Mechanisms into the development of fatty liver disease: role of bile acids, Betaine and MHY908

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MECHANISMS INTO THE DEVELOPMENT OF FATTY LIVER DISEASE: ROLE OF BILE ACIDS, BETAINE AND MHY908

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Author’s Declaration

I declare that all the material covered in this thesis is my own work. The present work was carried out in accordance with the guidelines and regulations of the University of Westminster.

Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is an emerging epidemic worldwide. It is a metabolic condition that etiologically parallels with obesity, type 2 diabetes, and the metabolic syndrome. However, the pathogenesis of NAFLD remains elusive, though it is known free fatty acids such as palmitate promote liver injury. Therefore, there is a pressing need to find a potent therapeutic approach for NAFLD. Firstly, this study aimed to investigate the lipotoxicity and mitochondrial dysfunction attributable to palmitic acid. Subsequent investigations focused on investigating possible alternative therapies for NAFLD such as bile acids, betaine and a PPARα/γ dual agonist MHY908.

Method: Human hepatoma cell line, VL17A cells were treated with saturated fatty acid palmitic acid (PA) with a range of concentrations between 10 µM - 300 µM for 24 h, 48 h and 72 h after which lipid accumulation, cell toxicity, oxidative stress and mitochondrial function were assessed. Further investigations were employed with bile acids chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA), betaine and MHY908 to determine whether lipotoxicity induced by PA would be attenuated and therefore could be a potential therapy for NAFLD.

Results: PA treatment showed a detrimental effect on cell viability in a time and dose dependant manner, where longer incubation time and higher concentrations of PA exhibited the greatest cytotoxicity. At 72 h, 300 µM PA led to a 90% decrease in cell viability ($p < 0.001$). Lipid accumulation also increased with higher doses, 250 µM PA treatment led to a 21% increase in lipid accumulation ($p < 0.001$). PA induced oxidative stress and mitochondrial dysfunction at higher concentration. At 300 µM PA treatment led to increases in ROS at 30 minutes (+46%, $p <0.001$) and 1 h (+61%, $p <0.001$). Lower PA doses 50 µM and 100 µM PA were employed in subsequent studies. UDCA exhibited hepatoprotective effects when co-incubated with PA predominantly at 24 h with 30 µM treatment; cell viability (UDCA +13%, $p < 0.05$), lipid accumulation (UDCA -47%, $p < 0.001$), ROS levels and mitochondrial function improved in all parameters investigated. CDCA showed less hepatoprotective effects in comparison to UDCA. Betaine 20µM displayed anti-oxidative properties and attenuated PA induced lipotoxicity as cell viability increased +41%, $p < 0.001$ but there was no significant effect on lipid accumulation. PPARα/γ dual agonist MHY908 also showed beneficial effect with 1 µM treatment where cell viability increased by 26% ($P < 0.001$), lipid accumulation decreased by 13% ($p < 0.001$), ROS generation had a less significant effect as levels decreased by 3% though mitochondrial function improved.

Conclusion: This study determined the saturated fatty acid PA induced cytotoxicity and lipid accumulation in hepatocytes. This may be due to the increased activity of the lipogenic pathway which consequently promotes oxidative stress and mitochondrial dysfunction. Alternative therapies such as UDCA, betaine and MHY908 attenuated PA induced lipotoxicity and improved mitochondrial function. However, further work aims to elucidate the protein expression and gene expression of transcriptional factors involved in the molecular pathways to gain a better understanding of fatty liver disease progression and development of novel therapies to treat NAFLD.

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Dedications

My parents Khalid and Sammiya, I dedicate this thesis to you as without your guidance, sacrifices and hard-work this PhD would not have been possible. You are the anchor of our family and I thank you for all your love and support.

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List of Abbreviations

ACC1	Acetyl-CoA carboxylase- 1
ACC2	Acetyl-CoA carboxylase-2
ALT	Alanine aminotransferase
AMPK	AMP- activated protein kinase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CA	Cholic acid
CAR	Constitutive Androstane Receptor
CDCA	Chenodexoycholic acid
ChREBP	Carbohydrate response element binding protein
CPT-1	Carnitine palmitoyl transferase-1
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Sterol 7α-hydroxylase
CYP27A1	Sterol 27α-hydroxylase
CYP2E1	Cytochrome P450 2E1
DCA	Deoxycholic acid
DCFDA	2′,7′-dichlorofluorescin diacetate
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
ETC	Electron transport chain

ELISA	Enzyme-linked immunoabsorbent assay
FA	Fatty acid
FADH2	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FCS	Foetal calf serum
FFA	Free fatty acid
FGF15/19	Fibroblast growth factor 15/19
FXR	Farnesoid X receptor
HepG2	Human hepatoma cell
HCC	Hepatocellular carcinoma
IR	Insulin resistance
JNK	C-Jun-N-terminal Kinase
LCA	Lithocholic acid
LCFA	Long chain fatty acid
MCD	Methionine- choline deficient diet
MHY908	2-[4-(5-chlorobenzo [d] thiazol-2-yl) phenoxy]-methylpropanoic acid
MPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MTT	Thiazolyl blue tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
OCA	Obeticholic acid

OCR	Oxygen consumption rate
PA	Palmitic acid
PBS	Phosphate buffered saline
PNPLA3	Patatin-like phospholipase -3
PPAR	Peroxisome proliferator- activated receptors
PXR	Pregnane X receptor
ROS	Reactive oxygen species
SAM	S-Adenosyl methionine
SAH	S-adenosyl homocysteine
SCD1	Stearoyl-CoA desaturase-1
SEM	Standard error of mean
SHP	Small heterodimer partner
SREBP1c	Sterol regulatory binding protein- 1c
TG	Triglyceride
TNFα	Tumour necrosis factor α
TZD	Thiazolidinedione
UDCA	Ursodeoxycholic acid
VDR	Vitamin D receptor

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Chapter 1

Introduction

1.1. Introduction

1.1. Non-alcoholic fatty liver disease: definition, histology and spectrum of disease

Non-alcoholic fatty liver disease (NAFLD) is a metabolic condition that etiologically parallels with obesity, type 2 diabetes, and the metabolic syndrome. NAFLD is defined as excessive fat accumulation in hepatocyte (steatosis) exceeding 5% of the liver weight; in the absence of aetiologies such as high alcohol intake (≥ 30g a day in men or ≥ 20g a day in women) (European Association for the Study of the Liver (EASL, 2016), drugs and hepatitis C virus. (Lonardo et al., 2015; Ghaemi et al., 2013). Generally, NAFLD is an umbrella term that comprises of liver pathology from simple steatosis to non-alcoholic steatohepatitis (NASH); the latter is associated with more severe liver damage such as hepatic ballooning and fibrosis and can lead onto fibrosis/cirrhosis (Takashi and Fukusato, 2014).

Histologically steatosis in NAFLD is macro-vesicular and hepatocytes have a characteristic foamy appearance. These occur in small patches or are observed in single hepatocytes and are primarily situated in zone 3 of a lobule. However, there is an accumulating body of evidence that steatosis, while reversible, is not benign, and may actually 'prime' the liver to progress to more severe states including steatohepatitis, fibrosis and hepatocellular carcinoma (HCC) (Mantena et al., 2009). In fact, in the UK, over the last decade (between 2000-2002 and 2009-2011) HCC rates have increased 45% in males and 33% in females (Liver Cancer UK, 2014). The evidence suggests that the prevalence of liver disease is rapidly increasing; but thus far there is no single putative therapeutic approach (Figure 1.1).

Furthermore, according to a meta-analysis carried out by Singh et al., (2015), over time, 34% of all NAFLD subjects showed NAFLD progression. In NASH there is evidence of steatosis, ballooning and lobular inflammation (EASL 2016). Ballooning indicates more progressive disease and is also linked with fibrosis (Caldwell and Argo., 2010). Other hallmarks that can be observed in NASH are mega-mitochondria, portal inflammation and Mallory-Denk bodies. (EASL, 2016). The histological changes and severity of NAFLD and NASH can be graded with NASH Activity Score.

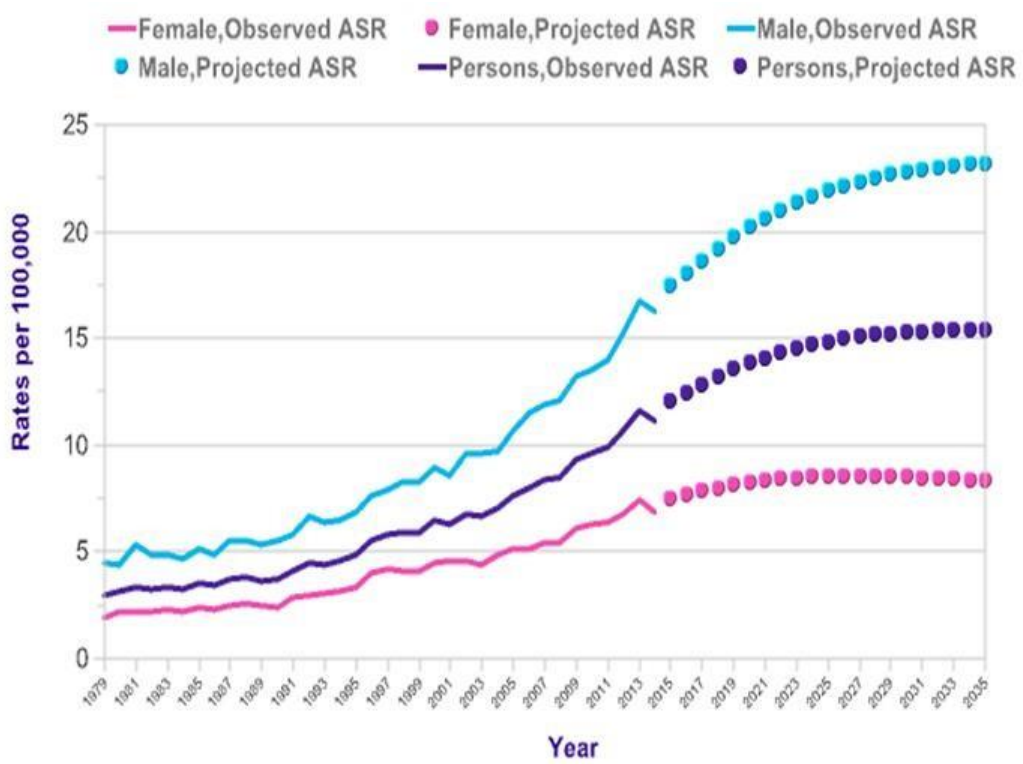


Figure 1.1. Liver Cancer observed and projected age-standardised incidence rates (1979-2035), by sex, UK. (Cancer Research UK, Dec 2018).

1.2. Epidemiology and global burden of disease

While prolonged, heavy alcohol intake is a recognised common cause of alcoholic liver disease and is estimated to be the third foremost cause of all preventable deaths worldwide, the occurrence of NAFLD is rapidly increasing and is now the most common form of chronic liver disease worldwide (McNear and Harrison., 2009; Lomanaco et al., 2013). In fact, since 1980, the global prevalence rate of NAFLD has doubled and is currently estimated at 25% (Younossi et al., 2016). The global burden of NAFLD is high, affecting one in four adults, with substantial geographic variation in prevalence (Figure 1.2)(Rinella and Charlton, 2016).

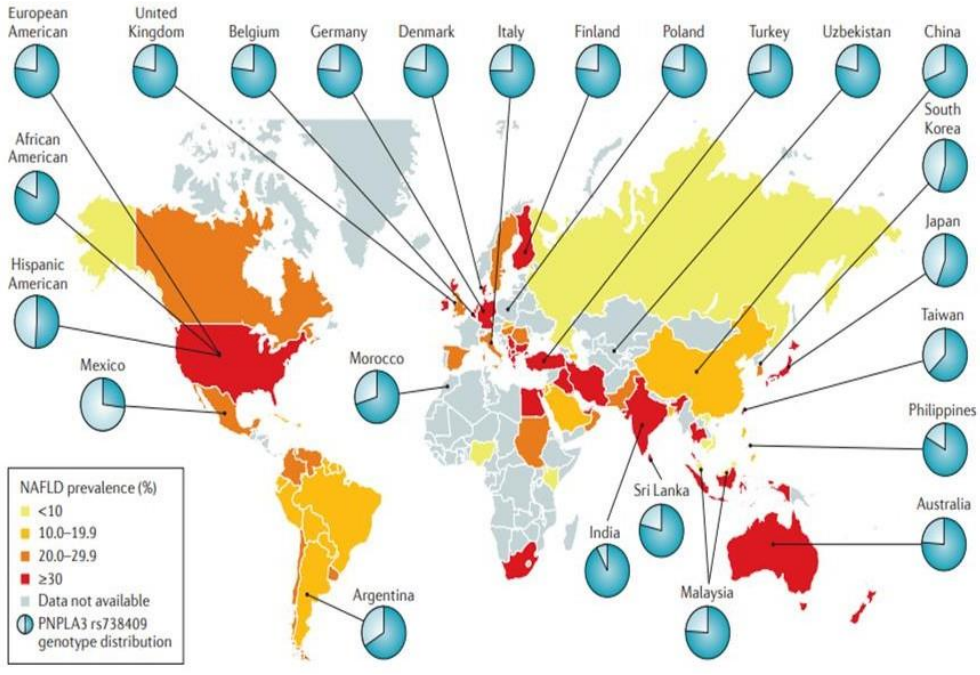


Figure 1.2. Global map showing the worldwide variation in the prevalence of NAFLD (Younossi et al., 2016).

The rise in the incidence of NAFLD in Western and developed countries is related to the widespread presence of risk factors including the use of drugs, sedentary lifestyle and a manifestation of metabolic disorders (Mavrogiannaki and Migdalis, 2013). Furthermore, a meta-analysis study with 8.5 million adults across 22 countries from 1989- 2015 showed the global prevalence rate of NAFLD is highest in the middle- east (32%) and in South America (31%) and the lowest in Africa (14%) (Table 1.1) (Younossi et al., 2017).

NAFLD is an emerging epidemic in light of its two major predisposing factors; a surge in both obesity and diabetes rates with a pooled prevalence of obesity reported as 51% worldwide (Younossi et al., 2016) (Duvnjak et al., 2009). This is due to sedentary lifestyle and poor dietary changes. (Duvnjak et al., 2009; Chitturi et al., 2007).

As aforementioned, the incidenceof NAFLD is significantly increased in diabetics (up to 63%) and in the morbidly obese (McNear and Harrison., 2009). Given the epidemic of obesity, attributable to the content of fat/excess calories in modern diet, it is estimated 42 million children were obese in 2010 and this figure is expected to rise to almost 60 million by 2020 (Onis et al., 2010). In addition to this, in the USA, annual medical costs are estimated at $292 billion and the projected cost of caring for patients is expected to increase by 18% by 2035 accompanied by a decline in the quality of life of NAFLD

patients. These projections foresee a continued worsening in prevalence, with a continued trend in obesity. Thus alternative therapeutic options are needed to manage or prevent this chronic liver disease.

Table 1.1 The prevalence of NAFLD in different regions of the world (Younossi et al., 2016).

Region	Number of Studies in this population	Prevalence of NAFLD in these studies (%)
Africa	2	**13.5**
Asia	14	**27.4**
Europe	11	**23.7**
Middle East	3	**31.8**
North America	13	**24.1**
South America	2	**30.5**
Overall	45	**25.2**

1.3. Hepatic *De Novo* Lipogenesis

The liver synthesises endogenous lipids by a complex cytosolic pathway known as *de novo* lipogenesis. In NAFLD patients, *de novo* lipogenesis is increased in the fasting state and fails to respond to changes in dietary state. In a fasting state, a decrease in insulin levels stimulates adipocyte triglyceride (TG) hydrolase, thereby releasing free fatty acids (FFAs) that are transported into the liver. The rate of *de novo* lipogenesis is regulated primarily at the transcriptional level by the transcriptional factor, sterol regulatory element binding protein 1-c (SREBP1c).

SREBP1c is a transcriptional factor which belongs to a family of sterol regulator element binding proteins that predominantly play a key role in the lipogenesis and gluconeogenesis pathways (Moslehi and Hamidi-zad., 2018). Mammals possess two genes of the SREBP family, this includes SREBP1 and SREBP2. The SREBP1 has two isoforms SREBP1a which is abundantly expressed in the spleen and intestine and SREBP1c is expressed predominantly in the adrenal gland, adipose tissue and in the liver (Quan et al., 2012; Moon., 2017).

SREBP1-c regulates cholesterol and triglyceride synthesis by activation of the transcription of genes required for lipogenesis. Under conditions of insulin resistance, insulin stimulates hepatic SREBP1c transcription, resulting in increased rates of fatty acid synthesis (Sanders and Griffin, 2016). In transgenic mice, SREBP1c overexpression leads to the development of hepatic steatosis (Shimomura et al., 1999). The upregulation of SREBP1c activates acetyl-CoA carboxylase-2 (ACC2) that produces malonyl-CoA at the mitochondrial membrane. The increase of malonyl-CoA causes a decreased oxidation of fatty acids due to the inhibition of carnitine palmitoyl transferase-1 (CPT-1), which 'shuttles' fatty acids into the mitochondria (Softic et al., 2017). The absence of ACC2 in knockout mice enhanced the activity of CPT-1 and caused an increased rate of fatty acid oxidation.

Furthermore, in NAFLD patients in response to feeding, hepatic SREBP1c levels were 33% higher in comparison to control subjects (Pettinelli et al., 2011). A study conducted by Moon et al on ob/ob mice, a mouse model of insulin resistance, with a deletion in the SREBP1c gene, demonstrated a 50% decline in hepatic triglyceride levels. This indicates the significance of the expression of SREBP1c in the ob/ob mice in the development of hepatic steatosis (Moon et al., 2017). In summary, SREBP1c is over expressed as it binds to its lipogenic genes such as acetyl-CoA carboxylase-1 (ACC1), fatty acid synthase (FAS), and Stearoyl-CoA desaturase-1 (SCD1) and stimulates hepatic lipogenesis.

1.4. Fatty acid Oxidation

Oxidation of FAs occurs within the mitochondria, peroxisomes, and the endoplasmic reticulum. It enables the degradation of activated FAs to acetyl-CoA. FAs are activated by acyl-CoA-synthetase to acyl-CoA in the cytosol, which is crucial for enabling FAs to cross membranes and enter organelles. Short- and medium-chain FAs pass the mitochondrial membrane without activation. However, activated long chain fatty acids (LCFAs) are shuttled across the membrane via carnitine CPT1 (Nassir and Ibdah, 2014). In the fed state, FA oxidation is inhibited, and *de novo* lipogenesis is promoted, allowing for storage and distribution of lipids. Generally, short-, medium-, and long-chain FAs are oxidized within the mitochondria (β-oxidation), however, very-long-chain FAs are oxidized within peroxisomes (Berlanga et al., 2014). In FA overload, cytochrome P450 (CYP4A)-dependent oxidation of LCFAs occurs in the endoplasmic reticulum (ER) and induces reactive oxygen species (ROS) and lipid peroxidation. During the process of β-oxidation, electrons are indirectly donated to the electron transport chain to drive ATP synthesis.

