes of the respiratory chain [complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome c reductase) and complex IV (cytochrome c oxidase)], complex V (ATP synthase) and two electron carriers, coenzyme Q and cytochrome C. Source: Adapted from www.Boundless.com.

1.3.5 Role of cannabinoids receptors (CB1, CB2)

Cannabinoids are chemical compounds that exist in three different forms: phytocannabinoids, which can be found in some plants and cannabis; endocannabinoids, which are synthesised naturally in the human body; and ultimately, synthetic cannabinoids, which can be chemically synthesised. Two types of cannabinoid receptors exist: CB1 and CB2. CB1 receptors are highly expressed in the brain and the central nervous system also has been found located in some glands like pituitary gland and organs as the liver and pancreas, whereas CB2 receptors can be found in the cells of the immune system and haematopoietic cells (Rice et al., 1997; Pagotto et al., 2001; Purohit et al., 2009; Purohit et al., 2010; Alswat, 2013). CB1 and CB2 receptors have also been identified in the human liver, and it is believed that the activation of these two receptors is implicated in the development of fatty liver disease (Julien et al., 2005; Teixeira-Clerc et al., 2006; Mendez-Sanchez et al., 2007; Purohit et al., 2010; Alswat, 2013).

Alswat (2013) stated that 66 cannabinoids can be isolated from cannabis. In cannabis sativa L, tetrahydrocannabinol (THC), is the principle active component, and besides its therapeutic effect, it is also implicated in the production of an undesirable effect following the activation of CB1 receptors in the central nervous system. Cannabidiol (CBD) is another active constituent in cannabis extract (up to 40%), which can exhibit a pharmacological effect without any side effects (Alswat, 2013).

As Purohit et al. (2010) demonstrated, obesity and the high consumption of fat and alcohol play fundamental roles in increasing the synthesis of 2-arachidonoylglycerol (2-AG) and archidonoylethanolamide (anandamide), both of which are considered the main discovered endogenous endocannabinoids. The elevated level of these endocannabinoids will activate CB1 receptors and stimulate the expression of SREBP1c and its target enzymes, FAS and acetyl-CoA carboxylase-1, thus decreasing the regulation of the rate-limiting enzyme in fatty acid β-oxidation and carnitine palmitoyltransferase-1. Accordingly, the *de novo* synthesis of fatty acids will increase, whereas the oxidation of fatty acids will decrease, leading to the development of fatty liver. High fat consumption also contributes to the development of fatty liver and NAFLD via the activation or upregulation of CB2 receptors, however, their role in NAFLD is still not clear and needs further investigation. Interestingly, it has also been reported that CB1 and CB2 receptors act as profibrogenic and antifibrogenic activators, respectively (Figure. 1.3) (Julien et al., 2005; Teixeira-Clerc et al., 2006; Purohit et al., 2010). Inhibition of CB1 receptors can be a promising treatment approach and may have a potential therapeutic effect in managing NAFLD (Alswat, 2013). Indeed, understanding this mechanism can significantly help in finding a reliable treatment for this disease by inhibiting CB1 receptors to prevent the prognosis and development of fatty liver disease.

1.4 Fatty acid classification and omega 6 and 3 metabolism

Most natural fatty acids consist of an even carbon chain with carboxyl (COOH) and methyl (CH3) terminals (Kelly and Scheibling, 2012). Two classes of fatty acids exist: saturated and unsaturated. Stearic (18:0) and palmitic (16:0) acids

are examples of saturated fatty acids, which have no double bonds in their carbon chains and are considered the major dietary saturated fatty acids found in the blood circulation. They account for approximately 30-33% of plasma fatty acids. Saturated fatty acids are non-essential fatty acids, which can be synthesised in the body. They are found in meat, butter, whole milk, dairy products, palm oil and coconut oil (Schaefer et al., 2000).

On the other hand, unsaturated fatty acids are essential fatty acids, and most of these acids must be obtained from one's diet, as they cannot be produced in the body, especially if the double bond needs to be placed in carbon 6 or 9. They are subdivided into two groups according to the number of double bonds in their carbon chains. Monounsaturated fatty acids constitute about 20% of plasma fatty acids, and each has only one double bond, such as omega 9 oleic acid (18:1n9), with the double bond being located at carbon 9 from the methyl terminal (Schaefer et al., 2000). Monounsaturated fatty acids can be found in avocados as well as in canola and olive oil. Polyunsaturated fatty acids constitute the second class of unsaturated fatty acids, such as omega 6 and omega 3, which each having more than one double bond that is separated by a methylene group (CH2). The double bond in the carbon chain usually allows the chain to kink in a 37-degree angle and consequently improves the fluidity of the cellular membrane. In addition, most natural unsaturated fatty acids have a cis configuration, where the two hydrogen atoms lie on the same side of the carbon chain, which is counter to the trans configuration, where the two hydrogen atoms occur on opposite sides of the hydrocarbon chain. This happens as a result of hydrogenation (Calder., 2008; Wall et al., 2010; Schaefer et al., 2000).

The importance of omega 6 and 3 is centered on regulating various biological functions, such as regulating blood clotting and blood pressure (Wall et al., 2010). They are also considered a major component of the cell membrane, where they maintain the fluidity of the membrane, (Das, 2005). Omega 6 and 3 also play significant roles in the functioning and development of the brain and nervous system as well as in regulating inflammatory and immune responses (Williams, 2000).

The difference between omega 6 and 3 is the position of the first double bond from the methyl end of the fatty acid chain. Linoleic acid (LA, 18:2) and α-linolenic acid (ALA, 18:3) are the precursors of omega 6 and omega 3, respectively, and they cannot be produced in the body due to the lack of desaturase enzymes, which are required in their production. Cereals, egg, butter, whole grain breads, nuts and vegetable oil, such as corn and sunflower oil, are the main sources of omega 6, whereas omega 3 is found in green leafy vegetables, canola, walnut, flaxseeds and fish (El-Badry et al., 2007; Simopoulos, 2008; Rustichelli et al., 2009; Kelly and Scheibling, 2012; Patterson et al., 2012; Elhardallou et al., 2014). It has been found that omega 3 and omega 6 fatty acids are correlated with the pathogenesis of NAFLD due to the dietary imbalance between them where the omega 6 concentration is higher than omega 3 (Santoro et al., 2012). However, the exact pathogenic mechanism for this is unclear. Therefore, it is important to understand and to identify how they affect intracellular lipid metabolism and NAFLD pathogenesis.

Omega 6 and 3 metabolism

Briefly, LA and ALA are metabolised mainly in the liver but also in some other tissues by adding one double bond by the delta 6-desaturase enzyme to the carboxyl end of the chain as the first and rate-limiting step in this pathway. Both fatty acids continue to elongate through the enzyme elongation of very long chain fatty acids 5 (ELOVL 5), and they desaturate to a longer fatty acid chain. Thus, LA generates arachidonic acid (AA, 20:4), whereas ALA is metabolised to eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Figure 1.5) (Simopoulos, 2008; Candela, et al., 2011)

Omega 6 and omega 3 compete for the desaturation enzymes, but both delta-5 and delta-6 desaturase enzymes prefer the desaturation of omega 3 fatty acids rather than those of omega 6. Therefore, the high dietary intake of LA interferes with the elongation and desaturation of ALA (El-Badry et al., 2007; Simopoulos, 2008; Candela, et al., 2011). It is known that delta-6 desaturase decreases with age and in some cases in hypertensive and diabetic individuals as well as in premature infants, where the ability to produce EPA and DHA is limited (Simopoulos, 2008). It is also known that hormones such as glucagon, thyroxin, epinephrine and glucocorticoids can suppress delta 5 and delta 6 desaturase enzymes, in contrast to insulin, which is considered a good stimulator of these enzymes (Nakamura et al., 1994). Although LA, ALA and their derivatives are vital cellular membrane components, mammalian cells are unable to synthesise or to convert omega 6 to omega 3 due to the lack of omega 3 desaturase enzymes (Simopoulos, 2008).

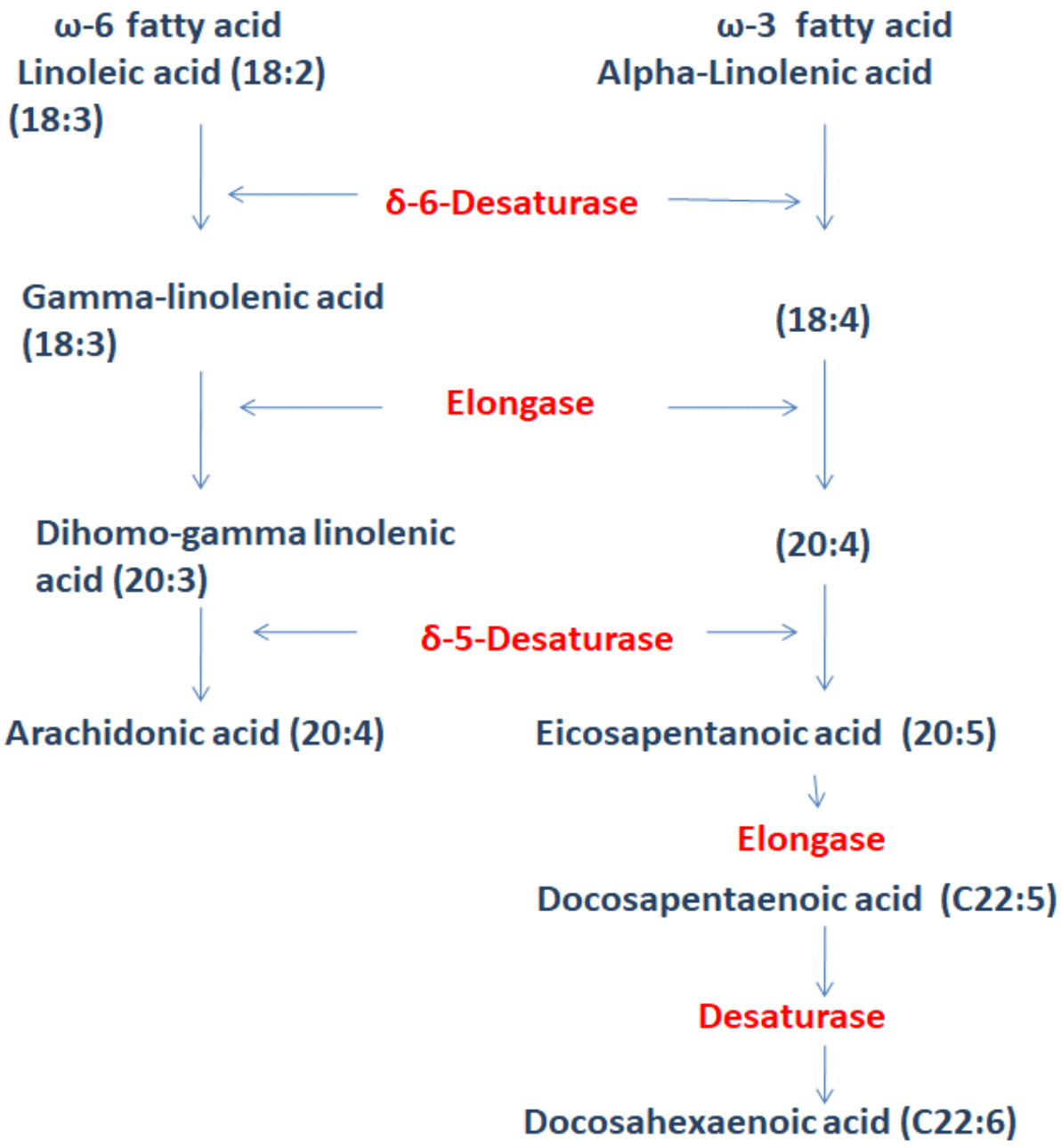

Figure 1.5: Metabolism of Omega 6 & 3 fatty acids. Essential linoleic acid and alpha-Linolenic acid cannot be synthesised in the human body and must be obtained from diet. Delta 6-Desaturase is the rate limiting enzyme in this pathway. Both fatty acids are metabolised via a series of desaturation (double bond addition) and elongation (two carbon atoms addition) reactions to longer derivatives.

1.5 Role of omega 6 and 3 in NAFLD development

DHA, EPA and AA are all incorporated into lipid bilayers of cell membranes, with changes in the proportions of these fatty acids affecting membrane fluidity (Candela et al., 2011; Patterson et al., 2012). Patterson et al. (2012) mentioned that the lack of ELOVL 5 enzyme leads to the accumulation of ALA and LA derivatives (γ-linolenic acid [18:3]) and consequently causes a decrease in the production of AA (omega 6), EPA and DHA (omega 3). The accumulation of LA derivatives results in the activation of the SRBP1c transcriptional factor, which is known to stimulate the synthesis of lipid and be associated with the development of liver steatosis. It has also been demonstrated that both delta-6 and delta-5 desaturase enzymes have low activity in the liver of NAFLD patients with obesity (Araya et al., 2010).

Therefore, the inadequate intake of dietary omega 3 (ALA) or any defect in the desaturation and elongation enzymes, as well as an imbalance with omega 6 linoleic acid, will affect the metabolism of omega 3 and decrease the production of DHA and EPA (Simopoulos, 2003). All of these factors increase the ratio of omega 6/3 and, as mentioned earlier, upregulate the transcriptional factor SREBP1c, which, in turn, activates some lipogenic genes involved in the synthesis of fatty acids, such as SCD1 and FAS (Yahagi et al., 2002). In the meantime, PPAR will be downregulated and supresses the oxidation of fatty acids and consequently deactivates the transcription of some genes involved in the degradation of fatty acids, such as acyl-CoA oxidase and carnitine palmitoyl transferase-1 in the mitochondria. These changes will then stimulate the accumulation of TG in the liver and result in the development of NAFLD (Figure. 1.6) (Kersten et al., 1999; Levy et al., 2004; El-Badry et al., 2007).

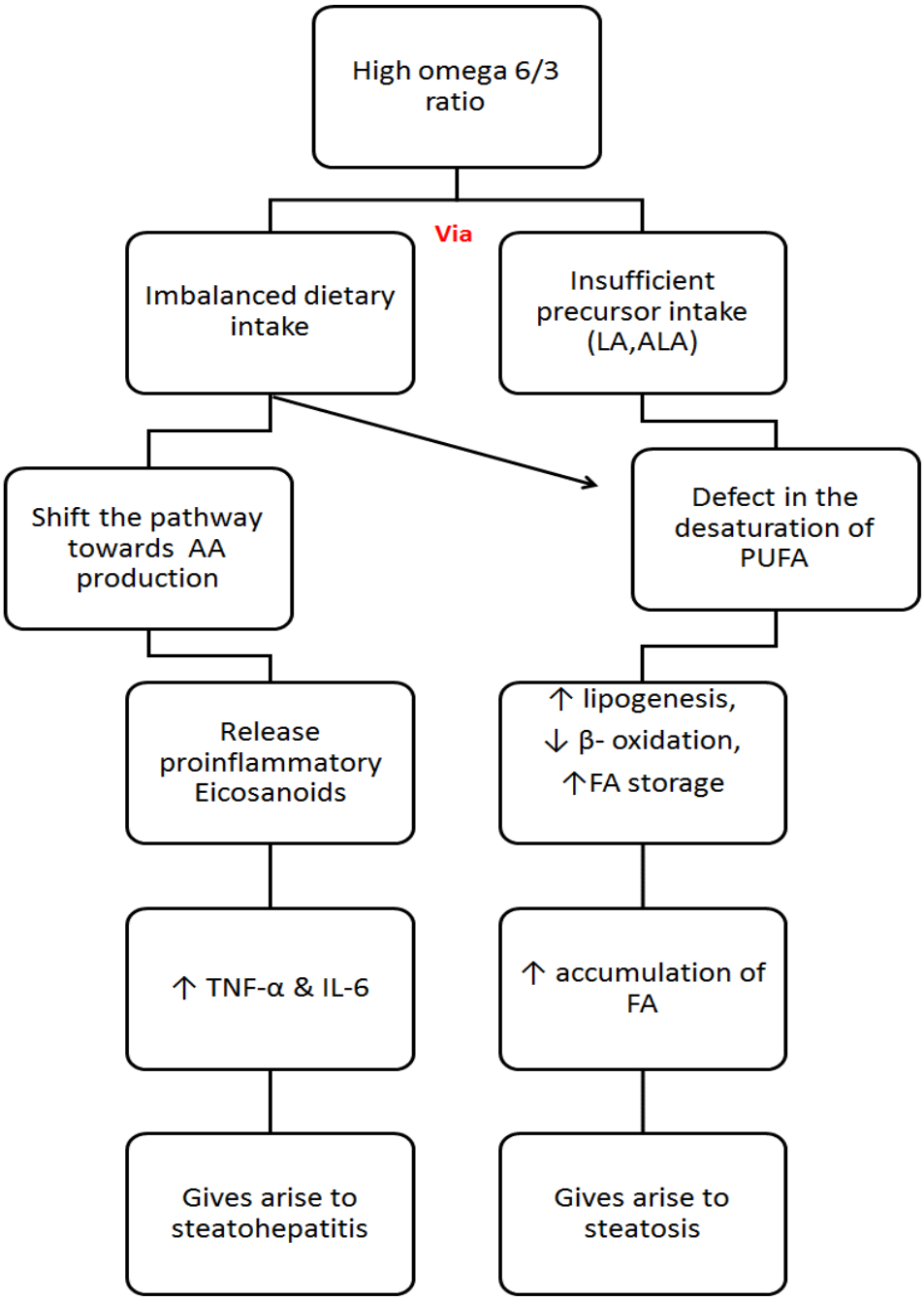

Figure 1.6: Role of increased omega 6/3 ratio in NAFLD.
High dietary consumption of omega 6 or insufficient LA, ALA intake, causes an increase in omega 6/3 ratio. Elevated levels of LA are metabolised by desaturase enzyme which in turn, shifts the pathway and increases the production of AA. As a result, this stimulates the production of some pro-inflammatory eicosanoids. The production of these pro-inflammatory mediators will then activate the release of inflammatory cytokines (TNF-α and IL-6) causing liver inflammation and fibrosis. High omega 6/3 ratio can also cause a defect in the desaturase activity and consequently, increases FA synthesis and storage with a decrease in the oxidation of FA which then activate and deactivate SREBP1-c and PPARα, respectively. FA then accumulates in the hepatocytes and gives arise to steatosis. AA, arachidonic acid; PUFA, Polyunsaturated fatty acid; FA, fatty acid; TNF-α, tumour necrosis factor alpha, IL-6, interlukin-6.

The recommended ratio of omega 6/3 is 1:1 to 2-4:1. Currently, omega 6 is more highly consumed in Western societies than omega 3, reflecting a higher ratio of omega 6/3 (20-30:1) one that is considerably greater than the recommended ratio (Candela et al., 2011). This imbalance can be normalised through increasing the dietary consumption of EPA and DHA, which will then to a certain extent replace excess omega 6 fatty acids, especially AA, in the cellular membrane of hepatocytes, erythrocytes, platelets, neutrophils and monocytes (Simopoulos, 2003). Indeed, by increasing the omega 6/3 ratio, eicosanoid metabolites will be largely produced from AA rather than from EPA. Eicosanoids are metabolic products of omega 6 and 3; they include thromboxanes, prostaglandins, lipoxins, leukotrienes and hydroxy fatty acids. Small amounts of eicosanoids have important biological roles; however, large quantities of these products produced from AA by the high consumption of omega 6 will act as a pro-inflammatory molecule, thus contributing to many inflammatory diseases, such as NAFLD. Meanwhile, EPA and DHA eicosanoid products have an anti-inflammatory effect that can antagonise the effect of the pro-inflammatory eicosanoids produced from AA (Rustichelli et al., 2009; Simopoulos, 2008; Patterson et al., 2012). Indeed, the low level of omega 3, which occurs in the hepatic tissue of NAFLD, reflects the trend of fatty acids towards fatty acid storage in the form of triacylglycerol and lipogenesis, instead of undergoing ß-oxidation. Thus, a high omega 6/3 ratio contributes to the development of NAFLD due to the derangement of the liver to handle and metabolise lipids (Araya et al., 2004; Patterson et al., 2012).

Omega 6 and 3 play crucial roles in cell signalling as well as in gene expression (Patterson et al., 2012). Therefore, dietary omega 6/3 ratio imbalance contributes

to the pathogenesis of many diseases, such as hypertension, coronary disease, arthritis and diabetes (Candela et al., 2011; Simopoulos, 2008). However, few studies have examined the exact ratio of omega 6/3 that can be implicated in the pathogenesis of NAFLD. Whether an increased omega 6/3 ratio could enhance mitochondrial dysfunction is yet to be determined. Also, what roles could cannabinoids play in the development of NAFLD? This warrants further investigation into such mechanisms involved in the pathogenies of NAFLD.

1.6 Alcoholic liver disease (ALD)

Heavy and prolonged alcohol consumption plays a major role in the prevalence of alcoholic liver disease (ALD) and many other diseases, such as some malignant neoplasm and heart diseases in Western countries (Popova et al., 2007). ALD has different stages; hepatic steatosis is the first stage, arising in 90% of alcohol consumers in response to heavy alcohol consumption (Arteel et al., 2003; Méndez-Sánchez et al., 2005). Although this stage is reversible and asymptomatic, the continued intake of alcohol causes 20-30% of cases with simple steatosis to evolve into alcoholic steatohepatitis, which is characterised by inflammation and fibrosis. This can then develop into cirrhosis, an irreversible stage (in 16% of patients). It may later develop into hepatocarcinoma (Figure 1.7) (Deleuran et al., 2012; Pateria et al., 2013; Nassir and Ibdah, 2014). Similarly to NAFLD, the pathogenesis of ALD is still not fully understood, reflecting the urgent need for further investigation into the mechanisms involved in the development of ALD.

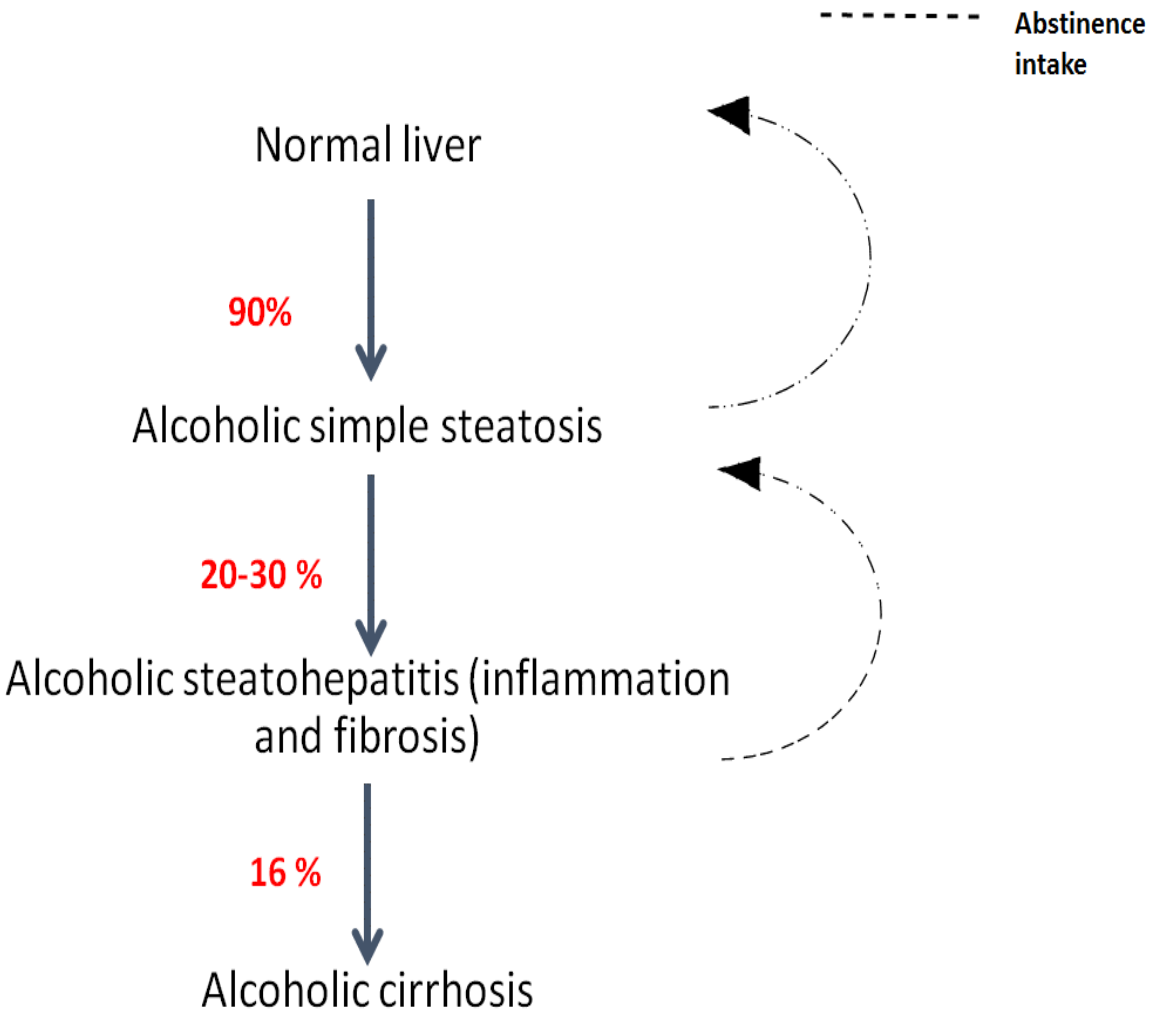

Figure 1.7: Pathogenesis of alcoholic liver disease and percentage among heavy drinkers.

About 76 million out of two billion people worldwide, who regularly consume alcohol at the rate of an average of 13 g/day, are diagnosed with disorders involving alcohol use. It also has been found that a high average rate of consumption of alcohol in England is highly associated with 200 conditions ranging from acute and chronic, such as cardiovascular diseases and cancers. Treating them comes at an estimated cost of £2.7 billion of the direct annual health care expenditure by the National Health Services in the United Kingdom

(UK), which soared to £3.5 billion between 2011 and 2012 (British Medical Association Board of Science, 2008; Gao and Bataller, 2011; Waszkiewicz et al., 2012; Knott et al., 2015).

As indicated in a report that WHO published in 2011 on alcohol and health, around 4.5% of the global burden of injury and disease was correlated to alcohol consumption, with the rates being about 7.4% and 1.4% for males and females, respectively. Beer, wine and spirits are the most commonly consumed types of alcohol, in ascending order. Regardless of the type of alcohol drink consumed, ALD is considered the prime cause of chronic liver disease and has a central causative role leading to increased mortality rates worldwide due to its ability to develop into fibrosis and cirrhosis. In 2004, around 3.8% of all deaths were related to alcohol consumption. According to a report published by the National Institute of Alcohol Abuse and Alcoholism, liver cirrhosis was ranked as the 12th major cause of mortality in the United States with a total death number of 29,925 in 2007, with 48% of this number being correlated to alcohol use (Gao and Bataller, 2011; Waszkiewicz et al., 2012).

It seems that alcohol intake elevates with urbanisation and industrialisation of the country. As the WHO stated, consumers in Eastern Europe are the biggest alcohol consumers in the world; there, the average of pure alcohol intake per capita is 12.2 L/year, whereas the eastern region of the Mediterranean and the Middle East showed the lowest percentages with an average of 0.7 L/year/capita (Roswall and Weiderpass, 2015).

1.7 Alcohol metabolism

Alcohol is an organic compound that can be metabolised in the body by two different pathways: oxidative and non-oxidative. Oxidative alcohol metabolism occurs mainly in the liver, in the cytosol of the hepatocyte. In this pathway, alcohol is metabolised by an enzyme called alcohol dehydrogenase (ADH) into acetaldehyde, which is considered a highly toxic and reactive molecule and is accompanied by NAD reduction into NADH. Other enzymes are also involved in the oxidation of alcohol, such as the microsomal ethanol oxidising system (MEOS), known as cytochrome P450 isoenzymes. Cytochrome P450 comprises a group of enzymes that includes CYP2E1, 3A4 and 1A2, and it is predominantly found in the endoplasmic reticulum and contributes to alcohol metabolism. Generally, with chronic alcohol consumption and/or when the concentration of alcohol is elevated, CYP2E1 is induced, resulting in oxidising the excessive amount of alcohol to acetaldehyde as well as generating ROS, which contributes to ER stress (Ji, 2008; Leung and Nieto, 2013). Catalase, another enzyme located in the peroxisomes, plays a minor role in metabolising ethanol to acetaldehyde. Finally, acetaldehyde oxidation occurs in the mitochondria through the enzyme aldehyde dehydrogenase (ALDH2), which has the highest affinity to acetaldehyde among the 19 ALDH mammalian genes, producing acetate and another NADH molecule, which, in turn, can be oxidised by the mitochondrial ETC (Lemasters, et al., 2012; Waszkiewicz et al., 2012; Zakhari, 2013). On the other hand, acetate is released into the bloodstream and can thus be metabolised further by peripheral tissues to form carbon dioxide, water or fatty acids (Figure 1.8) (Caballería, 2003).

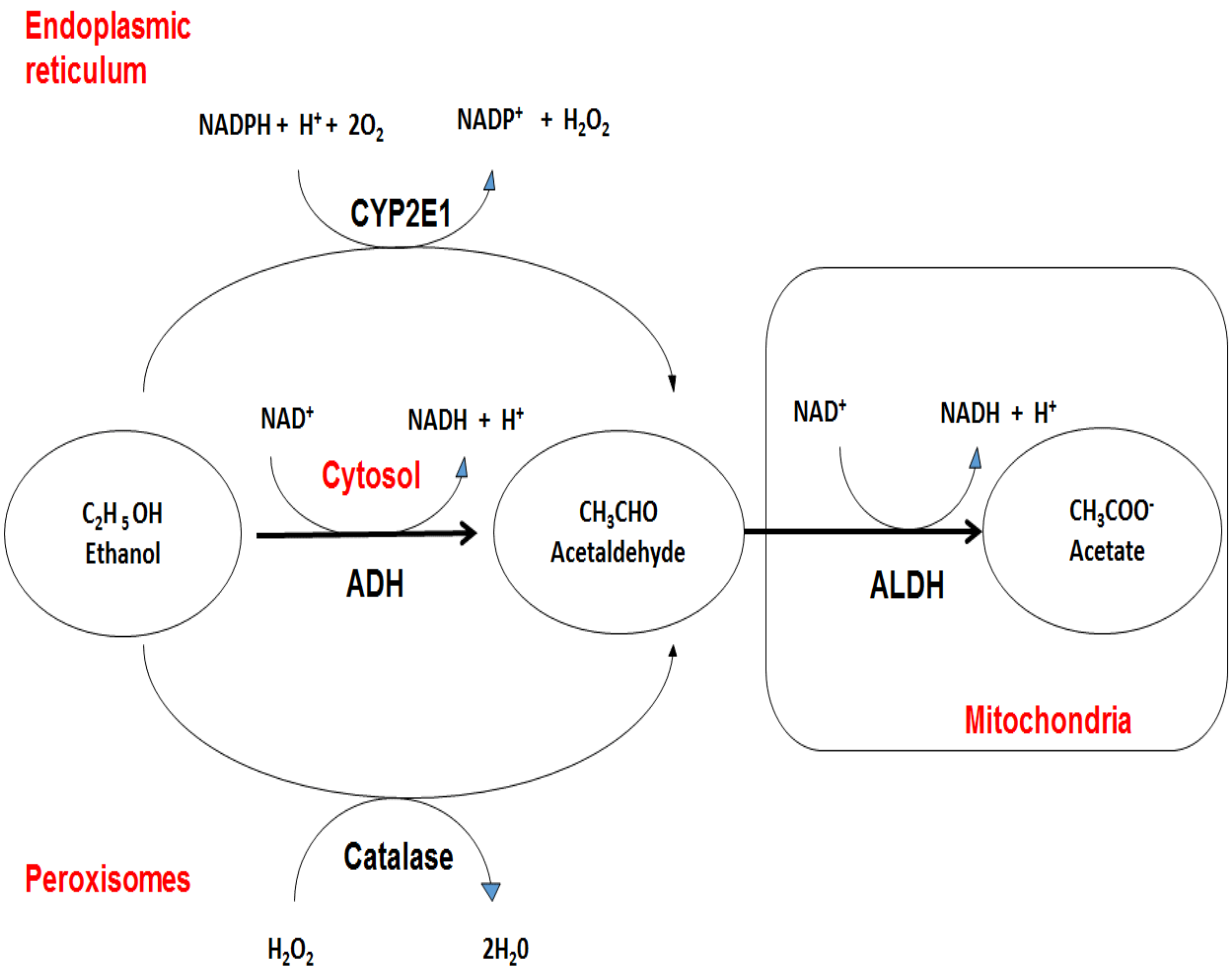

Figure 1.8: Oxidative alcohol metabolism. Alcohol is mainly metabolised into acetaldehyde by alcohol dehydrogenase (ADH) in the cytoplasm and the microsomal cytochrome P450 2E1 (CYP2E1) in the endoplasmic reticulum. However, to minor extent, alcohol also converted in the peroxisomes by catalase to acetaldehyde with a production of H2O. Acetaldehyde then metabolised into acetate by the mitochondrial acetaldehyde dehydrogenase (ALDH). NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; H_2O_2, hydrogen peroxide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide phosphate.