Similarly, the activity of the enzyme CYP2E1 is noteworthy in NAFLD as it is involved in the oxidation of ethanol and fatty acids. CYP2E1 participates in an uncoupling reaction of oxygen with NADPH and as a result generates ROS such as hydrogen peroxide and superoxide anion radicals which contributes to mitochondrial dysfunction in hepatocytes in NAFLD patients. Studies of a mouse model of the methionine- choline deficient diet (MCD) have shown a consistent trend of elevated CYP2E1 expression which correlates with increased expression of the enzyme in NAFLD (Weltman et al., 1996).

Peroxisome proliferator-activated receptors (PPARs) play a vital role in FA oxidation by regulating the expression of genes involved in mitochondrial FA and peroxisome FA oxidation. In the liver, activated PPARα can prevent and decrease hepatic fat storage. In a prolonged fasting state in an animal study of PPARα knockout mice, animals exhibited severe hepatic steatosis (Mello et al., 2016).

PPARα also regulates inflammatory processes, primarily by preventing inflammatory gene expression. Hepatic PPARα activation has been repeatedly shown to reduce hepatic inflammation elicited by acute exposure to cytokines and other compounds. Furthermore, PPARα activation plays a role in the modulation of hepatic steatosis due to its effects on upregulation of FA oxidation genes, reduction in the toxicity of FAs, and its anti-inflammatory effects (Berlanga et al., 2014). Clinical studies in NAFLD subjects, validated lower expression of hepatic PPARα. However, PPARα expression was found to increase in NAFLD subjects secondary to lifestyle intervention or bariatric surgery.

The hepatic expression of PPARα is downregulated in response to lipid overload attributable to ingestion of a high fat diet. Consequently, this leads to an increase in lipogenesis and hence the increased expression of SREBP1c and subsequently a decrease in fatty acid oxidation which causes cell death due to increased lipotoxicity in the liver.

At present there are no putative drugs available for the treatment of NAFLD but one of the most commonly targeted class of compounds are the PPAR family, in particular PPAR α/γ dual agonists have been developed to treat metabolic disorders. Of particular interest is a PPAR α/γ dual agonist 2-[4-(5-chlorobenzo [d] thiazol-2-yl) phenoxy]-2-methylpropanoic acid (MHY908) studied by Park et al on a db/db mouse model. Male db/db mice were placed on a calorie restricted diet and administered MHY908 (1 mg or 3 mg/kg/day) mixed in food for 4 weeks (Park et al., 2013). Results reported improvements in hepatic steatosis by increased CPT-1 levels in the mice liver. Additionally, MHY908

enhanced the binding and transcriptional activity of PPAR α and γ in hepatic cells, decreased serum triglyceride, glucose and insulin levels (Park et al., 2013). The study shows the beneficial effects of MHY908 as a PPAR α/γ dual agonist in treating metabolic disorders such as type II diabetes and possibly NAFLD.

1.5. Pathogenesis of NAFLD: Multi-hit Theory

The underlying mechanism for the progression of NAFLD remains elusive and is multifactorial. Various theories have been proposed of which the 'two- hits hypothesis' by Day and James (1998) was considered as the principal theory for the development of NASH. According to this 'two-hit hypothesis' the 'first-hit' is the hepatic accumulation of triglycerides (steatosis), secondary to obesity, high fat diet and insulin resistance. As a result, these factors increase the livers vulnerability to further injury, this includes, oxidative stress and endoplasmic reticulum stress which cause inflammation, fibrosis and cellular death in the liver (Day and James, 1998).

However, more recently it has become evident that the hypothesis is too simplistic and genomic studies have shown the significance of patatin-like phospholipase 3 (PNPLA3) gene polymorphism in NAFLD. This genetic polymorphism can promote the development and progression of NAFLD to NASH without inflammation and fibrosis. Therefore, a multiple-hit hypothesis has now replaced the out-dated 'two-hit hypothesis' for the progression of NAFLD (Figure 1.3).

Although the pathogenesis of NAFLD remains unclear, it is known abnormalities in lipid metabolism such as (1) increased *de novo* lipogenesis in hepatocytes; (2) decreased rate of β-oxidation in the liver and (3) decreased export of TGs, result in fat accumulation in the liver.

1.5.1. 'First hit': steatosis

Steatosis arises as a result of excessive ingestion of carbohydrates and fat leading to elevated levels of glucose and free fatty acids (FFA) in the blood which promote the development of insulin resistance (Raszeja- Wyszomirska et al., 2008). In patients with insulin resistance the hormone lipase in the adipocytes is not completely supressed by insulin. Thus, in the plasma, these FFAs circulate in non-esterified form and insulin resistance favours high rates of FFA flux to the liver from increased adipose tissue lipolysis. Consequently, there is an increase in hepatic lipogenic gene expression facilitated by sterol regulatory element binding protein (SREBP1c) and carbohydrate response element binding

protein (ChREBP) (Lomonaco et al., 2013). As a result of this, there is an increase in hepatic triglyceride (TG) synthesis accompanied with a decreased export of very low density lipoprotein (Kawano and Cohen, 2013).

Insulin resistance and obesity are two key factors in the progression of NAFLD therefore systemic insulin resistance enhances the flux of FFAs to the liver which triggers enhanced *de novo* lipogenesis and induces hepatic steatosis, a ‘first hit’ of NAFLD. C-Jun-N-terminal Kinase (JNK) plays a pivotal role in hepatic insulin resistance through serine phosphorylation of the insulin receptor, insulin receptor substrate- 1, leading to down-regulation of tyrosine phosphorylation of these molecules which is required for normal insulin signalling (Kodama and Brenner, 2008).

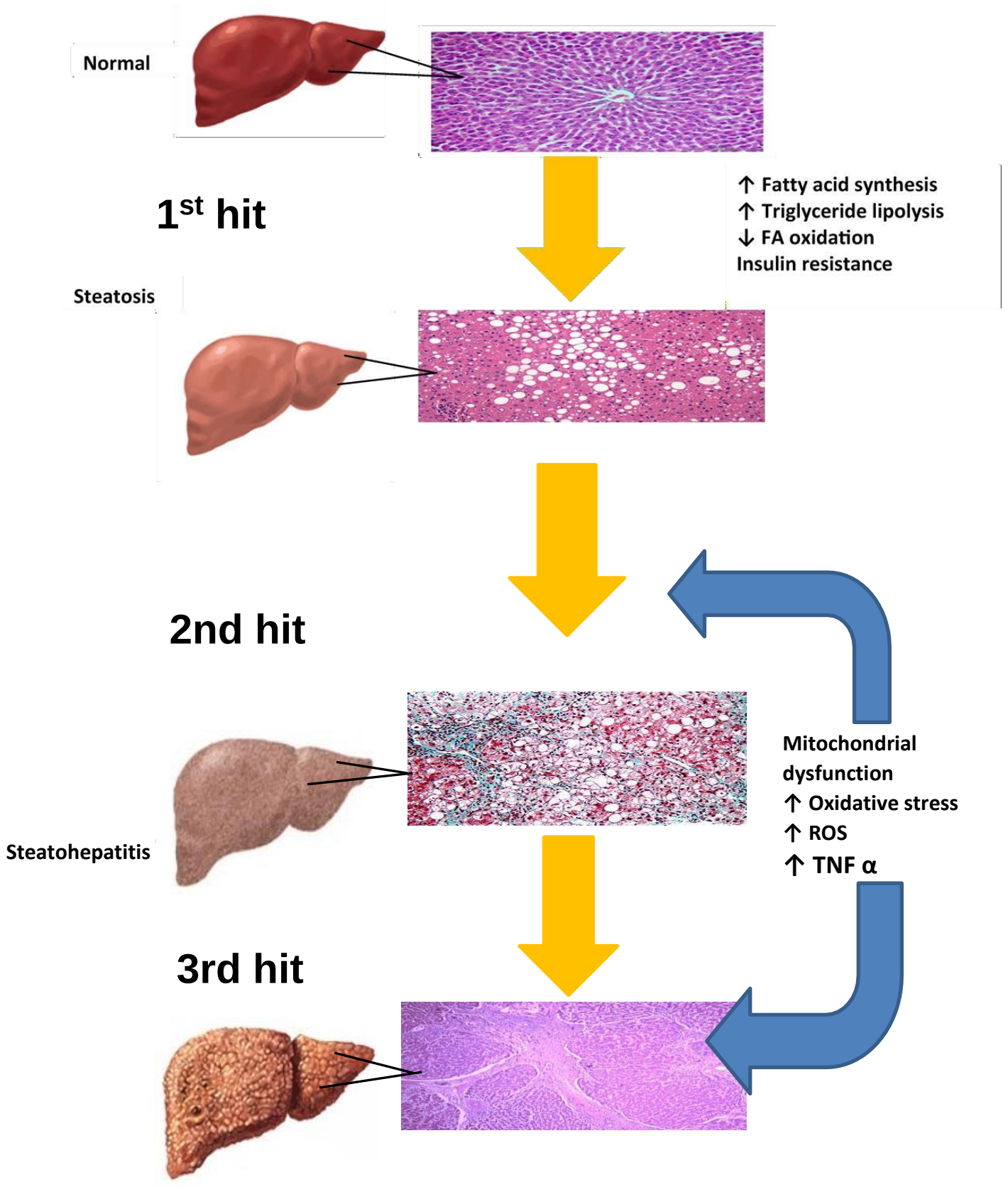


Figure 1.3. NAFLD 'multiple-hit' theory.

The progression of NAFLD to NASH is multifactorial. The 'first hit' consists of hepatic accumulation of fatty acids which is closely associated with insulin resistance. Consequently, this primes the liver to further injury or 'seconds hits' which trigger mitochondrial dysfunction and oxidative stress. Furthermore, the third hit leads to the development of cirrhosis as a result of sustained inflammation in the liver. (Adapted from Cohen et al., 2011).

1.5.2. Second Hit: Mitochondrial dysfunction

The liver is the metabolic hub of the human body and plays a crucial role of maintaining blood glucose and energy homeostasis. Roles undertaken by the liver include but are not limited to the metabolism of glucose and fatty acids, hormone production and detoxification of toxic metabolites (Rui et al., 2014). To orchestrate these biological processes, the hepatocytes require a substantial amount of energy in the form of adenosine triphosphate (ATP). In fact, a clinical study by Schmidt et al, reported ATP is produced at a rate of 30 mM/min with the bulk synthesised from oxidative phosphorylation. Hepatocytes are densely packed with approximately 800 mitochondria occupying 18% of the entire liver cell volume (Wei et al., 2008). Thus, abnormal mitochondria and ATP production are considered as hallmarks of fatty liver disease (Auger et al., 2015).

Hepatic mitochondria in NAFLD exhibit structural and molecular abnormalities such as decreased ATP production and deformed cristae of the mitochondria (Caldwell et al., 1999; Einer et al., 2017). As aforementioned, the mitochondria serve as the cell powerhouse to generate ATP and to efficiently participate in liver metabolism especially as it is the major site for β-oxidation of fatty acids and oxidative phosphorylation. In NAFLD, continuous deposition of free fatty acids in the hepatocytes and dysregulation of lipid metabolism over-burdens the mitochondria and therefore fails to prevent lipotoxicity. Tedoro et al (2008) demonstrated this using a choline-deficient NAFLD model, which reported elevated levels of oxidative phosphorylation efficiency at 12 weeks but reduced ability at 16 weeks. At 16 weeks, the mitochondria presented with modifications in the electron transport chain (ETC) complexes and membrane potential and decreases in ATP synthesis (Tedoro et al., 2008). The ability of the mitochondria to overcome the increased FFA levels was entirely lost in progressive stages of the disease. In these stages, disease development was augmented by CPT-1 downregulation, diminished mitochondrial fatty acid oxidation, and chronic ATP depletion in hepatocytes (Serviddio et al., 2008).

In addition to this, the progression of fatty liver disease is mirrored by pro-inflammatory cytokines such as tumour necrosis factor α (TNFα), a marker of chronic inflammation that contributes to mitochondrial dysfunction in the hepatocytes. Patients with NASH have been found to have increased blood TNFα levels, (36% higher serum TNFα) than in control subjects (Perez et al., 2003). Similarly, in an ob/ob

mice model TNFα levels in liver tissue were 20-fold higher than in normal mice (Ruiz et al., 2006). TNFα prompts the swelling of the mitochondrial membrane which consequently bursts leading to a disturbance in the ETC complexes I and III (Wei et al., 2008).

The ETC is composed of a respiratory chain which encompasses five multi-protein complexes (I-V) as well as two electron carriers (coenzyme Q and cytochrome C) (Figure 1.4). The primary function of the ETC is to generate energy in the form of ATP under aerobic conditions, a process known as oxidative phosphorylation. In NAFLD, over stimulation of mitochondrial fatty acid oxidation and Krebs cycle results in an increase in reducing agents to the ETC and as a consequence promotes superoxide production (Aharoni-Simon et al., 2011).

Complexes I and III are key sites of superoxide generation as a result of electron leakage in the inner mitochondrial membrane (Nassir and Ibdah, 2014). Studies have highlighted two additional mitochondrial enzymes, namely 2-oxoglutarate dehydrogenase and glycerol 3-phosphate dehydrogenase as these may be important to sustain the mitochondrial redox potential (Quinlan et al., 2013). Furthermore, superoxide undergoes an enzymatic reaction mediated by NADPH oxidase, nitric oxide synthase and is converted to hydrogen peroxide which initiates inflammatory signalling responses which contributes to mitochondrial damage. Reactions such as these may contribute to the increased ROS production in NAFLD patients (Mantena et al., 2009).

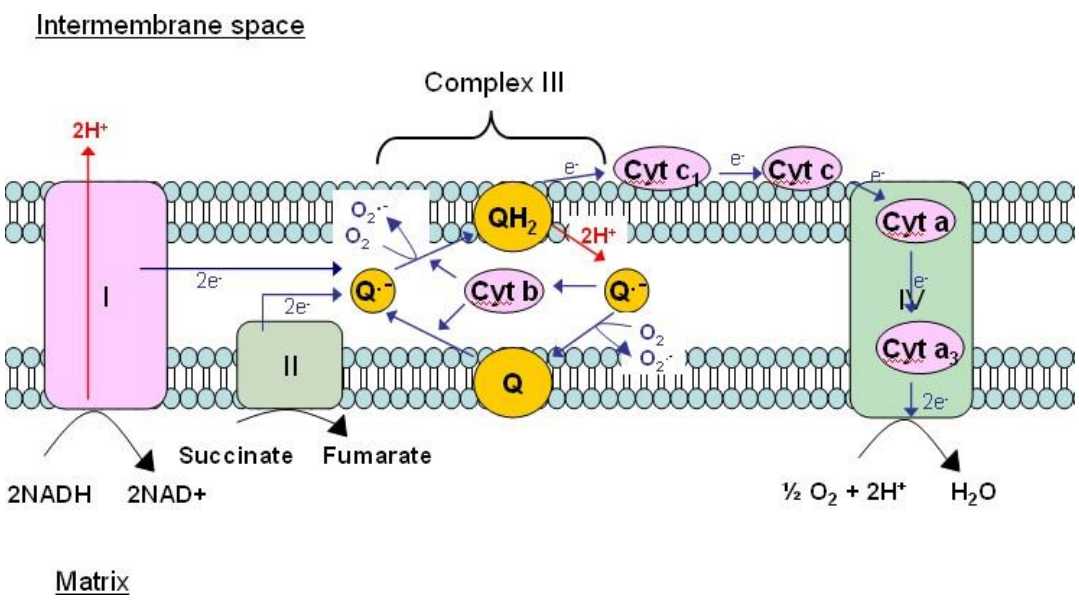


Figure 1.4. The electron transport chain. The passage of electrons through a series of reactions catalysed by four enzymes, or 'complexes', resulting in the formation of ATP at complex V (not shown). NAD+; Nicotinamide adenine dinucleotide; NADH: reduced NAD+; cyt c; cytochrome c. (Adapted from www.Boundless.com).

1.5.3. 3rd hit: Patatin-like phospholipase 3 (PNPLA3) gene

Genome-wide association studies have documented the role of patatin-like phospholipase-3 (PNPLA3) single nucleotide polymorphisms in the progression of NAFLD (Anstee and Day, 2013). The PNPLA3 gene encodes a protein adiponutrin which exhibits triglyceride hydrolase activity that is associated in lipid metabolism processes and is abundantly expressed on the lipid membranes of hepatocytes and the endoplasmic reticulum (Anstee and Day, 2013).

In 2008, Romeo et al., reported the first genetic variant of PNPLA3 (an allele Ile148Met) with robust evidence for the development of NAFLD. It was established from this study, individuals in Western populations were vulnerable to fatty liver disease and the allele was linked to inflammation and fibrosis in the liver. It was reported homozygous carriers of the allele had a 73% higher liver fat content compared to non-carriers (Romeo et al., 2008). It can be concluded genetic variants do indeed impact hepatic FFAs flux, oxidative stress and are determinants in the pathogenesis of NAFLD.

1.5.4. Summary of NAFLD pathology

The pathology of NAFLD and its development remains a complex process, which by no means can be limited to the ‘two-hit’ hypotheses. The exact underlying molecular mechanisms in the development of NAFLD still remain unclear, however in Figure 1.5 the main mechanisms are outlined. Furthermore, there is an abundance of evidence for the diverse and multifactorial processes involved in NAFLD and NASH progression such as the genetic determinants, oxidative stress and mitochondrial dysfunction to name a few, thus the multi hit theory deems more apt to understand the vast pathways involved in fatty liver disease.

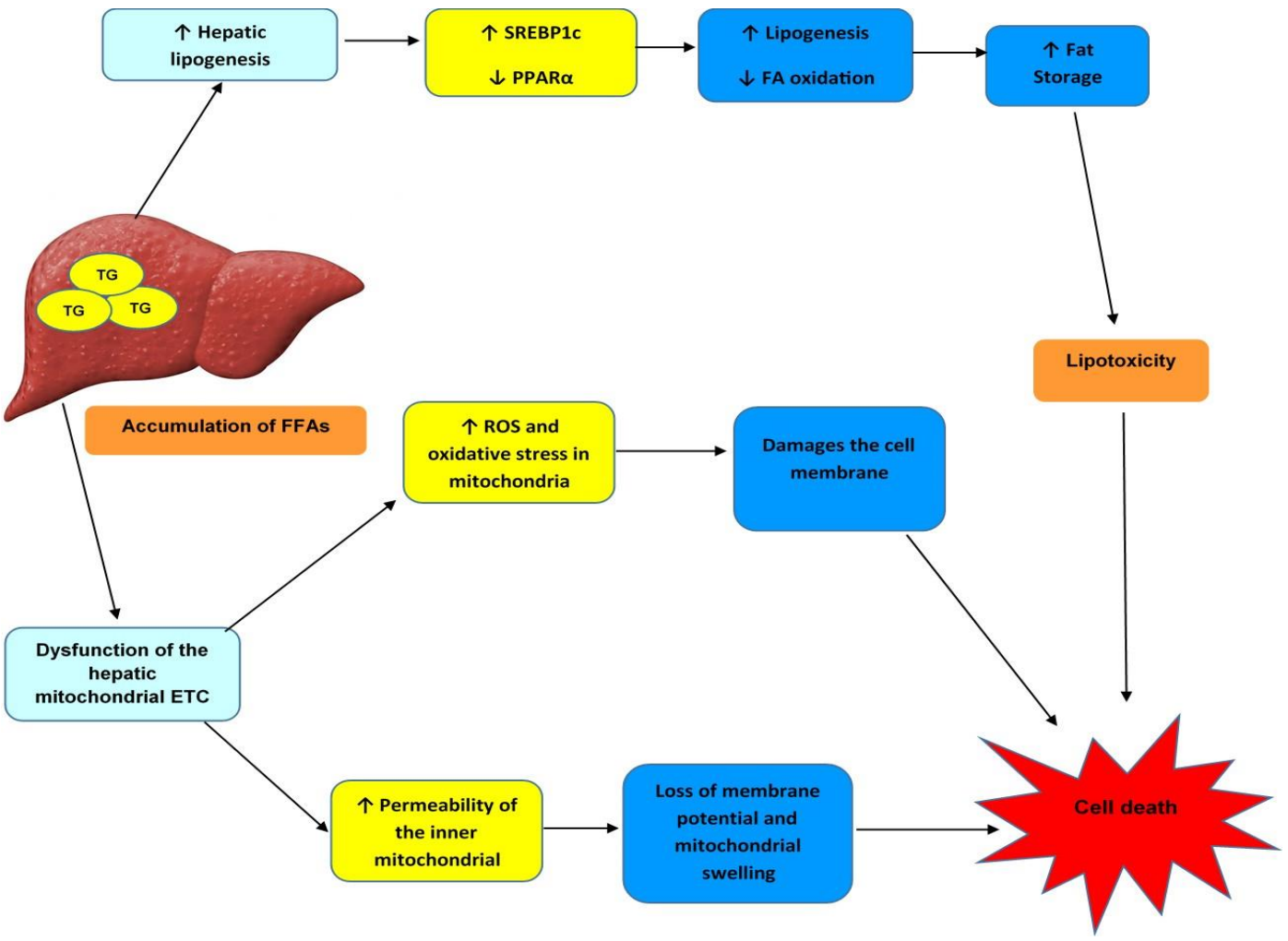


Figure 1.5. Mechanisms into NAFLD.