Non-oxidative ethanol metabolism is mediated by the esterification of ethanol and fatty acids or fatty acyl-CoA into fatty acid ethyl esters (FAEE). Non-oxidative ethanol metabolism oxidises only about 1% of ethanol by FAEE synthase enzymes found in the cytosol and microsomes of the liver, pancreas, heart and brain. These organs can easily be damaged by ethanol and its non-oxidative (FAEE) metabolite, as well as oxidative metabolites (acetaldehyde) and generated ROS. FAEE can destabilise the cellular and lysosomal membranes by increasing the fluidity of the membrane, consequently leading to mitochondrial oxidative phosphorylation uncoupling in the ETC followed by a reduction in the ATP produced (Table 1.2) (Waszkiewicz et al., 2012).

Alcohol metabolism can exert a significant fluctuation and elevation in the ratio of NADH/NAD through the production of acetaldehyde and acetic acid, causing an extravagant reduction in the level of the nicotinamid adenine dinucliotide (NAD). Under normal conditions, the approximate ratios of NADH/NAD in the hepatic cytosol and mitochondria are 700:1 and 7–8:1, respectively. However, chronic alcohol consumption leads to an elevation of this ratio and disturbs the metabolism of several pathways, such as carbohydrate, uric acid, lipids and protein metabolism. Elevated NADH/NAD ratios also influence the modulation of gene expression, cell death and mitochondrial permeability transition opening (MPT); the latter leads to mitochondrial swelling, loss of the mitochondrial membrane potential and subsequently reduced adenosine triphosphate (ATP) synthesis (Table 1.2). Acute or chronic alcohol consumption can increase the availability of NADH, which drives the mitochondrial ETC, leading to increased electron leakage at complexes I and III and a subsequent elevation in ROS

formation (Cunningham and Bailey, 2001; Waszkiewicz et al., 2012; Zakhari, 2013).

The formation of ROS, which is mediated by the CYP2E1 isoenzyme, is also generated by various reactions, such as lipid metabolism and NADPH oxidase; however, the majority is via the mitochondrial ETC (Waszkiewicz et al., 2012; Leung et al., 2013; Zakhari, 2013). Epigenetic changes caused by oxidative alcohol metabolism can be induced by several mechanisms, such as high NADH/NAD ratio and via the production of acetate, acetaldehyde and ROS (Zakhari, 2013). However, due to the electrophilic nature of acetaldehyde, it can covalently bind to molecules, such as protein, lipids and DNA, changing the homeostasis of the cell by altering the protein structure and promoting DNA mutations as well as inducing ER stress (Table 1.2) (Ceni et al., 2014; Ji, 2015).

Deduced from what has been discussed on alcohol metabolism, alcohol metabolites (acetaldehyde, acetate, FAEE) are all involved in the pathogenesis of ALD due to their harmful impact on the cellular haemostasis. There are also studies pointed at the role of fatty acids in the pathogenesis of ALD and how the amount and the type of fatty acids are involved in alcoholic liver injury (Tsukamoto et al., 1986; Nanji and Hiller-Sturmhofel., 1997; Caro and Cederbaum., 2007). In addition, increasing the ratio of NADH/NAD and the production of ROS, which, in turn, increases either the mitochondrial oxidative stress or ER stress, are all engaged in distracting the mechanisms involved in ALD.

Table 1.2: ***Key molecular alterations following alcohol metabolism***

Metabolite or Molecule	**Mechanism**	**Effect**
Acetaldehyde	Binding to membrane protein, DNA or lipid	Changing the fluidity of the membrane by changing the cellular homeostasis Inducing ER stress causing steatohepatitis
FAEE	Causing oxidative phosphorylation uncoupling ATP synthesis reduction	Destabilization of the cellular membrane Increasing the permeability of the membrane
NADH/NAD ratio	ETC disruption resulting in electron leakage and ATP synthesis reduction	Increasing the cellular membrane permeability
ROS	Production of reactive lipid molecules	Damage the membrane of the cell, and change the membrane stabilisation.

ER- endoplasmic reticulum; NADH/NAD- nicotinamide adenine dinucleotide (reduced/oxidised form); FAEE- fatty acid ethyl ester; ROS- reactive oxygen species; ETC- electron transport chain; ATP- adinosin triphosphate.

1.8 Aims

The aim of this project was to investigate the mechanisms involved in fatty liver disease development, with particular attention focused on studying the role of omega 6/3 ratio in NAFLD pathogenesis, alongside mitochondrial function and lipid biogenesis.

1.9 Objectives:

1. To characterise the effect of fatty acids (saturated/ unsaturated) and omega 3 and 6 fatty acids ratio on lipotoxicity and lipid accumulation in HepG2 cells
2. To determine the effect of omega 6/3 ratio on lipid metabolisms and cannabinoid system
3. To investigate the effect of alcohol and omega 6/3 ratio on mitochondrial function

Chapter 2

Materials and Methods

2.1 Materials

Triglyceride Assay Kit was obtained from Universal Biologicals (Cambridge, United Kingdom [UK]). Tumour necrosis factor (TNF-α) was obtained from Cayman (Cambridge, UK). Heat-inactivated foetal calf serum (FCS) was obtained from Biosera (Ringmer, UK). Phosphate buffered saline (PBS), L-glutamine, penicillin-streptomycin mix and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Lonza Ltd (Wokingham, UK). 1x Trypsin in DPBS was from First Link Ltd (Birmingham, UK). Sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), Oil red O, 2′,7′-dichlorofluorescin diacetate (DCFDA), palmitic acid (PA), stearic acid (SA), linoleic acid (LA), α-Linolenic acid (ALA), arachidonic acid (AA), docosahexaenoic acid (DHA) and D-(+)-Glucose were acquired from Sigma-Aldrich (Gillingham, UK). Dimethyl sulphoxide (DMSO), formaldehyde and isopropanol were obtained from Fisher Scientific UK (Loughborough, UK). XF Cell Mito Stress Test Kit and Seahorse XF Assay Medium were obtained from Agilent Technologies (Craven Arms, UK).

2.2 Methods

2.2.1 Cell culture

Human hepatoma cell lines VA-13 and VL-17A, which are HepG2 cells that over-express ADH, and CYP2E1 and ADH respectively, were generously given by Dr. Dahn Clemens (University of Nebraska, Nebraska, United States of America [USA]). Cells were cultured in a high-glucose (25 mM) DMEM supplemented with penicillin (100 u/ml) and streptomycin (100 mg/ml), L-Glutamine (2 mM), 10% FCS and sodium pyruvate (1 mM) (Chandrasekaran et al., 2011; Gyamfi et al.,

2012) at 37ºC in 5% CO_2 atmosphere. VL-17A cells were also grown in the presence of Plasmocin Prophylatic 5-µg/mL DMEM for the initial four weeks prior to their culturing in a high glucose medium. The medium was then changed after 3–4 days from the seeding day. At a 70% confluence, cells were trypsinised and split, then treated with alcohol or various fatty acids (individual or a mixture) with a range of different concentrations (Donohue et al, 2006; Gyamfi et al., 2012). Briefly, fatty acids were prepared as described by Gyamfi et al. (2012) in DMSO as a 8 mM stock. Alcohol or fatty acids were diluted in 1% FCS low-glucose (1 g/L) DMEM to the required concentrations. Fatty acids were in free form with a concentration of 0.02% bovine serum albumin in the FCS.

2.2.2 Determination of cellular lipotoxicity

2.2.2.1 MTT assay

Cell viability was assessed by the MTT method with a slight modification (Chuturgoon et al., 2015). Either VA-13 or VL-17A cells were seeded overnight in a 96-well plate ($2.5x10^4$ cells/ 200 µl DMEM per well) and then treated with the corresponding concentration of individual saturated fatty acids SA or PA (20, 80, 160 µM) or individual unsaturated fatty acids LA, ALA, AA or DHA (0.5–300 µM). Cells were also treated with a mixture of the fatty acids LA/ALA or AA/DHA at various ratios (1:1, 4:1, 15:1 and 25:1 ratio) or a mixture of AA (40 µM) with alcohol (100 mM) for six, 24, 48 and 72 h. Vehicle control was included as 0.5% DMSO. After 2 h of incubation with MTT (5 mg/ml) at 37ºC, the reagent was removed and cells were incubated with DMSO (100 µl/well) for 15 min at room temperature. Cell proliferation was then determined by assaying the ability of the succinate dehydrogenase enzyme in the mitochondria to reduce the MTT to a

blue formazan crystal. This colour intensity was measured at 550 nm using a VersaMax microplate reader (Molecular Devices, Wokingham, UK). The data was expressed as percentage from the control group.

2.2.3 Determination of steatosis

2.2.3.1 Oil red O assay

To quantify the amount of lipid accumulation following fatty acid treatment, cells ($5x10^4$ cells/well) were seeded and treated in a 96-well plate as described above for 24, 48 and 72 h. Cells were then fixed for 1 h with 10% formalin at room temperature. After fixation, the cells were washed two times with ice cold PBS and stained with 60% oil red solution (3 mg/mL in isopropanol) for another 1 h at room temperature. Then, the cells were washed twice for a second time with ice cold PBS and once with distilled water then incubated for 5 min with DMSO. Next, the absorbance was measured calorimetrically at 510 nm with a VersaMax microplate reader (Molecular Devices, Wokingham, UK). The data are reported as percentages from the control (Gyamfi et al., 2012).

2.2.3.2 Measurement of intracellular triglyceride content

The triglyceride content in VL-17A cells was measured using an EnzyChrom Triglyceride Assay Kit (BioAssay Systems, USA) (Guo et al., 2011). Briefly, cells were seeded at a density of 2 x 10^5 cell/ 2 mL in a 24-well plate overnight. After the cells adhered, the cells were treated in triplicate for 24, 48 and 72 h with four different AA/DHA ratios ranging from 1:1 to 25:1 using 1% FCS DMEM, along with a vehicle control containing 0.5% DMSO as a final concentration. The triglyceride content was then determined following the manufacturer's

instructions after the cells had been washed and trypsinised (Gyamfi et al., 2012). The protein concentration was also measured using a Bio-Rad protein assay kit according to the manufacturer's instructions. The triglyceride content was expressed as milligram triglyceride per milligram protein.

2.2.4 Western blot

Following treatment as described above with different fatty acid ratios for a 24 h, the expression of cannabinoid receptors 1 and 2 (CB1, CB2) and the transcriptional factors (sterol regulatory element-binding protein (SREBP1), peroxisome proliferator-activated receptor (PPAR-α), Stearoyl-CoA desaturase (SCD1)) was determined. VL17A cell pellets were treated with a Protease Inhibitor Cocktail (1 ml/10^8 cells/ml) (Sigma-Aldrich, Gillingham, UK) to avoid the degradation of the intact protein by endogenous enzymes, such as phosphatase and protease. Cells were then lysed with 1% Triton 100x, and the protein concentration was measured using a Bio-Rad protein assay kit by following the manufacturer's instructions (Bio-Rad Laboratories, Hemel Hempstead, UK). Protein from cell lysate was then prepared by adding 5x SDS Protein Sample Buffer containing 30% glycerol, 0.31M Tris, 10% SDS and 0.5% bromo phenol blue. Protein (20-40 µg) was loaded on 10% SDS-PAGE gels (Thermo Scientific Pierce, UK). The gel was electrophoresed for 90 min at 120 v and then electro-blotted onto a nitrocellulose membrane (0.45 µm) for 1 h at 350 A. To evaluate the transfer quality and the equality of band intensity, the membrane was stained with Ponceau S solution before being blocked for 1 h with BSA blocking buffer ([1%] in 1% 1x Tween-PBS [TPBS] for all antibodies except β-actin with [5%] BSA) at room temperature and then washed three times with 1X TPBS prior to

being incubated with a primary antibody overnight. An immunoblot analysis of proteins was carried out using primary antibodies; these are as follows: mouse anti-human SREBP-1 (1:250) (Santa Cruz Biotechnology, Santa Cruz, UK) and SCD1 (1:4000) monoclonal antibodies. Rabbit polyclonal antibody recognising PPAR alpha (1:2000) and CB2 (1:2000), as well as goat polyclonal antibody recognising CB1 (1:4000) (Santa Cruz Biotechnology, Santa Cruz, UK) were used.

After the incubation, the membrane was washed three times with 1X TPBS and then incubated with rabbit anti-mouse (for actin, SCD1 and SREBP-1), goat anti-rabbit (for PPARα and CB2) and rabbit anti-goat (for CB1) IgG peroxidase as a secondary antibody for 1h at room temperature (Table 2.1). Bands were imaged on x-ray film after the membrane was incubated with West PICO Chemiluminescent Substrate (ThermoFisher, Cramlington, UK) and normalised to actin (Patel et al., 2007). All films were scanned by a Bio-Rad GS-800 Calibrated Densitometer (Bio-Rad, Hemel Hempstead, UK). Secondary antibodies were all obtained from Sigma Aldrich, (Gillingham, UK). Primary antibodies SCD1, CB2, PPAR alpha and β-actin antibodies were purchased from Abcam (Cambridge, UK).

Table 2.1 ***Concentrations of optimised primary and secondary antibodies***

Concentration / Antibody	Primary antibody	Secondary antibody
SREBP1	1:250	1:2000
PPAR-α	1:2000	1:4000
SCD1	1:4000	1:10000
CB1	1:750	1:2000
CB2	1:2000	1:8000

2.2.5 Determination of ROS

The level of intracellular ROS was investigated in VL-17A cells by using DCFDA. Briefly, the cells were seeded at a concentration of 1 or 5 x 10^4 cell/ 200-µl DMEM in a 96-well plate at 37ºC for an overnight period. The cells were then treated separately with either different omega 6/3 ratios (1:1–25:1) or a mixture of alcohol (100 mM) and omega 6/3 ratios (1:1–25:1) for 30 min–2 h. Following the incubation, the cells were incubated with 1 µM DCFDA diluted in PBS for 45 min at 37ºC. The intensity of fluorescence was determined by FLUOstar OPTIMA (Jencons-PLS, Bedfordshire, UK) at an excitation of 485 nm and an emission of 535 nm. The intracellular ROS level was indicated by the fluorescence level, and the results are expressed as percentage from the control.

2.2.6 Measurement of mitochondrial stress

To evaluate the mitochondrial respiration rate, an XF Cell Mito Stress Test Kit was used following the manufacturer's instructions. In brief, VL-17A cells were seeded on SeaHorse 24-well cell culture microplates (Agilent Technologies, Craven Arms, UK) at a density of 1.5 x 10^4 cells/100µL (10% FCS) DMEM and were incubated at 37ºC, 5% CO_2. After two hours of adherence, 150 µL of DMEM (10% FCS) was added to the cultured cells and re-incubated for an overnight period, then treated for 24 h with 500 µL per well of 10% FCS DMEM containing 4 different ratios of omega 6/3 (1:1 to 25:1) along with a vehicle control containing (0.5% DMSO). On the day of treatment, 1 mL per well of XF Calibrant Solution (Agilent Technologies, Craven Arms, UK) was added into the 24-well SeaHorse Utility Plate topped with a SeaHorse Sensor Cartridge (Agilent Technologies, Craven Arms, UK) and dehydrated overnight at 37ºC with 0% CO_2 atmosphere. Following the incubation, Seahorse XF Assay Medium without glucose was augmented with 25 mM of glucose and sodium pyruvate (1 mM) and adjusted at pH 7.4. Next, cells were washed twice with 500 µL of the above prepared SeaHorse XF medium then incubated with 500 µL SeaHorse medium for 45 min at 37ºC without CO_2 to de-gas and to allow CO_2 diffusion from the cells and medium. The oxygen consumption rate of the cells was then monitored using a SeaHorse XF analyser (Agilent Technologies, Craven Arms, UK) after being calibrated with the sensor cartridge containing Mito stress drugs—oligomycin (1 µM) to inhibit ATP synthase; FCCP (1 µM), an uncoupling agent; and an antimycin/Rotenone mixture (0.5 µM)—to inhibit oxidative phosphorylation and electron transfer, respectively. Seahorse cell Mito stress parameters were calculated following manufacture's protocol as outlined below (Table 2.2) (Figure

2.1). following OCR measurement, cells were lysed using 1% Triton X-100 and protein content was measured using Bio-Rad protein assay kit for normalisation. The data are expressed as percentages from the control.

Table 2.2: Calculations of Seahorse cell Mito stress test parameters

Parameters	**Equations**
Non-mitochondrial respiration	Minimum measurement following Rotenone/Antimycin injection
Basal respiration	(Average of measurements before Oligomycin injection) _ (Non-mitochondrial respiration)
Maximal respiration	(Highest rate measurement after FCCP injection) _ (Non-mitochondrial respiration)
Proton (H^+) leak	(Lowest rate measurement after Oligomycin injection) _ (Non-mitochondrial respiration)
ATP production	(average rate measurement prior Oligomycin injection) _ (lowest rate measurement after Oligomycin injection)

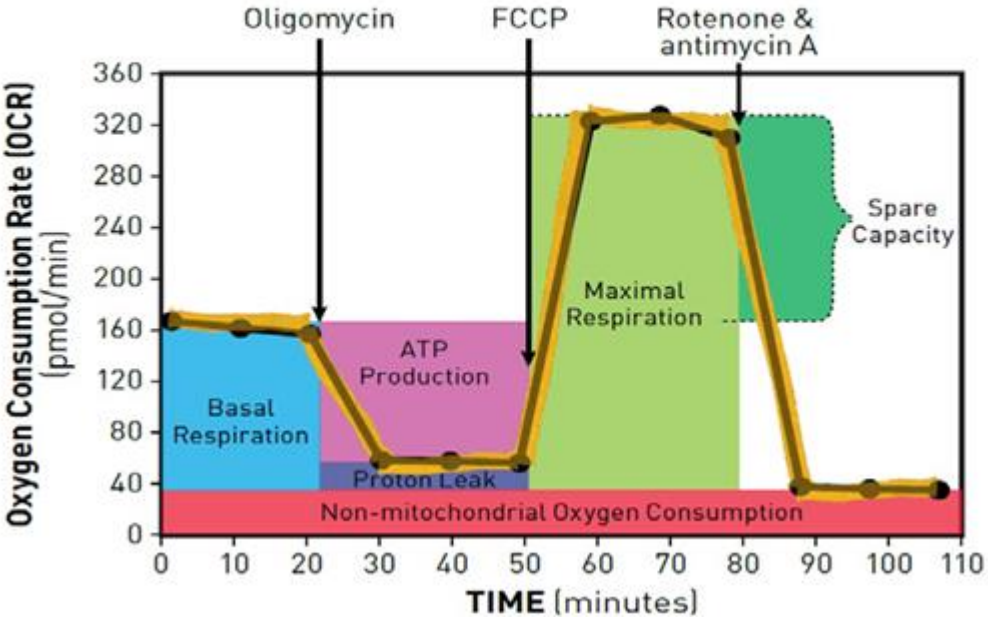

Figure 2.1: Seahorse Mito stress test parameters. Source: For more information on Agilent Seahorse XF cells Mito stress parameters and kinetics see http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/xf-cell-mito-stress-test-report-generator

2.2.7 Liver function test and TNF-α cytokine measurements in ALD patients

Liver function profile in alcoholic patients includes the following tests: Bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), Gamma Glutamyltransaminase (GGT), albumin and total protein. These were kindly measured in the serum of normal individuals (n= 15) and alcoholic patients (IRAS project ID 135979) (n= 63) by Dr. Vinood Patel using an Ilab Aries Clinical chemistry analyser.

TNFα inflammatory cytokine was measured using TNFα (human) EIA Kits following manufacture's protocol. Briefly, serum was incubated with acetylcholinesterase:Fab' conjugate for overnight at 4ºC in a 96-well plate coated with monoclonal antibody specific for TNFα to bind TNFα in the well. Next day, wells were washed five times with a washing buffer then incubated for 2 h in the dark with EIlman's reagent. Colour intensity was measured spectrophotometrically by FLUOstar OPTIMA (Jencons-PLS, Bedfordshire, UK) at 410 nm and cytokine concentration was expressed as pg/mL.

2.2.8 Statistical analysis

SPSS version 23.0 was used to perform one-way ANOVA to analyse the data, followed by post hoc multiple comparisons using Tukey's test. The data are reported as the mean ± standard error of mean (SEM), and the differences were considered significant if the p value ≤ 0.05.

Chapter 3

Results

3. Results

The first series of experiments involved determining the comparative effect of different types of fatty acids, saturated, unsaturated and mixture of fatty acids, as well as studying the concentration and period of treatment on cell viability and lipid accumulation.

3.1 Effect of saturated palmitic acid on cell viability and steatosis:

Palmitic acid is a C16 saturated fatty acid that has previously been reported to induce fatty liver and lipotoxicity (Wang et al., 2006; Oh *et al*., 2012). To confirm whether similar effects were observed in VA-13 cells and that this cell line could be used as a model of NAFLD, cell viability was assessed using the MTT assay after treating the cells with different palmitic acid concentrations (20, 80, and 160 µM) for 24, 48, and 72 h. The results showed that increasing the concentration and incubation time of palmitic acid (PA) treatment led to increasing cell death, especially at 80 and 160 µM, at these time points. The 160 µM treatment caused a significantly greater drop (-59% at 24 h and -53% at 48 h) in cell viability than the 80 µM treatment (-35% at 24 h and -34% at 48 h) ($P < 0.001$) when compared to control. There was further reduction in the cell viability with the 160 µM treatment at 72 h (-62%, $P < 0.001$) when compared to control at 72 h (Figure 3.1A-C).

With increasing concentration and incubation time PA treatment significantly led to an increase in lipid accumulation. After 24 h of 80 µM palmitic acid treatment, lipid accumulation was slightly elevated by 17%, whereas the increase at 160 µM (24%) was significantly higher ($P < 0.05$) than the control (Figure 3.1D). At 48 h,

lipid accumulation correlated closely to higher palmitic acid concentrations when compared to control, where 20 µM, (28%, $P < 0.05$;) 80 µM (39%, $P < 0.01$) and at 160 µM (96%, $P < 0.001$) (Figure 3.1E). After 72 h a similar pattern was observed, whereby increasing palmitic acid concentration was accompanied by an increase in lipid accumulation with a 16% increase at 20 µM ($P>0.05$), +45% at 80 µM and +55% at 160 µM when compared to the control ($P < 0.05$) (Figure 3.1F). However, among all time points, lipid accumulation was most apparent after 48 h than at other time points.

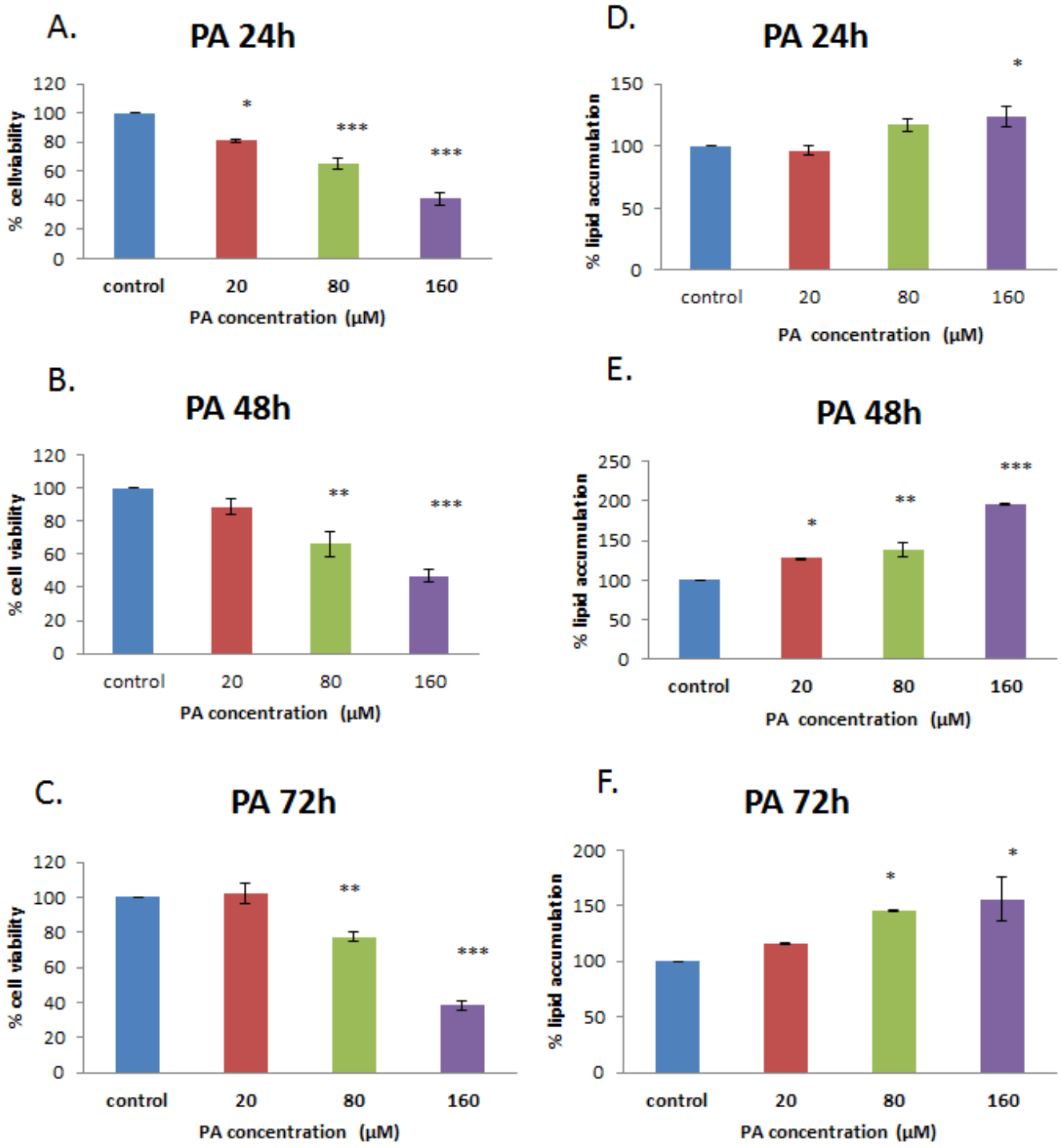

Figure 3.1: Effect of palmitic acid on the viability of VA-13 cells (A-C) and lipid accumulation (D-F) after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.05, **P < 0.01, and ***P < 0.001 as compared to the control**.** PA, Palmitic acid.

3.2 Effect of saturated stearic acid on cell viability and steatosis:

The viability of the cells was also assessed after treatment with three different concentrations of the saturated C:18 fatty acid stearic acid (20, 80, and 160 µM) for 24, 48, and 72 h. In contrast to PA, after 24 and 48 h of treatment, the 20 µM and 80 µM stearic acid treatment had no effect on cell viability ($P > 0.05$). However, the 160 µM treatment caused a large drop in cell viability (by -48% and -36% after 24 and 48 h, respectively) ($P < 0.01$) (Figure 3.2A, B). The effect at 72 h was considerably greater where cell viability gradually decreased at higher concentrations when compared to the control (-23% at 20 µM, -44% at 80 µM and -67% at 160 µM; $P < 0.001$) (Figure 3.2C).

Again, in contrast to PA treatment, neither increasing the exposure time nor raising the concentration of stearic acid caused any change in lipid accumulation ($P > 0.05$) (Figure 3.2D-F).

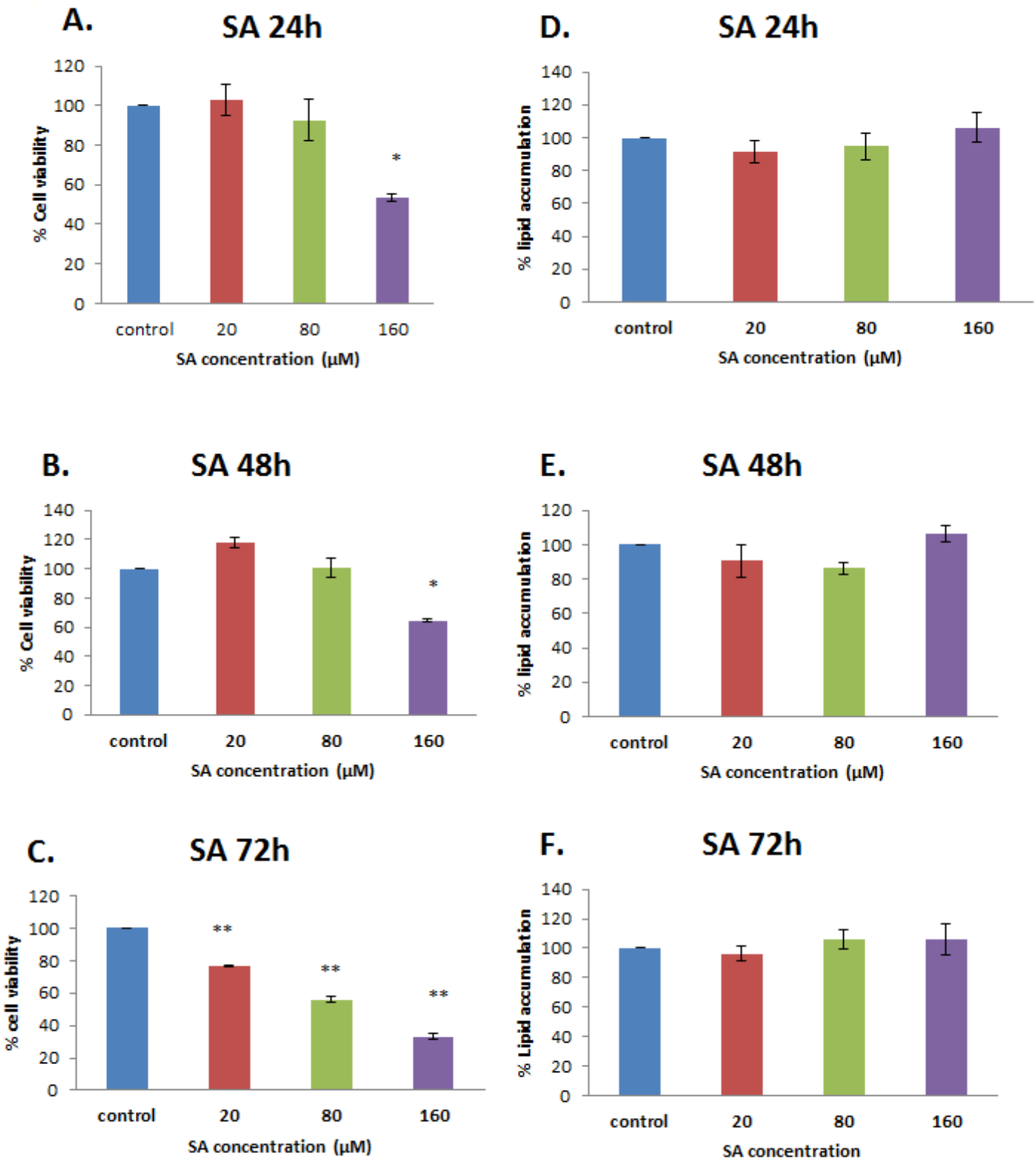

Figure 3.2: Effect of stearic acid on the viability of VA-13 cells & lipid accumulation after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.01 and **P < 0.001 as compared to the control. SA, Stearic acid.

3.3 Effect of unsaturated omega 6 linoleic acid on cell viability and steatosis:

As discussed in section 1.5, omega 6 fatty acids are thought to be detrimental to liver function leading to the development of NALFD. However, less information is known about the precursors to AA. Here, cell viability was analysed after treatment with various concentrations of omega 6 linoleic acid (LA) (10, 30, 50, 100, 200, and 300 µM) at 24, 48 and 72 h. At 24 h, compared to that in the control, cell viability at 200 µM dropped significantly (-37%; $P < 0.001$) and further decreased at 300 µM (-48%; $P < 0.001$) (Figure 3.3A). A lower decline was observed at 48 h with 300 µM (-22%; $P < 0.05$), while after 72 h, there was a significant drop in the 200 µM group (-30%) than in the 50 µM and 100 µM groups (-28%) ($P < 0.05$) (Figure 3.3B, C).