The accumulation of hepatic free fatty acids and triglycerides is a hallmark of NAFLD and is attributable to sedentary lifestyle and ingestion of high fat diet. Lipid overload in the hepatocytes stimulates the activation of SREBP1c and as a result elevates fatty acid synthesis. On the other hand, the activity of PPARα is significantly reduced which results in a decline in fatty acid oxidation. Consequently, this leads to cell death attributed to lipotoxicity caused by an increase of fat. Similarly, a vital mechanism that can also lead to cell death is the disruption of the mitochondrial electron chain by destabilising the inner mitochondrial membrane. As a result of this the membrane potential is imbalanced and causes the mitochondrial membrane to swell due to electron leakage and oxidative phosphorylation uncoupling of the ETC, which then reduces the production of ATP. A dysfunctional ETC also increases the synthesis of ROS which can lead to cell death. (Compiled by the author from several sources).

1.6. Role of bile acids

Bile acids produced in the liver as an end product of cholesterol catabolism were originally categorised as physiological detergents that facilitated the metabolism of dietary lipids and lipid soluble vitamins (A, D, E and K), and the hepatobiliary secretion of endogenous metabolites and xenobiotics (Li et al., 2013; Nie et al., 2012). However, recent interest in bile acids over the past few years has shed new light on their roles in the synthetic and regulatory metabolic pathways, pertaining to lipid, carbohydrate and cholesterol regulation acting as indispensable signalling molecules co- ordinating these networks of biological processes (Chiang et al., 2009).

Whilst bile acids chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), cholic Acid (CA) can negatively feedback their own production, they can also act as endogenous ligands for nuclear receptors to facilitate this regulation (Hylemon et al., 2012; Vlahcevic et al., 1996; Parks et al., 1999). The nuclear receptor, Farnesoid X Receptor (FXR; NR1H4) was the first bile acid receptor discovered, followed by other nuclear receptors in the NR1I subfamily, namely Constitutive Androstane Receptor (CAR; NR1I3), Pregnane X Receptor (PXR; NR1I2) and Vitamin D Receptor (VDR; NR1I1) (Makishima et al., 1999; Hylemon et al., 2012; Staudinger et al., 2001).

In terms of nuclear receptor activation, PXR and VDR are stimulated by lithocholic acid (EC50 of approximately 100 nM), which is a hydrophobic bile acid derived from the 7-dehydroxylation of CDCA by intestinal bacteria (Li et al., 2013) (Hylemon et al., 2012). FXR though can be stimulated to varying degrees by most bile acids (CDCA>LCA=DCA>CA), but with the highest potency by CDCA, with an EC50 of approximately 10 μM (Li et al., 2013). Conversely, constitutive androstane receptor is not triggered directly by bile acids but nonetheless is vital for controlling detoxification and transport of bile acids (Gao et al., 2011; Claudel et al., 2011). Of all the nuclear receptors, VDR is expressed broadly across different tissue types. However, FXR and PXR are found abundantly, mainly in tissues in direct contact with bile acids for example in the intestine and liver (Li et al., 2013).

FXR is now considered as a “master regulator of bile acid metabolism” as it is involved in all phases of the biosynthetic pathway, affecting gene expression of ileal bile acid binding protein, small heterodimer partner, phospholipid transfer protein, ABC transporters and apolipoprotein c-II (Chiang, 1998; Mazuy et al., 2014; Russell, 2003). Activation of these nuclear receptors leads to a reduction

in bile acid synthesis, promotion of lipid oxidation and drug metabolism and transport, as well as affecting cholesterol metabolism (Russell, 2003). Conversely dysregulation of bile acid metabolism has a significant impact on inflammatory and metabolic disorders, such NAFLD, diabetes, and obesity (Porez et al., 2012).

1.6.1. Bile acid synthetic pathways

The metabolism of bile acids is tightly controlled, where approximately 95% of the 3 g bile acid body pool that is secreted daily into the intestine is reabsorbed via the enterohepatic circulation, with a small amount excreted in the faeces (200-600 mg/day). Bile acids that are lost are replaced by *de novo* hepatic synthesis derived from cholesterol catabolism (approximately 500 mg) (Chiang, 2009; Russell and Setchell, 1992).

Hepatic bile acid synthesis begins from cholesterol catabolism involving a 17 -step enzymatic pathway (Li et al., 2013; Russell and Setchell, 1992). The synthesis of bile acids is a complex process catalysed by several cytochrome P450 enzymes and involves two major bile acid biosynthetic pathways, the classic and alternative pathway (Hylemon et al., 2012; Li et al., 2013). In the classic pathway, cholesterol is converted to 7α-hydroxycholesterol by the microsomal cytochrome P450 enzyme, Cholesterol 7α-hydroxylase (CYP7A1), which is also the rate-limiting step (Vlahcevic et al., 1996). In humans, the immediate by product of these pathways are the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) (Russell, 2003). The proportion of CA to CDCA synthesised is approximately equal and is regulated by the microsomal enzyme sterol 12-hydroxylase (CYP8B1) (Figure 1.6). The alternative pathway is carried out by mitochondrial sterol 27-hydroxylase (CYP27A1). This pathway occurs in the liver, macrophages and several tissue types in the body and predominantly synthesise CDCA (Hofmann, 2004). The import of cholesterol to the inner mitochondrial membrane, via soluble cholesterol binding protein is thought to be the rate-limiting step in the alternative pathway (Pandak et al., 2002).

Subsequently, bile acids are conjugated to amino acids, typically taurine or glycine via the enzymes bile acid transferase and bile acid coenzyme A synthase, with the proportion of glycine to taurine conjugates estimated as 3 to 1 (Hofmann and Hagey, 2008). Conjugation of bile acids is an important process as it prepares the bile acids for effective detoxification, enhances their amphipathicity and

solubility properties, which consequently leads to impermeability to cell membranes and reduced bile acid toxicity.

However, some conjugated bile acids are deconjugated in the intestine by anaerobic bacteria converting CA and CDCA via 7α-dehydroxylase to the secondary bile acids, lithocholic acid (LCA) and deoxycholic acid (DCA), respectively (Table 1.2). Both of these bile acids are thought to have toxic properties, with DCA causing colon cancer for example (Bernstein et al., 2011). Whilst most DCA is reabsorbed in the colon and LCA is excreted in faeces, around 4% of LCA is transported to the liver where it is conjugated by amidation and sulfation and subsequently excreted into bile. Furthermore, sulfation of hydrophobic bile acids by sulfotransferase 2A1 is a key route for bile acid detoxification (Hofmann, 2004).

Table 1.2. Principle bile acids found in the liver and intestine.

Primary	**Secondary**	**Conjugated**
Cholic acid (CA)	Deoxycholic acid (DCA)	Glycocholic acid
Chenodeoxycholic acid (CDCA)	Lithocholic acid (LCA) Ursodeoxycholic acid (UDCA)	Taurocholic acid Glycodeoxycholic acid Taurodeoxycholic acid Glycolithocholic acid Taurolithocholic acid Glycochenodeoxycholic acid Taurochenodeoxycholic acid

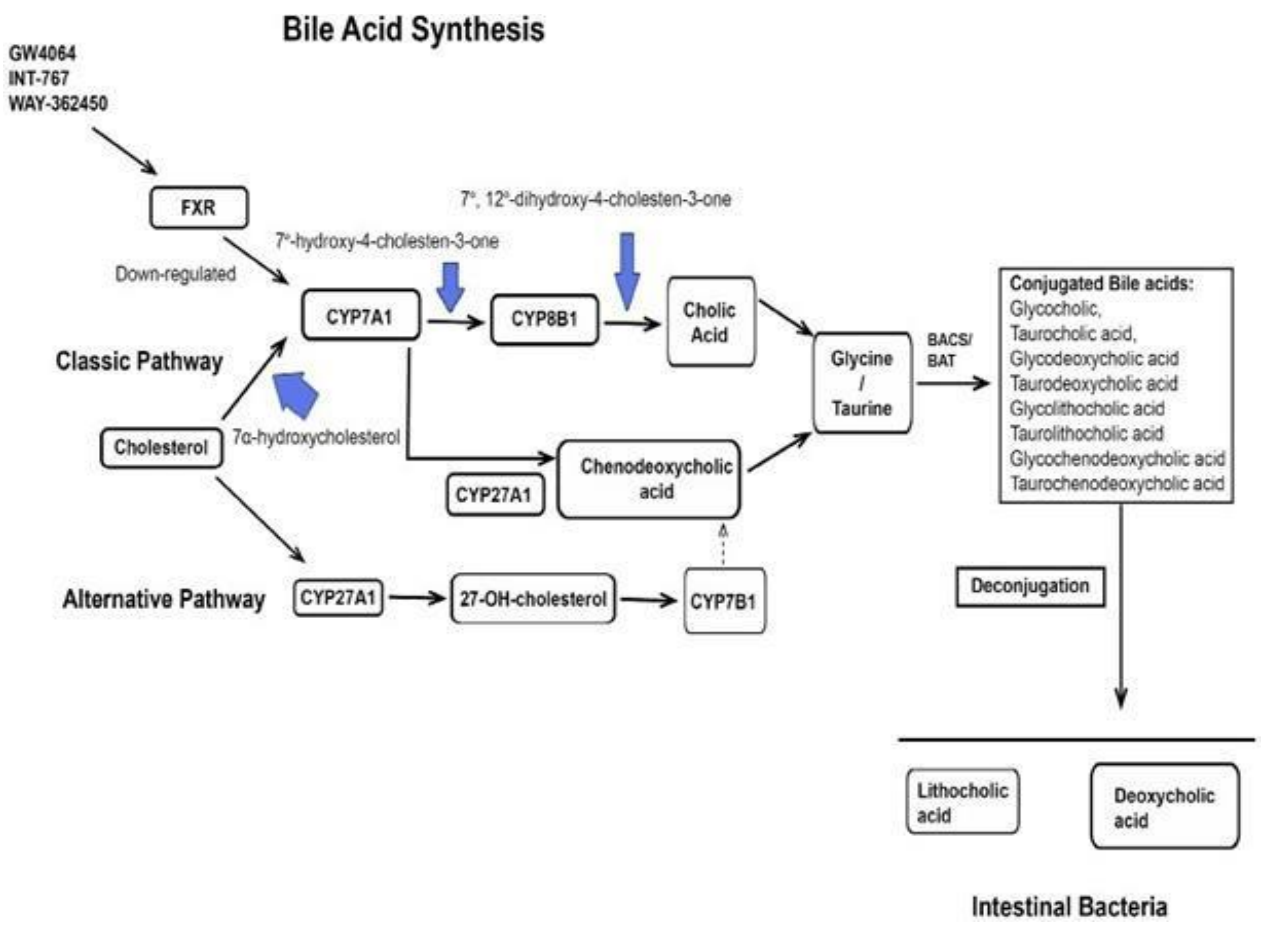


Figure 1.6. Bile acid synthesis.

Catabolism of cholesterol produces the primary bile acids Cholic Acid (CA) and Chenodeoxycholic acid (CDCA) through one of two pathways in the liver. Key regulated enzymes in both pathways are shown. In the classic pathway, Cholesterol-7α-hydroxylase (CYP7A1) catalyses the first rate-limiting step to convert cholesterol to 27- hydroxycholesterol. However, sterol 27α-hydroxylase (CYP27A1) initiates the alternative pathway. Oxysterols, such as sterol 7α-hydroxylase (CYP7B1) produced in peripheral tissues are transported to hepatocytes and converted to CDCA and CA in the alternative pathway. CA synthesis is tightly regulated by 7α-hydroxylase (CYP8B1) in the classic pathway. In the intestine, CA and CDCA are conjugated with Glycine (G) or Taurine (T) by the enzymes Bile Acid Transferase (BAT) and Bile acid coenzyme A synthase (BACS). Some conjugated bile acids such as glycocholic acid, taurocholic acid, glycodeoxycholic acid, taurodeoxycholic acid, glycolithocholic acid, taurolithocholic acid, glycochenodeoxycholic acid and taurochenodeoxycholic acid are de-conjugated and subsequently dehydroxylated at the 7α-position by bacterial enzymes and converted to the secondary bile acids, Deoxycholic acid (DCA) and Lithocholic acid (LCA) respectively. Farnesoid X receptor is activated by agonists such as GW4064, INT-767 and WAY-362450 leading to reduced expression of CYP7A1 and lower levels of the bile acids. (Adapted from Russell, 2009).

1.6.2. Bile acid transport in the enterohepatic circulation

Increased bile acid concentration in the hepatocytes is the driving force of phosphatidylcholine, cholesterol and bile acid transport through the basolateral membrane, and removal of bile acids at the canalicular membrane into the biliary system (Boyer, 1980). Conjugated bile acids are actively transported by the canalicular bile salt export pump and stored in the gallbladder (Ananthanarayanan *et al*., 2001; Childs *et al.,* 2005). Furthermore, mutations in the bile salt export pump gene leads to familial cholestatic syndrome, with the accumulation of toxic hydrophobic bile acids leading to cirrhosis in some cases (Strautnieks *et al*., 1997). The proportion of these molecules, phospholipids, cholesterol and bile acids is tightly controlled by forming mixed micelles in the bile to (i) boost cholesterol solubility, (ii) decrease bile acid toxicity in the bile duct, and (iii) during digestion facilitate the uptake of nutrients into enterocytes. Excess cholesterol and/or hydrophobic bile salts causes saturated bile accumulation, which can consequently lead to the formation of cholesterol gallstones in the biliary system and gallbladder.

At the brush border membrane of the ileum 95% of bile acids are actively reabsorbed by the sodium dependent transporter. Upon absorption, bile acids bind to the ileal bile acid binding protein and diffuse across the basolateral membrane for release into the blood circulation via the heterodimeric organic solute transporters (Dawson *et al*., 2005). Completing this cycle, uptake of reabsorbed bile acids to the liver is mediated by Na+ dependent taurocholate co-transport peptide located in the sinusoidal membrane. Sinusoidal membranes function to efflux bile acids into the circulatory system and therefore express bile acid efflux transporters such as multidrug resistance protein (Hylemon *et al.,* 2012). During cholestasis it has been recognised that these sinusoidal membranes are triggered and could play a fundamental role in the protection of liver injury when bile acids accumulate excessively in hepatocytes (Li *et al*., 2013).

Evidence suggests bile acids have the ability to initiate the production of the metabolic hormone fibroblast growth factor 15/19 (FGF-15 in mice, FGF-19 homolog in humans) in the ileocyte through the action of a functional FXR domain. Fibroblast growth factor 15/19 is transported to the liver where it subsequently binds to its cognate tyrosine kinase receptor. This results in the activation of the c-Jun N- terminal kinases 1/2 signalling pathway and down-regulation of CYP7A1 and bile acid synthesis (Figure 1.7). Interestingly, it is reported that cognate tyrosine kinase receptor β-klotho

null mice have raised CYP7A1 mRNA levels and a larger bile acid pool, and that bile acids and cytokines (TNF-α) are also capable of triggering the c-Jun N-terminal kinases 1/2 signalling pathway and as a result down-regulate CYP7A1 MRNA (Song et al., 2009).

1.7. Therapeutic approach of bile acids for non-alcoholic fatty liver disease

To date there are two major categories of NAFLD therapies: (i) lifestyle intervention (weight loss, physical exercise) and (ii) pharmaceutical therapies (insulin sensitizers, lipid-lowering agents) (Durazzo *et al.,* 2012). Considering insulin resistance plays a core role in the pathogenesis of NASH, glitazones and peroxisome proliferator- activated receptor γ agonists used in the treatment of type 2 diabetes have been comprehensively studied (Ratziu and Pienar, 2011).

In particular, thiazolidinedione (TZD), an insulin sensitizer has been shown to improve hepatic biochemical and histological parameters in patients (Aithal *et al*., 2008). Similarly, treatment with the TZD pioglitazone led to a reduction in circulating aspartate aminotransferase (AST) activities by 30-58%, improved hepatic insulin sensitivity, and reversed steatosis in NASH patients (Ratziu and Pienar, 2008). TZDs also reduced the expression of genes linked with inflammation such as interleukin-6 and TNF-α and possessed regulatory properties through proliferator-activated receptor γ activation of adipokines, the latter possessing crucial roles in the pathogenesis of NAFLD (Sharma and Staels, 2007). Furthermore, in diabetic patients with NASH, a phase II double blind trial demonstrated that pioglitazone significantly decreased steatosis, inflammation and ballooning necrosis (Belfort *et al*., 2006).

However, there is variable data from TZDs studies regarding regression of fibrosis (Ratziu and Pienar, 2008). A meta- analysis showed that pioglitazone, but not rosiglitazone reduced fibrosis (Boettcher *et al.,* 2012). However, a large trial comparing ‘pioglitazone versus Vitamin E for the treatment of non-diabetic patients with NASH showed improved histology in NASH patients who received pioglitazone but no significant improvement in fibrosis stage for either treatment (Sanyal *et al.,* 2010). Despite the promising results of these pioglitazone studies, the adverse effects of TZD such as weight gain, increased bladder cancer risk and cardiovascular morbidity has limited the use of pioglitazone in patients with NASH (Baran and Akyuz, 2014). Although vitamin E (400 IU/day) demonstrated some benefit to NASH subjects, as shown by reduced inflammation, other reports

suggest vitamin E supplementation is associated with cardiac morbidity including heart failure and haemorrhagic stroke (Lavine *et al*., 2011; Sanyal *et al*., 2010; (Saremi and Arora, 2010).

Overall it seems that targeting insulin resistance and oxidative stress is crucial but not adequate for effectively treating NASH, signifying the need for wider hepatoprotective agents. Emerging evidence suggests beneficial properties of bile acids and their derivatives in the treatment of NAFLD by regulating lipid and glucose pathways, reducing inflammation and lowering hepatic triglyceride levels (Li et al., 2013). Agonists have been developed targeting FXR receptors as a therapeutic approach in NAFLD as discussed below.