Treating the cells with omega 6 LA (10-300 µM) for 24, 48, and 72 h did not show any statistically significant increase in lipid accumulation. Although there was a slight increase in lipid accumulation after 48 and 72 h, this increase was not significant compared to the control ($P > 0.05$) (Figure 3.3D-F).

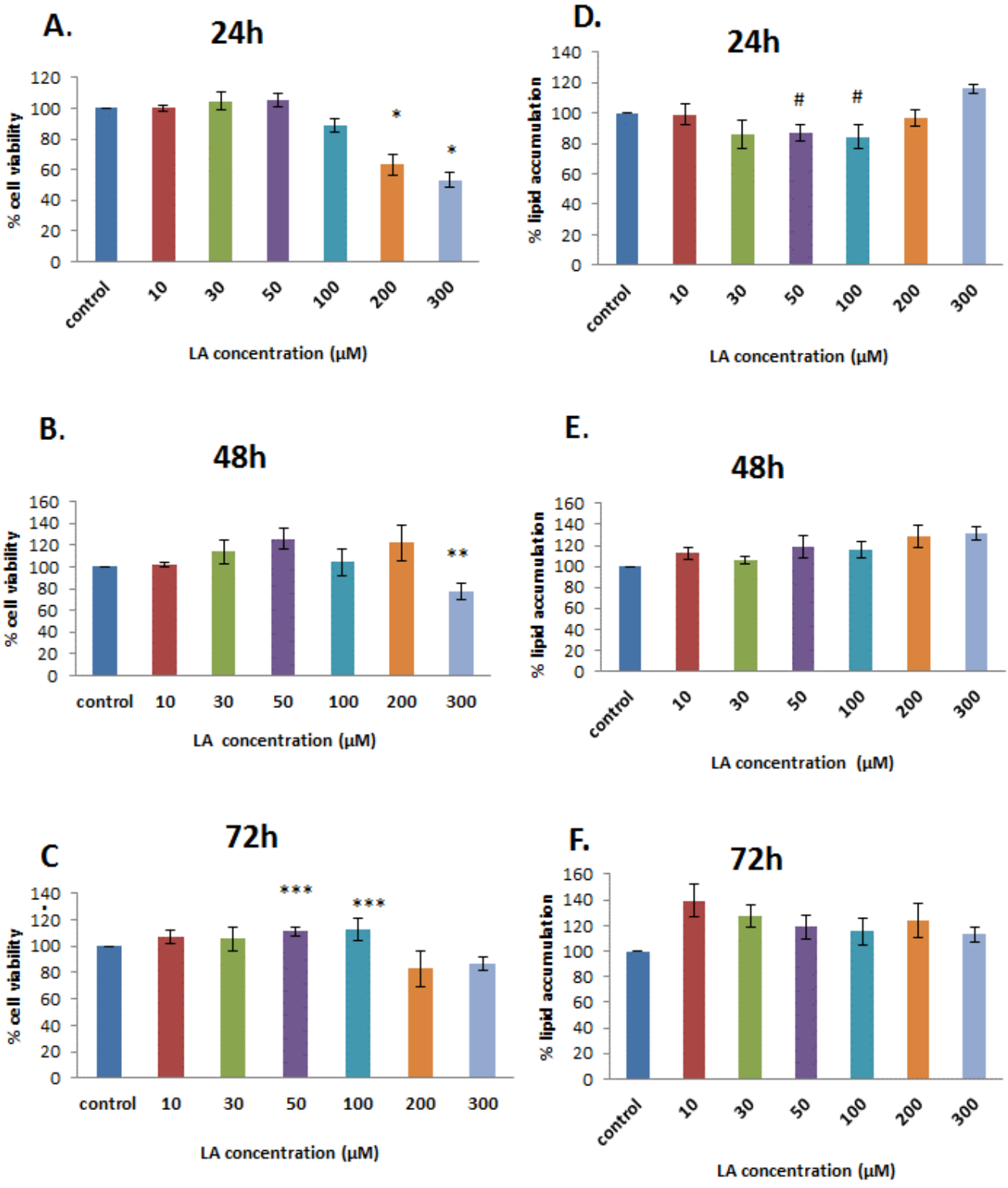

Figure 3.3: Effect of linoleic acid on the viability of VA-13 cells & lipid accumulation after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 4 determinations of 6 replicates per treatment). *P < 0.001 as compared to the control; **P < 0.05 as compared to the value at 50 µM; ***P < 0.05 as compared to the value at 200 µM; # P < 0.05 as compared to the value at 300 µM. LA, linoleic acid.

3.4 Effect of unsaturated omega 6 arachidonic acid on cell viability and steatosis:

Cell viability was analysed after treatment with various concentrations of omega 6 arachidonic acid (AA) (10, 20, 30, and 80 µM) for 24, 48, and 72 h. 80 µM treatment caused a significant decrease in cell viability after 24 and 48 h (-21%, $P < 0.05$ and -20% $P < 0.01$, respectively) (Figure 3.4A, B). However, at 72 h, there was a gradual, significant reduction at 20 µM (-18%; $P < 0.05$) followed by a significant gross reduction of 69% at 30 µM and 96% at 80 µM (Figure 3.4A-C).

No significant change was observed after 24 h of treatment in lipid accumulation. However, after 48 h, the 30 µM and 80 µM AA treatments caused a 37% and 40% ($P < 0.05$) increase in lipid accumulation, respectively. However, after 72 h, this effect was reversed, whereby at 10 µM and 20 µM lipid accumulation was reduced by -21% and -41%, respectively ($P < 0.01$), and similarly at 30 µM and 80 µM AA (-36% and -43%, respectively, $P < 0.01$). (Figure 3.4D-F).

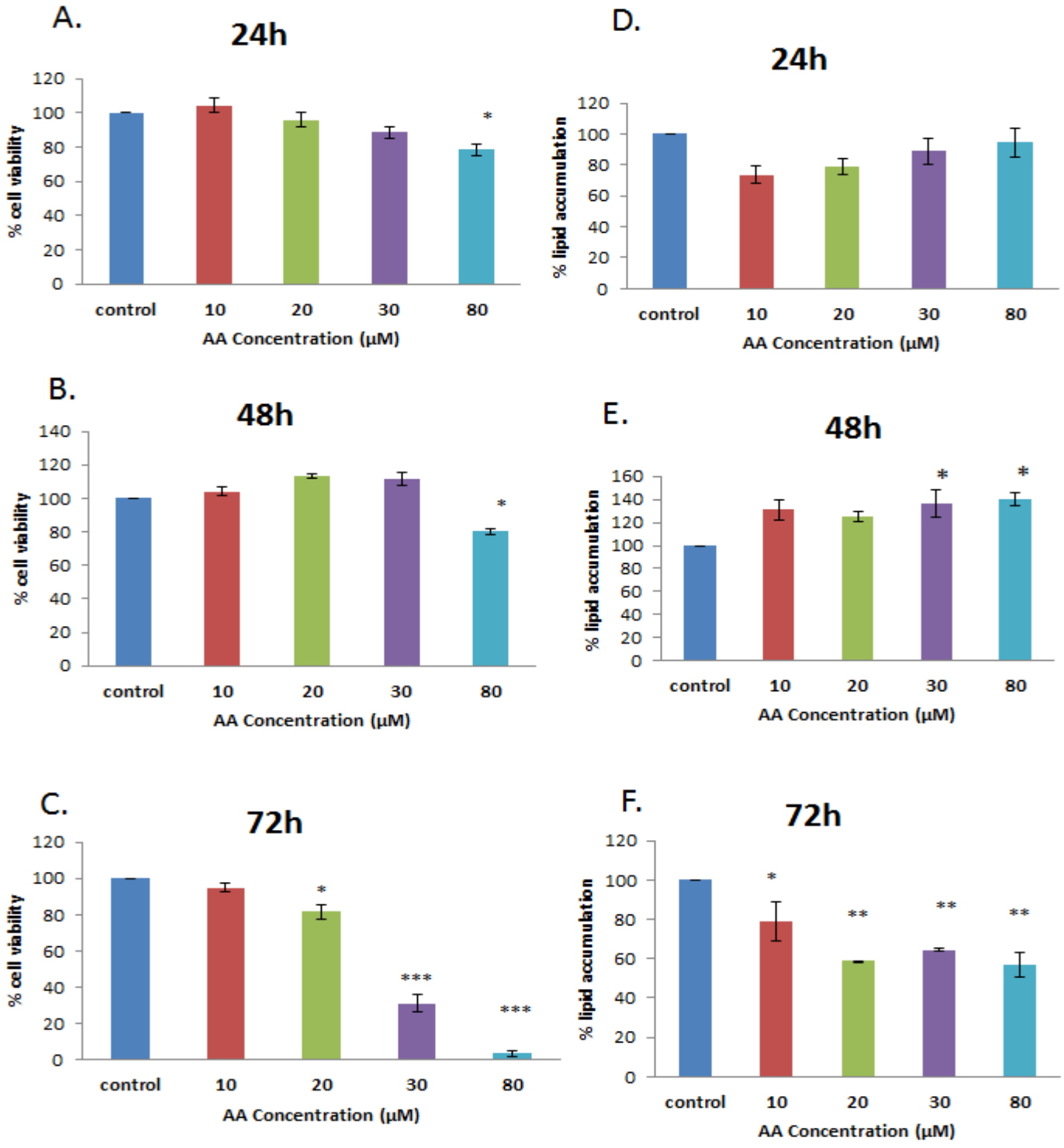

Figure 3.4: Effect of arachidonic acid on the viability of VL-17A cells & lipid accumulation after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.05, **P < 0.01, and ***P < 0.001 as compared to the control. AA, arachidonic acid.

3.5 Effect of unsaturated omega 3 α-linolenic acid and docosahexaenoic acid on cell viability and steatosis:

To ascertain the effects of omega 3 fatty acids, α-linolenic acid (ALA) (0.4, 0.66, 1, 2.5, 5, and 10 µM) and docosahexaenoic acid (DHA) (1.2, 2, 3, 7.5, 15, and 30 µM) were used separately at a range of concentrations to study cell viability and lipid accumulation at 24, 48, and 72 h (Figure 3.5). Cell viability was not affected by an increase in the ALA concentration at any time point. (Figure 3.5A-C). ALA treatments also did not show any marked change in lipid accumulation. Although there was a slight increase at 10 µM (21%), this change was not significant when compared to the control ($P > 0.05$) (Figure 3.5D-F).

In addition, neither increasing the incubation time nor raising the concentration of DHA had any significant effect on cell viability (Figure 3.6A-C) or lipid accumulation when compared to the control ($P > 0.05$) (Figure 3.6D-F).

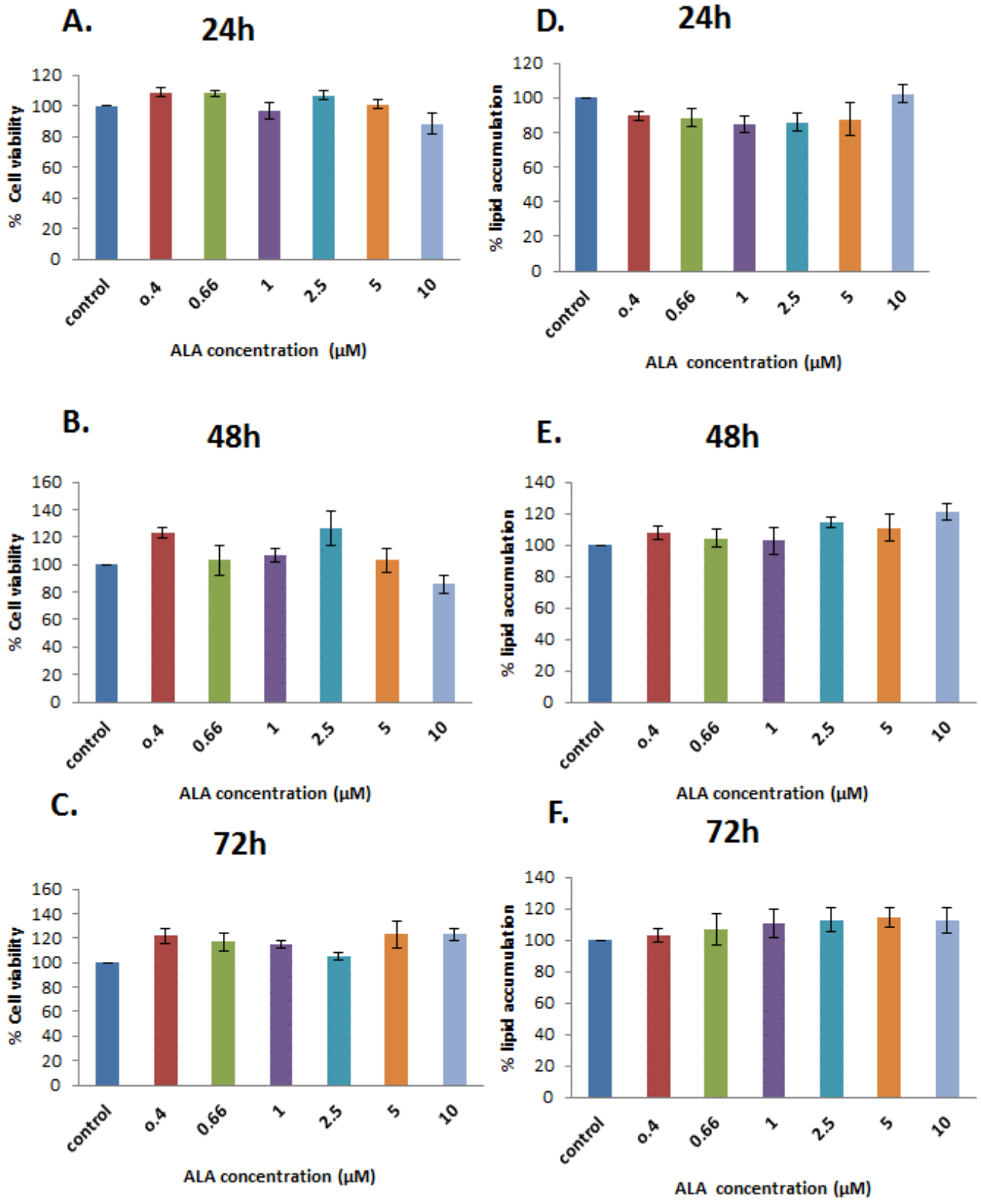

Figure 3.5: Effect of alpha linolenic acid on the viability of VA-13 cells & lipid accumulation after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). P > 0.05 as compared to the control. ALA, alpha linolenic acid.

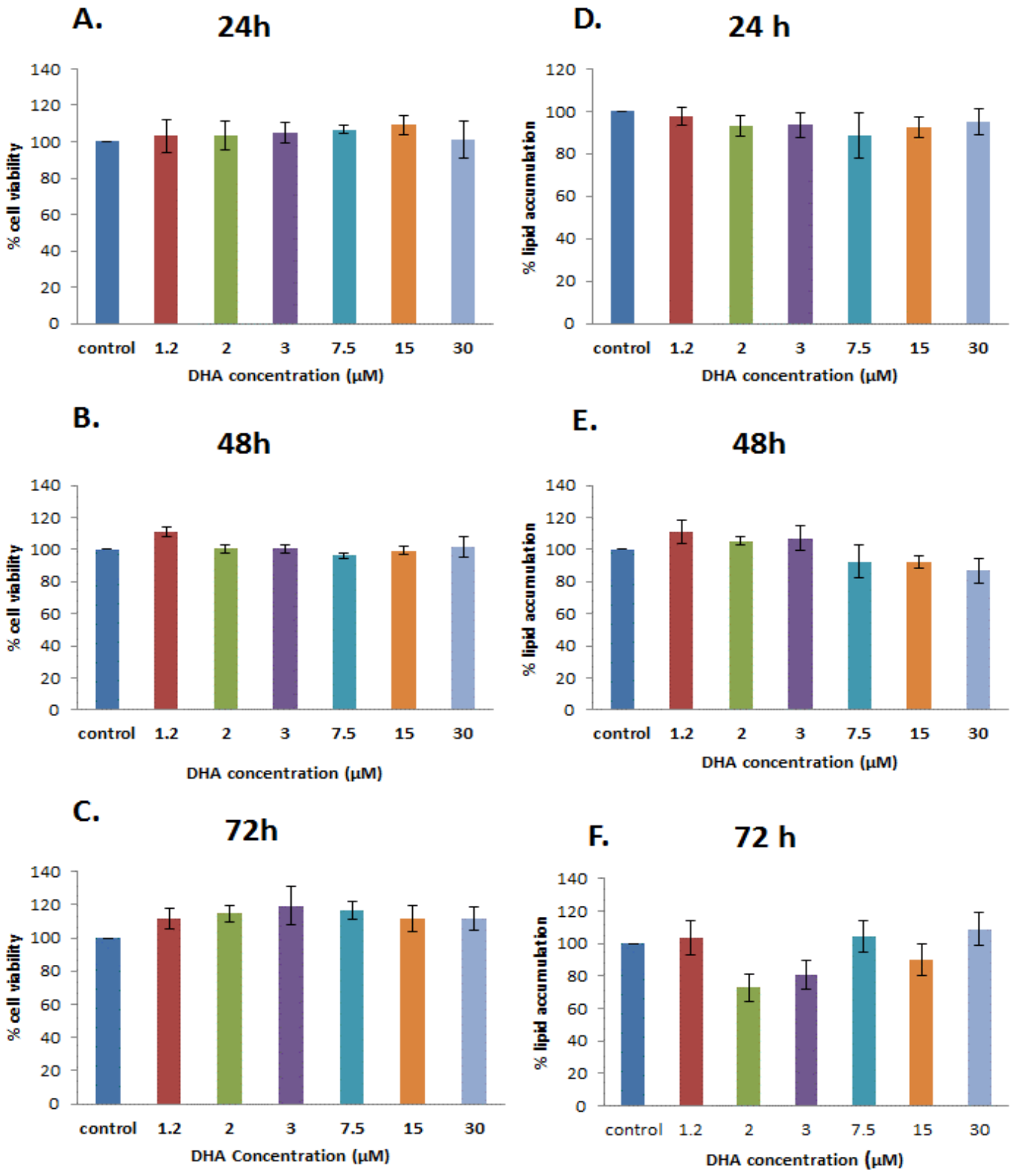

Figure 3.6: Effect of docosahexaenoic acid (DHA) on cell viability & lipid accumulation in VL-17A cells after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). P > 0.05 as compared to the control.

3.6 Effect of increasing omega LA/ALA (6/3) ratio on cell viability and lipid accumulation:

As previously discussed (1.5) increased omega 6/3 ratios are associated with the development of NAFLD. To study the effect of treating VA-13 cells with different LA/ALA ratios (range 1:1–25:1) on the cell viability, cells were incubated at various concentrations (1.2–30 μM) for 24, 48, and 72 h. Cell viability did not change significantly at any ratios after 24 h. However, after 48 h, cell viability dropped when the ratio increased to 15:1 (-25%) and 25:1 (-22%), while these reductions were not statistically significant compared to the control, they were lower when comparing these ratios to 1:1 (-42%, -39%, $P<0.05$, respectively) and when comparing the 15:1 ratio to 2:1 ratio (-39%, $P= 0.05$) (Figure 3.7A,B). After 72 h, increasing the LA/ALA ratio decreased the cell viability; 32% decrease was observed with 10:1 and 31% with 15:1 ($P = 0.08$ and $P = 0.05$, respectively) (Figure 3.7C).

To evaluate the effect of an increase in the LA/ALA ratio on lipid accumulation, cells were treated (as before) with six different ratios, ranging from 1:1 to 25:1 for 24, 48, and 72 h. Steatosis was not observed after 24 h; however, there was a non-significant reduction in lipid accumulation at 1:1 (-20%), 2:1 (-23%), 4:1 (-24%), and 15:1 (-23%); however, higher high ratios (10:1 and 25:1) did not cause any change. Increasing the incubation period to 48 h increased the lipid accumulation at 15:1 and 25:1 by 13% and 16%, respectively, compared to the control ($P>0.05$). The 72 h exposure treatments also failed to increase lipid accumulation (Figure 3.7 D-F).

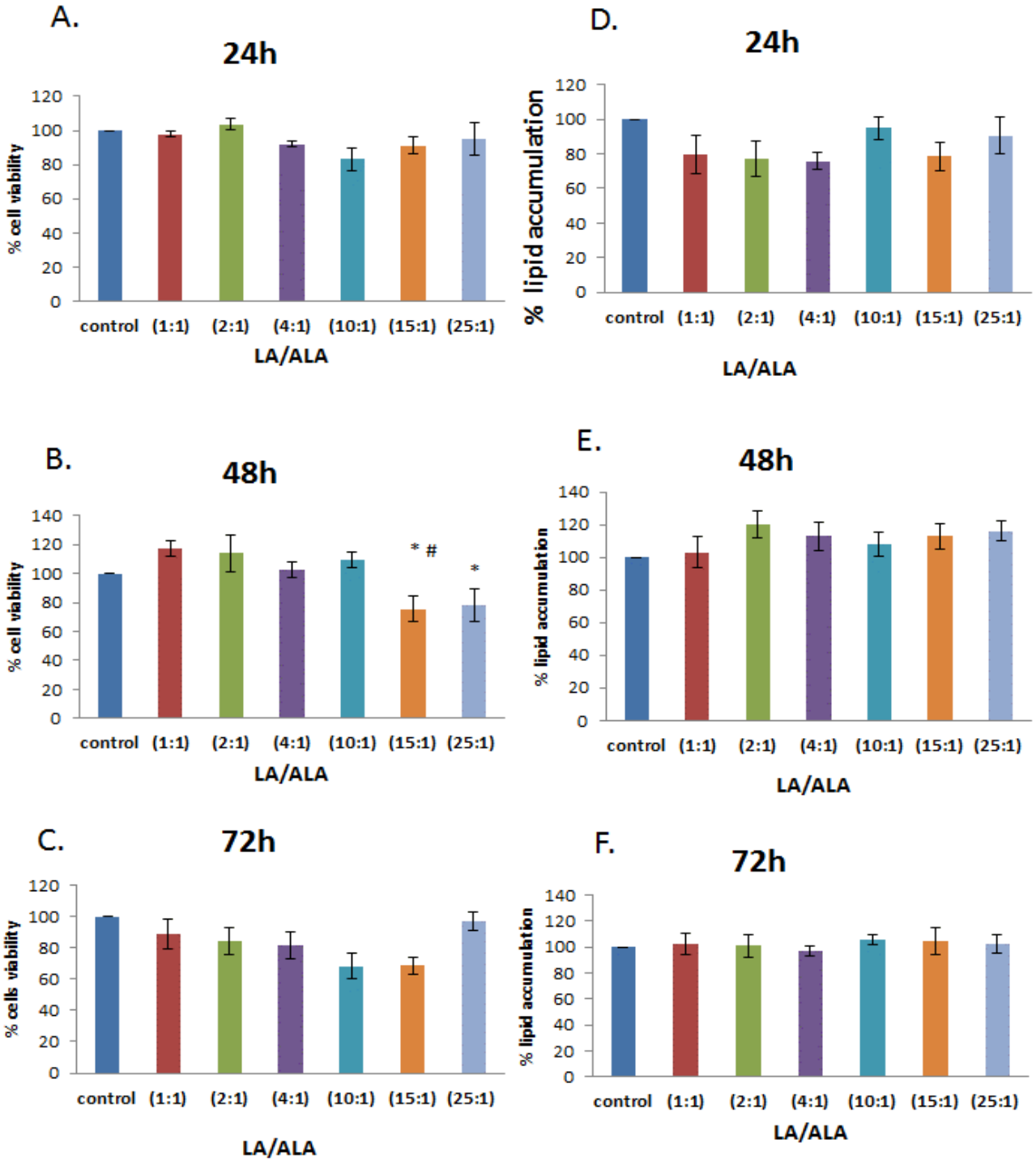

Figure 3.7: Effect of treatment with different concentrations of the LA/ALA ratio on cell viability & lipid accumulation in VA-13 cells after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3-5 determinations of 6 replicates per treatment) *P < 0.05 as compared to the value with the 1:1 ratio; #P < 0.05 as compared to the value with the 2:1 ratio. LA, Linoleic acid. ALA, alpha linolenic acid.

3.7 Effect of increasing the omega 6/3 (AA/DHA) ratio on cell viability and steatosis:

Cells were treated with different ratios of both, AA and DHA (range: 1:1–25:1) for 24, 48, and 72 h. Data showed that increasing the ratio of AA/DHA (omega 6/3), had a significant negative effect on cell viability. High ratios (15:1 and 25:1) of AA/DHA significantly reduced cell viability. After 24 h, a ratio of 15:1 caused a 17% reduction in cell viability ($P < 0.05$), which increased considerably at 25:1 where the reduction was 82% ($P < 0.001$) when compared to the control. Interestingly, when compared to 1:1 and 4:1 the viability was still low at 15:1 (-23%, $P < 0.01$ and -21%, $P < 0.05$, respectively) and at 25:1 (-88% and 86%, respectively, $P < 0.001$).

At 48 h, only the 25:1 ratio caused a significant reduction in cell viability (-51%, -51%, -57%; $P < 0.001$) as compared to the control, 1:1, and 4:1, respectively (Figure 3.8A-C). At 72 h a marked reduction in viability occurred with 15:1 and 25:1 ratio treatment (-91%, $P < 0.001$ and 89%, $P < 0.001$, respectively) as compared to the control. Furthermore, cell viability considerably dropped at 15:1 and 25:1 (-104% and -102%, $P < 0.001$, respectively) when compared to that at 1:1, with similar effects when 15:1 and 25:1 ratio was compared to 4:1 (-87%, -82%, $P < 0.001$, respectively).

Increasing the ratio of AA/DHA (omega 6/3) slightly increased lipid accumulation at 24 h with 15:1 and 25:1 ratios, with no effect at 48 h (Figure 3.8D-E). In contrast, lipid accumulation decreased significantly at all ratios at 72 h, with greatest reductions -32% at 15:1 ($P < 0.01$) and -53% at 25:1 ($P < 0.001$) (Figure 3.8F).

To obtain a second method for determining steatosis, the TG concentration was quantified per mg protein of cell lysate. This more accurately compensates for any changes in the cell density which may be affected by the treatment conditions and/or rapid cell death.

TG level was measured after treating VL-17A cells with different omega 6/3 ratio (AA/DHA) for 24, 48 and 72h. Data showed a slight decrease (-12%) in lipid level after 24h with 15:1 ratio while there was a significant rise (42%; $P< 0.01$) with 25:1 compared to control. On the other hand, 1:1 ratio indicated a significant reduction (-43%, $P< 0.01$) in the level of lipid but 4:1 did not show any change (1%, $P> 0.05$) after 24h. Comparing all ratios to 1:1, there was a pronounced increase in the level of TG with 4:1 ratio (75%, $P< 0.01$) where this increase rocketed to 248% ($P< 0.001$) at 25:1 ratio. This effect decreased although the level of lipid was still high (53%, $P> 0.05$) at 15:1 compared to 1:1 ratio (Figure 3.9A).

After 48h, results indicated a gradual increase in the concentration of TG with a significant effect at 4:1 and 15:1, (117%, $P< 0.05$ and 177%, $P< 0.01$, respectively) whereas 25:1 ratio showed less significant increase in lipid level (91%, $P< 0.05$) (Figure 3.9B). When compared to 1:1 ratio, there was also a significant elevation in lipid concentration at 4:1 and 15:1 only (91% and 143%, $P< 0.01$, respectively). On the other hand, 25:1 ratio showed a significant increase (68%, $P< 0.05$) compared to 1:1 ratio and showed a rapid decline in TG concentration compared to 15:1 (-31%, $P= 0.05$) (Figure 3.9B). After 72h, lipid concentration decreased most with 15:1 (-31%, $P> 0.05$) ratio when compared to

the control, while all other ratios showed a slight decrease ($P > 0.05$) (Figure 3.9C).

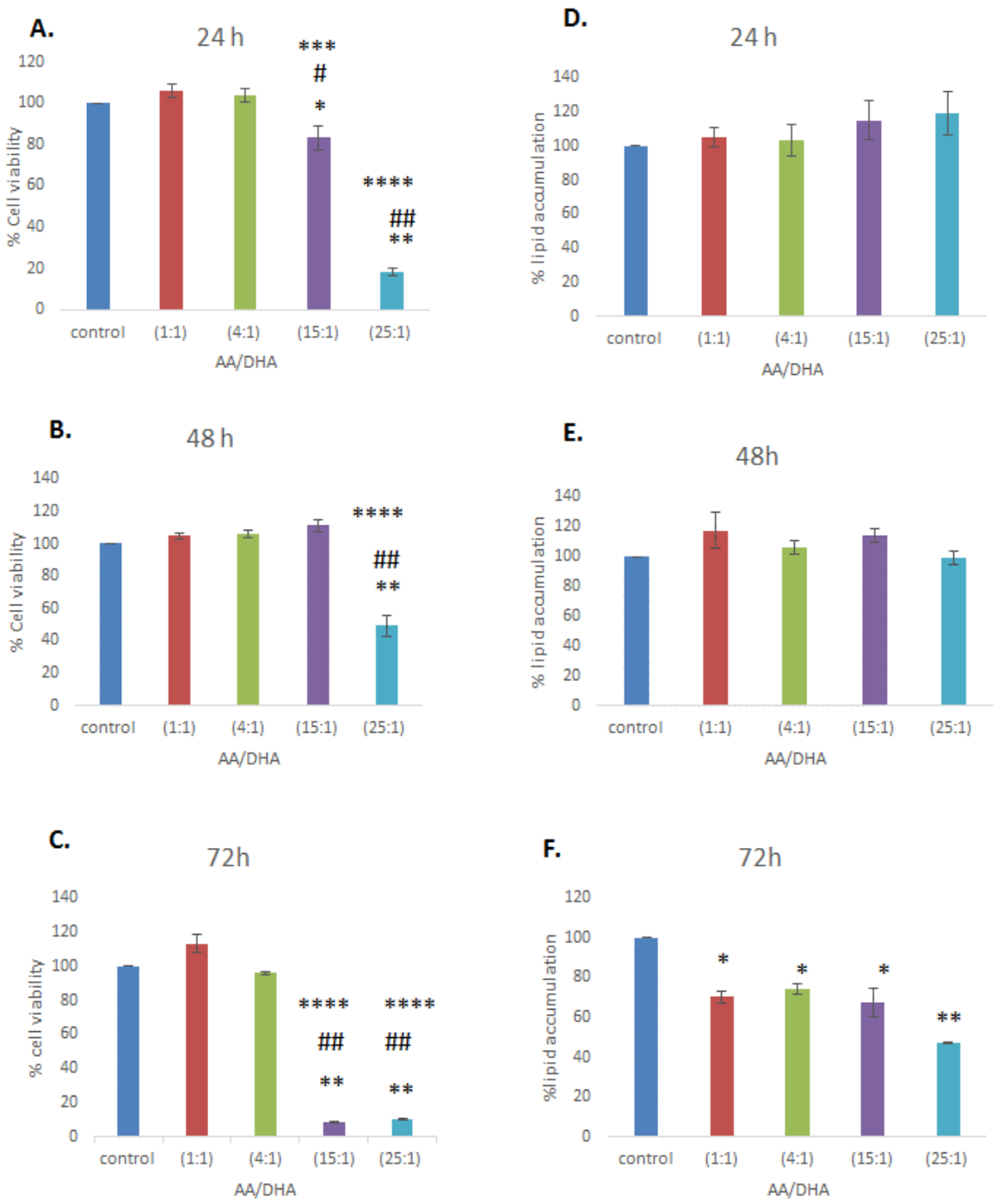

Figure 3.8: Effect of treatment with different concentrations of AA/DHA ratios on cell viability & lipid accumulation in VL-17A cells after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.01; **P < 0.001 as compared to the control; $^{\#}$P < 0.01 and $^{\#\#}$P < 0.001 as compared to the value with the 1:1 ratio; ***P < 0.01 and ****P < 0.001 as compared to the value with the 4:1 ratio. AA, arachidonic acid. DHA, docosahexaenoic acid.

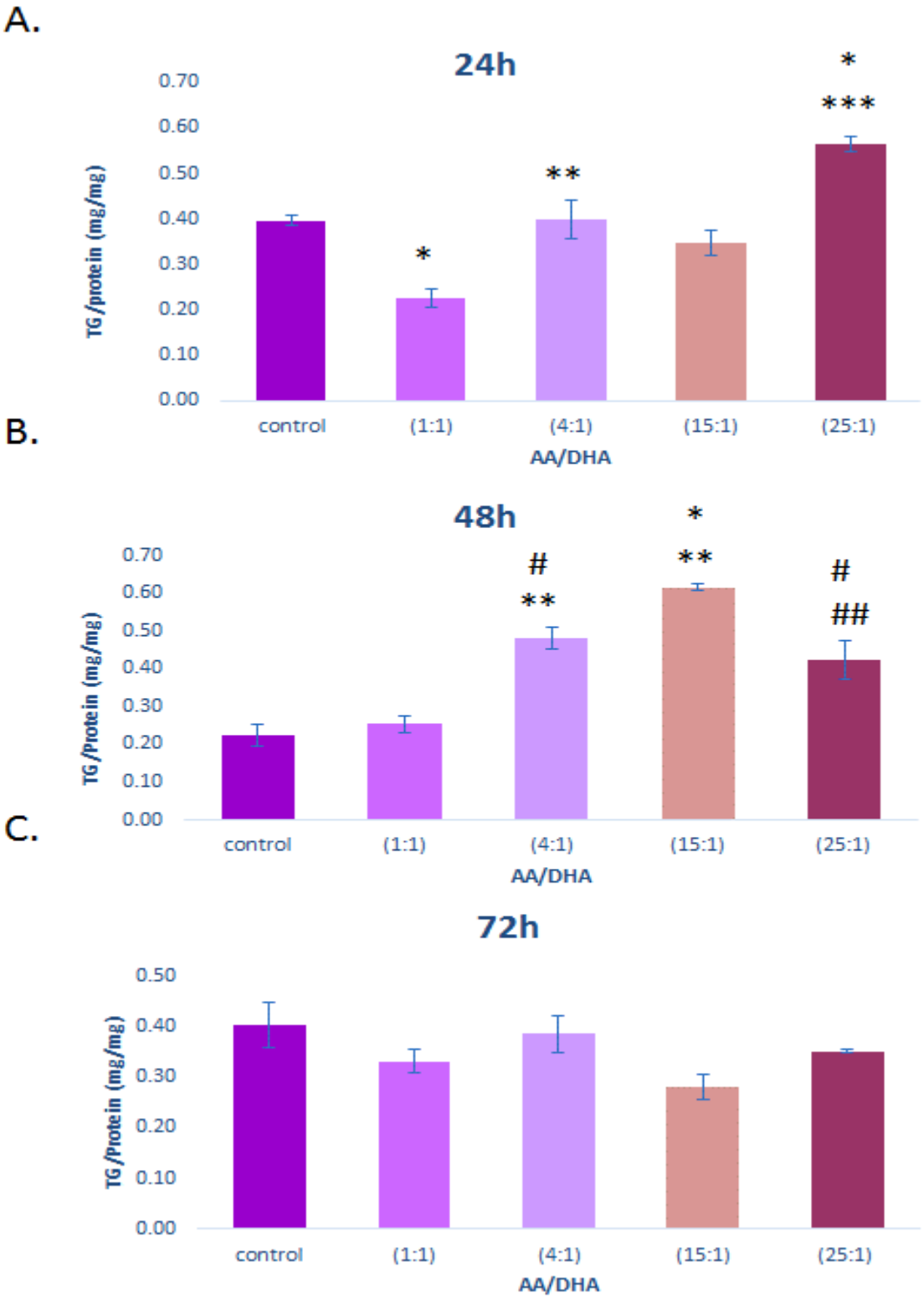

Figure 3.9: Effect of treatment with different concentrations of AA/DHA ratio on steatosis in VL-17A cells after 24, 48, and 72 h. The results are presented as mean ± SEM (n = 5 determinations of 3 replicates per treatment), and the levels of the intracellular TG are expressed as mg/mg protein. *P < 0.01 and #P<0.05 as compared to the control; ##P<0.05, **P < 0.01 and ***P < 0.001 as compared to the value with the 1:1 ratio. TG, triglyceride. AA, arachidonic acid. DHA, docosahexaenoic acid

3.8 Effect of increasing the omega 6/3 (AA/DHA) ratio on the expression of PPAR-α, SREBP1, and SCD1:

The expression of key transcriptional genes involved in FA metabolism was determined using Western blotting after treating VL-17A cells with different omega AA/DHA ratios ranging from 1:1 to 25:1 for 24 h. The expression of PPAR-α significantly decreased at 15:1 and 25:1 (-29% and -35%, respectively; $P < 0.05$) in the treated VL-17A cells compared to that in the control. The AA/DHA ratios caused greater reduction in the PPAR-α expression at 15:1 and 25:1 (-35%; $P < 0.05$ and -40%; $P < 0.01$) when compared to that at the 1:1 ratio (Figure 3.10A-B).

Treating the cells with increasing ratios of AA/DHA for 24 h caused a slight decrease in the SREBP1 expression at 4:1 and 15:1 (-29% and -16%, respectively; $P > 0.05$) when compared to the control. On the other hand, the SREBP1 expression was significantly decreased only at 4:1 as compared to that at 1:1 (-35%, $P < 0.05$). In contrast, at 15:1 and 25:1 ratios, there was non-significant increase in the expression of this gene when compared to that at 4:1 (13% and 21%, respectively; $P > 0.05$) (Figure 3.11A). SCD1 expression was assessed using the VL-17A cells treated with various AA/DHA ratios. The SCD1 expression gradually increased with an increase in the AA/DHA ratio. Interestingly, this elevation was significant at 25:1 as compared to that in the control, and at the 1:1 and 4:1 ratios (33%, 38%, and 34%; respectively; $P < 0.05$). However, the increase in SCD1 expression at the 15:1 ratio was not statistically significant as compared to that in the control, and that at the 1:1 and 4:1 ratios (22%, 27%, and 23%, respectively; $P > 0.05$) (Figure 3.11B).

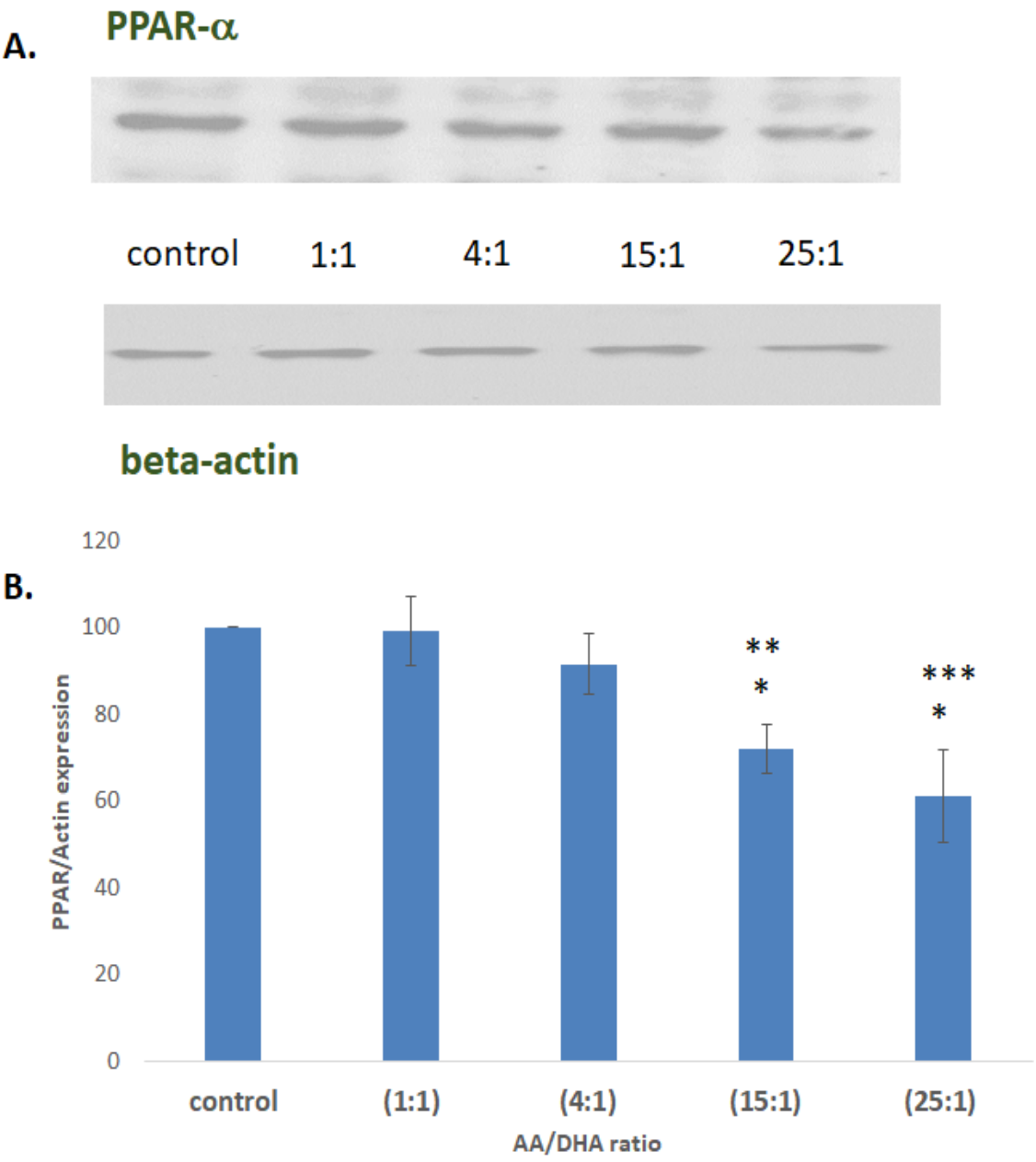

Figure 3.10: Effect of increasing AA/DHA ratios on the expression of PPAR alpha in VL-17A cells after 24 h. A. Immunoblot; B. PPAR/Actin expression. The results are presented as mean ± SEM (n = 3). *P < 0.05 as compared to B-actin (as a control); **P < 0.05 and *** P < 0.01 as compared to the value with the 1:1 ratio. AA, arachidonic acid. DHA, docosahexaenoic acid.

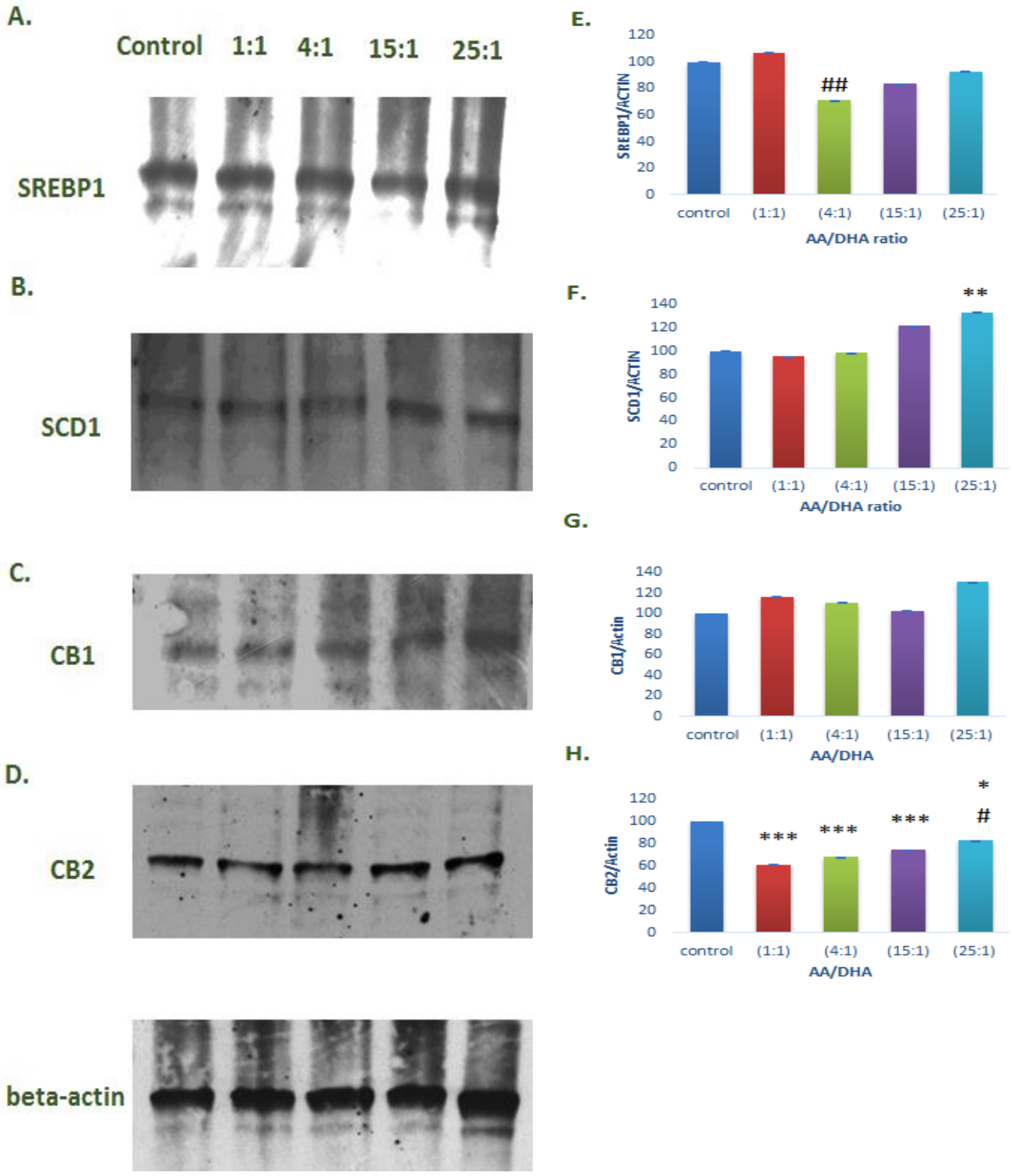

Figure 3.11: Effect of AA/DHA ratios (1:1, 4:1, 15:1, and 25:1) on lipogenic markers (SREBP1 & SCD1) and cannabinoids receptors (CB1 & CB2) in VL-17A cells after 24 hr. A-D. Representative Western blots. E-H. Expression of proteins relative to beta actin. The results are presented as mean ± SEM (n = 3). *P < 0.05 as compared to the control and 4:1 ratio; **P < 0.05 as compared to the control, 1:1, and 4:1 ratio. ***P < 0.001 as compared to the control; $^{\#}$P < 0.01, $^{\#\#}$P < 0.05 as compared to the value with 1:1 ratio. AA, arachidonic acid. DHA, docosahexaenoic acid.

3.9 Effect of increasing the omega AA/DHA ratio on the expression of CB1 and CB2 receptors:

The expression of cannabinoids receptors, CB1 and CB2 were evaluated after treating the VL-17A cells with increasing omega AA/DHA ratios (range 1:1–25:1) for 24 h. There was an increase in the CB1 expression with all ratios except 4:1 when compared to that in the control; however, this effect was not statically significant (17% at 1:1, 11% at 15:1, and 31% at 25:1; $P > 0.05$) (Figure 3.11C).

A low expression of CB2 was observed with all ratios in general, and particularly at the 1:1 ratio (-39%, $P < 0.001$), with significant decreased CB2 expression at 4:1 (-32%, P<0.001), 15:1 (-26%, $P < 0.001$) and 25:1 (-17%, $P < 0.05$) compared to control. However, compared to 1:1 ratio, the CB2 expression increased slightly at 4:1, a 13%, (non-significant) increase in the CB2 expression at 15:1, and interestingly was increased significantly by 22% ($P < 0.01$) at 25:1 (Figure 3.11D).

3.10 Effect of increasing the omega AA/DHA ratio on ROS production:

Increased oxidative stress is a known feature in NAFLD (Seth et al., 2011). However, no information is known on how high AA/DHA ratios affect ROS. ROS production was evaluated in the VL-17A cells treated with different omega AA/DHA ratios (range 1:1–25:1) for 30 min, 1 h, and 2 h. After 30 min, all ratios caused a significant elevation in the ROS levels (Figure 3.12). The 1:1 and 15:1 ratios increased the ROS levels by 74% ($P < 0.05$), while the 4:1 and 25:1 ratios led to an 88% and 104% increase, respectively ($P < 0.01$) (Figure 3.12A). A 1 h incubation did not show any significant change in the ROS levels at 1:1 or 4:1 ratios, compared to the control. The 4:1 ratio caused a slight increase in the ROS

levels (23%, P>0.05). The higher omega ratios, 15:1 and 25:1, caused a marked increase in the ROS levels. With the 15:1 ratio, ROS levels increased by 117%, 120%, and 93% compared to the control, 1:1 and 4:1, respectively ($P < 0.001$). Whereas, the 25:1 ratio caused 96% and 99% ($P < 0.001$) increase in the ROS levels compared to that in the control and at 1:1, respectively, and 72% ($P < 0.01$) compared to that at 4:1 (Figure 3.12B).

ROS levels were generally lower at the 2 h time point compared to 30 min and 1 h. However, ROS production rose significantly by 32% ($P < 0.05$) and 48% ($P < 0.01$) at the 15:1 ratio compared to that with the control and 4:1 ratio, respectively. ROS levels were also moderately increased by 25% at the 25:1 ratio, when compared to the 4:1 ratio ($P < 0.05$) (Figure 3.13).

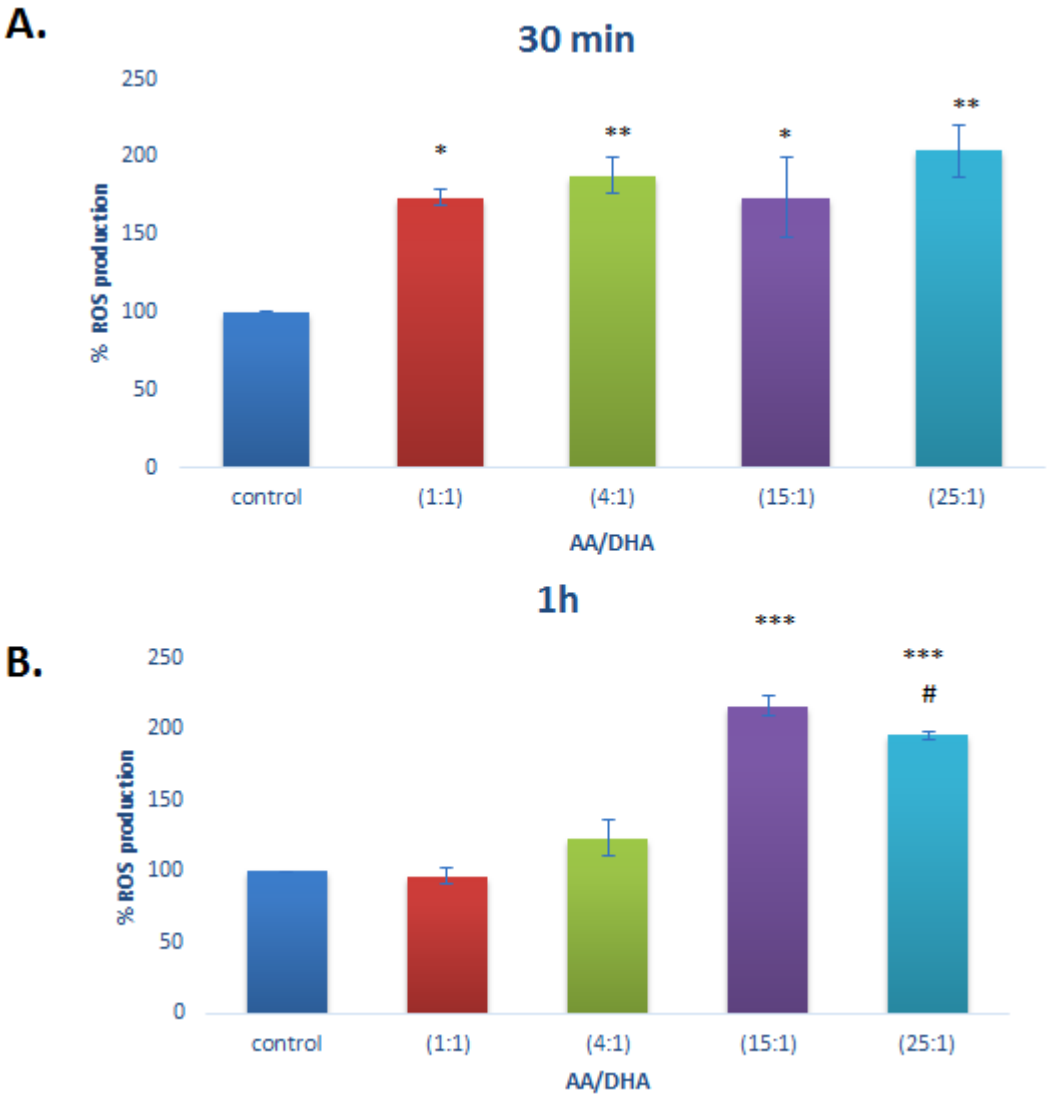

Figure 3.12: Effect of increasing AA/DHA ratios on reactive oxygen species (ROS) production in VL-17A cells at 30 min and 1 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.05 and **P < 0.01 as compared to the control; *** P < 0.001 as compared to the control and the 1:1 and 4:1 ratios; $^{\#}$P < 0.01 as compared to the value with the 4:1 ratio. AA, arachidonic acid. DHA, docosahexaenoic acid.

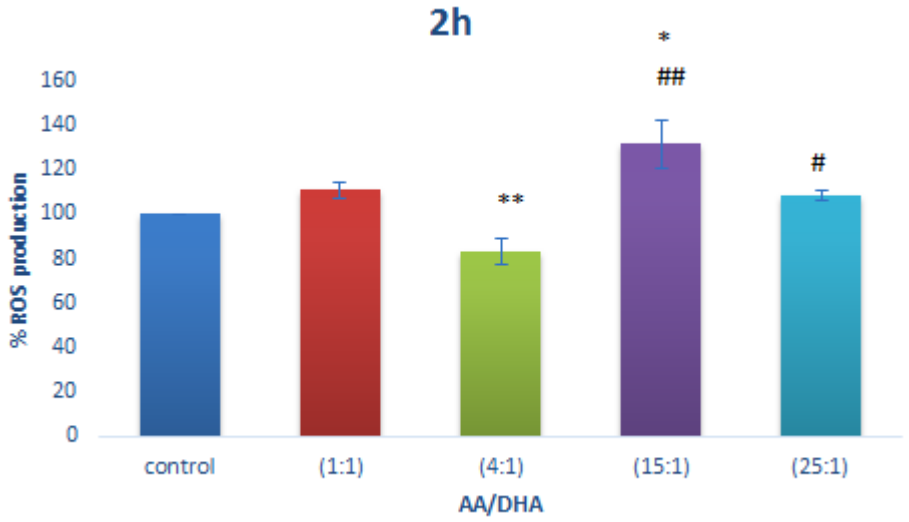

Figure 3.13: Effect of increasing AA/DHA ratios on reactive oxygen species (ROS) production in VL-17A cells at 2 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.05 as compared to the control; **P < 0.05 as compared to the value with 1:1; $^{\#\#}$P < 0.01 as compared to the 4:1 ratio; $^{\#}$P < 0.05 as compared to the value with the 4:1 ratio. AA, arachidonic acid. DHA, docosahexaenoic acid.

3.11 Effect of increasing the AA/DHA ratio on oxygen consumption rate:

Various studies have implicated perturbed mitochondrial function in the pathogenesis of NAFLD (Gentile and Pagliassotti., 2008; Dowman et al., 2010), which also can lead onto the more severe stages in disease development. Here, the respiratory function of the mitochondria was evaluated by measuring the oxygen consumption rate (OCR) using the Seahorse XF24 analyser after treating the VL-17A cells with various omega AA/DHA ratios, ranging from 1:1 to 25:1 for 24 h. The results showed that the function of the ETC was affected by an increase in the omega AA/DHA ratio (Figure 3.14B). Our data showed a gradual, remarkable decrease in basal respiration with an increase in the AA/DHA ratio. Thus, basal respiration decreased by 20% ($P < 0.05$) and 25% ($P < 0.01$) at 1:1 and 4:1, respectively, and by 38% and 40% at 15:1 and 25:1, respectively, as

compared to the control (P < 0.001) (Figure 3.15A). Results also indicated significant reductions in the basal respiration at 15:1 and 25:1 as compared to 1:1 (19% and 21%, respectively, P< 0.05) (Figure 3.15A).

ATP production was also significantly affected and reduced by an increase in the ratio of AA/DHA and decreased by 25% and 33% (P < 0.01) at 1:1 and 4:1, respectively. Furthermore, ATP production was reduced further at 15:1 and 25:1 ratios (-44% and -41%, respectively, P < 0.001) (Figure 3.15B).

The proton leak reduced significantly (P < 0.001) at only high AA/DHA ratios (15:1 [41%] and 25:1 [50%]) as compared to the control; however, only the 25:1 ratio caused a significant decrease in proton leak (31%) (P < 0.01) as compared to the 1:1 ratio (Figure 3.15C). Maximal respiration as well as spare respiratory capacity showed a gradual and remarkable reduction with an increase in the AA/DHA ratio. Thus, maximal respiration reduced gradually by 35% (P < 0.01), 44%, 54%, and 56% (P < 0.001) (Figure 3.15D) for 1:1, 4:1, 15:1 and 25:1, respectively. A similar pattern occurred with the spare respiratory capacity which was significantly reduced by 52%, 56%, 67%, and 68% (P < 0.001) with increasing AA/DHA ratios compared to the control (Figure 3.15E).

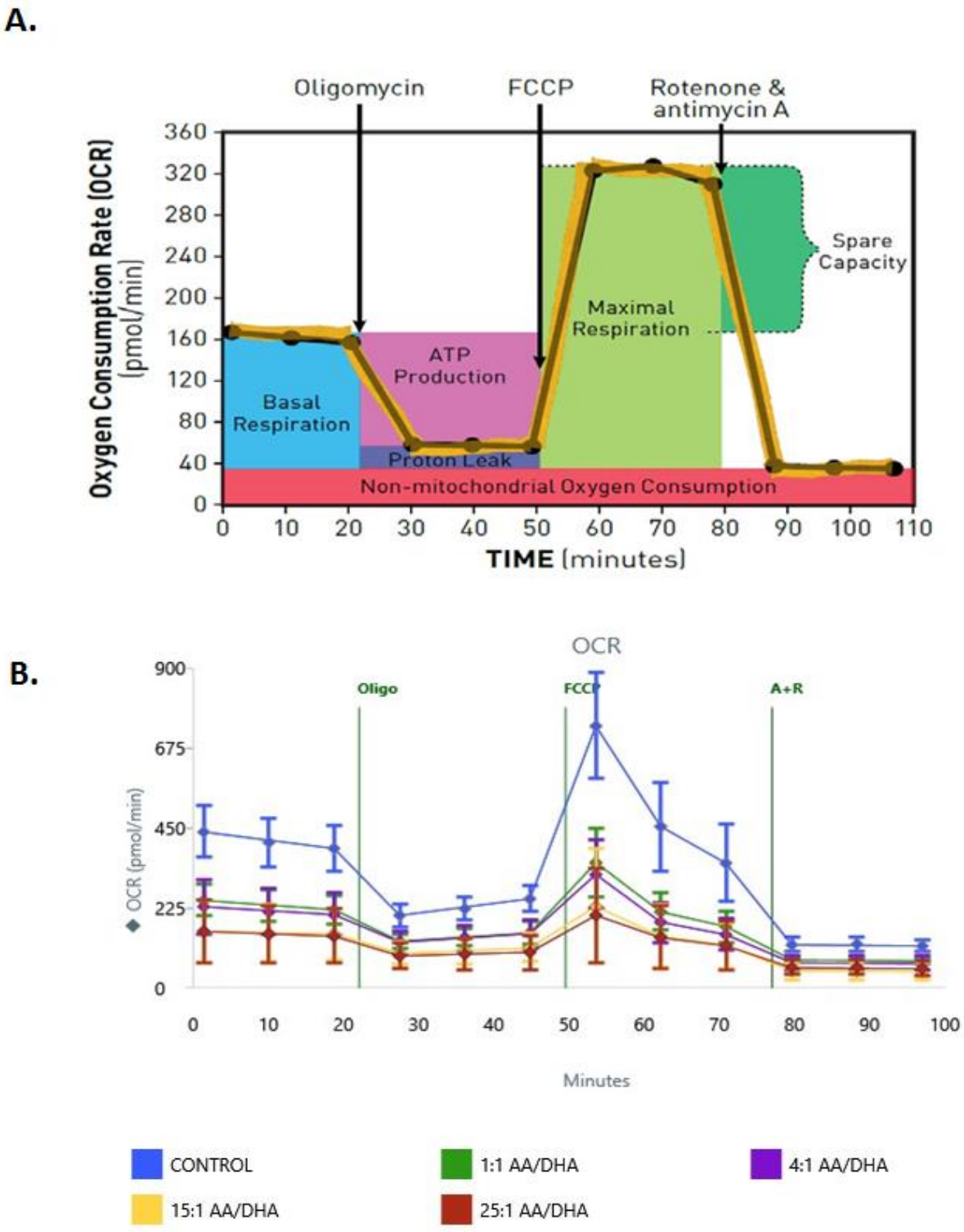

Figure 3.14: Effect of high AA/DHA ratio on the Seahorse Mito stress test parameters in VL-17A cells. **A. Representative result. B. Model.** AA, arachidonic acid; DHA docosahexaenoic acid.

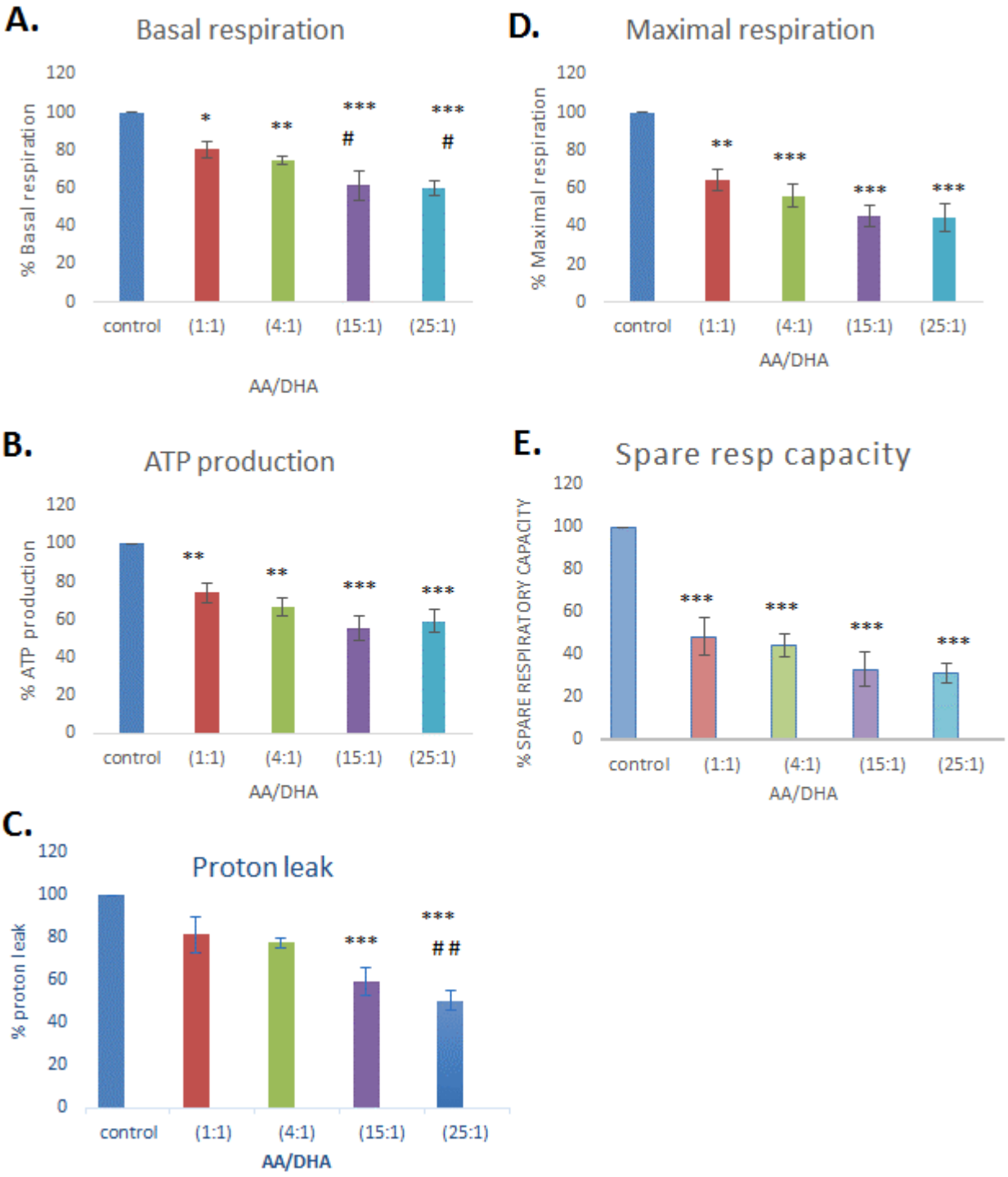

Figure 3.15: **Effect increasing AA/DHA ratios on oxygen utilization in VL-17A cells treated for 24 h**. The results are presented as mean ± SEM (n = 4-5 determinations, of 4 replicates per treatment). *P < 0.05, **P < 0.01, and ***P < 0.001 as com-pared to the control; $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01 as compared to the value with the 1:1 ratio. AA, arachidonic acid. DHA, docosahexaenoic acid.