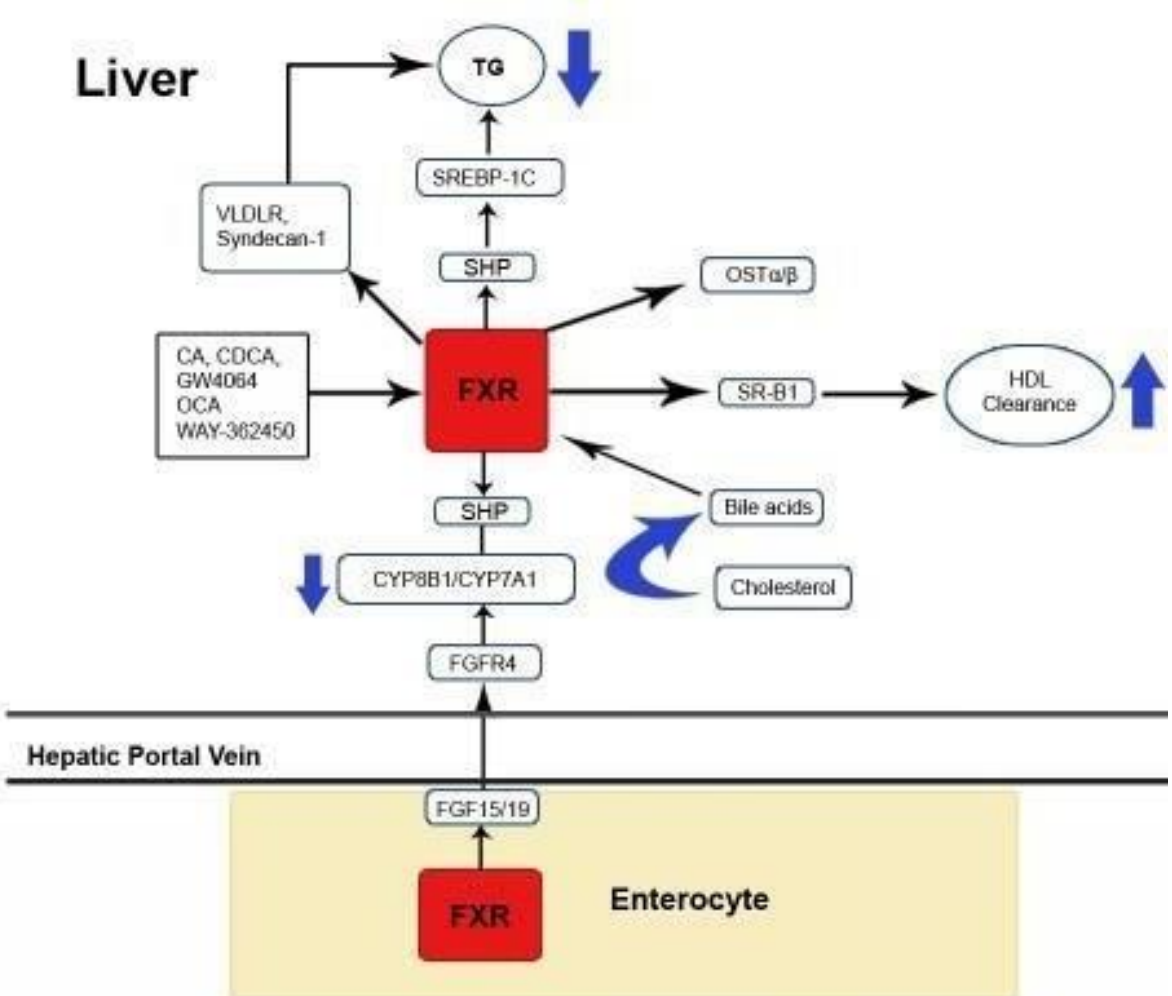


Figure 1.7. Overview of FXR regulation

Bile acids Cholic Acid (CA) and Chenodeoxycholic acid (CDCA) and FXR agonists such as GW4064, OCA and WAY362450 activate hepatic and intestinal FXR to regulate genes vital for bile acid metabolism. The activation of hepatic FXR lowers plasma cholesterol and Triglyceride (TG) synthesis via several pathways. For instance, Short Heterodimer Protein (SHP)-dependent inhibition of Sterol Regulatory Element Binding Protein-1c (SREBP-1c) leads to the suppression of hepatic TG. Furthermore, FXR suppresses bile acid synthesis by reducing CYP7A1 and CYP8B1 expression via SHP. Stimulation of Very Low Density Lipoprotein Receptor (VLDLR) and syndecan-1 also promotes the clearance of TG lipoproteins, and similarly Scavenger receptor class B1 (SR-B1) promotes the clearance of High Density Lipoprotein (HDL) in the liver. In the enterocytes bile acids bind to FXR and as a result elevate the expression of two transporters, organic solute transporter alpha and beta (OSTα, OSTβ) that facilitate bile acid transport into the hepatic portal vein. Induction of intestinal FXR increases the expression of fibroblast growth factor 15/19 (FGF15/19) into the hepatic portal vein. The subsequent binding of FGF15/19 to the hepatic cell-surface receptor fibroblast growth factor receptor 4 (FGFR4) triggers the JNK pathway leading to the suppression of CYP7A1 and CYP8B1 hence a decrease in bile acid synthesis. (Adapted from Chiang et al., 2009).

1.8. Role of FXR in NAFLD

Evidence of the vital role that FXR plays in NAFLD has been observed in FXR null mice. Here FXR null mice present with hepatic steatosis, inflammation, elevated bile acid levels, hyperlipidaemia and fibrosis (Yang et al., 2010). Furthermore, in FXR deficient mice fed a 1% cholesterol diet, mice exhibited severe muscle wastage, raised hepatic cholesterol content and a 23-fold greater increase in hepatic triglyceride. Additionally, excessive levels of bile acids correlated to 30% mortality in FXR deficient mice by day 7, which was attributable to liver failure (Sinal et al., 2000). Gut derived lipopolysaccharide also plays a crucial role in the pathogenesis of NASH (Abu-Shahnab and Quigley, 2010). Increased lipopolysaccharide stimulates nuclear factor kappa β, which acts to recruit inflammatory cells, thus promoting inflammation, fibrosis and carcinogenesis in advanced NAFLD (Elsharkawy and Mann, 2007).

In FXR null mice, inflammation was characterised by increased circulating TNF-α, interleukin-1β levels, and hepatic bile acid levels, as well as spontaneous hepatocellular carcinoma at 12 months (Kim et al., 2007). Although these studies indicate a causal link between elevated bile acids and inflammation, FXR activation has been shown to suppress the nuclear factor kappa β pathway, as well as the inflammatory cytokines and cyclooxygenase- 2 in hepatocytes (Carr and Reid, 2015).

FXR activation also regulates carbohydrate metabolism (Stayrook et al., 2005). Administration of cholic acid to C57BL/6J mice led to a decrease in fasting glucose concentration and reduced expression of the phosphoenolpyruvate carboxykinase gene in the liver, and also in HepG2 cells incubated with CDCA (Yamagata et al., 2004; Adorini et al., 2012). Other studies found that administration of the FXR agonist GW4064 in a diabetic rat model mediated the activation of hepatic FXR and as a result this reduced phosphoenolpyruvate expression, reversed hyperglycaemia and normalised glycogen storage (Yamagata et al., 2004; Langhi et al., 2008). Thus, FXR is thought to regulate gluconeogenesis through its key enzyme phosphoenolpyruvate carboxykinase (Ma et al., 2013). These studies suggest activated FXR normalises lipid and glucose metabolism and prevents inflammation, thus such properties of FXR make it a novel therapeutic approach in NAFLD treatment.

1.9. FXR agonists in NAFLD and NASH

A variety of studies have targeted FXR to modulate the metabolism of lipids, carbohydrates and bile acids in NASH, with the synthetic agonist GW4064 widely studied (Zhang et al., 2006). Here, FXR deficient mice show both systemic and hepatic insulin resistance, which can be normalised by giving the FXR agonist GW4064 (Cariou et al., 2006). In a diabetic mouse model lacking leptin receptors, treatment with GW4064 for 5 days led to a substantial decrease in the plasma levels of glucose and triglycerides (Zhang et al., 2006). Whereas administration of GW4064 normalised steatosis and serum triglycerides levels in aged mice possibly due to the reduction in endoplasmic reticulum stress (Xiong et al., 2014). Finally, GW4064 reduced bile acid synthesis, and increased bile acid export in a model of cholestasis (Liu et al., 2003). In alternate models of NASH, C57BL/6 mice fed a methionine and choline deficient diet and treated with the FXR agonist WAY-362450 for 4 weeks showed a decline in serum AST and ALT activities, improved liver histology and decreased inflammatory cell infiltration and fibrosis (Zhang et al., 2009). These studies indicate that FXR agonists may be useful for the treatment of NASH and related liver disorders by normalising carbohydrate, lipid and bile acid metabolism.

Obeticholic acid (OCA) is a synthetic bile acid analogue, also known as INT-747 and the 6α-ethyl derivative of CDCA and was originally identified in 2002 for its hepatoprotective and anti-cholestatic properties in a model of cholestasis, protecting hepatocytes against acute necrosis triggered by LCA (Pellicciari et al., 2002). Interestingly, OCA synthesised through the addition of the ethyl group to CDCA exhibits 100-fold greater agonistic activity than CDCA and this potency has been confirmed by the analysis of the co-crystal structure of the FXR ligand-binding domain (Mi et al., 2003).

OCA has been studied in a rabbit model of the metabolic syndrome and in the Zucker rat model of obesity where administration of OCA improved glucose and insulin tolerance and decreased steatohepatitis (Vignozzi et al., 2011; Cipriani et al., 2010). Furthermore, FXR activation by OCA decreased hepatic expression of genes involved in fatty acid synthesis including SREBP1c, reduced TNF-α levels and elevated peroxisome- proliferator activated receptor alpha expression, which therefore led to an improvement in the NASH phenotype (Carr and Reid, 2015; Cipriani et al., 2010). OCA therapy also reduced inflammation in a FXR deficient model of autoimmune hepatitis and prevented hepatic stellate cell activation by inhibiting osteopontin production (Fiorucci et al., 2004;

Mencarelli et al., 2009). In the thioacetamide rat model of fibrosis, OCA prevented fibrosis progression, reversed fibrosis and cirrhosis development, and significantly reduced portal hypertension (Carr and Reid, 2015). Therefore, these preclinical findings have established the anti-inflammatory and anti-fibrotic properties of OCA mediated by FXR, as a candidate agent for NASH/NAFLD treatment.

1.10. FXR agonists in clinical studies

To date there are only a handful of studies investigating FXR agonists in NAFLD. A small study of patients with type II diabetes and NAFLD showed a marked improvement in insulin sensitivity of 28% when given 25 mg of OCA for a short 6 week period (Mudaliar et al., 2013). Furthermore, endogenous levels of bile acid decreased, as well as markers of inflammation and fibrosis (Mudaliar et al., 2013). However, the largest clinical trial to date 'Farnesoid X receptor ligand Obeticholic Acid in Non-Alcoholic Steatohepatitis' (FLINT) study has been partially reported in NASH patients receiving oral OCA 25 mg for 72 weeks (Neuschwander et al., 2014). Preliminary findings indicated a 45% improvement in liver histology compared to 21% of the placebo group (Neuschwander et al., 2014).

Similar to the previous study in diabetics, lower activities of ALT in the group were noted as well as an increase in serum alkaline phosphatase activities, despite decreased gamma-glutamyl transferase levels. Pruritus an adverse effect of OCA treatment was observed in 23% of the OCA group compared with 6% of the placebo group, thus these OCA treated patients may require symptom management (Neuschwander et al., 2014). Despite these promising results, longer term clinical studies are required to explore the impact of OCA as well as other FXR agonists in the treatment of NASH/ NAFLD. There is some evidence indicating that OCA treatment leads to a significant increase (~20%) in LDL-cholesterol levels, which was apparent in the FLINT study, the earlier study on diabetes and NAFLD and in the treatment of diarrhoea (Walters et al., 2015). Authors from the FLINT study suggested that this atherosclerosis risk requires careful monitoring and OCA treatment withdrawn in these cases. This clearly may have a negative impact for the long-term usage of OCA, despite the clinical benefit on liver histology parameters.

One of the original bile acids used clinically for NASH was UCDA. This has been comprehensively reviewed, but earlier studies did not report an improvement in liver histology or liver enzymes after 2 years of treatment at a low dose of 13- 15 mg/kg or 6 months at a high dose of 28-32 mg/kg, but was effective at a high dose after 1 year of treatment (Lindor et al., 2004; Adams et al., 2010; Ratziu et al., 2011). Whilst the long-term benefit of UCDA requires confirmation, a taurine conjugate of UCDA called tauroursodeoxycholic acid may have potential use, as this has shown to prevent NASH progression by decreasing endoplasmic reticulum stress (Cho et al., 2014).

1. 11. Alternative therapies

Alternative treatment options may offer immediate solutions such as exercise weight loss programmes, insulin sensitizers or bariatric surgery, which has proven to reduce steatosis, inflammation and moderate amounts of fibrosis in NAFLD patients. However, like OCA, the long-term benefit of bariatric surgery still requires investigation. In addition to these therapies, antioxidant approaches have shown encouraging results in reducing lipid accumulation in the liver. Betaine (trimethylglycine), a methyl donor derived from the oxidation of dietary sources of choline may be a potentially effective treatment for NAFLD (Abdelmalek et al., 2009).

Animal models of NAFLD have used high carbohydrate and fat diets or methyl donor deficiency diets (lacking in methionine, choline and folic acid) to induce NAFLD. Betaine lowers hepatic S-adenosylmethionine (SAM), the major methyl donor, and increases levels of both homocysteine and S-adenosylhomocysteine (SAH). Betaine reduces circulating levels of homocysteine by facilitating its conversion to methionine, which also lowers SAH. Betaine is the only alternate source of methyl groups for the conversion of homocysteine to methionine. Furthermore, betaine can also substitute for SAM as a methyl donor for the direct methylation of phosphatidylethanolamine. This pathway serves as an alternative route of phosphatidylcholine formation. From these functions, betaine has derived its beneficial effects on metabolism and lessens defects in the methylation pathway caused by deficiencies or impaired function of the folate cycle.

Although the relationship between plasma/serum betaine and NAFLD is not well understood, a study by Konstantinova et al (2008) showed the concentration of plasma betaine is inversely associated with serum non-HDL cholesterol, triglyceride, BMI and is positively associated with HDL and physical

activity. Animal models have established that betaine could substitute for choline and function as an effective dietary methyl donor when dietary methionine is insufficient.

Novel therapeutic approaches to manage the chronic liver disease epidemic are becoming essential. As mentioned, bile acid-activated FXR regulates key homeostatic mechanisms including carbohydrate, lipid, and bile acid metabolism whilst also inhibiting inflammatory and fibrogenic responses. However, the precise mechanisms are still being investigated as well as the impact of intestinal bile acids, on FXR activation. Despite this, pre-clinical and clinical evidence support the notion that therapy with the first-in-class FXR agonist obeticholic acid has the potential to effectively and safely treat chronic liver diseases such as NAFLD.

1. 12. Aims

The aim of this project is to investigate the mechanisms of action involved in fatty liver disease development and the role of bile acids (ursodeoxycholic acid, chenodexoycholic acid) and betaine in attenuating fatty liver disease.

1.13. Objectives

1. To characterise the effect of saturated fatty acid (palmitic acid) on lipotoxicity and lipid accumulation in VL17A cells.

2. To determine the mechanism of action of bile acids (ursodeoxycholic acid, chenodeoxycholic acid), betaine and MHY908 on prevention of lipotoxicity and lipid accumulation in VL17A cells.

1.14. Hypothesis

Bile acids (ursodeoxycholic acid and chenodeoxycholic acid), betaine and MHY908 are hepatoprotective and can be used as an alternative therapeutic approach to attenuate lipid accumulation in NAFLD patients.

Chapter 2

Materials & Methods

2. Materials & Methods

2.1. Materials

Phosphate buffered saline (PBS), L-glutamine, penicillin-streptomycin mix and Dulbecco's Modified Eagle Medium (DMEM) were bought from Lonza Ltd (Wokingham, UK). 1x Trypsin in DPBS was purchased from First Link Ltd (Birmingham, UK). Heat-inactivated foetal calf serum (FCS) was obtained from Biosera (Ringmer, UK). Sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), Oil red O, 2′,7′-dichlorofluorescin diacetate (DCFDA), palmitic acid (PA), ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA), D-(+)-Glucose were acquired from SigmaAldrich (Gillingham, UK). Dimethyl sulfoxide (DMSO), formaldehyde and isopropanol were obtained from Fisher Scientific UK (Loughborough, UK). Triglyceride Assay Kit was obtained from Universal Biologicals (Cambridge, United Kingdom [UK]), XF Cell Mito Stress Test Kit and Seahorse XF Assay Medium were obtained from Agilent Technologies (Craven Arms, UK). Nuclear extraction kit and SREBP1 transcription factor assay kit were purchased from Cayman Chemicals (Michigan, USA).

2.2. Methods

2.2.1. Cell culture

Human hepatoma cell line (HepG2), VL-17A cells (kindly donated by Dr Clemens, University of Nebraska) which over-express the cytochrome enzyme Cytochrome P450 2E1 (CYP2E1) and alcohol dehydrogenase (ADH) were cultured in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) (Lonza, UK) supplemented with 10% fetal calf serum (FCS) (Lonza, UK), L-Glutamine (2 mM) (Lonza, UK), penicillin (100 units/mL) (Lonza, UK) and sodium pyruvate (1mM) (Sigma Aldrich, Gillingham, UK) (Chandrasekaran *et al.,* 2011; Gyamfi *et al*., 2012). VL17A cells were grown at 37ºC in a humidified atmosphere of 95% air and 5% CO2. Cells were also grown in the presence of the antibiotic Plasmocin Prophylactic (5 µg/mL) for 4 weeks to eliminate potential microbial contamination. The culture medium was changed every 3-4 days.

At 70-80% confluence, cells were passaged by trypsinisation for 5 minutes. To neutralise the trypsin, 10% FCS DMEM was added and centrifugation was performed at 500 rotations per minute (rpm) for

5 minutes at room temperature. The supernatant was removed, and fresh DMEM was added to the cell pellet and the cells were plated at the required density for fatty acid studies. Cells were treated individually or were co-incubated with fatty acid or bile acids at a range of concentrations and time points. Briefly, fatty acids were prepared as described by Gyamfi *et al*. (2012) and similarly bile acids with some modifications, in DMSO as stock and subsequently the bile acids and fatty acids were diluted in 1% FCS, low glucose (1 g/L) DMEM to the required concentrations.

2.2.1.2. Liquid nitrogen storage and cell revival

VL17A cells were stored in cryogenic vials in 90% FCS and 10 % dimethyl sulphoxide (DMSO) in the Cryo 1°C freezing container, at -80 ºC for 24 hours and then transferred to a liquid nitrogen cell bank for long term storage. When reviving the cells from liquid nitrogen, frozen vials were thawed in a water bath for 2 minutes at 37 ºC. To wash away the DMSO, 1 ml of warm maintenance media (10% FCS DMEM) was added and the contents were transferred to a 15 mL centrifuge tube. Cells were centrifuged at 1000 rpm for 5 min at room temperature. The supernatant was discarded and the cell pellet was re-suspended in maintenance medium and seeded in a 25 cm^2 flask containing warm maintenance medium to initiate cell growth.