3.12. Effect of alcohol and arachidonic acid on cell viability and steatosis:

Only a few studies have examined the interaction between alcohol and fatty acids (Gyamfi et al., 2012). It is possible additional factors such as alcohol increases the risk for disease development in NAFLD. There is no data on this interaction with omega 6/3 ratios. VL-17A cells were treated separately with alcohol (100 mM), AA (40 µM) and a mixture alcohol (100 mM plus AA 40 µM) for 6, 24, 48, and 72 h respectively, to investigate the effect on cell viability and lipid accumulation. Data showed that the cell viability remained unchanged after 6 h; however, it reduced significantly after 48 and 72 h with individual AA treatment (-57% and -65%; $P < 0.001$, respectively) and alcohol/AA mixture (-63% and -64%; $P < 0.001$, respectively). In contrast, at 24 h, there was a slight non-significant decrease in the cell viability with alcohol/AA mixture (-14%, $P = 0.06$) (Figure 3.16A-D). On the other hand, there was no increase in lipid accumulation at any time point. However, lipid accumulation decreased significantly at 48 and 72 h with individual AA (-18%; $P < 0.001$ and -54%; $P < 0.01$, respectively) and alcohol (-18% and -64%; $P < 0.001$, respectively) treatment as well as with the alcohol/AA mixture (-10%; $P < 0.01$ and -27%; $P < 0.05$, respectively) (Figure 3.16E-H).

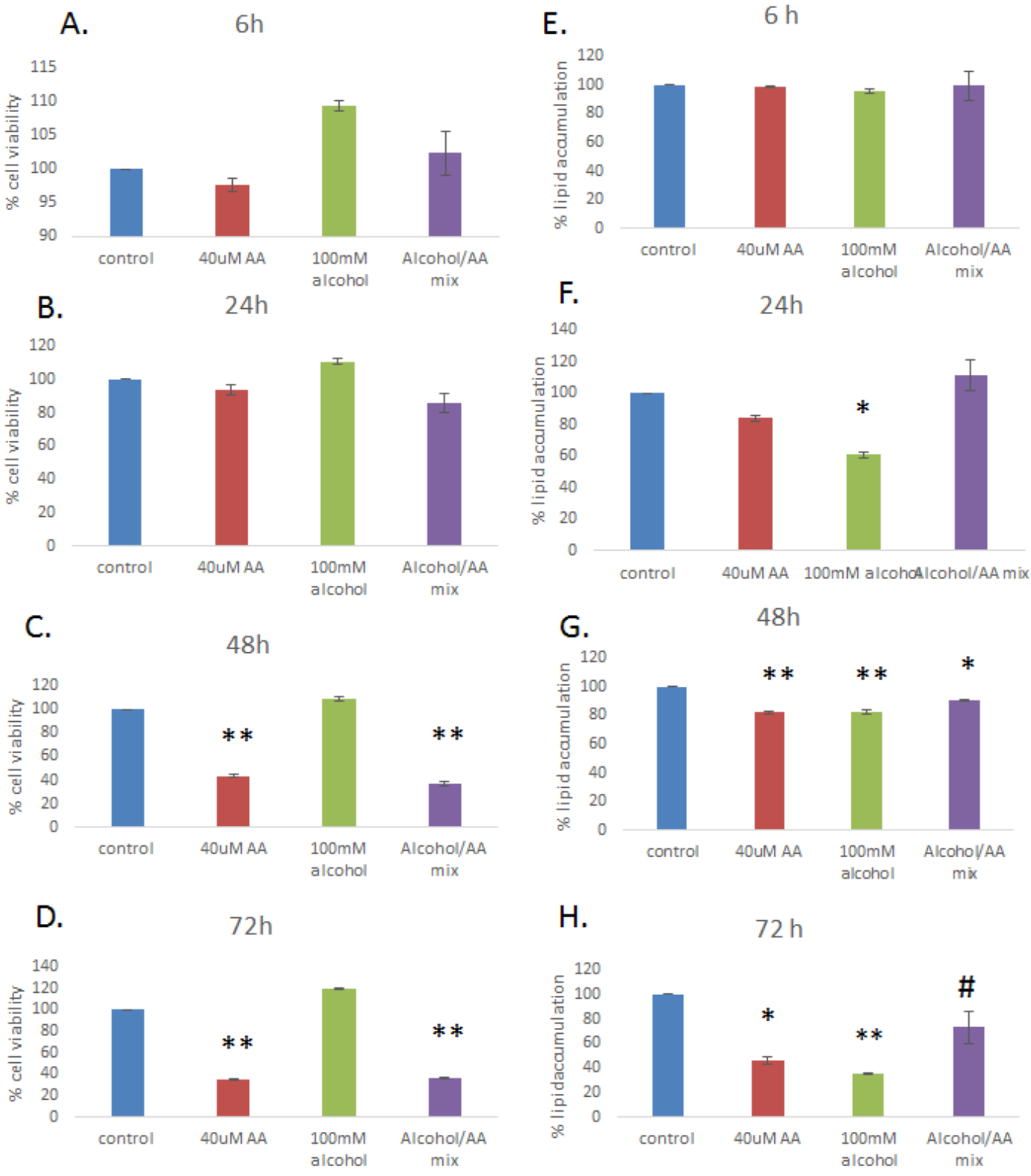

Figure 3.16: Effect of AA and/or alcohol on cell viability (A-D) & lipid accumulation (E-H) in VL-17A cells after 6, 24, 48, and 72 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). # P < 0.05, *P < 0.01, and **P < 0.001 as compared to the control. AA, arachidonic acid.

3.13 Effect of the AA/DHA and alcohol mixture on cell viability:

VL-17A cells were exposed to different AA/DHA ratios (range 1:1–25:1) in the presence of (100 mM) alcohol for 24 h. Our data did not show any significant changes in the cell viability ($P > 0.05$). However, we found that cell viability significantly decreased at 15:1 (23%) and 25:1 (24%) ratios when compared to that at the alcohol/1:1 ratio only ($P < 0.05$) (Figure 3.17).

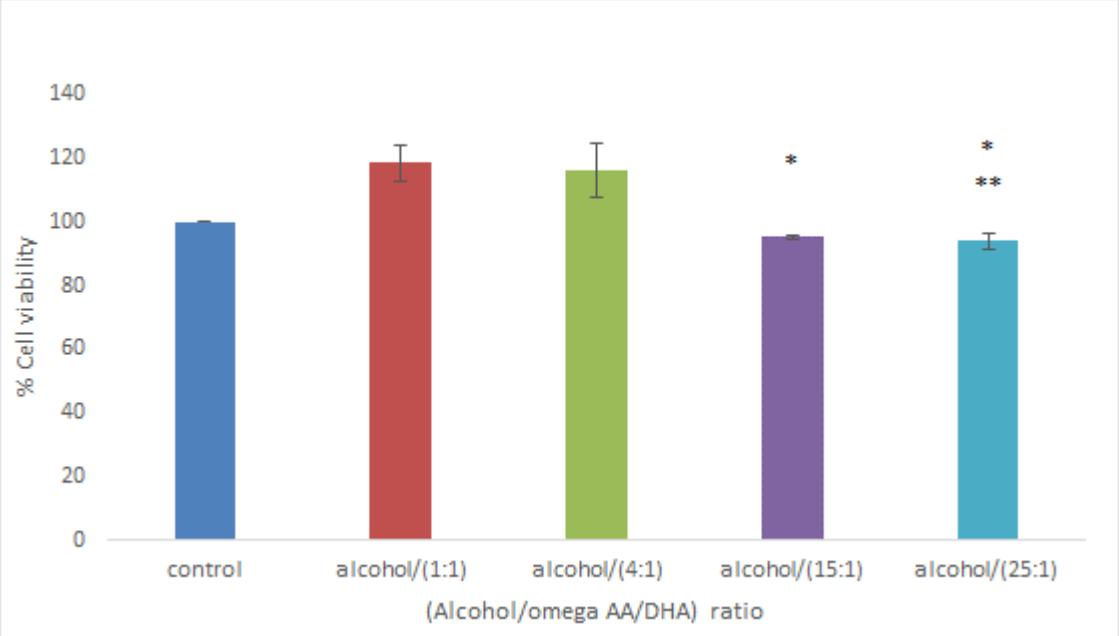

Figure 3.17: Effect of increasing AA/DHA ratios in the presence of alcohol on cell viability in VL-17A cells after 24 h. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *$P < 0.05$ as compared to alcohol/1:1; **$P < 0.05$ as compared to alcohol/4:1. AA, arachidonic acid; DHA, docosahexaenoic acid.

3.14 Effect of (AA/DHA) ratio and alcohol mixture on ROS production:

The production of ROS was measured after treating the VL-17A cells with four different ω-6/ω-3 (AA/DHA) ratios in the presence of alcohol (100 mM) for 30 min, 1 h, and 2 h. There was no significant change with any ratio at the 30-min time point (Figure 3.18A). None of the ratios showed a significant difference as compared to the control after 1 h; however, only the 4:1 alcohol/omega ratio led to a significant, 39% ($P < 0.05$) reduction in ROS production. On the other hand, the 15:1 ratio caused an increase in the ROS levels; however, this elevation did not achieve statistical significance as compared to control (11%), but was significant when compared to that at the 4:1 ratio (Figure 3.18B).

Only the higher alcohol/omega ratios (15:1 and 25:1), particularly the 25:1 ratio, stimulated ROS production after 2 h and caused a significant rise in the ROS levels. This elevation with the 15:1 and 25:1 ratios was observed mainly when compared to that in the control (49% and 73%; $P < 0.01$, respectively). However, when compared to the 1:1 and 4:1 ratios, the mixture of alcohol/15:1 ratio increased the ROS production (28%; $P > 0.05$ and 33%; $P < 0.05$, respectively); in addition, the ROS level elevation was significantly higher with the alcohol/ 25:1 ratio (52% and 57%; $P < 0.01$, respectively) as compared to the 1:1 and 4:1 ratios (Figure 3.19).

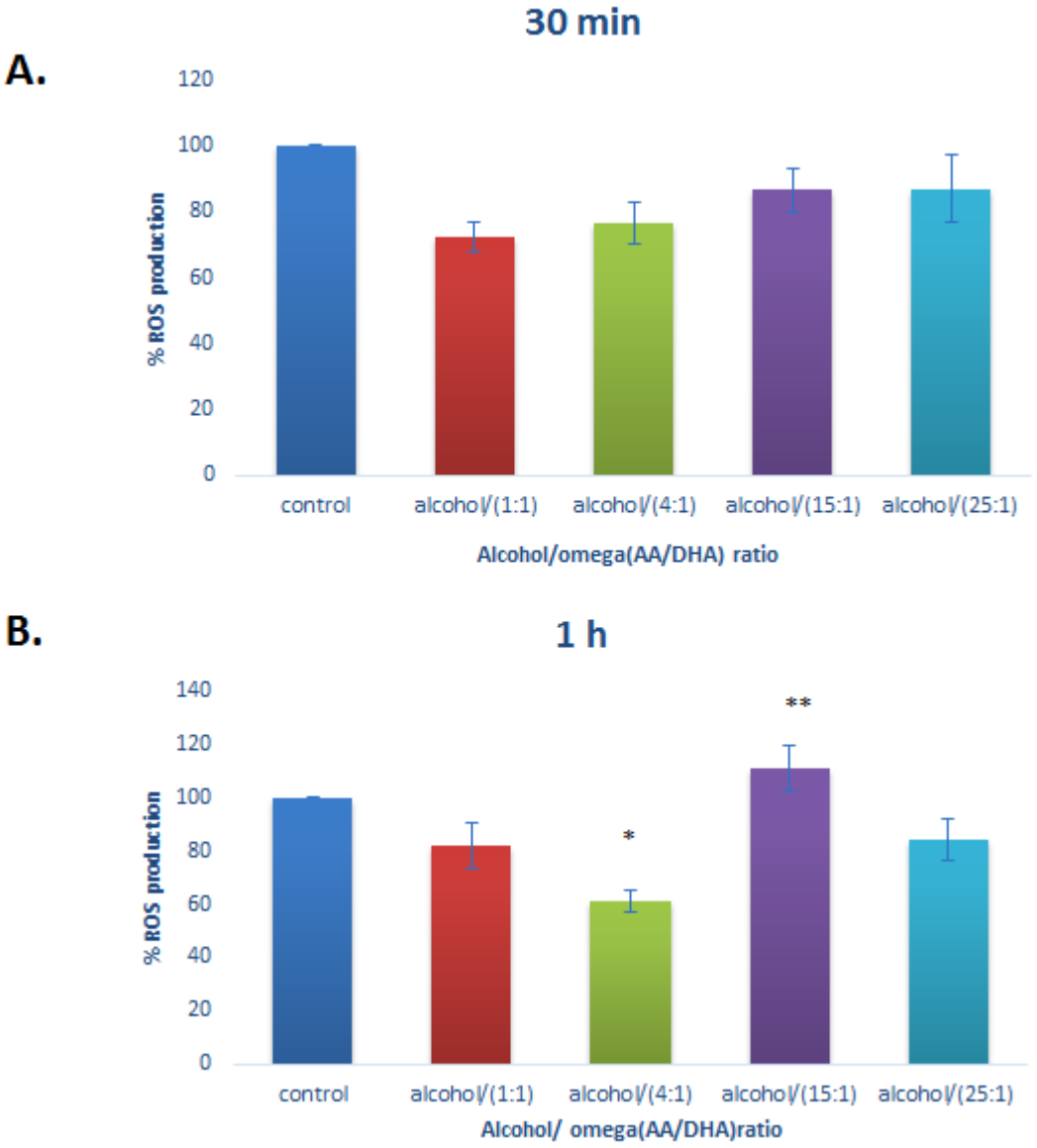

Figure 3.18: Effect of increasing AA/DHA ratios in the presence of alcohol on ROS production in VL-17A cells after 30 min and 1 h treatment. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.05 as compared to the control; **P < 0.01 as compared to the value with 4:1 ratio in the presence of alcohol. AA, arachidonic acid. DHA, docosahexaenoic acid.

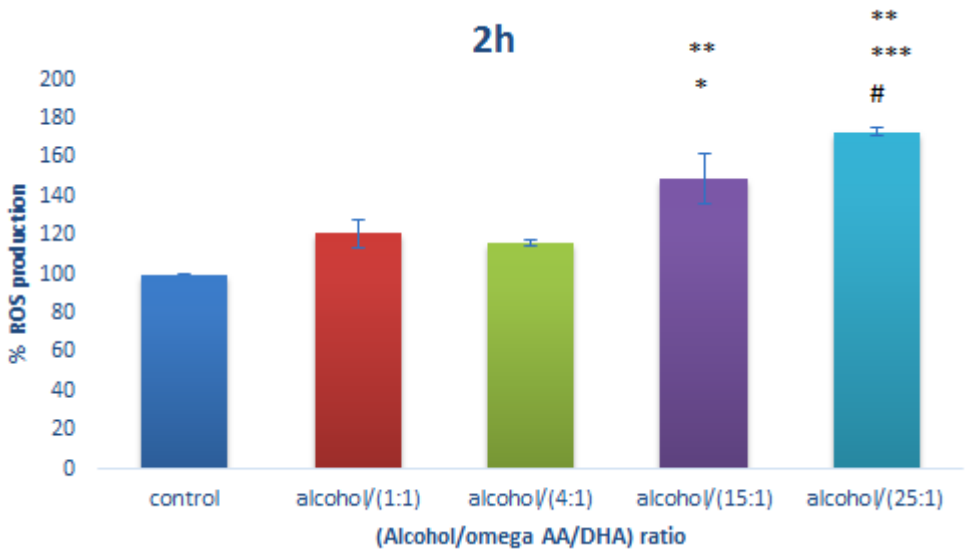

Figure 3.19: Effect of increasing AA/DHA ratios in the presence of alcohol on ROS production in VL-17A cells after 2 hr treatment. The results are presented as mean ± SEM (n = 3 determinations of 6 replicates per treatment). *P < 0.05 as compared to the value with 4:1 ratio in the presence of alcohol; **P < 0.01 as compared to the control; ***P < 0.01 as compared to the value with 4:1 ratio in the presence of alcohol; $^{\#}$P < 0.01 as compared to the value with 1:1 ratio in the presence of alcohol. AA, arachidonic acid. DHA, docosahexaenoic acid.

3.15 Effect of chronic alcohol consumption on TNFα and liver function profile (LFT):

There are many similarities between the pathological mechanisms between ALD and NAFLD, with hepatic inflammation are one of the prime manifestations. TNFα is a cytokine which is believed to be highly released in inflammation associated with ALD (DeSantis et al., 2013). Liver injury in ALD is normally accompanied by an increase in the markers of the liver function test and some cytokines (Zhang et al., 2017). To prove the above theory, the measurement of LFT and pro-inflammatory cytokine (TNFα) in the serum of alcoholic patient was performed. Data showed a significant increase in the concentration of AST, ALT, ALP and GGT (Figure 3.21). In addition, serum TNFα concentration was also high but this increase did not achieve statistical significance as compared to control (P >0.05) (Figure 3.20).

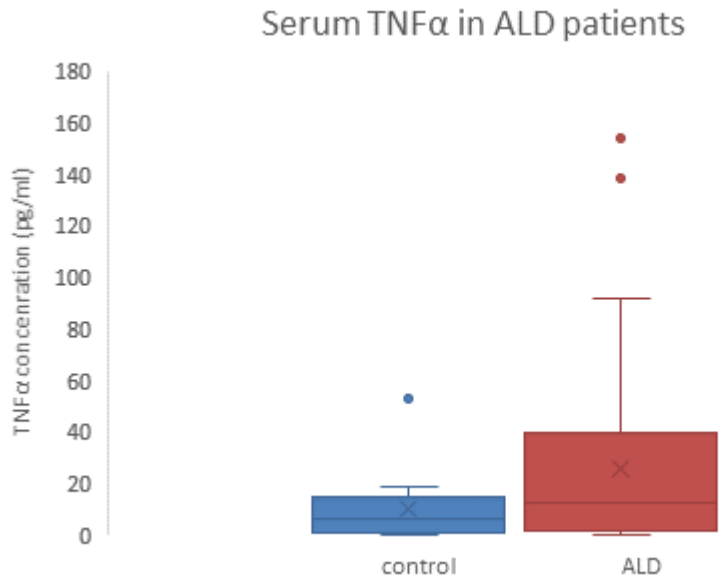

Figure 3.20: Box and Whisker plot of serum TNFα in ALD patients. TNFα, tumour necrosis factor; ALD, alcoholic liver disease. Data is Control n=15; ALD n=63.

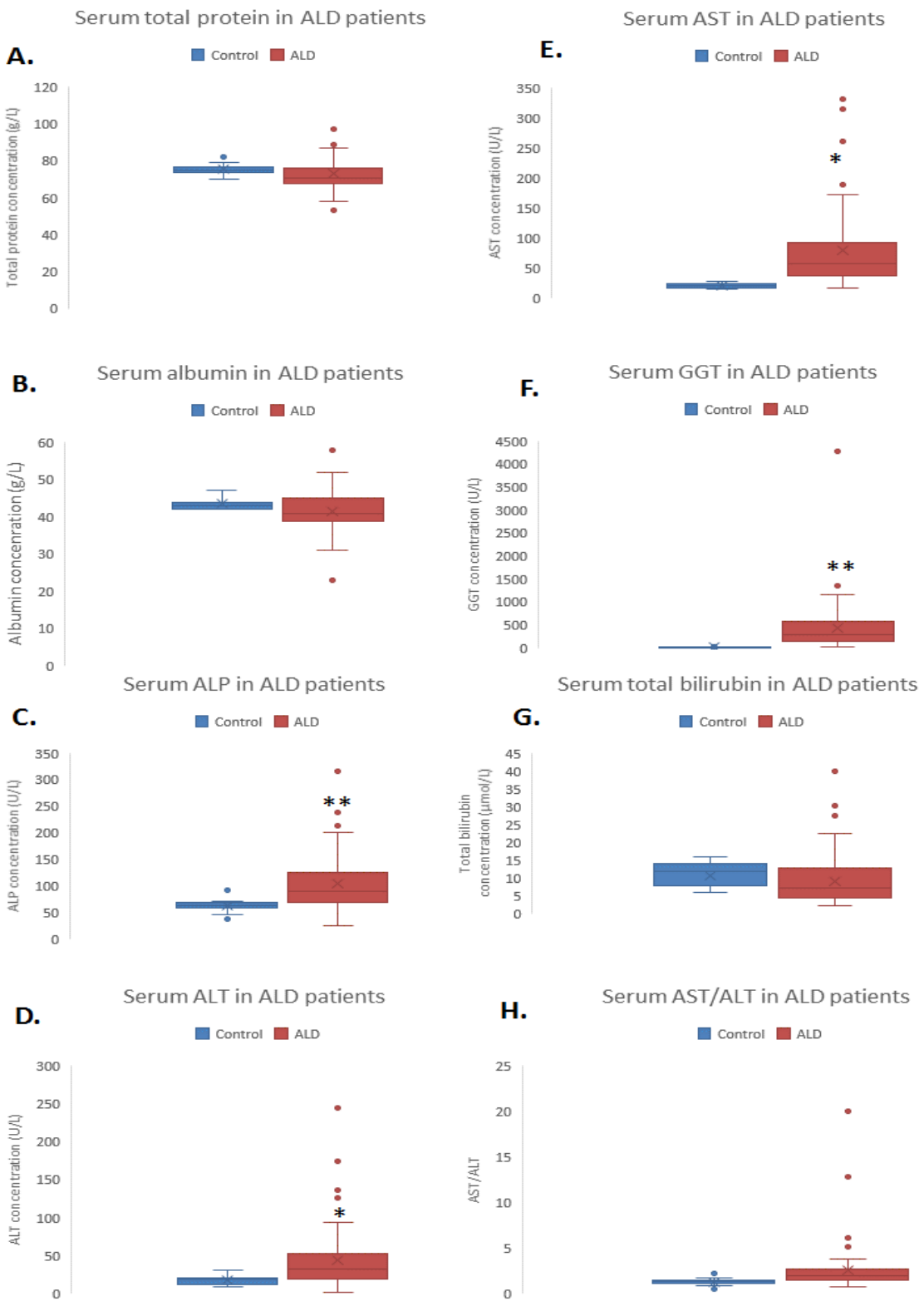

Figure 3.21: Effect of chronic alcohol consumption on liver injury markers in ALD patients. ALT, alanine aminotransferase; AST, aspartate; ALP, alkaline phosphatase; total bilirubin; GGT, gamma glutamyltransferase. Data is Control n=15; ALD n=63. *P <0.01, **P <0.001.

Chapter 4

Discussion

4. Discussion

Non-alcoholic fatty liver disease (NAFLD) is one of the main types of chronic liver disease in obese children and adults (Rinella and Sanyal, 2016). Its characteristics include an excessive increase in hepatic triglyceride (TG) and cholesterol (simple steatosis). If not controlled, these can lead to steatohepatitis (Bae et al., 2017). As previously mentioned, hepatic steatosis is the prime manifestation of NAFLD. In addition, as stated earlier in chapter 1 (section 1.3.4), free fatty acids have a vital role in the development of NAFLD. Many studies discuss the relationship between high dietary consumption of omega-6 and a significant reduction in omega-3 consumption with the development of many diseases. These include cardiovascular disease, cancer (such as colorectal and breast cancer) and arthritis (Simopoulos, 2002; Candela et al., 2011).

This study aimed to characterise the effect of various fatty acids (PA, SA, LA, ALA, DHA and AA) individually or as ratios (LA/ALA and AA/DHA) on lipotoxicity, liver steatosis, oxidative stress and mitochondrial function using HepG2 (VL-17A and VA-13) cells. A further aim was to demonstrate the particular implication of omega-6/3 ratios in the pathogenesis of NAFLD by investigating the effect on lipid metabolism as well as the cannabinoid system. In addition, this project examined the interaction of fatty acids and alcohol on cell toxicity and reactive oxygen species (ROS) production as well as alcohol alone on hepatic toxicity.

4.1 Effect of saturated fatty acids

To confirm that HepG2 cells could be used as a model for examining steatosis and lipotoxicity, fatty acids PA and SA were studied at various time points (Figures 3.1 and 3.2). Here the storage of TG by oil red staining indicated that PA

but not SA after 48 and 72 h showed a significant increase in lipid accumulation while both fatty acids caused cell toxicity. These results are consistent with a study that showed a toxic effect of PA and SA on SK-Hep-1 cells but at a higher concentration (250 µM and 500 µM, respectively) (Oh *et al*., 2012), than the present study (up to 160 µM). The results are also in relatively good agreement with other studies carried out by Zhang et al. (2011; 2012) where PA and SA (250 µM) treatment significantly increased cell death of rat liver H4IIE cells and of primary rat hepatocytes, respectively.

Gambino et al. (2016) found that the levels of PA and monounsaturated oleic acid were the highest circulating free fatty acids in patients with NAFLD. It is believed that the destabilisation of the lipid bilayer of the cellular membrane due to the unusual incorporation of saturated phospholipids. These form from excess unesterified saturated fatty acids and leads to endoplasmic reticulum (ER) stress (Spector and Yorek, 1985; Borradaile et al., 2006). Increased ER stress is associated with impairment of the unfolded protein response (UPR), which is required to restore ER homeostasis (Akazawa et al., 2010; Zhang et al., 2011; Zambo et al., 2013). Another study further supports this, showing that treatment of Wistar rats with a diet high in saturated fatty acids correlates highly with steatosis and ER stress (Wang et al., 2006).

The significant elevation in lipid accumulation by PA can also arise via the activation of lipogenic enzymes. As mentioned previously (see section 1.3.2), the SCD1 enzyme is responsible for the conversion of PA and SA to monounsaturated fatty acids. Another factor is the activation of nuclear receptors such as the liver X receptor (LXR) and the pregnane X receptor (PXR). When both are highly expressed in the liver, this leads to the overexpression of SCD1 and lipogenesis (Zhang et al., 2013). For example, treatment of neonatal cardiac

myocytes with PA and SA led to increased SCD1 expression, induced the accumulation of lipids and suppressed fatty acid oxidation (Matsui et al., 2012).

4.2 Effect of omega-6 fatty acids

Previous work from our laboratory (Gyamfi et al., 2012) demonstrated that PA at 80 and 160 µM caused less damage to HepG2 cells than unsaturated fatty acids. PA had a mild damaging effect due to the low effect on ROS production and mitochondrial dysfunction. This suggests that unsaturated fatty acids may pose greater cell toxicity than saturated fatty acids.

Omega-6 fatty acids LA and AA showed a cytotoxic effect on HepG2 cells, with AA expressing higher toxicity than LA (Figures 3.3 and 3.4) and greater than PA treatment by 72 h. After 24 h, LA (200–300 µM) showed a significant reduction in the viability of the cells. However, this decrease was only slightly significant after 48 h and non-significant at 72 h compared to AA. This indicates the lower toxicity effect of LA on HepG2 cells. Muller et al. (2010) observed similar findings where a significant reduction in the viability of rat primary hepatocytes occurred after 48 h of treatment with LA (100 µM), but in the presence of high glucose and insulin. An increase in oxidative stress as well as lipid accumulation resulted possibly due to the low cellular nitric oxide (NO) concentration. Many studies establish that NO acts as an antioxidant by binding to superoxide anion radicals and also limits fatty acid synthesis in the liver (Halliwell et al., 1999; Goldstein et al., 2000; Schild et al., 2006). Muller et al. (2010) found that LA reduced the concentration of cellular NO. It concomitantly increased both oxidative stress and the synthesis of fatty acids and consequently TG accumulation in hepatocytes. In contrast, this study did not show a significant increase in lipid accumulation with LA, but there was a marked increase with AA especially after 48 h than with LA (Figures 3.3 and 3.4).

However, this rise with AA reduced significantly compared to control after 72 h, which suggests the high toxicity of AA or its immediate metabolites.

A natural mechanism known as inflammation normally protects the human body from long-term injury. Inflammation produces physiological inflammatory mediators to defend the body. However, these mediators can be harmful and damage tissues if released inappropriately or in a higher proportion than normal levels (Maciejewska et al., 2015). As mentioned previously (see section 1.5), eicosanoids produced from polyunsaturated fatty acids LA and AA are among the main inflammatory mediators (Calder, 2010). Lipoxygenase (LOX) and cyclooxygenase (COX) can metabolise AA enzymatically to produce certain products. These include lipoxins (LX), leukotrienes (LT), hydroxyeicosatetraenoic acids (HETE), prostaglandins (PG) and hydroperoxyeicosatetraenoic acids (HPETE). Conversely, only LOX or non-enzymatic oxidative pathways can metabolise LA to produce the oxidative stress markers 9- and 13-hydroxyoctadecadienoic acids (9-HODE and 13-HODE) (Maciejewska et al., 2015). Therefore, the body metabolises AA and LA to a variety of inflammatory mediators considered causative components of steatohepatitis.

However, several studies show AA produces stronger mediators than LA, which plays a key role in causing liver inflammation (Patterson et al., 2012; Kirack et al., 2015; Maciejewska et al., 2015). For example, the levels of 9-HODE and 5-HETE mediators that LA and AA produced respectively were higher in patients with stage II NAFLD than in those with stage I. This could result from the higher activity of the 5-LOX enzyme (Maciejewska et al., 2015). In addition, LA eicosanoids (9-HODE and 13-HODE) were elevated in patients with non-alcoholic steatohepatitis (NASH) compared to those with simple steatosis (Feldstein et al., 2010). These studies confirm that the level of proinflammatory eicosanoids

produced from either AA or LA were high. Consequently, knowing the proinflammatory trait of AA and LA eicosanoids may explain the reduced viability of the cells in this study after the administration of AA and LA. Overall, these findings reflect the greater toxicity of AA compared with LA. In agreement with this work, a study that treated VA-13 cells for 24 h with AA showed a reduction in the level of cellular adenosine triphosphate (ATP) and mitochondrial membrane potential. This also showed an increase in ROS production as well as lipid accumulation, demonstrating the cytotoxic effects of AA (Gyamfi et al., 2012).

It is interesting to note that cytochrome P450 (CYP) epoxygenases can also metabolise AA to epoxyeicosatrienoic acid (EET) eicosanoids. CYP is highly expressed in the liver and serves as a target for peroxisome proliferator-activated receptor alpha (PPAR-α) (Suzuki-Kemuriyama et al., 2016; Wells et al., 2016). EETs are potent and protective anti-inflammatory metabolites that soluble epoxide hydrolase (sEH), a broadly distributed cellular enzyme, can rapidly convert to a less active eicosanoid, dihydroxyeicosatrienoic acid (DHET) (Wells et al., 2016). The functional relevance of CYP epoxygenases in the pathogenesis of NAFLD remains unclear. However, some studies mentioned that the dysregulation of CYP epoxygenases and the suppression of the biosynthesis of circulating and hepatic EETs are a consequence of NASH (Schuck et al., 2014; Wells et al., 2016).

On the other hand, ablation of one of the CYP 450 homologs, 4A14 (CYP4A14) which is involved in the hydroxylation of AA, correlates with attenuating hepatic fibrosis and inflammation in a CYP4A14-deficient mice model. This was also overexpressed in NAFLD patients and NAFLD murine models (Zhang et al., 2017). Interestingly, Wells et al. (2016) found that the level of free anti-inflammatory EET increased after the induction of NASH in mice and were also

high in patients with NASH. They also demonstrated that the genetic disruption of sEH enzyme in mice elevated the concentration of EETs and attenuated the previously induced steatosis and inflammation. It can be concluded from everything mentioned above that not all the elevated metabolites of AA lead to hepatic inflammation. However, some can be therapeutic and can attenuate the manifestations of NAFLD.

4.3 Effect of omega-3 fatty acids

Several studies have evaluated the efficacy of omega-3 *in vivo* and *in vitro* using omega-3 fatty acids as a treatment for proven NAFLD cases (Zhang et al., 2011; Bae et al., 2017; Hodson et al., 2017). However, one of the objectives of the current study was to know the effect of single fatty acids on VL-17A cells. Therefore, untreated cells were exposed to different omega-3 concentrations to indicate whether this class of fatty acids had the ability to reduce lipid content in untreated cells. It is common knowledge that omega-3 fatty acids (ALA and DHA) are more protective than omega-6 ones. Many studies report that omega-3 fatty acids (DHA and EPA) are low in patients with hepatic steatosis, which may suggest the efficacy of omega-3 in preventing and treating steatosis (Araya et al., 2004; Allard et al., 2008; El-Badry et al., 2007; Patterson et al., 2012). The present study corroborated this where there was no observed cytotoxicity or change in lipid accumulation (Figures 3.5 and 3.6).

Omega-3 derivatives (EPA and DHA) seem to act as activators of PPAR*α*. PPAR*α* has a role in promoting lipid oxidation, which can be associated to the protective effect of the omega-3 family. In one study, ALA reversed the effect SA elicited and reduced apoptosis as well as ER stress in rat hepatocytes (Zhang et

al., 2011). This protective effect of ALA may be due to the conversion of ALA to its derivatives (EPA and DHA). It may also result from modulating some metabolic processes directly through the activation of some receptors such as PPARα or regulating inflammatory mediator pathways (Hanke et al., 2013).

Another possible mechanism is the expression of SCD1, which converts saturated fatty acids to unsaturated fatty acids. Caputo et al. (2014) found that DHA reduced the activation of liver X receptor alpha (LXRα). Consequently, this decreased the activity of SREBP-1c and the binding of this transcriptional factor (SREBP1-c) to SCD1. In turn, this led to reduced TG synthesis and accumulation of lipids in hepatocytes. Furthermore, Kheder et al. (2016) agreed with other studies and showed that vitamin D3 and DHA either separately or as a mixture were able to down- and upregulate the activity of SREBP and PPARα, respectively. In addition, they reduced the production of tumor necrosis factor alpha (TNFα, a proinflammatory cytokine from macrophages), which can induce hepatic stellate cells to develop fibrosis. This is consistent with a study conducted on children with NAFLD and vitamin D deficiency. Here again, administering both DHA and vitamin D improved insulin resistance and reduced the activation of stellate cells and consequently collagen content (Della Corte et al., 2016).

From the data presented and documented above, it is likely that this protective impact of omega-3 fatty acids can be due to their effect on the activity of SREBP and PPARα. As stated these helps metabolise fatty acids by modulating the accumulation of lipids in the hepatocytes. This can take place through the inhibition of fat biosynthesis and consequently suppress the development of steatosis (Suzuki-Kemuriyama et al., 2016).

Several studies have reported that EPA and DHA have the ability to improve the pathological features of NASH. Moreover, EPA is more functional than DHA in

reducing hepatic steatosis by decreasing the expression of lipogenic genes involved in fatty acid and TG synthesis. These include fatty acid synthase (FAS), glycerol-3-phosphate acyltransferase (GPAT1, an enzyme involved in TG synthesis), and elongation of very long-chain fatty acids (Elovl6). The latter is a microsomal enzyme that plays a role in the elongation of saturated and monousaturated fatty acids, mainly C16 to C18, and serves as a target enzyme for SREBP in the liver. In contrast, these studies found DHA more efficient in attenuating the level of ROS, fibrosis and hepatic inflammation (Matsuzaka et al., 2012; Depner et al., 2013; Suzuki-Kemuriyama et al., 2016).

The expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits can also serve as a marker for oxidative stress. The belief is that this enzyme has a key role in the pathogenesis of NASH, especially in fibrosis (De Minicis and Brenner, 2007). NADPH oxidase can generate a superoxide via transferring an electron from NADPH along the cellular membrane to bind to molecular oxygen and form a superoxide anion. In turn, superoxide dismutase can convert the anion to hydrogen peroxide. However, DHA has more potential than EPA in attenuating the expression of the NADPH oxidase subunits involved in oxidative stress (Depner et al., 2013).

In conclusion, all the mechanisms mentioned above can help in understanding how omega-3 fatty acids can be protective and attenuate the pathological manifestations of NAFLD. This in turn can clarify in this study, why both ALA and DHA did not cause any lipotoxicity or lipid accumulation when they were used as a treatment.

4.4 Effect of increased omega 6/3 ratio on cellular toxicity and steatosis

In this study, it was hypothesised that high omega 6/3 ratios will cause high cell toxicity and lipid accumulation. Here, LA/ALA and AA/DHA findings were not entirely consistent with other research. These studies showed a link between an increased ω6/ω3 ratio and the development of hepatic steatosis (Araya et al., 2004; Puri et al., 2007). Thus, treatment with LA/ALA ratios did not affect the viability of cells (Figure 3.7A-C) nor cause an increase in the accumulation of lipids (Figure 3.7D-F) This could be due to the low concentration of LA used in this study. In contrast, AA/DHA ratio findings showed a significant decrease in cell viability after 24, 48 and 72 h when the ratios increased (Figure 3.8A-C). Unexpectedly, the oil red method data did not show a significant increase in lipid accumulation, and at 72 h, some ratios showing a significant decrease (Figure 3.8D-F).

Enzymatic quantification of the concentration of intracellular TG helped confirm the data above. This method expresses the lipid content in relation to cell protein content. In contrast to the previous data the oil red method provided, results indicated a significant elevation in lipid concentration, especially after 24 h and 48 h (Figure 3.9A-B). These results agree with other studies where the omega 6/3 ratio was high in the livers of patients with NAFLD exhibiting steatosis and NASH (Araya et al., 2004; Puri et al, 2007). These findings are also similar to a study by Enos et al. (2015) in which they fed male mice a high-fat diet with different omega AA/EPA and AA/DHA ratios (1:1, 5:1, 10:1 and 20:1). This caused hepatomegaly with a significant increase in hepatic lipid accumulation.

Nutritional factors along with increased oxidative stress and factors like the development of insulin resistance play a crucial role in changing hepatic metabolism and developing steatosis (Farrell and Larter, 2006; Musso et al.,

2009; Gormaz et al., 2010). Therefore, overload of FA in the liver correlates with the rise in the mitochondrial oxidation of FA. Consequently, increased hepatic oxidative stress leads to the progression of steatohepatitis (Valenzuela and Videla, 2011). This theory is consistent with this study in which high AA/DHA ratios increased the intracellular amount of lipids. However, a discussion and evaluation of oxidative stress in this study and the production of ROS will occur later in this chapter.

The dysregulation of lipid metabolism in the liver can be another factor in the development of steatosis. In this research, the increase in the concentration of TG with high AA/DHA ratios can be also due to low DHA and high AA concentrations. Depletion of omega-3 seems to increase the lipogenic capacity by favouring TG and FA synthesis. This causes derangement in the oxidation of FA and decreases the export of TG from the liver. Subsequently, this downregulates PPARα and activates SREBP, which in turn promotes the development of steatosis and inflammation (Valenzuela and Videla, 2011).

As mentioned previously, the significant decrease in cell viability can be due to the increase of AA metabolites, which are considered as strong mediators of liver inflammation. Conversely, EPA and DHA can antagonise the effect of these mediators. Furthermore, in a study by Suzuki-Kemuriyama et al. (2016), EPA and DHA were able to suppress the synthesis and metabolism of ω6 fatty acids and reduce the content of AA. This in turn can clarify the reason behind the low viability of the cells in this study with high omega 6/3 ratios and consequently the development of steatosis.

Therefore, further work must take place to evaluate the effect of high omega 6/3 ratios on the expression of omega 6 inflammatory products. This may then

provide valuable information into how high omega 6/3 ratios can induce liver inflammation, which is the prime manifestation of NASH.

4.5 Effect of high AA/DHA ratios on hepatic lipid metabolism

This study aimed to investigate the mechanism behind the elevated amount of lipid in liver cells. To this end, the protein expression of SREBP-1c, SCD1 and PPARα and cannabinoid receptors (CB1 and CB2) involved in hepatic lipid metabolism was determined. The present study presumed that high AA/DHA ratios will lead to changes in the expression of these transcriptional factors. Unpredictably, treating VL-17A cells with different AA/DHA ratios for 24 h did not cause an expected increase in the expression of SREBP-1c, a lipogenic factor known to promote the synthesis of lipids. Thus, these findings contrast with those in other studies. For example, Zhang et al. (2014) found that the expression of SREBP-1c was upregulated in HepG2 cells after treating the cells with a LXR agonist. This was the TO901317 drug, known for activating the SREBP-1c promoter. In addition, this upregulation led to an increase in the expression of SCD1 after infecting HepG2 cells with an adenovirus expressing SREBP-1c. Also, mice fed a high-fat diet with different ratios of omega 6/3 led to increased SREBP-1c expression (Enos et al., 2015).

Moreover, supplementation of omega-3 fatty acid in *ob/ob* mice downregulated the expression of SREBP-1 and consequently liver lipogenesis. It also reduced the levels of serum fatty acids, insulin and glucose (Sekiya et al., 2003). This study illustrated that a low omega-3 fatty acid level usually upregulates the expression of SREBP-1. This is similar to the current study where omega-3 fatty acids were not in balance with omega-6 ones. Further support for this notion

comes from feeding mice a diet lacking omega-3 fatty acids. This activates SREBP-1c and lipogenesis as compared with control mice (Pachikian et al., 2011). However, this project's findings agree with a study conducted on a Chinese population with NAFLD. This study assessed the impact of four single-nucleotide polymorphisms (SNPs) in SREBP-1c on NAFLD vulnerability. They also found that these four SNPs in SREBP-1c did not correlate with the risk of NAFLD in the Chinese Han population (Peng et al., 2016). Furthermore, the results demonstrated that SNP alleles in SREBP-1c vary between different populations with various genetic backgrounds. It is also important to state that NAFLD is a multifactorial disease that develops due to different environmental factors and genetic changes.

However, the current findings relate to work by Shimomura et al. (1997) where HepG2 cells, diploid human fibroblasts and mouse embryo fibroblasts produce more SREBP-1a than SREBP-1c. Conversely, the SREBP-1c isoform is more predominant in human liver and adipose tissue than in cultured cells. After incubating HepG2 cells with either a medium supplemented or lacking sterol, RNA was isolated and both treated cells showed higher SREBP-1a transcripts than SREBP-1c. This variance was even higher in the sterol-deficient medium (Shimomura et al., 1997). This may explain the lack of expression change in SREBP-1c in the present study.

Although these findings showed no change in the expression of SREBP-1c especially with high ratios, SCD1 expression, a lipogenic enzyme, was up-regulated at 15:1 and this effect was even more pronounced at 25:1 ratio (Figure 3.11F). As mentioned previously in chapter 1 (see section 1.3), polyunsaturated fatty acids constitute a factor involved in the regulation of SCD1 expression. This

can occur either directly by binding to LXR or indirectly through the activation of SREBP-1c via LXR. LXR is a member of the nuclear hormone receptor family and is highly abundant in the liver and other organs such as the kidney and spleen. Furthermore, it has the ability to regulate cholesterol and fatty acid homeostasis and can be activated by sterol and FFAs (DeBose-Boyd et al., 2001; Chuu et al., 2007; Zhang et al., 2014). Therefore, this study can attribute the high expression of the SCD1 enzyme to its binding to LXR and not to the activation of SREBP-1c since the expression of the latter did not indicate any change. These findings are consistent with those in which SREBP-1c null mice treated with the LXR agonist TO901317 showed a slight increase in SCD1 expression (Zhang et al., 2014). Researchers in another study fed mice a diet enriched in saturated and omega-6 fatty acids and low in omega-3. Results showed a high liver expression of SREBP-1 and SCD1 while PPARα showed limited expression (Sealls et al., 2008). Yet these findings partly disagree with what Caputo et al. (2014) reported. That study showed polyunsaturated fatty acids (AA, DHA and EPA) can inhibit LXRα activation. Consequently, they can inhibit the activation of SREBP-1 and SCD1. However, the current study can accept Caputo et al.'s (2014) findings in terms of DHA but not for AA.

The elevation of the omega 6/3 ratio in the present study increased the expression of SCD1. Consequently, this agrees with what Miyazaki et al. (2007) found, where SCD1-deficient mice fed a high fat-diet protected these animals from the onset of hepatic steatosis. This reflects the role of SCD1 in the development of steatosis. Thus, further work is needed to identify the role of a high omega 6/3 ratio in regulating the activity of LXR.

Most studies evaluate the expression of SREBP along with PPARα in studying the lipogenic pathways. Thus, to study the impact of high AA/DHA ratios on lipid

metabolism, the effect of these ratios on the expression of PPARα was evaluated. This transcriptional factor stimulates FA oxidation by activating enzymes such as acyl-CoA oxidase (ACO) in the peroxisome and carnitine palmitoyltransferase I (CPT1) in the mitochondria (El-Badry et al., 2007). As hypothesised in this study, high ratios of AA/DHA (15:1 and 25:1) significantly downregulated the expression of PPAR-α (Figure 3.10B). The supplementation of omega-3 fatty acids (EPA and DHA) in mice fed a high-fat diet led to upregulated expression of PPARα. Consequently, this prevented hepatic steatosis and inflammation (Tapia et al., 2014). This theory can explain the low expression of PPARα in this study where omega-3 fatty acids were not in balance and were lower than omega-6.

Overall, these findings showed that high AA/DHA ratios affected both fatty acid oxidation and lipogenesis by decreasing PPARα expression and upregulating SCD1 expression, respectively. The latter, possibly activated in this study by LXR and not SREBP1-c because the expression of SREBP1c did not change. This calls for further investigations on the effect of AA/DHA ratios on LXR activity or to study the impact of these ratios on the expression of SREBP1-c using animal liver instead. However, these findings are consistent with data from TG assays in which lipid levels increased by increasing the AA/DHA ratio.

4.6 Effect of high AA/DHA ratios on the cannabinoids system

As mentioned in chapter 1 (section 1.3.5), high fatty acids can increase the level of endocannabinoids and consequently activate the expression of CB1 and CB2. To some extent, this is consistent with this study's findings where CB1 expression increased slightly with high AA/DHA ratios. However, this elevation was not significant, which is possibly due to the short incubation time. As described earlier, the expression of SCD1 and not SREBP-1c increased while PPARα

decreased. This may be the reason behind the slight increase in CB1 receptor expression with high AA/DHA ratios.

Osei-Hyiaman et al. (2005) found that after feeding CB1-deficient mice and wild-type mice a high-fat diet for 14 weeks, only the latter developed steatosis. Moreover, the results showed an elevated CB1 expression in the wild-type group while the level of the endogenous cannabinoid anandamide, which activates CB1 receptors, increased in both groups. Furthermore, activating CB1 receptors via a CB1 agonist in wild-type mice fed a chow diet increased the expression of SREPB1-c and lipogenic enzymes (involved in fatty acid synthesis). It can be concluded from their study that a high-fat diet activates CB1 receptors via increasing the level of anandamide. This consequently leads to fatty liver development by upregulating the expression of SREBP-1c and lipogenic enzymes.

In another study, the researchers also investigated the impact of a high-fat diet on metabolic pathways using wild-type mice and liver CB1-deficient mice (Osei-Hyiaman et al., 2008). Here, both mice species developed obesity but the liver CB1-deficient mice developed less hyperglycaemia, insulin resistance, dyslipidaemia and steatosis than the wild-type mice. This suggests that the activation of endocannabinoids induces the expression of CB1 receptors causing some metabolic and hormonal alterations and consequently contributes in the development of steatosis.

Unlike the moderate number of studies conducted on CB1 receptors and their role in the development of fatty liver, the expression and the role of CB2 receptors in the development of NAFLD is still unclear and under-investigated. Compared with the control and in contrast to the current findings, high AA/DHA ratios did not result in increased expression of CB2. Instead, the opposite occurred, with all

ratios (1:1–25:1) showing a significant reduction in CB2 expression, particularly with 1:1 and 4:1 ratios. However, comparing all ratios to 1:1 showed an increase in CB2 expression, which was significant at the 25:1 ratio. Thus, our study agrees with Mendez-Sanchez et al. (2007), who showed upregulation of the expression of CB2 receptors in the livers of NAFLD patients characterised by steatosis and steatohepatitis. Moreover, feeding mice a high-fat diet for 15 weeks caused fatty liver and increased TG levels. Conversely, CB2 knockout mice fed the same diet showed less hepatic steatosis (Deveaux et al., 2009). Julien et al. (2005) also demonstrated that CB2 receptors are not expressed in normal livers but only in cirrhotic livers and are upregulated in hepatic fibrogenic cells. The researchers surmised that activated CB2 receptors act as anti-fibrogenic factors by triggering apoptosis and growth inhibition like cyclooxygenase-2.

Altogether, these data indicate that CB2 receptors are mainly expressed in liver fibrogenic cells and cirrhotic livers but not in normal livers to counteract fibrosis. This can be the reason behind the low expression of CB2 in the present study. This can also be due to the short exposure of the cells to the treatments, assuming they may require longer time to induce severe injury with high AA/DHA ratios and consequently increase CB2 expression. This notion can be consistent with the present study's findings that the expression of CB2 receptors was significantly low at 1:1 and 4:1 ratios. Conversely, 15:1 and particularly 25:1 ratios expressed more CB2 receptors than the 1:1 ratio.

To our knowledge, this study has demonstrated for the first time that cannabinoid receptors correlate to high omega 6/3 ratios. However, further work can elucidate the effect of high AA/DHA ratios on the expression of CB2 receptors by evaluating inflammatory markers like TNFα and fibrogenic ones. Additional studies could also help identify the mechanism by which high AA/DHA ratios affect and

upregulate the expression of CB1. It would thus be useful to investigate the expression of other enzymes involved in either fatty acid oxidation like CPT1 or in lipogenesis such as FAS.

4.7 Effect of increased AA/DHA ratios on mitochondrial functions and ROS production

Research has established that free fatty acids correlate with mitochondrial dysfunction via the uncoupling of oxidative phosphorylation (Schonfeld and Wojtczak, 2008). The current study followed the concept above and the previous investigation on the effect of high AA/DHA ratios on lipid metabolism. To this end, the influence of polyunsaturated free fatty acids as a ratio (AA/DHA) on mitochondrial dysfunction was assessed by measuring the cellular oxygen consumption rate. Here, high AA/DHA ratios (15:1 and 25:1) led to mitochondrial function disturbance along with a significant decrease in mitochondrial respiration and the production of ATP (Figure 3.14). This impairment of mitochondrial oxidative respiration was accompanied by a reduction in the proton leak as well as the ability of the cells to achieve their maximal respiration and to respond to elevated energy demand.

The mechanism behind this mitochondrial dysfunction and ATP synthesis depletion could be due to the destabilisation of cytochrome c in the electron transport chain (ETC) by fatty acids. This in turn decreases the flow of electrons in the respiratory chain and beyond complex II, inducing the release of electrons from the mitochondrial membranes (Schonfeld and Wojtczak, 2008). Mitochondrial dysfunction due to the accumulation of fatty acids can also result from (as stated in Chapter 1 section 1.3.4) electron leakage. Also, can be due to the ability of fatty acids to increase the permeability of the mitochondrial

membrane by stimulating the opening of the mitochondrial permeability transition (MPT) pores in the inner mitochondrial membrane. This leads to mitochondrial depolarisation, loss of membrane potential by uncoupling and consequently swelling, leading to cell death (Jaeschke et al., 2002).

Proton leak is one of the parameters of mitochondrial function that reflects any damage to the mitochondria. In addition, the reduction of proton leak in this study can be attributed to the reduced electron flow in the ETC. This usually accomplished by proton pump from the mitochondrial matrix into the mitochondrial intermembrane space. Proton pump reduction will then lower protons entering through the channel of complex V and consequently decreases the production of ATP.

Overall, these data agree with the work by Gyamfi et al. (2012). This study found that AA reduced the mitochondrial membrane potential and ATP production more than PA in VA-13 cells. The present study used AA as the main source of omega-6 fatty acids. Several studies have reported altered mitochondrial function in fatty liver disease. Oliveira et al. (2006) showed disruption of mitochondrial function occurred in Wistar rats with fatty liver. Their study found mitochondrial dysfunction represented by a reduction in respiratory control rate, an increase in the oxygen consumption rate and an increase in oxidative stress. Petrosillo et al. (2007) showed similar results with reduced complex I activity in the livers of rats with fatty liver. This also correlated with an increase in the production of hydrogen peroxide (H_2O_2), which could explain the significant decrease in the complex I activity. Another study reported mitochondrial uncoupling in obese individuals with NASH and a respiration rate decreased by 30–40% (Koliaki et al., 2015).

Following the evaluation of mitochondrial function, this study assessed the effect of AA/DHA ratios on ROS production. As stated in chapter 1 (section 1.3.4), ROS

mainly produced from the mitochondria, and complex I and III are the main sites of electron leakage leading to ROS production (Schonfeld and Wojtczak, 2008). The increase in ROS production normally arises as a response to mitochondrial dysfunction (Leamy et al., 2013). This theory is consistent with the findings of this study, where high AA/DHA ratios caused mitochondrial dysfunction. The study also found that all the AA/DHA ratios increased the production of ROS after 30 min. But over time, this elevation at 1:1 and 4:1 went down to normal levels while with high ratios, ROS remained elevated, especially at the 15:1 ratio. This reduction in ROS at 1:1 and 4:1 could be due to the oxidation of fatty acids, coupled with the respiratory chain handling the excess release of electrons. This also facilitates the movement of electrons through the respiratory chain to reduce oxygen atoms into water instead of producing ROS.

The elevation in ROS levels with high AA/DHA ratios can be attributed to the inhibition of complex I and III via these ratios where the concentration of AA was higher and imbalanced with DHA. As it is known that (AA) acts as a strong inhibitor of complexes I and III of the respiratory chain (Cocco et al., 1999; Dymkowska et al 2006). In addition, the inhibition of these complexes prevents the flow of electrons through the respiratory chain. Consequently, electron leak from complex I and III will reduce the mitochondrial membrane potential and promote ROS production. This in turn increases oxidative stress and damages cell membranes by inducing the production of certain lipid diffusible molecules like malondialdehyde and 4-hydroxynonenal. These findings are in agreement with Gyamfi et al. (2012) where the production of ROS increased in VA-13 cells with AA. The researchers suggested that electron leakage from complex I caused this elevation in ROS production. Furthermore, Dymkowska et al. (2006) reported that AA, PA and oleic acid induced ROS production in rat liver AS-30D cells. They

demonstrated this can be as a result of the inhibition of complex I and III in the ETC via these fatty acids.

When inducing steatosis in HepG2 cells using high concentrations of PA and oleic acid, these conditions led to an increase in ROS production and a decline in ATP production (Zhang et al., 2011). Interestingly, when treating these steatotic cells with high concentrations of DHA or EPA omega-3 fatty acids, ROS production decreased and ATP production increased. On the other hand, in this study, DHA concentration was low and imbalanced with AA at high omega ratios. This in turn can explain the ROS increase and the reduction in ATP production. In addition, lipid accumulation normally accompanies an increase in the beta-oxidation of fatty acids. This in turn increases the production of acetyl coenzyme A (acetyl-coA) and its metabolism in the tricarboxylic acid (TCA) cycle. Consequently, this also increases the delivery of substrates like reduced nicotinamide adenine dinucleotide (NADH) to the ETC. The increase of these substrates stimulates the production of ROS. This can then be one of the mechanisms leading to an upsurge in ROS in this study with high AA/DHA ratios (Gusdon et al., 2014).

Studies have shown that the CYP2E1 enzyme seems to be involved in the pathogenesis of NAFLD. The enzyme has a role in the hydroxylation of omega fatty acids, and a high-fat diet can also induce it (Daly, 2013; Abdelmegeed et al., 2015). Research has also revealed that the CYP2E1 isoform is not only expressed in the ER but also in the mitochondria. Moreover, CYP2E1 is overexpressed in rat hepatocytes and patients with steatohepatitis (Weltman, et al. 1996; 1998; Abdelmegeed et al., 2015). This can be a possible cause of the increase in ROS production in this study with high AA/DHA ratios. The induction of CYP2E1 via a high-fat diet or alcohol increases the level of ROS and reactive nitrogen species like nitric oxide (NO). This in turn causes mitochondrial damage

by stimulating the peroxidation of the mitochondrial lipid membrane. Furthermore, it damages mitochondrial DNA and consequently components of the ETC. Ultimately, this causes mitochondrial dysfunction (Aubert et al., 2011; Abdelmegeed et al., 2015). However, other studies did not agree with this notion and report that there is no evidence of the contribution of CYP2E1 in the pathogenies of NAFLD. Thus, whether this enzyme intervenes in NAFLD pathogenesis for certain is still unclear (Daly, 2013).

Altogether, AA/DHA ratios caused mitochondrial dysfunction and reduced the production of ATP, which an increase in the production of ROS also accomplished. These findings suggest that high AA/DHA ratios caused a disturbance in the ETC by increasing the permeability of the inner mitochondrial membrane and the production of ROS. Consequently, this caused mitochondrial uncoupling and ended up causing mitochondrial dysfunction.

4.8 Effect of AA/DHA ratios in the presence of alcohol on cell viability and ROS production

To recap, this study assessed the effect of AA/DHA ratios on lipid metabolism, mitochondrial function and ROS production. The misuse of alcohol also plays a role in the development of fatty liver. Thus, it was also interesting to determine the impact of these ratios in the presence of alcohol on cell viability and ROS production. However, prior to the performance of these investigations, this study sought to understand the effect of alcohol when mixed with AA. Thus, the impact of an AA (40 µM) and alcohol (100 mM) mixture alone on cytotoxicity and lipid accumulation was evaluated. Findings showed that an AA/alcohol ratio during the first 6–24 h did not affect the viability of the cells nor lipid accumulation. However, after 48 and 72 h, there was a pronounced decrease in the viability of the cells

accompanying a slight reduction in lipid accumulation (Figure 3.16). These findings had a similar pattern of change when exposing the cells to AA alone, because treating the cells with alcohol only did not show any change in both parameters (Figure. 3.16).

This contrasts with Huang, et al. (2013), where they induced steatosis in mice with acute alcohol administration. This suggests the reduction in cell viability is most probably due to the effect of AA and not alcohol. As mentioned above, alcohol was not able to induce any cell toxicity or lipid accumulation. This may result from the fact that VL-17A cells overexpress CYP2E1 and alcohol dehydrogenase (ADH) enzymes. Both play roles in alcohol oxidation, and this may explain the rapid oxidation of alcohol. However, the proinflammatory effect of AA eicosanoids may affect this study's findings as stated in this chapter (section 4.2). These findings to some extent are consistent with Gyamfi et al. (2012). This study found both alcohol and AA alone caused a loss in mitochondrial membrane potential with a reduction in ATP production and an increase in lipid accumulation. Treating VA-13 cells with AA in the presence of alcohol (100 mM) further enhanced this effect. However, the difference in results could be because VA-13 cells overexpress the ADH enzyme only.

A study that Vatsalya et al. (2016) conducted on an alcoholic patients confirmed liver injury via high alanine transaminase (ALT) and aspartate transaminase (AST) levels. At the same time, they also measured the fatty acid profile, which showed an increase in the omega 6/3 ratio. This study sought to evaluate the effect of AA/DHA ratios in the presence of alcohol on cell viability. Thus, VL-17A cells were treated with alcohol and different AA/DHA ratios ranging from 1:1 to 25:1 for 24 h. No change in cell viability occurred when compared to controls. However, high AA/DHA ratios in the presence of alcohol caused a marked

reduction in cell viability when compared to 1:1 and 4:1 ratios in the presence of alcohol (Figure 3.17). On the other hand, greater lipotoxicity resulted after exposing VL-17A cells to the same AA/DHA ratios without alcohol.

Nevertheless, after investigating the effect of the same treatments on ROS production, it did not occur in the first 30 min to 1 h. Yet after 2 h, 15:1 and 25:1 ratios showed a significant increase in ROS (Figures 3.18 and 3.19). The excessive and prolonged consumption of alcohol is highly correlated with the onset of alcoholic liver disease (ALD). Chapter 1 (section 1.7) outlined some mechanisms in which alcohol can increase the level of ROS. Briefly, chronic alcohol consumption and high alcohol concentrations can induce the CYP2E1 enzyme in the endoplasmic reticulum to oxidise alcohol to acetaldehyde. ROS production accompanies this step, causing endoplasmic reticulum (ER) stress.

In addition, the cytosol mainly oxidises alcohol via the ADH enzyme into acetaldehyde. In turn, nicotinamide adenine dinucleotide (NAD) reduction to NADH accompanies this. Aldehyde dehydrogenase (ALDH2) then further oxidises acetaldehyde into acetate in the mitochondria with NAD reduction to NADH again. NADH will then be oxidised in the ETC, and the excessive production of NADH leads to an increase in the NADH:NAD ratio. This plays a role in the MPT, which in turn causes mitochondrial swelling leading to mitochondrial dysfunction. An elevated NADH:NAD ratio in the mitochondria also leads to ETC disturbance and increases electron leakage in complex I and III. Consequently, this increases ROS production, which in turn elevates the level of oxidative stress in the mitochondria (Akie et al., 2015).

In summary, whether this rise in ROS levels results from the effect of either AA/DHA or alcohol or as a result of both is still unclear. However, earlier this chapter (section 4.7) outlined the reason behind the increase in ROS production

via AA/DHA ratios. This study suggests that this effect is due to mitochondrial dysfunction. The increase in ROS levels may also result from the effect of alcohol via the influence of its metabolites in the ETC. Subsequently, this leads to mitochondrial dysfunction. Despite the effect of alcohol (100 mM), evaluating ROS production did not show any change at any time. Alcohol findings are not consistent with Bailey et al. (1999) and Stiuso et al (2016). In these studies, ethanol increased ROS production and reduced cell viability in rat hepatocytes and HepG2 cells. These data indicate that the increase in ROS levels in this study may result from high AA:DHA ratios and not due to the effect of alcohol. This may be because CYP2E1 and ADH enzymes in VL-17A cells rapidly metabolised the alcohol. In addition, the increase in ROS levels may be due to the activation of CYP2E1 via the high AA:DHA ratios as indicated in this chapter (4.7). A high-fat diet can be one factor inducing CYP2E1. Therefore, further work using different cell types, like VA-13, may elucidate the influence of AA:DHA ratios in the presence of alcohol in ALD development. Finally, no studies use differing ratios of omega 6/3 in the presence of alcohol studying mitochondrial function.

4.9 Chronic alcohol consumption and pro-inflammatory cytokines

ALD has a broad spectrum of liver injury ranging from liver steatosis to steatohepatitis. Inflammation characterises the latter, which can then develop to fibrosis and cirrhosis (Neuman, et al., 2015). Studies show that elevated levels of the pro-inflammatory cytokine TNF-α is a consequence of chronic alcohol consumption, which plays a crucial role in alcoholic hepatitis (Arteel, 2003; Neuman, et al., 2015). Excessive alcohol consumption induces the translocation of gut endotoxins to portal circulation by increasing gut permeability. This translocation occurs due to the intestinal epithelium damage resulting from

alcohol metabolites such as acetaldehyde (Jampana and Khan, 2011; Hartmann et al., 2012; Chassaing et al., 2014). This in turn increases the level of the bacterial products, lipopolysaccharides (LPS), in circulation and thus elevates the exposure of the liver to LPS. This exposure will then induce the activation of toll-like receptor 4 (TLR4), a vital constituent of the innate immune system. In turn, this will stimulate the activation of inflammatory cells like Kupffer cells (Jampana and Khan, 2011). The activation of Kupffer cells consequently induces liver inflammation and fibrosis by increasing the release of TNF-α (Kawaratani et al., 2017).

This study evaluated hepatic injury by measuring the concentration of the pro-inflammatory cytokine (TNFα) and some biomarkers of liver damage. The results showed an increase in the levels of all liver enzymes AST, ALT, ALP and GGT as well as the concentration of TNF-α (Figures 3.20 and 3.21). These findings agree with Das and Vasudevan (2005) in which alkaline phosphatise (ALP), AST, ALT and gamma-glutamyl transferase (GGT) were high in ALD patients. Other studies have also reported similar findings with elevated serum levels of TNFα, ALT and AST in rats exposed to binge alcohol for 16 weeks (Zhou et al., 2013) or a high-alcohol liquid diet (Peng et al. 2012). In summary, these studies demonstrated the relation between chronic alcohol consumption and its contribution to liver injury. Moreover, binge alcohol drinking also corresponds to elevated concentrations of liver enzymes and pro-inflammatory cytokines. The correlation of these markers with indices of steatosis and fibrosis would aid the usefulness of these liver markers in ALD pathogenesis.

Chapter 5

Conclusion and Future works

5.1 Conclusion

Fatty liver disease is one of the most prevalent diseases worldwide and can be caused by either excessive alcohol consumption (60 g/day) or a high dietary ratio (>15:1) of the omega 6 and 3 fatty acids. The development of fatty liver disease can lead onto hepatitis, fibrosis and cirrhosis. In NAFLD progression to end stage cirrhosis is also highly associated with hepatic carcinoma, the 4th most commonest cancer. Thus, understanding the early mechanisms in fatty liver disease is crucial to elucidate improved treatment modalities as well as preventing disease progression. The main objectives of this project were therefore to determine the effect of fatty acids on cell viability, lipid accumulation and mitochondrial function.