2.3. Determination of cellular lipotoxicity

2.3.1. MTT assay

Cell viability was assessed by the MTT assay using the Chuturgoon et al. (2015) method with some modification. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), a yellow tetrazolium salt is dehydrogenised by reductase enzymes in mitochondria of live cells to form purple coloured insoluble formazan crystals.

VL17A cells were seeded overnight in sterile 96 well plates (2.5 x 10^4 cells/well) and treatment media was then added to each well. For example, VL17A cells were exposed to the following concentrations of saturated palmitic acid (10 µM, 20 µM, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM, 250 µM and 300 µM) or with bile acids alone (UDCA: 30 µM, 150 µM, 300 µM and CDCA: 30 µM, 150 µM, 300 µM) or co- treatment of PA and bile acids for 24, 48 and 72 hours respectively at 37°C. Vehicle control

was included as 0.5% DMSO. On completion of the treatment exposure time, the treatment media were discarded and cells were incubated with MTT (5 mg/mL) for 2 hours at 37ºC. The media were discarded and 100 µL DMSO was added to each well and incubated at room temperature for 15 minutes on a vortex plate. The absorbance was read spectrophotometrically at 550 nm. The data are expressed as percentage from the control.

2.4. Determination of steatosis

2.4.1. Oil red O assay

To quantify lipid accumulation in VL17A cells following fatty acid treatment (PA: 10 µM, 20 µM, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM, 250 µM, 300 µM) and bile acid treatment (UDCA: 30 µM, 150 µM, 300 µM and CDCA: 30 µM, 150 µM, 300 µM), cells (5 x104 cells/well) were seeded and treated in 96 well plates as described above. Treatment media were discarded, and cells were washed thrice with iced PBS and then fixed with 10% formalin for 1 hour at room temperature. After fixation, cells were washed thrice with iced PBS and stained with Oil red O solution (60% working oil red solution, 3 mg/ml in isopropanol) (Sigma- Aldrich, Gillingham, UK) for 1 hour at room temperature. Cells were washed with distilled water to remove unbound stain. Lipid accumulation was measured by adding 200 µL DMSO to each well for 5 min and absorbance was read spectrophotometrically at 510 nm (Gyamfi et al., 2012). The data are expressed as a percentage from the control.

2.4.2. Intracellular triglyceride assay

The EnzyChrom triglyceride assay kit (BioAssay systems, USA) was used to quantify the intracellular triglyceride concentration in VL17A cells. Cells were seeded at a density of 2 x 10^5 cell/ 2 mL in a 24 well plate overnight to allow the cells to adhere. Following this, the cells were treated in triplicate for 24 hours with palmitic acid (50 µM) and bile acids (UDCA: 30 µM and CDCA: 30 µM). A vehicle control of DMSO with a final concentration of 0.5% was also added. The intracellular triglyceride content was then quantified following the manufacturer's protocol after the cells had been washed and trypsinised. Subsequently, the protein concentration was also measured using a Bio-Rad protein assay kit following the manufacturer's instructions. The triglyceride concentration was expressed as milligram triglyceride per milligram protein.

2.5. Protein estimation

VL17A cells were passaged and the cell pellets were collected in centrifuge tubes. To this 1 mL of Triton (x100) was added to breakdown the cell membrane to release protein and following this, were centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was collected and the protein assay was carried out using the Bio-Rad assay kit (Bio-Rad, UK). This is a dye-binding assay in which differential colour change of the dye occurs in response to various concentrations of protein. Standard protein solutions of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2 µg/ µL, blank and diluted samples were added in triplicate to a 96-well plate followed by 250 µL of the Bradford reagent. Absorbance was quantified at 550 nm with the FLUOstar OPTIMA (Jencons-PLS, Bedfordshire, UK) spectrophotometer and the protein concentration of the sample calculated from a standard calibration plot.

2.6. Measurement of intracellular ROS levels

The level of intracellular reactive oxygen species (ROS) was investigated in VL-17A cells by using 2′,7′-dichlorofluorescin diacetate (DCFDA). Briefly, the cells were seeded at a density of 15 x 10^4 cell/ 200-µL DMEM in a 96-well plate at 37ºC overnight. The cells were then treated separately with PA (10 µM, 20 µM, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM, 250 µM and 300 µM) or bile acids (UDCA: 30 µM, 150 µM, 300 µM and CDCA: 30 µM, 150 µM, 300 µM) for 30 min, 1 h and 2 h, respectively at 37°C. Following the incubation, the cells were further incubated with 1 µM DCFDA diluted in PBS for 45 min at 37ºC. The intensity of fluorescence was measured using 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 data are expressed as percentage of the control.

2.7. Seahorse assay

Several parameters analysing mitochondrial function were measured using the Seahorse XFe Analyzer (Figure 2.1) (Seahorse Biosciences, Massachusetts, USA). Previous research work in the laboratory was undertaken to optimise the conditions required to achieve optimal baseline readings. Briefly, VL17A cells were seeded at a range of densities (1 x10^4, 1.5 x 10^4 and 2 x10^4 cells/well) into a Seahorse XFe24 microplate using recommended seeding density from the Seahorse database and subsequently the Mito stress assay was carried out according to the manufacturers protocol. The optimal cell density for the experiments was determined as 1.5 x10^4 cells/well.

VL17A cells were seeded in 100 µL DMEM and incubated at 37°C, in 5% CO2 for 2 hours to adhere to the bottom of the plate, then topped up with 400 µL of DMEM and incubated overnight. The Seahorse XFe 24 analyser was warmed overnight and the assay cartridge soaked in 1 mL per well of XF calibrant overnight in a CO2 -free incubator at 37°C to hydrate the cartridges. Using the XF 24-well plate, wells A1, B4, C3 and D6 served as blank wells with no cells. After approximately 24 hours of incubation, the XF media for the assay was prepared by adding 10 mM glucose, 2 mM glutamine and 1 mM pyruvate to the media. The pH of the media was adjusted to 7.4 using 0.1 M NaOH and the media was warmed up in a water bath at 37°C. An hour prior to the experiment, the cells were washed twice with the prepared XF media, where 500 µL of media was added to the cells

and the plate was incubated in CO2 -free incubator at for at least 1 hour before the start of the experiment to allow the microplate to de-gas.

During incubation the software was set up as per the manufacturer's instructions. When prompted the sensor cartridge with the calibrant plate was loaded onto the analyser tray to be calibrated and equilibrated. Following this, the cell culture microplate was loaded, the tray door closed, and the assay started. Following the protocols already set up in the software, oxygen consumption rate (OCR) and extracellular cell acidification rate readings for each well were taken and recorded.

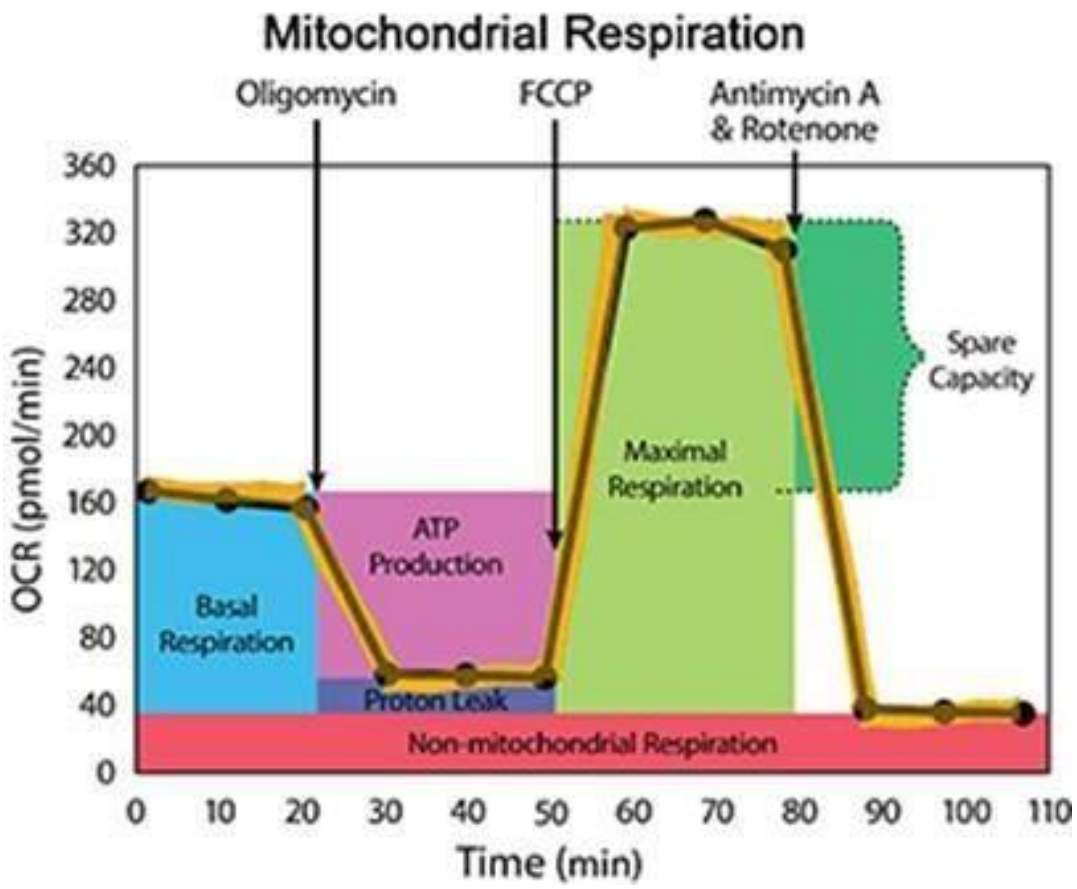


Figure 2.1. Seahorse Mito stress parameters.

2.7.1. The Mito Stress assay

An XF cell mito stress kit was used to determine the mitochondrial respiration rate as per the manufacturer's protocol (Agilent technologies, Craven Arms, UK). This assay involved the measurement of OCR of cells as a main parameter. After the injection of compounds (1 µM FCCP, 1 µM oligomycin and 0.5 µM antimycin A / rotenone mix) that are the modulators of the electron transport chain, parameters like the basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration were measured/calculated. VL17A cells were seeded overnight, the cells were treated with control, palmitic acid (100 µM) and bile acid, UDCA (30 µM), betaine (20 µM), and incubated in seahorse media for 24 h at 37°C and the mitostress assay performed on the cells following treatment.

An hour prior to the experiment, 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 loaded with Mito stress drugs, 59 µL of oligomycin (1 µM) to inhibit

ATP synthase; 62 µL FCCP (1 µM), an uncoupling agent; and 69 µL antimycin/rotenone mixture (0.5 µM) to inhibit oxidative phosphorylation and electron transfer, respectively. Following OCR measurement, results were normalised against the protein concentration.

2.7.2. Protein normalisation

OCR readings were normalised against protein. Here, cells were washed in ice cold PBS, and then lysed in 30 µL of RIPA buffer (Millipore, UK) for 5 minutes at room temperature. The protein concentration was determined for each well as described in Section 2.5.

2.7.3. Seahorse data analysis

The data was analysed using the Seahorse Wave 2.0 software, which allows for normalization of OCR values against protein concentration and were presented in a Mito stress report.

2.8. Sterol regulator element binding protein 1 ELISA Assay

A 96 well enzyme-linked immunoabsorbent assay (ELISA) was carried out to determine the expression of the transcriptional factor SREBP1 in fatty acid and bile acid (100 µM PA and 30 µM UDCA) treated nuclear extracts. A specific double stranded DNA (dsDNA) sequence containing the SREBP response element was immobilised onto the wells of a 96-well ELISA plate. SREBP-1 contained in a nuclear extract will bind specifically to the SREBP response element and SREBP-1 is detected by addition of a specific primary antibody directed against SREBP-1. A secondary antibody conjugated to HRP was added and the absorbance reading at 450 nm was taken and recorded.

2.8.1. Nuclear Extraction assay

To analyse the activity of SREBP1 in the treated VL17A cells, nuclear extracts were prepared. Briefly, 7 x10^7 VL17A cells were cultured in T75 flasks and treated with fatty acids or bile acid (100 µM PA and 30 µM UDCA), respectively. The adherent cells were collected in pre-chilled 15 mL centrifuge tubes by scraping using a sterile scraper. VL17A cells were centrifuged at 300 x g for 5 minutes at 4 ºC and subsequently the supernatant was discarded. The cell pellet was then re-suspended in 5 mL of ice cold PBS/phosphatase inhibitor solution and centrifuged for 5 minutes at 4 ºC and the step was repeated. Again, the supernatant was removed and 500 µL ice -cold 1X hypotonic buffer was added to the pellet and was mixed gently by pipetting. The re-suspended pellet was transferred to a 1.5 ml

microcentrifuge tube. Following this, the samples were incubated on ice for 15 minutes to allow the cells to swell and then 100 µL of 10% NP-40 assay reagent was added to the microcentrifuge tube.

Subsequently, the samples were centrifuged at 14,000 x g for 30 seconds and the pellet was resuspended in 100 µL ice-cold complete nuclear extraction buffer (1x). The samples were vortexed vigorously for 15 seconds and then placed on ice for 15 minutes. Following this the samples were centrifuged at 14,000 x g for 10 minutes at room temp and the supernatant which contains the nuclear extract was transferred to pre-chilled tubes. The protein concentration in the nuclear extracts was quantified by following the protocol outlined in Section 2.5.

2.8.2. SREBP1 transcription factor assay

Reagents for this assay were prepared according to manufacturer's protocol and the strips for the 96 well plate were set up. In each well, 90 µL of the complete transcription factor binding assay was added and followed by 10 µL of competitive dsDNA supplied in the kit to the well. Following this, 10 µL of the positive control was added to appropriate wells and 10 µL of the nuclear extracts were prepared containing SREBP1 and added to the relevant wells. The plate was incubated overnight at 4ºC without agitation.

On the following day, each well was washed 5 times with 200 µL of 1X wash buffer and then 100 µL of diluted SREBP1 antibody was added to each well except the blanks and incubated for 1 hour at room temperature. Again, each well was washed 5 times with 200 µL of 1X wash buffer and then incubated for one hour at room temperature with 100 µL of the diluted secondary antibody with the exception of the blank wells.

After incubation, each well was washed with 200 µL of 1X wash buffer followed by the addition of 100 µL of developing solution per well. The plate was incubated at room temperature for 45 minutes to allow the blue colour to develop in the assay for 45 minutes. Following this 100 µL of stop solution was added to each well whereby a colour change from blue to yellow was observed and the absorbance was read at 450 nm.

2.9 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 were presented as mean $\pm$ standard error of mean (SEM), and the differences were considered significant if the p value $\leq$ 0.05.

Chapter 3

Results

3. Results

As mentioned in Section 1.1., Non-alcoholic fatty liver disease (NAFLD) histopathologically ranges from simple steatosis to more severe pathologies such as fibrosis and hepatocellular carcinoma characterised by inflammation and fibrogenic changes in the hepatocytes.

While steatosis seems to be relatively benign, the accumulation of fat in the hepatocytes results in the increased susceptibility of lipotoxic damage in the liver. Mechanisms of increased sensitivity of steatosis in hepatocytes to lipotoxic stimuli should be understood to preserve the liver from further cellular injury.

Therefore *in vitro* models are useful to gain a fuller understanding of the effects of steatosis in the liver. The most abundant saturated fatty acid in a steatotic hepatocyte is palmitic acid (PA) C16:0 and previous studies have reported the induction of steatosis and lipotoxicity by PA (Oh et al., 2012; Wang et al., 2008).

The aim of the first set of dose response experiments was to verify the steatogenic and cytotoxic effects of PA at different concentrations and time points in VL17A cells and to determine whether this *in vitro* model could be used as a model of NAFLD.

3.1. Effect of palmitic acid (PA) on cell viability in VL17A cells

Cell viability of cells exposed to different concentrations of palmitic acid (10 μM, 20 μM, 30 μM, 50 μM, 100 μM, 150 μM, 200 μM, 250 μM and 300 μM) was examined using the MTT assay at 24, 48 and 72 hour time points.

Data showed a significant increase in cell viability at lower concentrations of PA 10 μM (+25%) and 20 μM (+23%) at 24 h, respectively ($p < 0.001$; Figure 3.1). However, at higher concentrations and longer incubation times of PA treatment there was a profound decrease in cell viability at all time points, especially with 150 μM and 300 μM ($p < 0.001$). As an example, at 24 h cell viability decreased by 80% at 300 μM PA treatment ($p < 0.001$; Figure 3.1) which further decreased when incubated for 48 h (-85%, $p < 0.001$) and 72 h (-90%, $p < 0.001$) respectively (Figure 3.1).

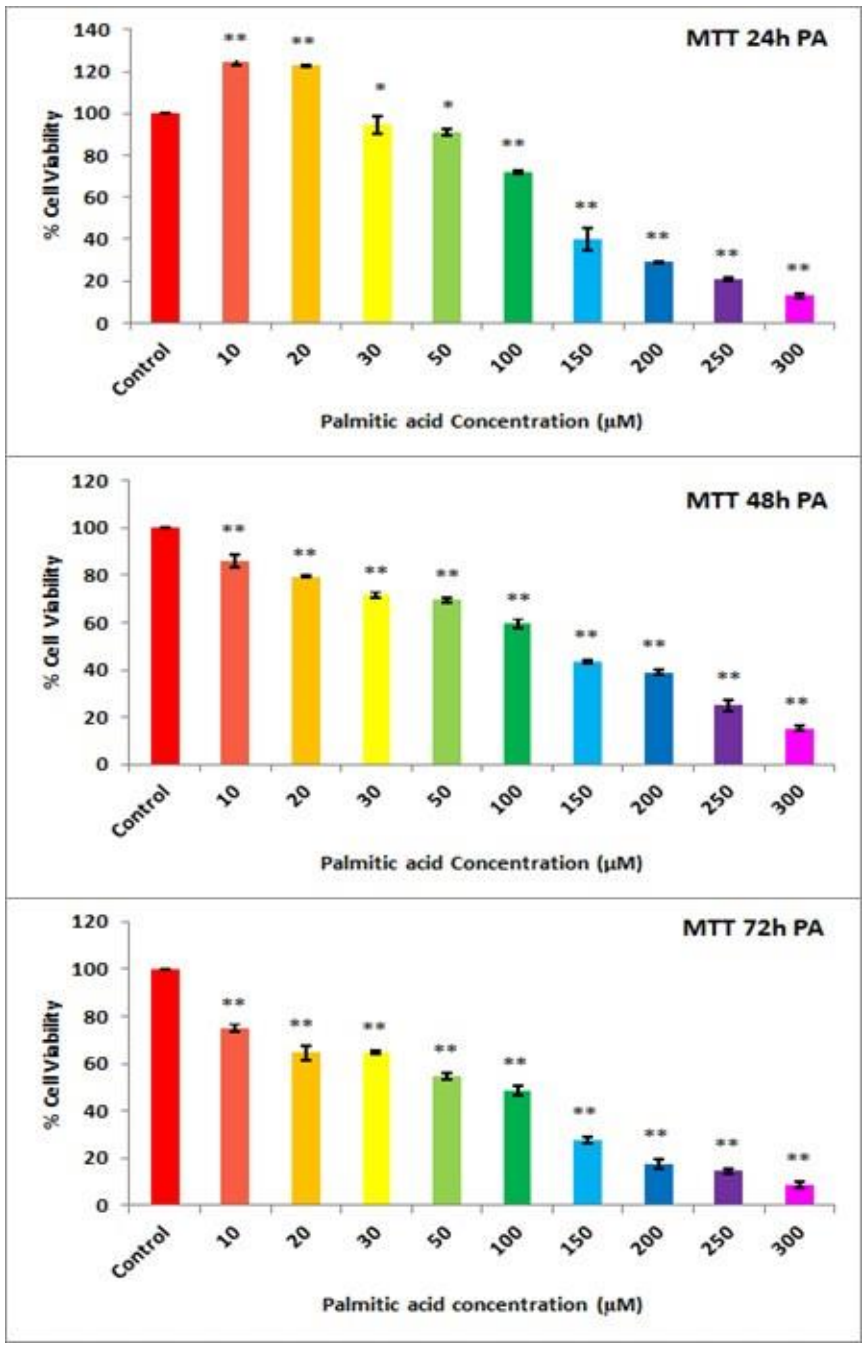
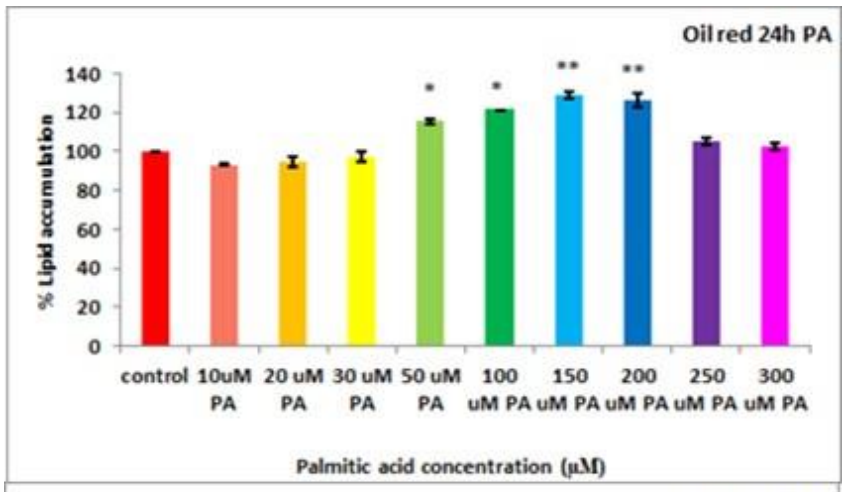


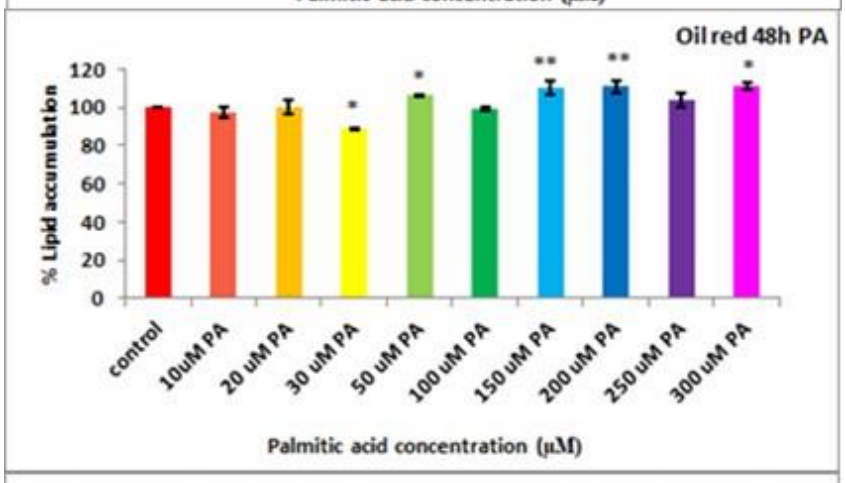
Figure 3.1. The effect of palmitic acid (PA) on the viability of VL17A cells. VL17A cells (25 x 103 cells/well) were seeded onto 96-well plates followed by fatty acid treatment with palmitic acid (PA) as described in the methods section. Cell viability was assessed after palmitic acid exposure at 24, 48 and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.001$ as compared to the control.

3.1.1. Effect of palmitic acid (PA) on steatosis

The effect of PA (10 µM, 20 µM, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM, 250 µM and 300 µM) on VL17A cells to induce lipid-overloading was evaluated by Oil red O staining, where lipid accumulation was quantified. With increasing concentration and incubation time PA treatment at 24 h led to a profound increase in lipid accumulation in VL17A cells between 50 µM (16%, $p < 0.05$) and 200 µM (26%, $p< 0.001$) when compared to control treated cells (Figure 3.2). However, interestingly the results showed at higher concentrations of PA treatment such as 250 µM leads to a 11% decrease in lipid accumulation in comparison to 50 µM PA treated VL17A cells.

At 48 h and 72 h lipid accumulation correlated closely to higher palmitic acid concentration when compared to controls. For example, at 48 h treatment, 30 µM, (-11%, $p <0.05$), 200 µM (+ 11%, $p < 0.05$) and 300 µM (+ 11%, $p <0.05$) (Figure 3.2). Similarly, at 72 h, an increase in lipid accumulation was observed at a higher concentration of PA treatment, where at 250 µM results showed a 21% increase ($p <0.001$; Figure 3.2).





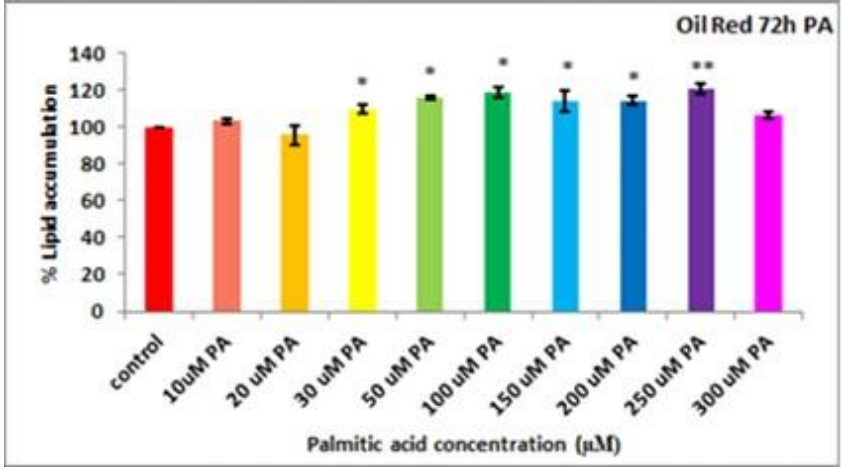


Figure 3.2. The effect of palmitic (PA) on lipid accumulation. VL17A cells ($50x10^3$ cells/well) were seeded onto 96-well plates followed by fatty acid treatment with palmitic acid (PA) to induce lipid over-loading as described in the methods section. Lipid accumulation was measured after palmitic acid exposure at 24, 48 and 72 h. The results are expressed as percentage of control and represented as the mean ± SEM (n=3). *$p < 0.05$, ** $p < 0.001$ compared to control.

3.1.2. Effect of palmitic acid (PA) on ROS production

In NAFLD, lipid accumulation in hepatocytes initiates increased oxidative stress. However, the effect of different doses of PA at different time points is yet to be elucidated in VL17A cells. VL17A cells were treated with a range of PA concentrations (10 µM, 20 µM, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM, 250 µM and 300 µM) to assess intracellular ROS levels at 30 min, 1 h and 2 h. Hydrogen peroxide (H_2O_2) and DMSO were included as vehicle controls (Figure 3.3).

Generally, at 30 minutes and 1 h time points, there was a gradual increase in ROS levels with increasing PA concentration. For example, at 30 mins VL17A cells treated with 50 µM PA the ROS levels measured at 10% higher than the control which further increased to 27% at 250 µM compared to control. At 3 h there was a less significant effect of palmitic acid on ROS levels (Figure 3.3).

In particular there was a significant increase in intracellular ROS levels after 30 minutes (+46%, $p < 0.001$) and 1 h (+61%, $p < 0.001$) incubation compared to control at 300 µM (Figure 3.3). However, results showed at 2 h time point that there is a less significant effect of palmitic acid in ROS production at 300 µM (+20%, $p < 0.05$) in comparison to lower time points (Figure 3.3).

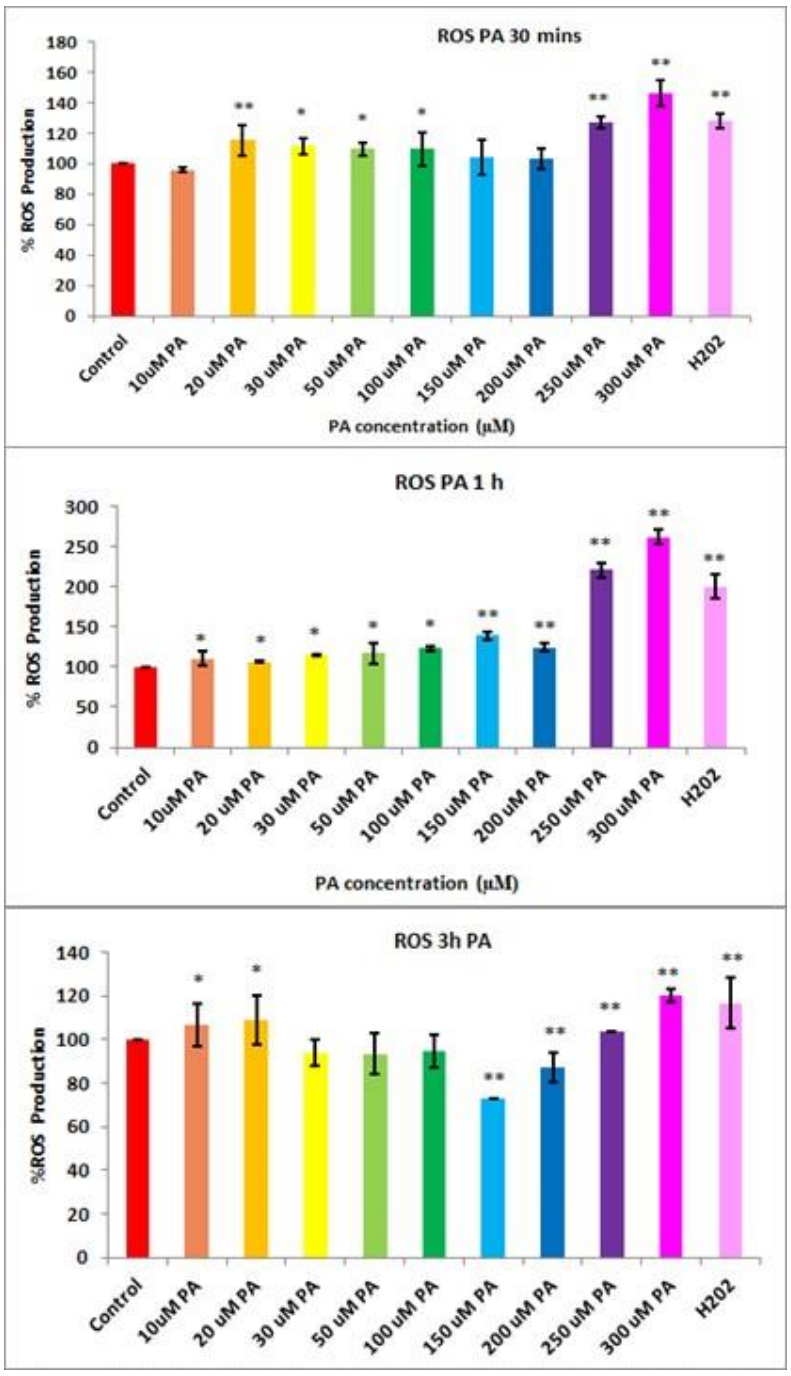


Figure 3.3. Effect of palmitic acid on ROS production

VL17A cells (15 x 10^3 cells/well) were seeded onto 96-well plates followed by fatty acid treatment to assess the cytotoxic properties of PA as described in the methods section. Intracellular ROS levels were measured after treatment exposure at 30 minutes, 1 h and 3 h. The results are expressed as percentage of control and represented mean ± SEM (n=5). *p < 0.05, **p <0.001 compared to control.

3.1.3. Palmitic acid dose response summary

Overall, VL17A treated with palmitic acid induced steatosis in VL17A and thus it can be used as an in *vitro* model of NAFLD. For further studies, the treatment concentration of 50 µM was chosen as this dose leads to lipid accumulation without over profound cytotoxcicity as deduced from the ROS and cell viability experiments.

3.2. Effect of bile acids on VL17A cells

Bile acids have emerged as signalling molecules that act on both hepatic and extrahepatic tissues to regulate lipogenesis and gluconeogenesis (Arab et al., 2017). They are endogenous ligands for nuclear receptors such as the farnesoid X receptor (FXR) to ameliorate the effects of NAFLD/ NASH such as insulin resistance and are proposed as a promising therapeutic approach.

The following experiments analysed the effect of primary bile acids chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) in a time and dose dependent response on cell viability and lipid accumulation in VL17A cells.

3.2.1. Effect of bile acids on cell viability on VL17A cells.

VL17A cells were treated with a range of bile acid concentrations (UDCA: 30 µM, 100 µM, 300 µM, CDCA: 30 µM, 100 µM and 300 µM) for 24 h, 48 h and 72 h.

Results showed cell viability increased at 30 µM for bile acids, UDCA (+41%, $p < 0.001$) and CDCA (+21%, $p < 0.001$) at 24 h. However, 300 µM CDCA exhibited greater cytotoxic effects at all time points (24 h: -66%, $p < 0.001$; 48 h: -82%, $p < 0.001$; and 72 h: -92%, $p < 0.001$) in comparison to control. As the concentration of bile acids increased with longer incubation time, the cell viability decreased for both bile acids (Figure 3.4).

At 24h, there was a profound increase in cell viability when treated with 30 µM UDCA (+41%, $p < 0.001$). However, the data showed a gradual decrease in cell viability at 24 h when treated with 100 µM (-18%, $p < 0.05$) and 300 µM (-52%, $p < 0.001$) of UDCA compared to the lower dose of 30 µM UDCA. CDCA exhibited greater cytotoxic effects on cell viability in comparison to UDCA at all time points and concentrations. In particular, at 300 µM CDCA treatment there was a 92% ($p < 0.05$) decrease in cell viability. Furthermore, at 48 h there was no significant effect of cell viability in the cells. Although, there was a similar pattern of decreasing cell viability with a higher UDCA or CDCA dose.

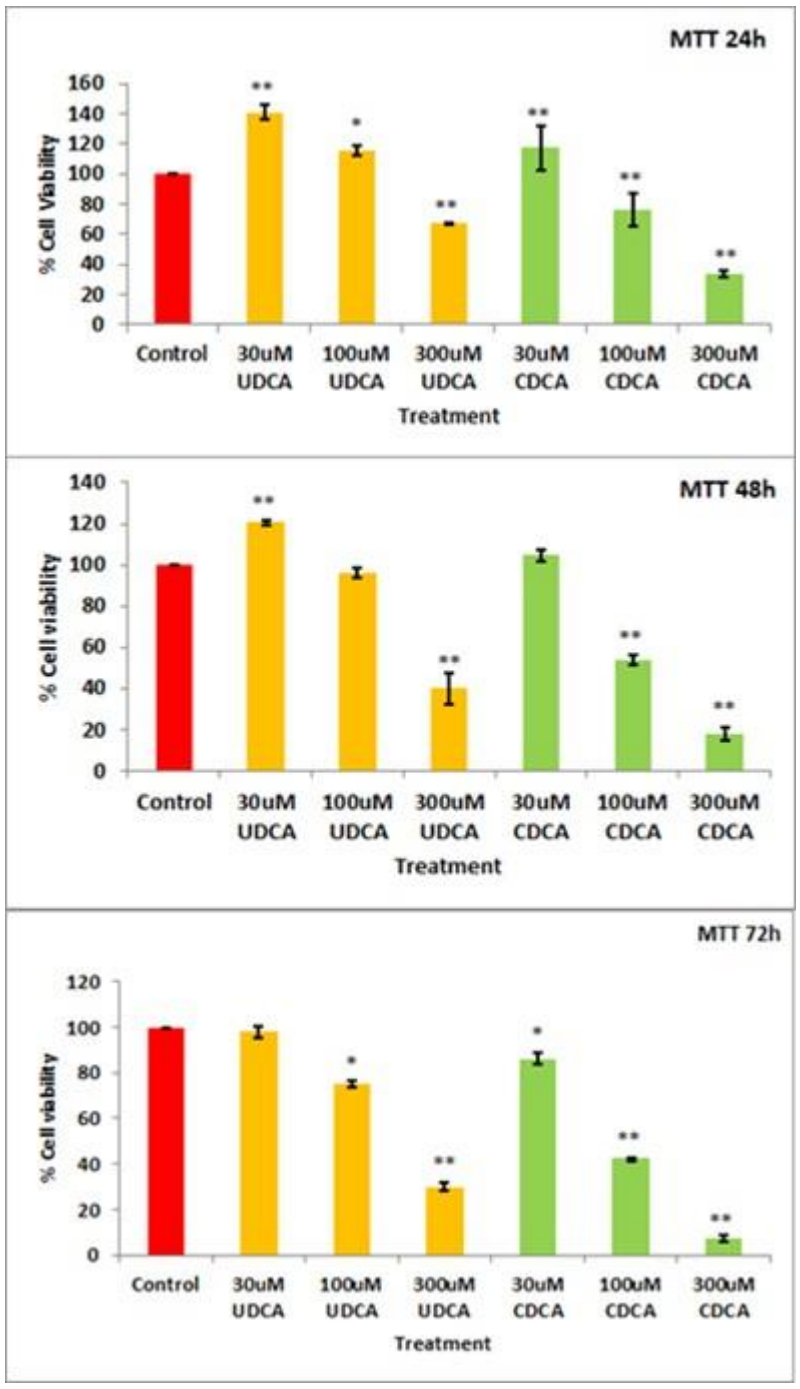


Figure 3.4. Effect of bile acids on cell viability.

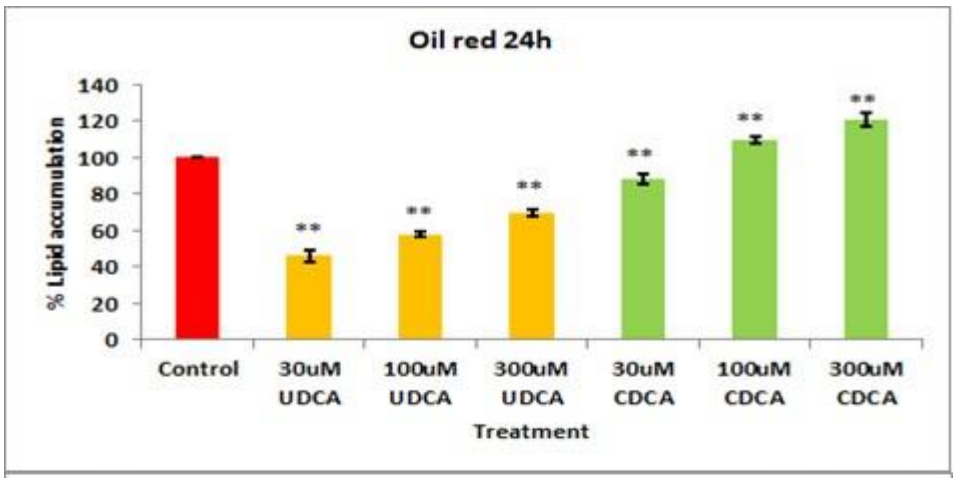
VL17A cells (25 x 10^3 cells/well) were seeded onto 96-well plates followed by bile acid treatment to assess the lipoprotective properties of the bile acids as described in the methods section. Cell viability was measured after treatment exposure at 24 h, 48 h and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=5). *$p < 0.05$, **$p < 0.001$ compared to control.