It was revealed in this study that omega 6 fatty acids have a deleterious impact on the liver cell. This was indicated by the higher reduction in the viability of the cells with high arachidonic acid (AA) concentrations (30 and 80 µM) especially at the 72h time point (Figure 3.4) than with linoleic acid (LA). This reduction with AA was accompanied by a significant increase in lipid accumulation at 48 h, which can be attributed to the high toxicity of AA possibly due to the effect of its proinflammatory eicosanoids (Patterson *et al*., 2012**;** Kirack *et al*., 2015; Maciejewska et al., 2015). Also, high level of lipid accumulation itself may lead to ER stress and cell death (Wang et al., 2006).

On the other hand, individual omega 3 fatty acids alpha linolenic acid (ALA) and docosahexaenoic acid (DHA) did not cause any change in either cell viability or lipid accumulation. This is because of their protective capability in reducing oxidative stress, attenuating the activity of lipogenic pathways such as SCD1 and SREBP as well as activating transcriptional factors involved in lipid oxidation such as PPARα. Several studies suggest a correlation between increased omega 6/3

ratio and the development of NAFLD (Araya et al., 2004; Patterson et al., 2012). Our findings are not entirely agreeable to other works whereby LA/ALA did not cause hepatic steatosis, reflecting the different experimental conditions or the need to increase the concentration of omega 6 in the ratios. However, AA/DHA ratio showed a greater impact with high ratios (15:1-25:1) on liver steatosis and cytotoxicity (Figure 3.8) which is consistent to other work (Araya et al., 2004; Puri et al., 2007; Enos et al., 2015).

It is known that the dysregulation of lipid metabolism in the liver plays a crucial role in the development of liver steatosis and which is a precursor to steatohepatitis. The imbalance of AA/DHA ratio and the low concentration of omega 3 (DHA) could be the reason behind the increased concentration of TG in this study with high AA/DHA ratios (15:1 and 25:1), since low DHA and high AA concentrations affects the oxidation of fatty acids and possible decreased export of TG from liver cells shifting the pathway toward TG synthesis.

In agreement with the above statement, in the present study the increase in lipid accumulation appeared to correlate with the expression of transcriptional factors involved in fat metabolism. Expression of SCD1, an enzyme involved in lipogenesis, but not SREBP1 was up-regulated with high AA/DHA ratios. This in turn, revealed that SCD1 is possibly not regulated by the activation of SREBP and more likely is up-regulated by its direct binding to LXR. High omega ratios were also able to down regulate the expression of PPARα and consequently the oxidation of fatty acids which in turn, gives arise to steatosis supporting the role of lipid dysregulation due to the imbalance in omega AA/DHA ratios.

It is also thought that high dietary fat affects the cannabinoid system and consequently, stimulates lipid synthesis by regulating the expression of key transcriptional genes involved in lipid metabolism. High AA/DHA ratios (15:1 and 25:1) was capable to induce hepatic steatosis and slightly increase the expression of CB1 although this did not achieve statistical significance, which possibly can be attributed to the short incubation time. Although CB2 receptor expression did not show any increase with all ratios relative to the control, expression of CB2 with 15:1 and 25:1 ratios were significantly higher than lower ratios (1:1). This result requires further investigation but does suggest some upregulation in CB2 expression but perhaps due to the short exposure time, is not sufficient to induce liver injury as it is believed that CB2 expression normally increases in advanced stages such as fibrosis and cirrhosis. However, what had been obtained here is consistence with the SCD1 and PPARα data in which high ratios induced lipid synthesis and stimulated the development of steatosis.

Liver injury is highly correlated with a high calorific diet, free fatty acid delivery to the liver and hepatic steatosis. High AA/DHA ratios were correlated with the induction of liver injury and mitochondrial dysfunction (Figure 3.14). The disturbance in mitochondrial function was represented by the low respiratory rate, decrease in ATP production, along with a reduction in the proton leak as well as an increase in ROS production. The impairment of mitochondrial oxidative respiration reduced the ability of the cell to achieve its maximal respiration and to respond to increased energy demand. The increase in ROS production and mitochondrial dysfunction could possibly come from electron leakage due to the destabilisation of cytochrome c by fatty acids in the ETC and consequently reduces the flow of the electron along the respiratory chain causing increased

release of electrons from the mitochondrial membrane. Also, it could be due to the ability of the fatty acids to increase the permeability of the mitochondrial membrane causing a reduction in the mitochondrial membrane potential, uncoupling which subsequently leads to mitochondrial swelling and a reduction in the production of ATP as it was indicated in this study. In addition, the increase in ROS production resulted as a consequence of mitochondrial dysfunction and the inhibition of complex I and III by AA in which the leakage of the electrons was increased and consequently the production of ROS (Figure 3.12A,B).

There is limited information on the interaction between alcohol and fatty acids. It is possible that some individuals are consuming high amounts of alcohol in addition to a high calorific diet, making them susceptible to higher degrees of liver injury due to the combined damaging effects. However, when alcohol was combined for 24 hr with increasing AA/DHA ratios no effect on cell viability was observed. Despite no increase in cell death there was a marked increase in the level of ROS with high AA/DHA ratios (15:1 and 25:1) in the presence of alcohol. This possibly was caused by the induction of CYP2E1 by the high fatty acid treatment producing ROS, and not alcohol as the latter alone did not show any effect on ROS. Furthermore, alcohol can be metabolised rapidly by both CYP2E1 and ADH enzymes reducing excess NADH production via ADH and thus limiting mitochondrial ROS production. Therefore, it seems that high AA/DHA ratios in the presence of alcohol induces CYP2E1 and consequently leads to increase the production of ROS causing mitochondrial dysfunction. Thus, these initial studies require further investigation to explore the possible combined effect of alcohol and fatty acids. It is possible that higher alcohol concentrations and longer exposure times are required to provide a more accurate information of this

interaction. Finally, many of the pathological mechanisms that occur with NAFLD also occur in ALD, such as gut permeability injury. Here it was observed that chronic alcohol consumption is highly correlated to the elevated level of liver injury markers as well as the pro-inflammatory cytokine, TNFα in ALD patients. It is possible that similar effects would be observed in NAFLD patients due to high AA/DHA ratios.

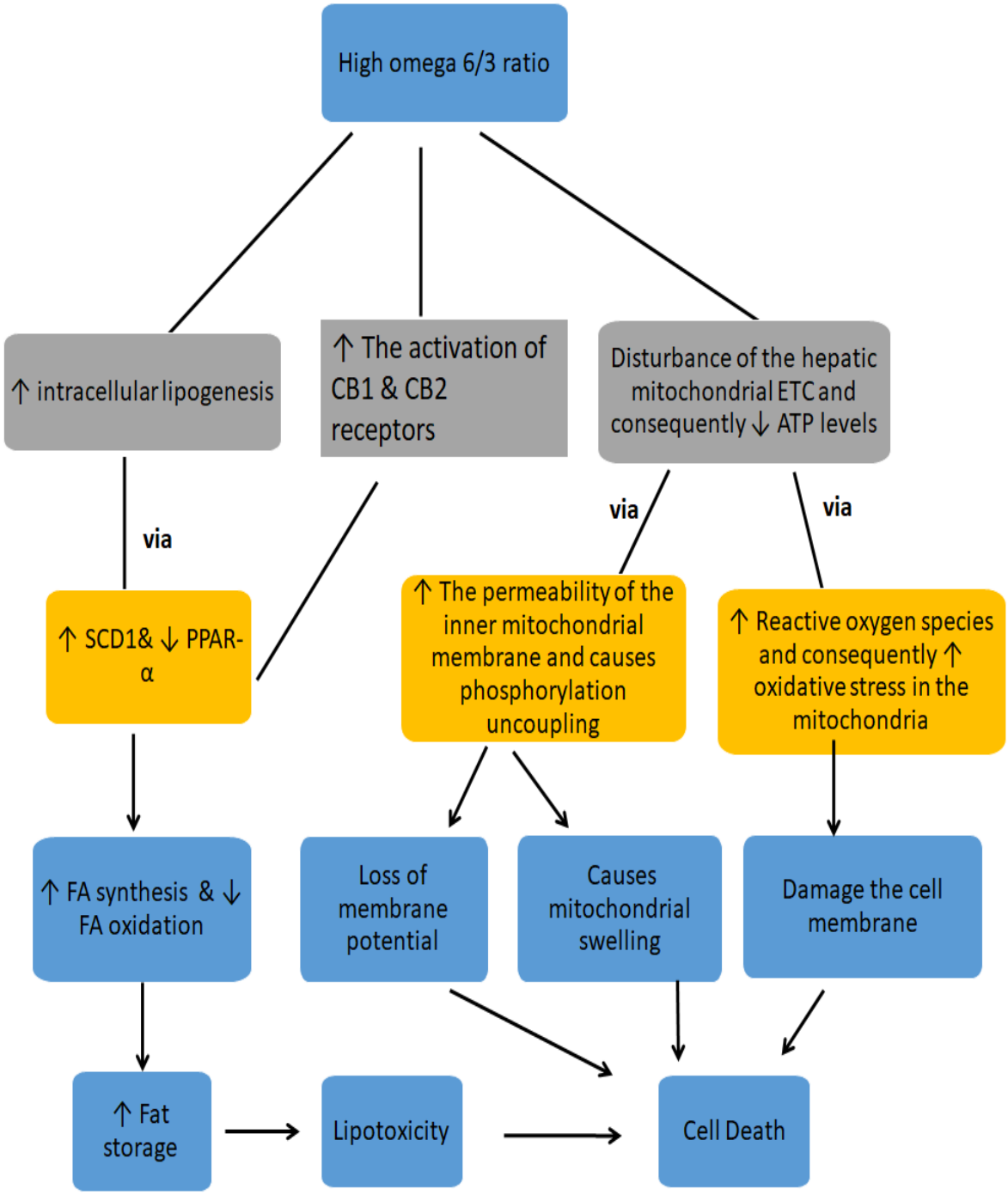

Figure 5.1: Pathways affected by high omega 6/3 ratio.

5.2 Future work

It has been demonstrated that saturated fatty acids PA and SA caused a reduction in cell viability whereas only PA showed an increase in lipid accumulation. Whilst this demonstrates the effect of saturated fatty acids, further studies are needed to investigate the effect of saturated fatty acids as a ratio with unsaturated fatty acids on cell toxicity and lipid accumulation. This can be also achieved by evaluating the expression of key transcriptional factors involved in lipid metabolism. These studies will provide further insight into the role of fatty acids in NAFLD.

With reference to the effect of omega fatty acids, it was illustrated that omega 6 AA was more toxic than LA, which can be due to its immediate pro-inflammatory mediators through the degradation of these fatty acids by lipoxygenase (LOX) and cyclo-oxygenase (COX) for AA and only LOX for LA. To further investigate the toxicity of these fatty acids, it would be helpful to evaluate the concentration of eicosanoids produced from AA and LA as well as the activity of LOX enzyme which is involved in metabolising AA and LA fatty acids. Data from omega 3, ALA and DHA fatty acids revealed the protective property of this type of fatty acids, as both ALA and DHA did not cause any lipotoxicity or lipid accumulation. This in turn, calls for a further work to study the effect of individual omega 3 fatty acids in NAFLD, their effect on the antioxidant-like nitric oxide (NO) and on the expression of transcriptional factors involved in lipid oxidation such as PPARα or its target enzymes, carnitine palmitoyltransferase-1.

As mentioned in the section on the effect of increased omega 6/3 ratio on cell viability, LA/ ALA ratios were not capable to induce cytotoxicity or increase the

accumulation of lipid which can be due to the low concentration of LA being used, since high concentrations of Individual LA (200 and 300 μM) was capable to induce lipotoxcity. This in turn, points to the need of increasing the concentration of LA in the LA/ALA ratios to show whether these ratios will induce any cytotoxicity or not. The capability of high AA/DHA (15:1 and 25:1) ratios to reduce cell viability occurs possibly due to inflammatory mediators of AA. Further work measuring these key proinflammatory mediators is required.

To further investigate lipid accumulation due to AA/DHA treatment, studies by Western blotting should investigate whether these ratios decrease the expression of vital enzymes involved in fatty acid oxidation such as carnitine palmitoyltransferase-1 or increase the expression of key enzymes involved in lipogenesis like Acetyl-CoA carboxylase or fatty acid synthase. It is also important to evaluate the expression of LXR as it is correlated to the activation of SREBP-1c. Moreover, AA/DHA ratios did not cause any change in the expression of SREBP1. Therefore, using another method other than western blotting to evaluate the expression of SREBP1c can possibly show the effect of the increased AA/DHA ratios on SREBP1c. These studies as well as evaluating the expression of endocannabinoids (2-arachidonoylglycerol (2-AG) and archidonoylethanolamide (anandamide) and inflammatory or fibrogenic markers will also help in clarifying the impact of these ratios on the activation of CB1 and CB2 receptors.

In regard to the effect of high AA/DHA ratios on mitochondrial function and ROS production. The findings of the current study showed a significant reduction in mitochondrial function with an increase in the production of ROS. A study looking

at the mitochondrial membrane potential by flow cytometry using rhodamine123 would also illuminate the potential impact of these ratios on mitochondrial function. Also, investigating the expression of CYP2E1 would be valuable in understanding the role of this enzyme in increasing the production of ROS with high AA/DHA ratios.

High AA/DHA ratios data in the presence of alcohol indicated a significant increase in the level of ROS and caused cellular cytotoxicity. However, these results could be as an effect of high AA/DHA ratio and not alcohol (100 mM) as the concentration of alcohol may be low and needs to be increased and further experimental investigations on the impact of these treatments using either different type of cells or higher alcohol concentration would be useful. The results of this study are encouraging and should be also validated by investigating the impact of AA/DHA ratios in the presence of alcohol on mitochondrial function and on the expression of fundamental enzymes involved in alcohol metabolism like CYP2E1, alcohol dehydrogenase or aldehyde dehydrogenase, to further elucidate mechanisms of alcoholic fatty liver development and prevention.

References:

Abdelmegeed, M., Ha, S., Choi, Y., Akbar, M., Song, J. (2015). Role of CYP2E1 in mitochondrial dysfunction and hepatic tissue injury in alcoholic and non-alcoholic diseases. *Current Molecular Pharmacology*.

Acın-Perez, R., Fernandez-Silva, P., Peleato, M., Perez-Martos, A., Enriquez, J. (2008). Respiratory Active Mitochondrial Supercomplexes. *Cell.* **32** (4), 529–539.

Adams, L., Angulo, P., Lindor, K., (2005). Nonalcoholic fatty liver disease. *Canadian Medical Association Journal*.**172** (7), 899–905.

Akazawa, Y., Cazanave, S., Mott, JL., Elmi, N., Bronk, SF., Kohno, S., Charlton, MR., Gores GJ. (2010). Palmitoleate attenuates palmitate-induced Bim and PUMA up-regulation and hepatocyte lipoapoptosis. *Journal of Hepatology.* **52** (4), 586–593.

Akie, T., Liu, L., Nam, M., Lei, S., Cooper, M. (2015). OXPHOS-Mediated Induction of NAD+ Promotes Complete Oxidation of Fatty Acids and Interdicts Non-Alcoholic Fatty Liver Disease. *PLoS One*. **10** (5), e0125617.

Allard, J., Aghdassi, E., Mohammed, S., Raman, M., Avand, G., Arendt, B., Jalali, P., Kandasamy, T., Prayitno, N., Sherman, M., Guindi, M., Ma, D., Heathcote, J. (2008). Nutritional assessment and hepatic fatty acid composition in non-alcoholic fatty liver disease (NAFLD): a cross-sectional study. *Journal of Hepatology*. **48** (2), 300-7.

Alswat, K., (2013). The Role of Endocannabinoids System in Fatty Liver Disease and Therapeutic Potentials. *Saudi Journal of Gastroenterology*.**19** (4), 144-151.

Araya, J., Rodrigo, R., Pettinelli, P., Araya, AV., Poniachik, J., Videla, L. (2010). Decreased liver fatty acid delta- 6 and delta- 5 desaturase activity in obese patients. *Obesity (Silver Spring).* **18** (7), 1460-3.

Araya, J., Rodrigo, R., Videla, L., Thielemann, L., Orellana, M., Pettinelli, P., Poniachik, J. (2004). Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clinical Science (London, England: 1979).* **106** (6), 635-43.

Arteel, G. (2003). Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology.* **124** (3), 778–790.

Arteel, G., Marsano, L., Mendez, C., Bentley, F., McClain, C. (2003). Advances in alcoholic liver disease. *Best Practice & Research Clinical Gastroenterology.* **17** (4), 625-647.

Aubert, J., Begriche, K., Knockaert, L., Robin, M., Fromenty, B. (2011). Increased expression of cytochrome P450 2E1 in non-alcoholic fatty liver disease: mechanisms and pathophysiological role. *Clinics and Research in Hepatology and Gastroenterology*. **35** (10), 630–7.

Bae, J., Park, J., Lee, J., Oh, B., Jang, S., Lee, Y., Han, Y., Ock, C., Cha, J., Hahm, K. (2017). Amelioration of non-alcoholic fatty liver disease with NPC1L1-targeted IgY or n-3 polyunsaturated fatty acids in mice. *Metabolism*. **66**, 32-44.

Bailey, S. and Cunningham, C., (2002). Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radial Biology and Medicine*. **32** (1), 11–16.

Bailey, S., Pietsch, E., Cunningham, C. (1999). Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III. *Free Radical Biology and Medicine*. **27** (7-8), 891–900.

Balaban, R., Nemoto, S., Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell*. **120** (4), 483-495.

Begriche, K., Igoudjil, A., Pessayre, D., Fromenty, B. (2006). Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion*. **6** (1), 1-28.

Berger, J., Moller, D. (2002). The mechanisms of action of PPARs. *Annual Review of Medicine*. **53**, 409–435.

Blednov, Y., Black, M., Benavidez, J., Stamatakis, E., Harris, R. (2016). PPAR Agonists: I. Role of Receptor Subunits in Alcohol Consumption in Male and Female Mice. *Alcoholism: Clinical and Experimental Research.* **40** (3), 553-62.

Borradaile, N., Han, X., Harp, J., Gale, S., Ory, D., Schaffer, J. (2006). Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *Journal of Lipid research*. **47** (12), 2726-37.

Boveris, A., Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochemical Journal.* **134** (3), 707-716.

British Medical Association Board of Science (2008). Alcohol misuse: tackling the UK epidemic. London. British Medical Association.

Caballería, J. (2003). Current concepts in alcohol metabolism. Annals of Hepatology. **2** (2), 60-68.

Calder, P. (2008). Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases. *Molecular Nutrition and Food Research.* **52** (8), 885–897.

Calder, P. (2010). Omega-3 fatty acids and inflammatory processes, *Nutriens*. **2** (3), 355–374.

Candela, C., Lopez, L., Kohen, V. (2011). Importance of a balanced omega 6/omega 3 ratio for the maintenance of health. *Clinical Nutrition and Dietetics Unit*. **26** (2), 323-329.

Caputo, M., De Rosa, M., Rescigno, T., Zirpoli, H., Vassallo, A., De Tommasi, N., Torino, G., Tecce, M. (2014). Binding of polyunsaturated fatty acids to LXRα and modulation of SREBP- 1 interaction with a specific SCD1 promoter element. *Cell Biochemistry and Function.* **32** (8), 637-46.

Caro, A., Cederbaum, A. (2007). Role of intracellular calcium and phospholipase A2 in arachidonic acid-induced toxicity in liver cells over expressing CYP2E1. *Archives of Biochemistry and Biophysics*. **457** (2), 252–263.

Ceni, E., Mello, T., Galli, A. (2014). Pathogenesis of alcoholic liver disease: role of oxidative metabolism. *World Journal of Gastroenterology*. **20** (47), 17756-72.

Chakravarty, K., Wu, SY., Chiang, C., Samols, D., Hanson, R. (2004). SREBP-1c and Sp1 Interact to Regulate Transcription of the Gene for Phosphoenolpyruvate Carboxykinase (GTP) in the Liver. *The journal of biological chemistry*. **279** (15), 15385–15395.

Chandrasekaran, K., Swaminathan, K., Kumar, S.M., Chatterjee, S., Clemens, D.L., Dey, A. (2011). Elevated glutathione level does not protect against chronic alcohol mediated apoptosis in recombinant human hepatoma cell line VL-17A over-expressing alcohol metabolizing enzymes – Alcohol dehydrogenase and Cytochrome P450 2E1. *Toxicology in Vitro.* **25** (4), 969-978.

Charlton, M., Burns, J., Pedersen, R., Watt, K., Heimbach, J., Dierkhising, R. (2011). Frequency and Outcomes of Liver Transplantation for Nonalcoholic Steatohepatitis in the United States. *Gastroenterology.* **141** (4), 1249-1253.

Chassaing B., Etienne-Mesmin, L., Gewirtz, A. (2014). Microbiota-liver axis in hepatic disease. *Hepatology*. **59** (1), 328–339.

Chigurupati, S., Dhanaraj, S., Balakumar, P. (2015). A step ahead of PPARγ full agonists to PPARγ partial agonists: therapeutic perspectives in the management of diabetic insulin resistance. *European Journal of Pharmacology*. 755, 50–57.

Chu, K., Miyazaki, M., Man, W., Ntambi, J. (2006). Stearoyl-coenzyme A desaturase 1 deficiency protects against hypertriglyceridemia and increases plasma high density lipoprotein cholesterol induced by liver X receptor activation. *Molecular and Cellular Biology*. **26** (18), 6786–6798.

Chuturgoon, A., Phulukdaree, A., Moodley, D. (2015). Fumonisin B1 inhibits apoptosis in HepG2 cells by inducing Birc- 8/ILP. *Toxicolgy Letters*. **235** (2), 67-74.

Chuu, C., Kokontis, J., Hiipakka, R., Liao, S. (2007). Modulation of liver X receptor signaling as novel therapy for prostate cancer. *Journal of Biomedical Sciences*. **14** (5), 543-53.

Cocco, T., Di Paola, M., Papa, S., Lorusso, M. (1999). Arachidonic acid interaction with the mitochondrial electron transport chain promotes reactive oxygen species generation. *Free Radical Biology and Medicine*. **27** (1-2), 51-9.

Cohen, J., Horton, J., Hobbs, H. (2011). Human fatty liver disease: old questions and new insights. *Science*. **332** (6037), 1519-23.

Cunningham, C., Bailey, M. (2001). Ethanol Consumption and Liver Mitochondria Function. *Biological Signals and Receptors*. **10** (3-4). 271–282.

Daly, A. (2013). Relevance of CYP2E1 to non-alcoholic fatty liver disease. *Sub cellular Biochemistry.* **67**,165-75.

Das, S., Vasudevan, D. (2005). Biochemical diagnosis of alcoholism. *Indian Journal of Clinical Biochemistry*. **20** (1), 35–42.

Day, C., James, O. (1998). Steatohepatitis: a tale of two "hits"?. *Gastroenterology*. **114** (4), 842-5.

De Minicis, S., Brenner, D. (2007). NOX in liver fibrosis. *Archives of Biochemistry and Biophysics*. **462** (2), 266-72.

DeBose-Boyd, R., Ou, J., Goldstein, J., Brown, M. (2001). Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proceedings of the National Academy of sciences of U S A***. 98** (4), 1477-82.

Deleuran, T., Grønbaek, H., Vilstrup, H., Jepsen, P. (2012). Cirrhosis and mortality risks of biopsy-verified alcoholic pure steatosis and steatohepatitis: a nationwide registry-based study. *Alimentary Pharmacology and Therapeutics.* **35** (11), 1336-1342.

Della Corte, C., Carpino, G., De Vito, R., De Stefanis, C., Alisi, A., Cianfarani, S., Overi, D., Mosca, A., Stronati, L., Cucchiara, S., Raponi, M., Gaudio, E., Byrne, C., Nobili, V. (2016). Docosahexanoic Acid Plus Vitamin D Treatment Improves Features of NAFLD in Children with Serum Vitamin D Deficiency: Results from a Single Centre Trial. *PLoS One.* **11** (12), e0168216.

Depner, C., Philbrick, K., Jump, D. (2013). Docosahexaenoic acid attenuates hepatic inflammation, oxidative stress, and fibrosis without decreasing hepatosteatosis in a Ldlr(-/-) mouse model of western diet-induced nonalcoholic steatohepatitis. *The Journal of Nutrition*. **143** (3), 315-23.

DeSantis, D., Ko, C., Liu, Y., Liu, X., Hise, A., Nunez, G., Croniger, C. (2013). Alcohol-induced liver injury is modulated by Nlrp3 and Nlrc4 inflammasomes in mice. *Mediators of Inflammation*. 2013: 751374.

Deveaux, V., Cadoudal, T., Ichigotani, Y., Teixeira-Clerc, F., Louvet, A., Manin, S., Nhieu, J., Belot, M., Zimmer, A., Even, P., Cani, P., Knauf, C., Burcelin, R.,

Bertola, A., Le Marchand-Brustel, Y., Gual, P., Mallat, A., Lotersztajn, S. (2009). Cannabinoid CB2 receptor potentiates obesity-associated inflammation, insulin resistance and hepatic steatosis. *PLoS One*. **4** (6), e5844.

Donohue, T., Osnaa, N., Clemens, D. (2006). Recombinant HepG2 cells that express alcohol dehydrogenase and cytochrome P450 2E1 as a model of alcohol-elicited cytotoxicity. *The International Journal of Biochemistry and cell Biology*. **38** (1), 92-101.

Dossi, C., Tapia, G., Espinosa, A., Videla, L., D'Espessailles, A. (2014). Reversal of high-fat diet-induced hepatic steatosis by n-3 LCPUFA: role of PPAR-α and SREBP-1c. *The Journal of Nutritional Biochemistry*. **25** (9), 977-84.

Dowman, J., Tomlinson, J., Newsome, P. (2010). Pathogenesis of non-alcoholic fatty liver disease. *An International Journal of Medicine.* **103** (2), 71-83.

Dymkowska, D., Szczepanowska, J., Wieckowski, M., Wojtczak, L. (2006). Short term and long-term effects of fatty acids in rat hepatoma AS-30D cells: the way to apoptosis. *Biochimica et Biophysica Acta.* **1763** (2),152-63.

Dyson, J., Anstee, Q., McPherson, S. (2014). Non-alcoholic fatty liver disease: a practical approach to diagnosis and staging. *Frontline Gastroenterology*. **5** (3), 211-218.

El-Badry, A., Graf, R., Clavien, P. (2007). Omega 3 – Omega 6: What is right for the liver?. *Journal of Hepatology*. **47** (5), 718-25.

Elhardallou, S., Elawad, A., Khairi, N., Gobouri, A., Dhahawi, H. (2014). A Review on Omega-3 and Omega-6 Essential Fatty Acids: Uses, Benefits and their Availability in Pumpkins (Cucurbita maxima) Seed and Desert Dates (Balanites aegyptiaca) Seed Kernel Oils. *Pakistan Journal of Biological Sciences*. **17** (12),1195-208.

Enos, R., Velázquez, K., McClellan, J., Cranford, T., Walla, M., Murphy, E. (2015). Lowering the dietary omega-6: omega-3 does not hinder nonalcoholic fatty-liver disease development in a murine model. *Nutrition Research*. **35** (5), 449-59.

Farrell, G., Larter, C. (2006). Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology*. **43** (2 Suppl 1), S99–S112.

Feldstein, A., Lopez, R., Tamimi, T., Yerian, L., Chung, Y., Berk, M., Zhang, R., McIntyre, T., Hazen, S. (2010). Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, *Journal of Lipid Research*. **51** (10), 3046–3054.

Fromenty, B., Robin, M., Igoudjil, A., Mansouri, A., Pessayre, D., (2004). The ins and outs of mitochondrial dysfunction in NASH. *Diabetes & Metabolism*. **30** (2), 121-138.

Gambino, R., Bugianesi, E., Rosso, C., Mezzabotta, L., Pinach, S., Alemanno, N., Saba, F., Cassader, M. (2016). Different Serum Free Fatty Acid Profiles in NAFLD Subjects and Healthy Controls after Oral Fat Load. *International Journal of Molecular Sciences*. **17** (4), 479.

Gao, B., Bataller, R. (2011). Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology.**141** (5), 1572-85.

Gentile, C., Pagliassotti, M. (2008). The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *National Institute of Health Public Access*. **19** (9), 567–576.

Goldstein, S., Czapski, G., Lind, J., Merènyl, G. (2000). Tyrosin nitration by simultaneous generation of NO and O2- under physiological conditions. How the radicals do the job. *The Journal of Biological Chemistry*. **275** (5), 3031–3036.

Gormaz, J., Rodrigo, R., Videla, L., Beems, M. (2010). Biosynthesis and bioavailability of long-chain polyunsaturated fatty acids in non-alcoholic fatty liver disease. *Progress in Lipid Research*. **49** (4), 407–419.

Grygiel-Górniak, B. (2014). Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications-a review. *Nutrition Journal.* **14**, 13-17.

Guo, H., Li, D., Ling, W., Feng, X., Xia, M. (2011). Anthocyanin inhibits high glucose-Induced hepatic mtGPAT1 activation and prevents fatty acid synthesis through PKC. J. *Lipid Researches*. **52**, 908–922.

Gusdon, A., Song, K., Qu, S. (2014). Nonalcoholic Fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. *Oxidative Medicine and Cellular Longevity*. 2014;2014:637027.

Gutiérrez-Juárez, R., Pocai, A., Mulas, C., Ono, H., Bhanot, S., Monia, B., Rossetti, L. (2006). Critical role of stearoyl-CoA desaturase–1 (SCD1) in the onset of diet-induced hepatic insulin resistance. *The Journal of Clinical Investigation*. **116** (6),1686-95.

Gyamfi D. and Patel V.B. (2009). Liver metabolism: Biochemical and molecular regulations. In: Preedy VR, Lakshman R, Srirajaskanthan R, Watson RR (ed). Nutrition, diet therapy, and the liver. CRC press, New York, 3-15.

Gyamfi, D., Everitt, H., Tewfik, I., Clemens, D., Patel, V., (2012). Hepatic mitochondrial dysfunction induced by fatty acids and ethanol. *Free Radical Biology and Medicine*. **53** (2012), 2131–2145.

Gyamfi, M., Wan, Y. (2010). Pathogenesis of alcoholic liver disease: the role of nuclear receptors*. Experimental Biology and Medicine*. **235** (5), 547-560.

Halliwell, B., Zhao, K., Whiteman, M. (1999). Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. *Free Radical Research*. **31** (6), 651 – 669.

Hanke, D., Zahradka, P., Mohankumar, S., Clark, J., Taylor, C. (2013). A diet high in α-linolenic acid and monounsaturated fatty acids attenuates hepatic steatosis and alters hepatic phospholipid fatty acid profile in diet-induced obese rats. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. **89** (6), 391-401.

Hartmann, P., Chen, W., Schnabl, B. (2012). The intestinal microbiome and the leaky gut as therapeutic targets in alcoholic liver disease. *Frontiers in Physiology*. 3, 402.

Hodson, L., Bhatia, L., Scorletti, E., Smith, D., Jackson, N., Shojaee-Moradie, F., Umpleby, M., Calder, P., Byrne, C. (2017). Docosahexaenoic acid enrichment in NAFLD is associated with improvements in hepatic metabolism and hepatic insulin sensitivity: a pilot study. European Journal of Clinical Nutrition. doi: 10.1038/ejcn.2017.9.

Huang, L., Wan, J., Wang, B., He, C., Ma, H., Li, T., Kang, J. (2013). Suppression of acute ethanol-induced hepatic steatosis by docosahexaenoic acid is associated with downregulation of stearoyl-CoA desaturase 1 and inflammatory cytokines. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. **88** (5), 347-53.

Ip, E., Farrell, G., Robertson, G., Hall, P., Kirsch, R., Leclercq, I. (2003). Central role of PPARalpha-dependent hepatic lipid turnover in dietary steatohepatitis in mice. *Hepatology*. **38** (1), 123-32.

Jaeschke, H., Gores, G., Cederbaum, A., Hinson, J., Pessayre, D., Lemasters, J. (2002). Mechanisms of hepatotoxicity. *Toxicological* Science. **65** (2),166–176.

Jampana, S., Khan, R. (2011). Pathogenesis of alcoholic hepatitis: Role of inflammatory signaling and oxidative stress. *World Journal of Hepatology*. **3** (5), 114-7.

Ji, C. (2008). Dissection of endoplasmic reticulum stress signalling in alcoholic and non-alcoholic liver injury. *Journal of Gastroenterology and Hepatology*. 1: S16-24.

Julien, B., Grenard, P., Teixeira-Clerc, F., Teixeira-Clerc, F., Van Nhieu, JT., Li, L., Karsak, M., Zimmer, A., Mallat, A., Lotersztajn, S. (2005). Antifibrogenic role of the cannabinoid receptor CB2 in the liver. *Gastroenterology*. **128** (3), 742–55.

Kawaratani, H., Moriya, K., Namisaki, T., Uejima, M., Kitade, M., Takeda, K., Okura, Y., Kaji, K., Takaya, H., Nishimura, N., Sato, S., Sawada, Y., Seki, K., Kubo, T., Mitoro, A., Yamao, J., Yoshiji, H. (2017). Therapeutic strategies for alcoholic liver disease: Focusing on inflammation and fibrosis (Review*). International Journal of Molecular Medicine.* 10.3892/ijmm.2017.3015.

Kelly, J., Scheibling, R. (2012). Fatty acids as dietary tracers in benthic food webs. *Marine Ecology Progress Series.* **446**, 1–22.

Kersten, S., Seydoux, J., Peters, J., Gonzalez, F., Desvergne, B., Wahli, W. (1999). Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *The Journal of Clinical Investigation*. **103** (11),1489–1498.

Kheder, R., Hobkirk, J., Stover, C. (2016). In vitro Modulation of the LPS-Induced Proinflammatory Profile of Hepatocytes and Macrophages- Approaches for Intervention in Obesity?. *Frontiers in Cellular and Developmental Biology*. **4**, 61.

Kim, H., Miyazaki, M., Ntambi, J. (2002). Dietary cholesterol opposes PUFA mediated repression of the stearoyl-CoA desaturase-1 gene by SREBP-1 independent mechanism. *Journal of Lipid Research*. **43** (10), 1750–1757.

Kirack, E., Özcan, F., Tuzcu, H., Elpek, G., Aslan, M. (2015). Analysis of polyunsaturated fatty acids and the omega-6 inflammatory pathway in hepatic ischemia/re-perfusion injury*. Molecular medicine reports*. **12** (3), 4149-56.

Knott, C., Coombs, N., Stamatakis, E., Biddulph, J. (2015). All cause mortality and the case for age specific alcohol consumption guidelines: pooled analyses of up to 10 population based cohorts. The BMJ. 350:h384.

Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W., Schlensak, M., Roden, M. (2015). Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metabolism*. **21** (5), 739–46.

Leamy, A., Egnatchik, R., Young, J. (2013). Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. *Progress in Lipid Research*. **52** (2013), 165–174.

Lemasters, J., Holmuhamedov, L., Czerny, C., Zhong, Z., Maldonado, N. (2012). Regulation of mitochondrial function by voltage dependent anion channels in ethanol metabolism and the Warburg effect. *Biochimica et Biophysica Acta*. **1818** (6),1536-44.

Leung, T., Nieto, N. (2013). CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *Journal of Hepatology*. **58** (2), 395-8.

Leung, T., Rajendran, R., Singh, S., Garva, R., Krstic-Demonacos, M., Demonacos, C. (2013). CytochromeP4502E1 (CYP2E1) regulates the response to oxidative stress and migration of breast cancer cells. *Breast Cancer Research*. **15** (6), R107.

Levy JR, Clore JN, Stevens W. (2004). Dietary n-3 polyunsaturated fatty acids decrease hepatic triglycerides in Fischer 344 rats. *Hepatology*. **39** (3), 608–616.

Liang, G., Yang, J., Horton, J., Hammer, R., Goldstein, J., Brown, M. (2002). Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element binding protein-1c. The *Journal of Biological Chemistry.* **277** (11), 9520–9528.

Maciejewska, D., Ossowski, P., Drozd, A., Ryterska, K., Jamioł-Milc, D., Banaszczak, M., Kaczorowska, M., Sabinicz, A., Raszeja-Wyszomirska, J., Stachowska, E. (2015). Metabolites of arachidonic acid and linoleic acid in early stages of non-alcoholic fatty liver disease--A pilot study. *Prostaglandins and Other Lipid Mediators.* **121** (Pt B),184-9.

Mantena, S., King, A., Andringa, K., Eccleston, H. Bailey, S., (2008). Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol-and obesity-induced fatty liver diseases. *Free Radical Biology and Medicine*. **44** (7), 1259-1272.

Mantena, S., Vaughn, D., Andringa, K., Eccleston,H., King, A., Abrams, G., Doeller, J., Kraus, D., Darley-Usmar, V., Bailey, S. (2009). High fat diet induces dysregulation of hepatic oxygen gradients and mitochondrial function in vivo. *Biochemical journal*. **417** (1), 183–193.

Matsui, H., Yokoyama, T., Sekiguchi, K., Iijima, D., Sunaga, H., Maniwa, M., Ueno, M., Iso, T., Arai, M., Kurabayashi, M. (2012). Stearoyl-CoA Desaturase-1 (SCD1) Augments Saturated Fatty Acid-Induced Lipid Accumulation and Inhibits Apoptosis in Cardiac Myocytes. *Plos One*. **7** (3), e33283.

Matsuzaka, T., Atsumi, A., Matsumori, R., Nie, T., Shinozaki, H., Suzuki-Kemuriyama, N., Kuba, M., Nakagawa, Y., Ishii, K., Shimada, M., Kobayashi, K., Yatoh, S., Takahashi, A., Takekoshi, K., Sone, H., Yahagi, N., Suzuki, H., Murata, S., Nakamuta, M., Yamada, N, Shimano, H. (2012). Elovl6 promotes nonalcoholic steatohepatitis. *Hepatology.* **56** (6), 2199-208.

McCullough, A.J. (2006). Pathophysiology of nonalcoholic steatohepatitis. *Journal of Clinical Gastroenterology.* **40,** S17-S29.

Méndez-Sánchez, N., Almeda-Valdés, P., Uribe, M. (2005). Alcoholic liver disease. An update. *Annals of Hepatology*. **4** (1) 32-42.

Mendez-Sanchez, N., Zamora-Valdes, D., Pichardo-Bahena, R., Barredo-Prieto, B., Ponciano-Rodriguez, G., Bermejo-Martínez, L., Chavez-Tapia, N., Baptista González, H., Uribe, M. (2007). Endocannabinoid receptor CB2 in non-alcoholic fatty liver disease. *Liver International*. **27** (2), 215–9.

Miyazaki, M., Flowers, M., Sampath, H., Chu, K., Otzelberger, C., Liu, X., Ntambi, J. (2007). Hepatic stearoyl-CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. *Cell Metab*olism. **6** (6), 484–96.

Mota, M., Banini, B., Cazanave, S., Sanyal, A. (2016). Molecular mechanisms of lipotoxicity and glucotoxicity in nonalcoholic fatty liver disease. *Metabolism*. **65** (8), 1049-61.

Mullen, E., Brown, R., Osborne, T., Shay, N. (2004). Soy Isoflavones Affect Sterol Regulatory Element Binding Proteins (SREBPs) and SREBP-Regulated Genes in HepG2 Cells. *The Journal of Nutrition.* **134** (11), 2942-7.

Müller, C., Gardemann, A., Keilhoff, G., Peter, D., Wiswedel, I., Kropf, S., Schild, L. (2010). Palmitate protects hepatocytes from oxidative stress and triacylglyceride accumulation by stimulation of nitric oxide synthesis in the presence of high glucose and insulin concentration*. Free Radical Research.* **44** (12), 1425–1434.

Murphy, M. (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal*. **417** (1), 1-13.

Musso, G., Gambino, R., Cassader, M. (2009). Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). Progress in Lipid Research. **48** (1), 1–26.

Nakamura, M., Tang, A., Villanueva, J., Halsted, C., Phinney, S. (1994). Selective reduction of delta 6 and delta 5 desaturase activities but not delta 9

desaturase in micropigs chronically fed ethanol. *The Journal of Clinical Investigation*. **93** (1), 450–454.

Nanji, A., Hiller-Sturmhofel, S. (1997). Apoptosis and necrosis: two types of cell death in alcoholic liver disease. *Alcohol Health Research World*. 21 (4), 325–330.

Nassir, F., Ibdah, J. (2014). Role of mitochondria in alcoholic liver disease. *World Journal of Gastroenterology*. **20** (9), 2136-2142.

Neschen, S., Morino, K., Dong, J., Wang-Fischer, Y., Cline, G., Romanelli, A., Rossbacher, J., Moore, I., Regittnig, W., Munoz, D., Kim, J., Shulman, G. (2007). n-3 Fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner. *Diabetes.* **56** (4), 1034–1041.

Neuman, M., Maor, Y., Nanau, R., Melzer, E., Mell, H., Opris, M., Cohen, L., Malnick, S. (2015). Alcoholic Liver Disease: Role of Cytokines. *Biomolecules*. **5** (3), 2023-34.

Ntambi, J., Miyazaki, M., Dobrzyn, A. (2004). Regulation of stearoyl-CoA desaturase expression. *Lipids.* **39** (11), 1061–1065.

Oh, J., Choi, J., Lee, J., Oh, S., Kim, H., Kim, B., Ma, J., Kim, S. (2012). Effects of palmitic acid on TNF-α-induced cytotoxicity in SK-Hep-1 cells. *Toxicology in Vitro.* **26** (6), 783-790.

Oliveira, C., Coelho, A., Barbeiro, H., Lima, V., Soriano, F., Ribeiro, C., Molan, N., Alves, V., Souza, H., Machado, M., Carrilho, F. (2006). Liver mitochondrial dysfunction and oxidative stress in the pathogenesis of experimental nonalcoholic fatty liver disease. *Brazilian Journal of Medical and Biological research*. **39** (2), 189-94.

Olusanya, T., Lesi, O., Adeyomoye, A., Fasanmade, O. (2016). Non alcoholic fatty liver disease in a Nigerian population with type II diabetes mellitus. *The Pan African Medical Journal*. 24.20.8181.

Osei-Hyiaman, D., DePetrillo, M., Pacher, P., Liu, J., Radaeva, S., Bátkai, S., Harvey-White, J., Mackie, K., Offertáler, L., Wang, L., Kunos, G. (2005). Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *The journal of clinical investigation*. **115** (5), 1298-305.

Osei-Hyiaman, D., Liu, J., Zhou, L., Godlewski, G., Harvey-White, J., Jeong, WI., Bátkai, S., Marsicano, G., Lutz, B., Buettner, C., Kunos, G. (2008). Hepatic CB1 receptor is required for development of diet-induced steatosis, dyslipidemia, and insulin and leptin resistance in mice. *The journal of clinical investigation.* **118** (9), 3160-9.

Pachikian, B., Essaghir, A., Demoulin, J., Neyrinck, A., Catry, E., De Backer, F., Dejeans, N., Dewulf, E., Sohet, F., Portois, L., Deldicque, L., Molendi-Coste, O., Leclercq, I., Francaux, M., Carpentier, Y., Foufelle, F., .Muccioli, G., Cani, P., Delzenne, N. (2011). Hepatic n-3 polyunsaturated fatty acid depletion promotes steatosis and insulin resistance in mice: genomic analysis of cellular targets. *PLoS One*. **6** (8), e23365.

Pagotto, U., Marsicano, G., Fezza, F., Theodoropoulou, M., Grübler, Y., Stalla, J., Arzberger, T., Milone, A., Losa, M., Di Marzo, V., Lutz, B., Stalla, GK. (2001). Normal human pituitary gland and pituitary adenomas express cannabinoid receptor type 1 and synthesize endogenous cannabinoids: first evidence for a direct role of cannabinoids on hormone modulation at the human pituitary level. *The journal of clinical endocrinology and metabolism.* **86** (6), 2687-96.

Papandreou, D., Andreou, E., (2015). Role of diet on non- alcoholic fatty liver disease: An updated narrative review. *World Journal of Hepatology.* **7** (3), 575-82.

Patel, V., Spencer, C., Young, T., Lively, M., Cunningham, C. (2007). Effects of 4-hydroxynonenal on mitochondrial hmg-coa synthase. *Free Radical Biology and Medicine*. **43** (11),1499-507.

Patel, J., Bettencourt, R., Cui, J., Salotti, J., Hooker, J., Bhatt, A., Hernandez, C., Nguyen, P., Aryafar, H., Valasek, M., Haufe, W., Hooker, C., Richards, L., Sirlin, C., Loomba, R. (2016). Association of noninvasive quantitative decline in liver fat content on MRI with histologic response in nonalcoholic steatohepatitis. *Therapeutic Advances in Gastroenterology*. **9** (5), 692-701.

Pateria, P., de Boer, B., MacQuillan, G. (2013). Liver abnormalities in drug and substance abusers. *Best Practice & Research Clinical Gastroenterology*. **27** (4), 577-596.

Patterson, E., Wall, R., Fitzgerald, G., Ross, R., Stanton, S. (2012). Health implications of high dietary omega- 6 polyunsaturated Fatty acids. *Journal of Nutrition and Metabolism.* 2012 539426.

Peng, J., Cui, T., Sun, Z., Huang, F., Chen, L., Xu, L., Feng, Q., Hu, Y. (2012). Effects of Puerariae Radix Extract on Endotoxin Receptors and TNF-α Expression Induced by Gut-Derived Endotoxin in Chronic Alcoholic Liver Injury. *Evidance-Based Complementary and Alternative Medecin*. 2012;2012:234987.

Peng, X., Chen, F., Liu, W., Hu, Z., Lin, X. (2016). Lack of association between SREBF-1c gene polymorphisms and risk of non-alcoholic fatty liver disease in a Chinese Han population. *Scientific Reports*. **6**, 32110.

Petrosillo, G., Portincasa, P., Grattagliano, I., Casanova, G., Matera, M., Ruggiero, F., Ferri, D., Paradies, G. (2007). Mitochondrial dysfunction in rat with nonalcoholic fatty liver Involvement of complex I, reactive oxygen species and cardiolipin. *Biochimica et Biophysica Acta*. **1767** (10), 1260-7.

Popova, S., Rehm, J., Patra, J., Zatonski, W. (2007). Comparing alcohol consumption in central and eastern Europe to other European countries. *Alcohol & Alcoholism.* **42** (5), 465–473.

Puri, P., Baillie, R., Wiest, M., Mirshahi, F., Choudhury, J., Cheung, O., Sargeant, C., Contos, M., Sanyal, A. (2007). A Lipidomic Analysis of Nonalcoholic Fatty Liver Disease. *Hepatology***. 46** (4), 1081-90.

Purohit, V., Gao, B., Song, B. (2009). Molecular mechanisms of alcoholic fatty liver. *Alcoholism, Clinical and Experimental Research*. **33** (2),191-205.

Purohit, V., Rapaka, R. Shurtleff, D. (2010). Role of Cannabinoids in the Development of Fatty Liver (Steatosis). *American Association of Pharmaceutical Scientists*.**12** (2), 233–237.

Quan, H., Kim, D., Chung, S. (2013). Caffeine attenuates lipid accumulation via activation of AMP-activated protein kinase signaling pathway in HepG2 cells. *BMB Reports*. **46** (4), 207-12.

Rao, M., Reddy, J. (2004). PPAR alpha in the pathogenesis of fatty liver disease. *Hepatology*. **40** (4), 783–786.

Rice, W., Shannon, J., Burton, F., Fiedeldey, D. (1997). Expression of a brain-type cannabinoid receptor (CB1) in alveolar Type II cells in the lung: regulation by hydrocortisone. *European journal of Pharmacology*. **327** (2-3), 227-32.

Rinella, M., Sanyal, A. (2016). Management of NAFLD: a stage-based approach. *Nature Reviews: Gastroenterology and Hepatology.* **13** (4), 196–205.

Roswall, N., Weiderpass, E. (2015). Alcohol as a Risk Factor for Cancer: *Existing Evidence in a Global Perspective Journal of Preventative Medicine and Public Health*. **48** (1), 1-9.

Rustichelli, C., Avallone, R., Campioli, E., Braghiroli, D., Parenti, C., Baraldi, M. (2009). HPLC analysis of n-3 and n-6 fatty acid levels in rat serum after chronic treatment with dietetic oils. *Journal of Food Lipids.* **16** (4), 422-435.

Santoro, N., Savoye, M., Kim, G., Marotto, K., Shaw, M., Pierpont, B., Caprio, S. (2012). Hepatic fat accumulation is modulated by the interaction between the rs738409 variant in the PNPLA3 gene and the dietary omega6/omega3 PUFA intake. *Public Library of Science One.* **7** (5), e37827.

Schaefer, E., Tsunoda, F., Diffenderfer, M., Polisecki, E., Thai, N., Asztalos, B. (2000). The Measurement of Lipids, Lipoproteins, Apolipoproteins, Fatty Acids, and Sterols, and Next Generation Sequencing for the Diagnosis and Treatment of Lipid Disorders. In: De Groot, L., Chrousos, G., Dungan, K., Feingold, K., Grossman, A., Hershman, J., Koch, C., Korbonits, M., McLachlan, R., New, M., Purnell, J., Rebar, R., Singer, F., Vinik, A. (ed). Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.;2000-2016 Mar 29.

Schild, L., Jaroscakova, I., Lendeckel, U., Wolf, G., Keilhoff, G. (2006). Neuronal nitric oxide synthase controls enzyme activity pattern of mitochondria and lipid metabolism. *FASEB Journal*. **20** (1), 145 – 147.

Schon, E., DiMauro, S., Hirano, M., Gilkerson, R. (2010). Therapeutic prospects for mitochondrial disease. *Journal of Trends Mol Med*. **16** (6), 268–276.

Schönfeld, P. Wojtczak, L. (2008). Fatty acids as modulators of the cellular production of reactive oxygen species. *Free Radical Biology and Medicine*. **45** (3), 231-241.

Schuck, R., Zha, W., Edin, M., Gruzdev, A., Vendrov, K., Miller, T., Xu, Z., Lih, F., DeGraff, L., Tomer, K., Jones, H., Makowski, L., Huang, L., Poloyac, S., Zeldin, D., Lee, C. (2014). The cytochrome P450 epoxygenase pathway

regulates the hepatic inflammatory response in fatty liver disease. *PLoS One*. **9** (10), e110162.

Schwimmer, J., Behling, C., Newbury, R., Deutsch, R., Nievergelt, C., Schork, N., Lavine, J. (2005). Histopathology of paediatric nonalcoholic fatty liver disease. *Hepatology*. **42** (3), 641–649.

Sealls, W., Gonzalez, M., Brosnan, M., Black, P., DiRusso, C. (2008). Dietary polyunsaturated fatty acids (C18:2 omega6 and C18:3 omega3) do not suppress hepatic lipogenesis. *Biochimica et Biophysica Acta*. **1781** (8), 406-14.

Seitz, H., and Becker, P. (2007). Alcohol Metabolism and Cancer Risk. Alcohol research and Health. **30** (1), 38-47.

Sekiya, M., Yahagi, N., Matsuzaka, T., Najima, Y., Nakakuki, M., Nagai, R., Ishibashi, S., Osuga, J., Yamada, N., Shimano, H. (2003). Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology*. **38**, 1529-1539.

Sertznig, P., Seifert, M., Tilgen, W., Reichrath, J. (2007). Present concepts and future outlook: function of peroxisome proliferator-activated receptors (PPARs) for pathogenesis, progression, and therapy of cancer. *Journal of Cellular Physiology*. **212** (1), 1–12.

Seth, D., Haber, P., Syn, W., Diehl, A., Day, C. (2011). Pathogenesis of alcohol-induced liver disease classical concepts and recent advances. *Journal of Gastroenterology and Hepatology*. **26** (1089), 1105; 2011.

Severson, T., Besur, S., Bonkovsky, H. (2016). Genetic factors that affect nonalcoholic fatty liver disease: A systematic clinical review. *World Journal of Gastroenterology*. **22** (29), 6742-56.

Shimomura, I., Shimano, H., Horton, D., Goldstein, L., Brown, S. (1997). Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *The Journal of Clinical Investigation*. **99** (5), 838-45.

Simopoulos, A.P. (2002). Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases. *Journal of the American College of Nutrition.* **21** (6), 495–505.

Simopoulos, A. (2003). Essential fatty acids in health and chronic diseases. *Forum of Nutrition*. **56**, 67–70.

Simopoulos, A. (2003). Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. *World Review of Nutrition and Dietetics.* **92**, 1–22.

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.* **233** (6), 674-88.

Spector, A., Yorek, M. (1985). Membrane lipid composition and cellular function. *Journal of Lipid research*. **26** (9),1015-35.

Stiuso, P., Bagarolo, M., Ilisso, C., Vanacore, D., Martino, E., Caraglia, M., Porcelli, M., Cacciapuoti, G. (2016). Protective Effect of Tyrosol and S-Adenosylmethionine against Ethanol-Induced Oxidative Stress of Hepg2 Cells Involves Sirtuin 1, P53 and Erk1/2 Signalling. *International Journal of Molecular Sciences.* **17** (5), pii: E622.

Suzuki-Kemuriyama, N., Matsuzaka, T., Kuba, M., Ohno, H., Han, S., Takeuchi, Y., Isaka, M., Kobayashi, K., Iwasaki, H., Yatoh, S., Suzuki, H., Miyajima, K., Nakae, D., Yahagi, N., Nakagawa, Y., Sone, H., Yamada, N., Shimano, H. (2016). Different Effects of Eicosapentaenoic and Docosahexaenoic Acids on

Atherogenic High-Fat Diet-Induced Non-Alcoholic Fatty Liver Disease in Mice. *PLoS One.* **11** (6), e0157580.

Takahashi, Y. Sugimoto, K., Inui, H., Fukusato, H. (2015). Current pharmacological therapies for nonalcoholic fatty liver disease/ nonalcoholic steatohepatitis. *World Journal of Gastroenterology.* **21** (13), 3777-85.

Tapia, G., Valenzuela, R., Espinosa, A., Romanque, P., Dossi, C., Gonzalez-Mañán, D., Videla, L., D'Espessailles, A. (2014). N-3 long-chain PUFA supplementation prevents high fat diet induced mouse liver steatosis and inflammation in relation to PPAR-α upregulation and NF-κB DNA binding abrogation. *Molecular Nutrition and Food Research*. **58** (6), 1333-41.

Teixeira-Clerc, F., Julien, B., Grenard, P., Tran Van Nhieu, J., Deveaux, V., Li, L., Serriere-Lanneau, V., Ledent, C., Mallat, A., Lotersztajn, S. (2006). CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nature Medicine.* **12** (6), 671–6.

Toyama, T., Nakamura, H., Harano, Y., Yamauchi, N., Morita, A., Kirishima, T., Minami, M., Itoh, Y., Okanoue, T. (2004). PPAR alpha ligands activate antioxidant enzymes and suppress hepatic fibrosis in rats. *Biochemical and Biophysical Research Communication*. **324** (2), 697-704.

Tsochatzis, E., Papatheodoridis, G. Archimandritis, A. (2009). Adipokines in non alcoholic steatohepatitis: from pathogenesis to implications in diagnosis and therapy. *Mediators of Inflammation*. Article ID 831670.

Tsukamoto, H., Towner, S., Ciofalo, L., French, S. (1986). Ethanol-induced liver fibrosis in rats fed high fat diet. *Hepatology.* **6** (5), 814–822.

Valenzuela, R., Videla, L. (2011). The importance of the long-chain polyunsaturated fatty acid n-6/n-3 ratio in development of non-alcoholic fatty liver associated with obesity. *Food and Function*. **2** (11), 644-8.

Vatsalya, V., Song, M., Schwandt, M., Cave, M., Barve, S., George, D., Ramchandani, V., McClain, C. (2016). Effects of Sex, Drinking History, and Omega-3 and Omega-6 Fatty Acids Dysregulation on the Onset of Liver Injury in Very Heavy Drinking Alcohol-Dependent Patients. *Alcoholism, Clinical and Experimental Research*. **40** (10), 2085-2093.

Vernon, G., Baranova, A., Younossi, Z. (2011). Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Alimentary Pharmacology and Therapeutics*. **34** (3), 274–285.

Vitto, M., Luz, G., Luciano, T., Marques, S., Souza, D., Pinho, R., Lira, F., Cintra, D., De Souza, C. (2012). Reversion of steatosis by SREBP-1c antisense oligonucleotide did not improve hepatic insulin action in diet induced obesity mice. *Hormone and Metabolic Research*. **44** (12), 885–890.

Wall, R., Ross, R., Fitzgerald, G., Stanton, C. (2010). Fatty acids from fish: the anti-inflammatory potential of long chain omega-3 fatty acids. *Nutrition Reviews*, **68** (5), 280–289.

Wang, D., Wei, Y., Pagliassotti, M. (2006). Saturated Fatty Acids Promote Endoplasmic Reticulum Stress and Liver Injury in Rats with Hepatic Steatosis. *Endocrinology*. **147** (2), 943-51.

Wang, J. and Liu, Y. (2003). Non-alcoholic fatty liver disease: the problems we are facing. *Hepatobiliary and Pancreatic Diseases International*. **2** (3), 334–337.

Wang, X., Cao, Y., Fu, Y., Guo, G., Zhang, X. (2011). Liver fatty acid composition in mice with or without non alcoholic fatty liver disease. *Lipids Health Dis.* **10**: 234.

Waszkiewicz, N., Szajda, S., Zalewska, A., Szulc, A., Kępka, A., Minarowska, A., Wojewódzka-Żelezniakowicz, M., Konarzewska, B., Chojnowska, S., Ladny, J.,

Zwierz, K. (2012). Alcohol abuse and glycol conjugate metabolism. *FOLIA HISTOCHEMICA ET CYTOBIOLOGICA*. **50** (1), 1-11.

Wells, M., Vendrov, K., Edin, M., Ferslew, B., Zha, W., Nguyen, B., Church, R., Lih, F., DeGraff, L., Brouwer, K., Barritt, A., Zeldin, D., Lee, C. (2016). Characterization of the Cytochrome P450 epoxyeicosanoid pathway in non-alcoholic steatohepatitis. *Prostaglandins and Other Lipid Mediators.* **125,** 19-29.

Weltman, M., Farrell, G., Hall, P., Ingelman-Sundberg, M., Liddle, C. (1998). Hepatic cytochrome P450 2E1 is increased in patients with non-alcoholic steatohepatitis. *Hepatology*. **27** (1), 128–133.

Weltman, M., Farrell, G., Liddle, C. (1996). Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation. *Gastroenterology*. **111** (6), 1645–1653.

Williams, C. (2000). Dietary fatty acids and human health. *Annals de Zootechnie.* **49** (3), 165-180.

Williams, C., Stengel, J., Asike, M., Torres, M., Shaw, J., Contreras, M., Landt, C., Harrison S., (2011). Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology*. **140** (1), 124-131.

Willson, T., Brown, P., Sternbach, D., Henke, B. (2000). The PPARs: from orphan receptors to drug discovery. *Journal of Medicinal Chemistry*. **43**, 527–550.

Wree, A., Kahraman, A., Gerken, G., Canbay, A. (2010). Obesity affects the liver—the link between adipocytes and hepatocytes. *Digestion (International Journal of Gastroenterology).* **83** (1–2), 124-133.

Yahagi, N., Shimano, H., Hasty, A., Matsuzaka, T., Ide, T., Yoshikawa, T., Amemiya-Kudo, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K.,

Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., Yamada, N. (2002). Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice. *The Journal of Biological Chemistry*. **277** (22), 19353–19357.

Yao, H., Qiao, YJ., Zhao, YL., Tao, XF., Xu, LN., Yin, LH., Qi, Y., Peng, JY. (2016). Herbal medicines and nonalcoholic fatty liver disease. *World Journal of Gastroenterology.* **22** (30), 6890-905.

Zámbó, V., Simon-Szabó, L., Szelényi, P., Kereszturi, E., Bánhegyi, G., Csala, M. (2013). Lipotoxicity in the liver. *World journal of hepatology*. **5** (10), 550-7.

Zakhari, S. (2013). Alcohol Metabolism and Epigenetics Changes*. Alcohol research: Current review*. **35** (1), 6-16.

Zhang, J., Wei, Y., Hu, B., Huang, M., Xie, W., Zhai, Y. (2013). Activation of Human Stearoyl-Coenzyme A Desaturase 1 Contributes to the Lipogenic Effect of PXR in HepG2 Cells. *Plos One*. **8** (7), e67959.

Zhang, X., Li, S., Zhou, Y., Su, W., Ruan, X., Wang, B., Zheng, F., Warner, M., Gustafsson, J., Guan, Y. (2017). Ablation of cytochrome P450 omega-hydroxylase 4A14 gene attenuates hepatic steatosis and fibrosis. *Proceeding of the National Academy of Science of U S A*. **114** (12), 3181-3185.

Zhang, X., Liu, J., Su, W., Wu, J., Wang, C., Kong, X., Gustafsson, J., Ding, J., Ma, X., Guan, Y. (2014). Liver X receptor activation increases hepatic fatty acid desaturation by the induction of SCD1 expression through an LXRα-SREBP1c-dependent mechanism. *Journal of Diabetes*. **6** (3), 212-20.

Zhang, X., Wang, H., Yin, P., Fan, H., Sun, L., Liu, Y. (2017). Flaxseed oil ameliorates alcoholic liver disease via anti-inflammation and modulating gut microbiota in mice. *Lipids in Health and Disease*. **16** (1), 44.

Zhang, Y., Dong, L., Yang, X., Shi, H., Zhang, L. (2011). α-Linolenic acid prevents endoplasmic reticulum stress-mediated apoptosis of stearic acid lipotoxicity on primary rat hepatocytes. *Lipids in Health and Disease.* **10**, 81.

Zhang, Y., Jiang, L., Hu, W., Zheng, Q., Xiang, W. (2011). Mitochondrial dysfunction during in vitro hepatocyte steatosis is reversed by omega-3 fatty acid-induced up-regulation of mitofusin 2. *Metabolism*. **60** (6), 767-75.

Zhang, Y., Xue, R., Zhang, Z., Yang, X., Shi, H. (2012). Palmitic and linoleic acids induce ER stress and apoptosis in hepatoma cells. *Lipids in Health and Disease.* **11**,1.

Zhou, J., Jiang, Z., Zhao, C., Zhen, Z., Wang, W., Nanji, A. (2013). Long-term binge and escalating ethanol exposure causes necroinflammation and fibrosis in rat liver. *Alcoholism, clinical and experimental research.* **37** (2), 213-22.

Online resources

World Gastroenterology Organisation (2012),

http://www.worldgastroenterology.org/guidelines/global-guidelines/nafld-nash/nafld-nash-english Accessed: 5 October 2016.

World health organisation.

http://gamapserver.who.int/mapLibrary/ (accessed 21 March 2017)

Boundless.com. Electron transport chain. Available at: https://www.boundless.com/biology/textbooks/boundless-biology-textbook/cellular-respiration-7/oxidative-phosphorylation-76/electron-transport-chain-362-11588/images/fig-ch07_04_01/ (accessed 16 October 2016).

Publications:

Ghazali, R., Patel, VB. (2016). *Alcohol Metabolism: General Aspects*. In: Patel, VB (ed). Molecular Aspects of Alcohol and Nutrition. Elsevier, London: 17-20.

Ghazali, R., Patel, VB. (2016). *Gene Expression in Alcoholism: An Overview*. In: Patel, VB (ed). Molecular Aspects of Alcohol and Nutrition. Elsevier, London: 225-228.

Ghazali, R., Bligh, A., Tewfik, I., Patel, VB. (2014). Abstract (Poster) No. 20: Mechanisms into the development of fatty liver disease: Prevention by plant products. In: Postgraduate fair abstracts.

Ghazali, R., Bligh, A., Tewfik, I., Patel, VB. (2015). Abstract (Poster) No. 20: Mechanisms into the development of fatty liver disease: role of free fatty acids. In: Postgraduate fair abstracts.

Ghazali, R., Bligh, A., Tewfik, I., Patel, VB. (2016). Abstract (oral) No. 5: Mechanisms into the development of fatty liver disease: role of free fatty acids and alcohol. In: Postgraduate fair abstracts.

Ghazali, R., Bligh, A., Tewfik, I., Patel, VB. (2017). Abstract (poster) No. P018: Role of omega fatty acids in fatty liver disease. In: British association for the study of the Liver (BASL).

Appendix

Appendix

Concentrations used in the preparation of omega 6/3 ratios

A. Concentrations of linoleic acid (LA) and alpha linolenic acid (ALA) used in the preparation of LA/ALA ratios:

LA/ALA ratio	LA concentration (μM)	ALA concentration (μM)
1:1	30	30
2:1	30	15
4:1	30	7.5
10:1	30	3
15:1	30	2
25:1	30	1.2

B. Concentrations of arachidonic acid (AA) and docosahexaenoic acid (DHA) used in the preparation of AA/DHA ratios:

AA/DHA ratio	AA concentration (μM)	DHA concentration (μM)
1:1	2	2
4:1	8	2
15:1	30	2
25:1	50	2