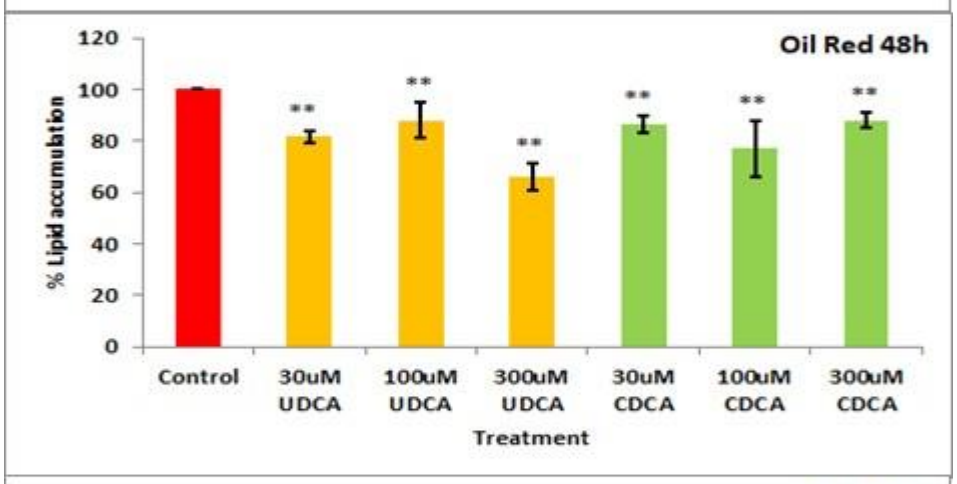
3.2.2. Effect of bile acids on steatosis

To analyse the hepatoprotective effects of bile acids, VL17A cells were treated with either UDCA or CDCA (30 µM, 100 µM and 300 µM) for 24h, 48 h and 72 h time points to measure lipid accumulation in the cells.

At 24 h an increase in the dose of UDCA treatment at led to a gradual increase in lipid accumulation in VL17A cells between 30 µM (46%), 100 µM (58%) and 300 µM (70%) however there was no significant effect when compared to control treated cells (Figure 3.5). Again, this was the same for VL17A cells treated with 30 µM and 100 µM CDCA at 24 h as lipid accumulation increased simultaneously with increasing doses of CDCA. Interestingly the results showed CDCA treated cells with 300 µM dose exhibited a profound increase (+ 21%, $p < 0.001$) in steatosis in VL17A cells.

At 48 h there was no significant effect noted in lipid accumulation for UDCA and CDCA treated cells when compared to control treated cells. At 72 h lipid accumulation correlated closely to higher CDCA concentration when compared to control. For example, at 300 µM CDCA treated cells had a 26% increase in lipid accumulation when compared to control treated cells ($p < 0.01$) (Figure 3.5). However, at 72 h UDCA treated cells did not have a significant effect on steatosis when compared to control.





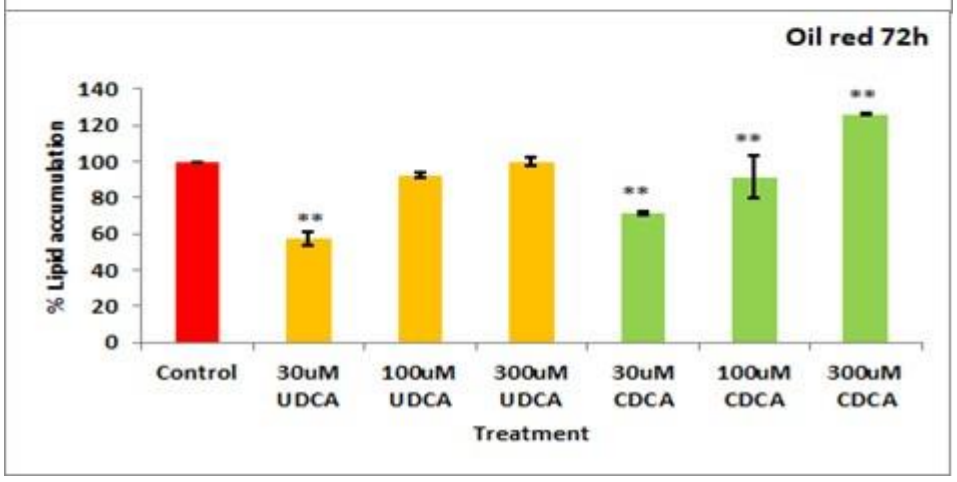


Figure 3.5. Effect of bile acids on lipid accumulation in VL17A cells

VL17A cells (50 x 10^3 cells/well) were seeded onto 96-well plates followed by bile acid treatment to assess the lipoprotective properties of the bile acids as described in the methods section. Intracellular lipid levels were measured after treatment exposure at 24 h, 48 h and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=5). *p < 0.05, **p <0.001 compared to control.

3.3. Effect of palmitic acid and bile acids co-treatment on VL17A cells

In the following series of experiments cells were incubated with bile acids and palmitic acid. From the dose response results, 30 µM UDCA and CDCA was used in subsequent experiments. This was to ascertain the putative protective effects of bile acids.

3.3.1. Effect of co-treatment of palmitic acid (PA) and bile acids (UDCA, CDCA) on cell viability.

To evaluate the lipoprotective effects of bile acids, VL17A cells were co-treated with palmitic acid (50 µM, 100 µM) and UDCA (30 µM) or CDCA (30 µM). Cell viability was assessed after 24, 48 and 72 h exposure time. At 24 h co- treatment with 50 µM PA + 30 µM UDCA, exhibited lipoprotective effects as cell viability significantly increased by 30% compared to VL17A cells treated with only 50 µM PA (Figure 3.6).

Similarly, co-treatment at 24 h with 100 µM PA + 30 µM UDCA also improved cell viability, although to a lesser effect than 50 µM PA +30 µM UDCA treatment (+18%) (Figure 3.6). Generally, at the lengthier incubation times of 48 h and 72 h, UDCA improved cell viability when co-treated with palmitic acid. For instance, at 48 h, 50 µM PA + 30 µM UDCA increased cell viability by 20% compared to cells treated with only 50 µM PA. However, data showed at 24h, UDCA exhibited the greatest lipoprotective effects as cell viability improved in all co-treated cells.

In contrast, CDCA exhibited less improvement in cell viability for all time points and treatments in comparison to UDCA. At 48 h, CDCA co-treatment led to the greatest improvement in cell viability compared to all other time points.

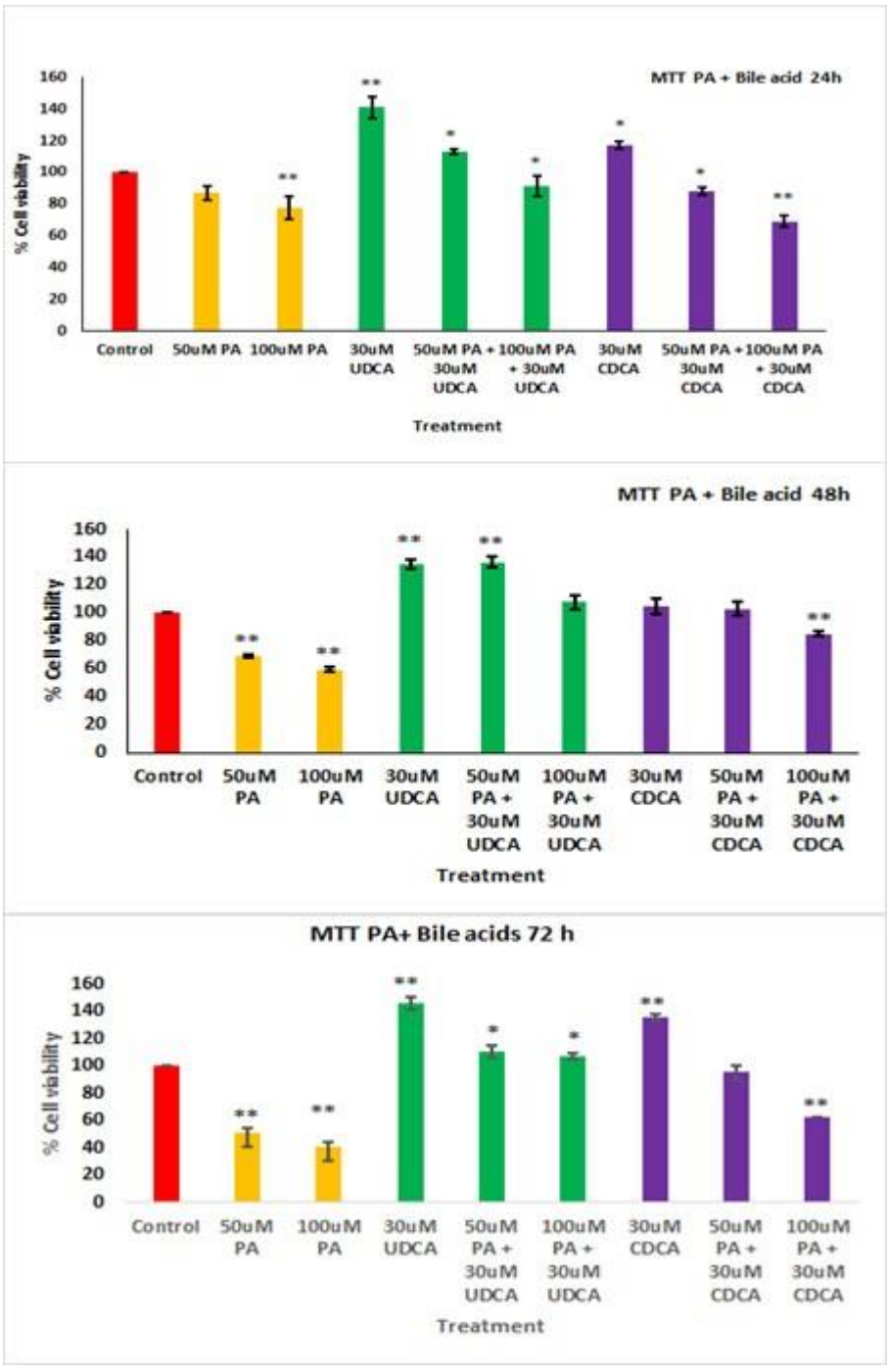


Figure 3.6. **Effect of co-treatment of palmitic acid (PA) and bile acids (UDCA, CDCA) on cell viability.**

VL17A cells (25 x 10^3 cells/well) were seeded onto 96-well plates followed by fatty acid (PA) and/ or bile acid treatment as described in the methods section. Cell viability was assessed after exposure at 24, 48 and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). * $p < 0.05$, * * $p < 0.001$ compared to the control.

3.3.2. Effect of co-treatment of palmitic acid (PA) and bile acids (UDCA, CDCA) on steatosis

VL17A cells were co-treated with palmitic acid and UDCA or CDCA to determine whether lipid accumulation could be prevented. Both bile acids exhibited lipoprotective properties at 24 h when co-incubated with 100 µM PA. A decrease of 40% in lipid accumulation occurred with UDCA ($p < 0.001$) and a 50% decrease when co-incubated with CDCA ($p < 0.001$; Figure 3.7). VL17A cells co-treated with 30 µM UDCA + 50 µM PA had a more pronounced decrease in lipid accumulation (-37%, $p < 0.001$) in comparison to all treatments at 24 h. Similarly, at 72 h co-treatment of both bile acids with 50 µM of PA resulted in a significant decrease in lipid accumulation (UDCA + PA, $p < 0.05$ and CDCA + PA; Figure 3.7).

To verify the occurrence of steatosis in VL17A cells, triglyceride (TG) concentration was quantified per mg protein of cell lysate. This method accurately compensates for variations in the cell density which may be affected by the treatment conditions and cell death.

TG concentration was measured after 24 h treatment of VL17A cells with PA 50 µM, UDCA 30 µM, UDCA 30 µM + PA 50 µM, CDCA 30 µM and CDCA 30 µM + PA 50 µM. Data showed VL17A cells treated with PA 50 µM showed a 36% ($p < 0.001$) increase in lipid accumulation compared to controls. In addition to this, cells co-incubated with UDCA 30 µM and PA 50 µM, there was a 27% ($p < 0.001$) decrease in triglyceride concentration (Figure 3.8.). Thus, it can be assumed that UDCA exhibited lipoprotective effects on VL17A cells.

On the other hand, CDCA treatment on its own lowered TG levels by 28% ($p < 0.001$) compared to control. Interestingly, CDCA co-incubated with PA 50 µM increased TG levels by 9% compared to CDCA treated cell on its own and 17% compared to controls (Figure 3.8).

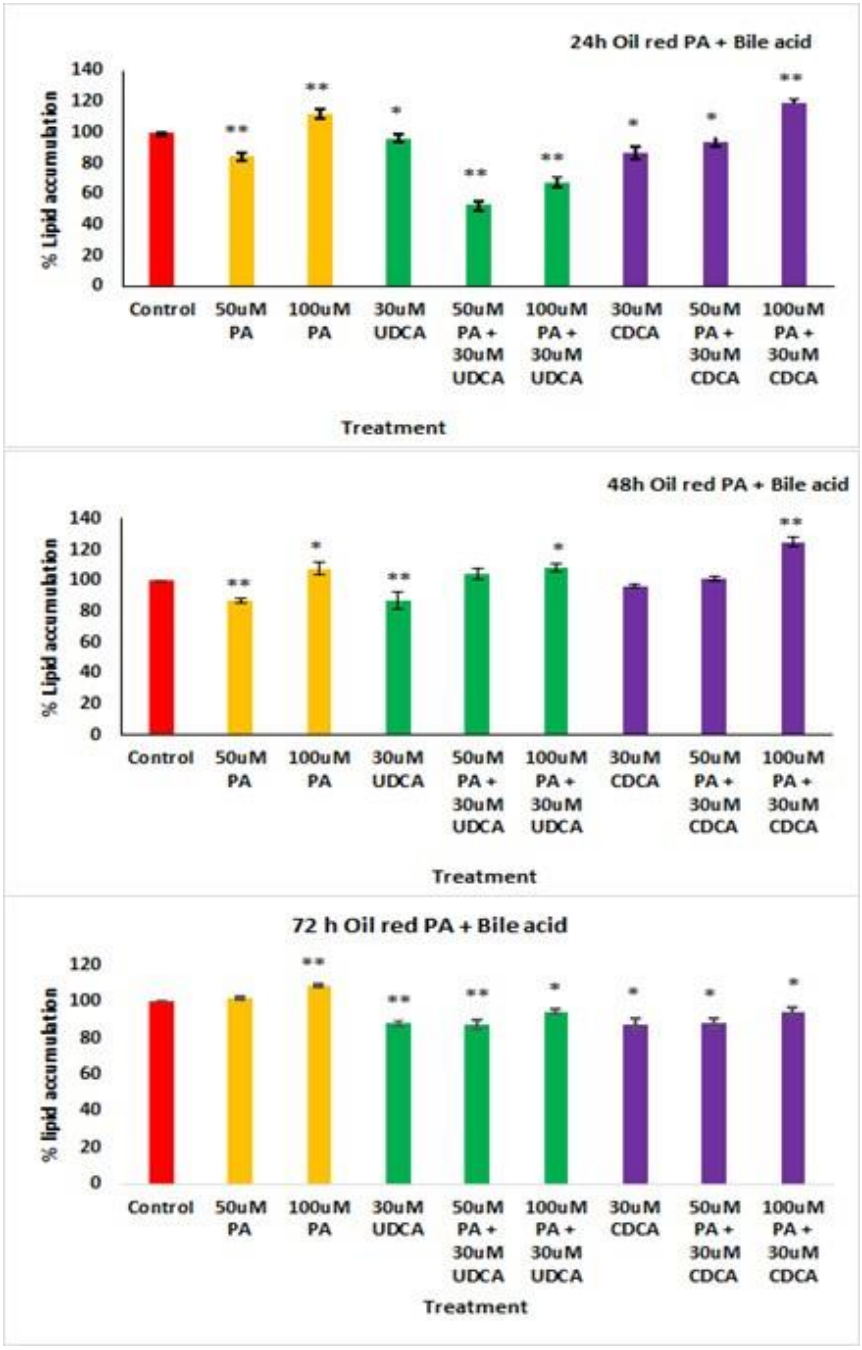


Figure 3.7. Effect of co-treatment of palmitic acid (PA) and bile acids (UDCA, CDCA) on steatosis

VL17A cells (50 x 10^3 cells/well) were seeded onto 96-well plates followed by fatty acid and bile acid treatment to assess the lipoprotective properties of the bile acids as described in the methods section. Lipid accumulation was measured after co-treatment exposure at 24, 48 and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). *$p < 0.05$, **$p < 0.001$ compared to control.

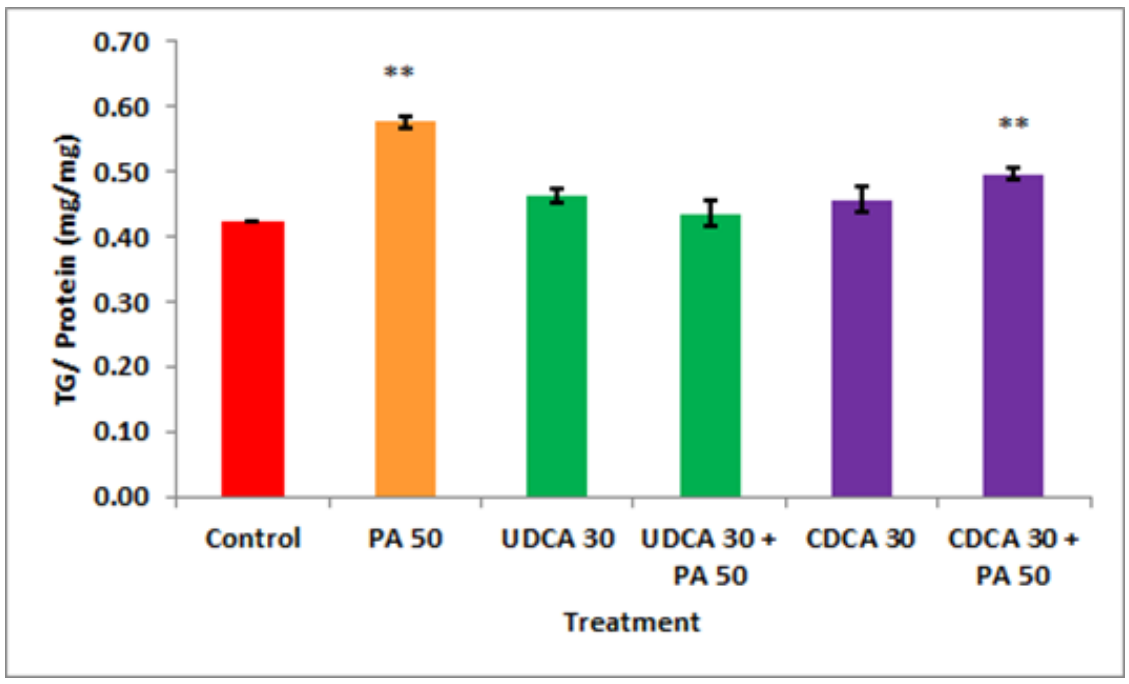


Figure 3.8. Effect of co-treatment of palmitic acid (PA) and bile acids (UDCA, CDCA) on steatosis and triglyceride levels.

VL17A cells (200,000 cells/well) were seeded onto 24-well plates followed by fatty acid and bile acid treatment to assess the lipoprotective properties of the bile acids as described in the methods section. Lipid accumulation was measured after co-treatment exposure at 24, 48 and 72 h. Intracellular TG levels are expressed as mg/mg protein. The results are expressed as mean ± SEM (n=3). **$p < 0.001$ compared to control.

3.3.3. Effect of co-treatment of palmitic acid and bile acid on ROS production

Increased oxidative stress is a well-established consequence of NAFLD, however the effect of palmitic acid co-incubated with bile acids on ROS production is unclear in VL17A cells. To evaluate the effects of ROS production, VL17A cells were co-treated with palmitic acid (50 µM) and UDCA (30 µM) or CDCA (30 µM) over 30 mins, 1 h and 2 h time points (Figure 3.9).

At 30 mins, there was no significant change in ROS levels when co-treated with PA and UDCA. However, PA and CDCA treated cells showed a 12% increase in ROS production ($p < 0.05$). Interestingly, UDCA exhibited lipoprotective properties at 1 h as results showed intracellular ROS levels decreased by 16% compared to control ($p < 0.001$). Lastly at 3 h, UDCA co-incubated with PA did not exhibit lipoprotective effects as results showed a slight increase in ROS production (+13%, $p < 0.05$; Figure 3.9).

Furthermore, Furthermore, CDCA did not exhibit lipoprotective effects at any time point. ROS levels increased significantly at all time points (+10% at 30 mins, $p < 0.001$; +343% at 1 hour, $p < 0.001$ and +47% at 3 hours, $p < 0.001$ respectively).

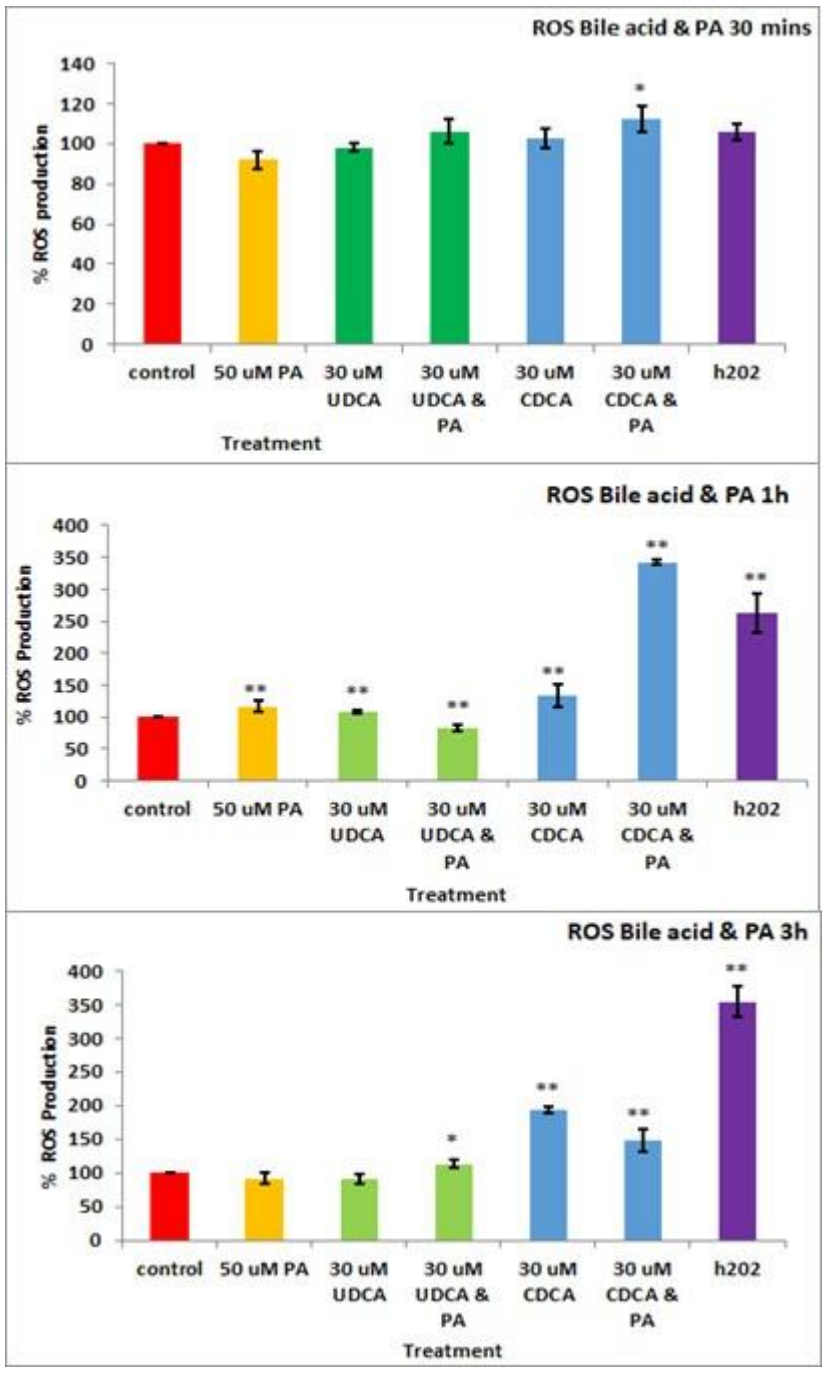


Figure 3.9. Effect of co-treatment of palmitic acid and bile acid on ROS production.

VL17A cells (15 x 10^3 cells/well) were seeded onto 96-well plates followed by bile acid and fatty acid treatment to assess the lipoprotective properties of the bile acids as described in the Methods section. Intracellular ROS levels were measured after treatment exposure at 30 minutes, 1 h and 3 h. The results are expressed as percentage of control and represented mean ± SEM (n=5). *p < 0.05, **p <0.001 compared to control.

3.3.4. Effect of palmitic acid (PA) and ursodeoxycholic acid (UDCA) co-treatment on SREBP1 expression

Sterol regulatory element binding protein (SREBP1) is a key transcriptional factor which primarily controls genes responsible for hepatic triglyceride synthesis and plays a role in the pathogenesis of NAFLD. To establish the effect of ursodeoxycholic acid (UDCA) on SREBP1 expression, two amounts of VL17A cell lysates (7.5 µg, Figure 3.10A and 15 µg, Figure 3.10B) were analysed after cells were treated with PA and UDCA for 24 h and analysed using an enzyme-linked immunosorbent assay (ELISA).

The expression of SREBP1 was significantly pronounced in PA treated cell lysates (+77%, $p < 0.001$ (7.5 µg), and +239%, $p < 0.001$ (15 µg)). However, when PA was co-treated with UDCA there was only a 5% decrease in SREBP1 expression in the 7.5 µg lysate (Figure 3.10A). On the other hand, SREBP1 expression decreased by 70% in the 15 µg lysate compared to PA treated lysates (Figure 3.10B). However, when compared to control, UDCA did have an increased expression of SREBP1 with 15 µg lysate.

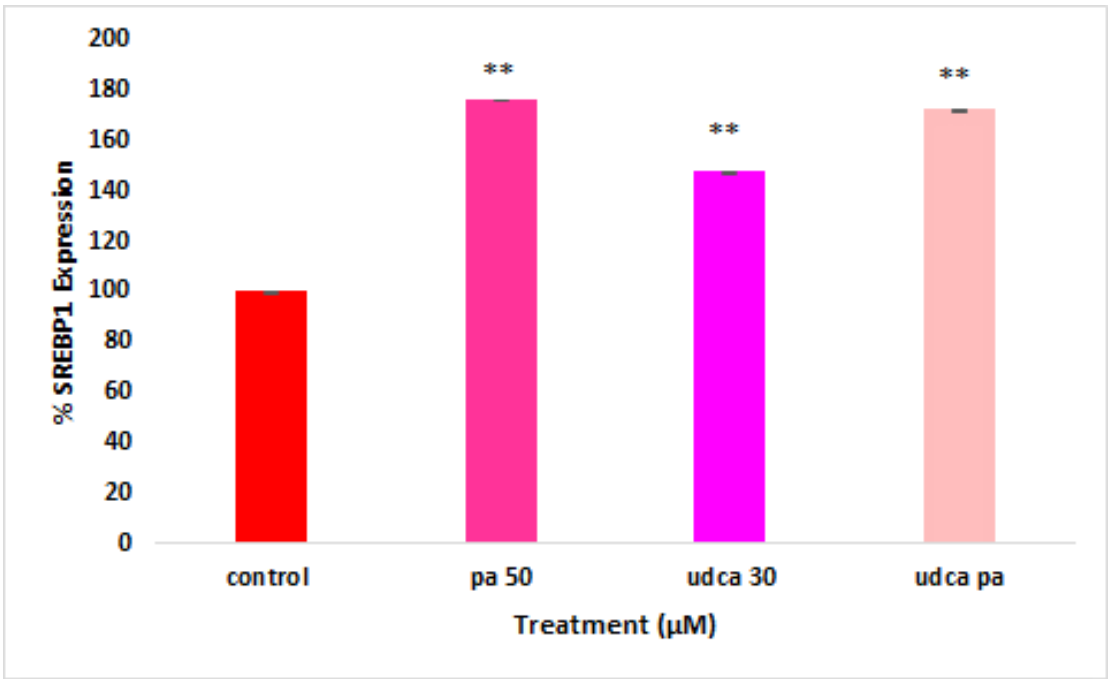


Figure 3.10A. Effect of PA and UDCA on SREBP1 expression.

The expression of the SREBP1 transcriptional factor was measured in VL17A cells treated with PA (50 µM) and UDCA (30 µM) for 24 h. Cell lysates (7.5 µg) were added to a 96-well ELISA plate to assess the lipoprotective properties of the bile acids. The results are expressed as percentage of control and represented mean ± SEM (n=3). **p <0.001 compared to control.

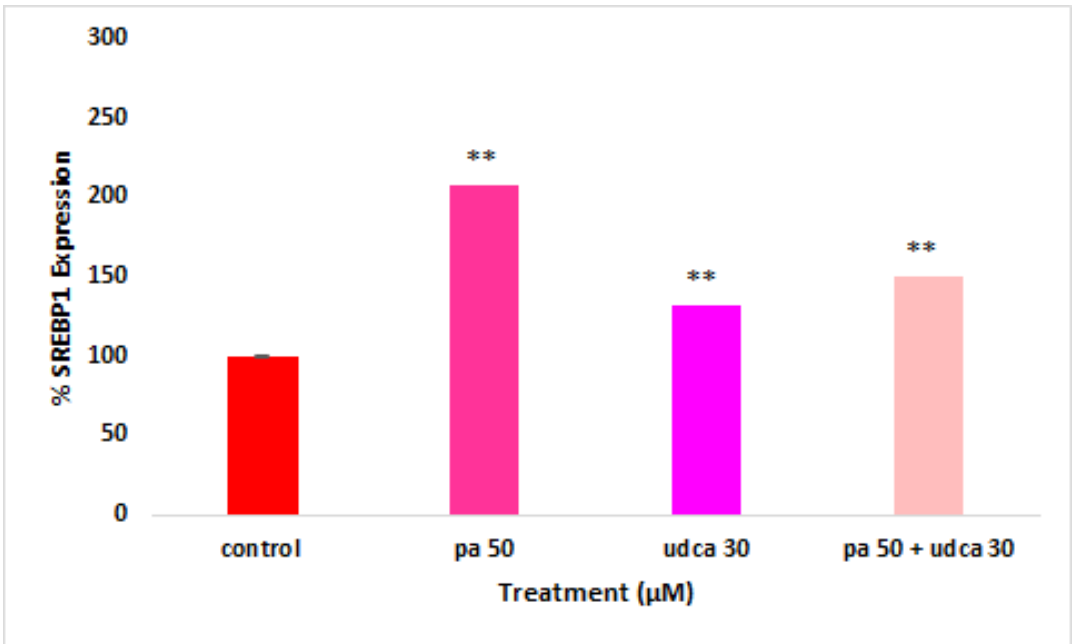


Figure 3.10B. Effect of PA and UDCA on SREBP1 expression.

The expression of the SREBP1 transcriptional factor was measured in VL17A cells treated with PA (50 µM) and UDCA (30 µM) for 24 h. Cell lysates (15 µg) were added to a 96-well ELISA plate to assess the lipoprotective properties of the bile acids. The results are expressed as percentage of control and represented mean ± SEM (n=3). **p <0.001 compared to control.

3.3.5. Effect of palmitic acid (PA) and ursodeoxycholic acid (UDCA) on oxygen consumption rate (OCR)

Under normal conditions, hepatic mitochondrial activity is increased to protect the hepatocytes from lipotoxicity exhibited by free fatty acid (FFA) deposition in the liver. In NAFLD, several studies have shed light on the diminished function of mitochondria which provokes metabolic dysfunction and consequently results in the progression of NAFLD to more severe pathologies (Wieckowsi et al., 2018).

To study the effect of palmitic acid (PA) and ursodeoxycholic acid (UDCA), VL17A cells were treated/ co-treated with PA (100 µM) and UDCA (30 µM) for 24 h and the respiratory function of the mitochondria were analysed by measuring the oxygen consumption rate (OCR) using the Seahorse XF24 analyser (Figure 3.11). Data showed that PA treatment (100 µM) had a detrimental effect on the function of the electron transport chain (ETC) in comparison to UDCA treatment (30 µM) and when VL17A cells were co-treated with PA and UDCA across all parameters measured.

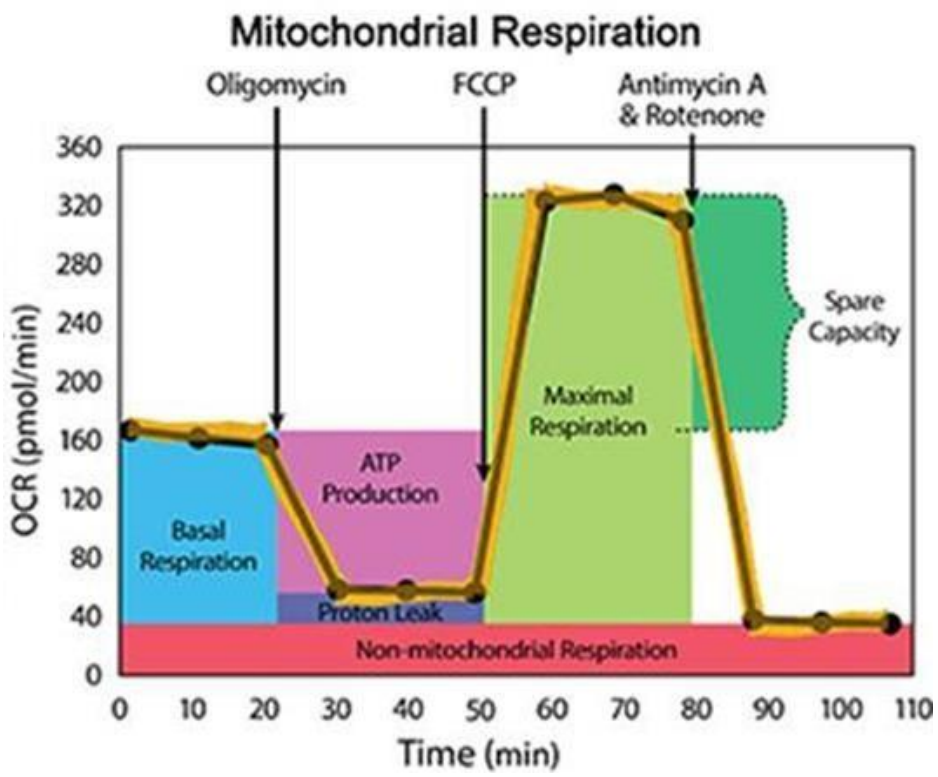
Results showed a significant decrease of 36% ($P < 0.001$) in basal respiration In VL17A cells when treated with PA only compared to the control. However, UDCA treated cells showed a 83% ($p < 0.001$) basal respiration rate as compared to control which was 19% ($p < 0.001$) higher than PA treated cells. Furthermore, co-treated VL17A cells with PA and UDCA had an improved basal respiration rate by 13% ($P < 0.001$) in comparison to PA treated cells only (Figure 3.12).

ATP production was significantly reduced by 32% ($p <0.001$) in PA treated cells from control. However, in the UDCA treated cells basal respiration was 89% ($p < 0.001$) compared to control. Also, co-treated cells with PA and UDCA showed a slight increase of 6% ($p < 0.001$) in basal respiration compared to PA treated cells (Figure 3.12).

The data for the following parameters; proton leak, maximal respiration and spare respiratory capacity showed a similar pattern with the treatments. The proton leak decreased by 29% ($p < 0.001$) in PA treated cells as compared to the control. Whereas UDCA treated cells showed a 17% ($p < 0.001$) less

decrease in proton leak rate as compared to control. On the other hand, co-treated cells with PA and UDCA showed an improved proton leak rate by 5% as compared to PA treated cells (Figure 3.12).

A



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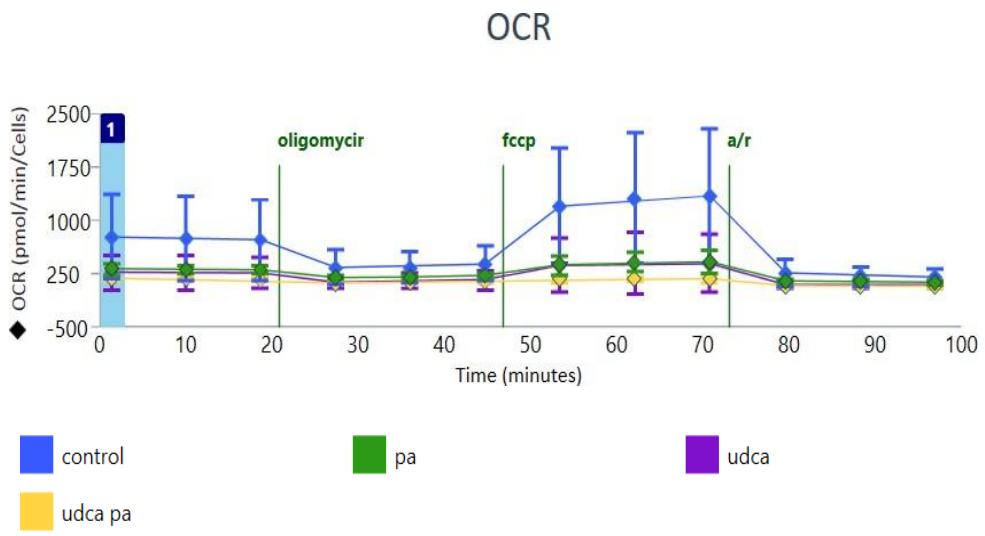


Figure 3.11. Effect of PA and UDCA on the Seahorse Mito stress test parameters. A. Representative result. B. Model. PA, palmitic acid; UDCA, ursodeoxycholic acid.

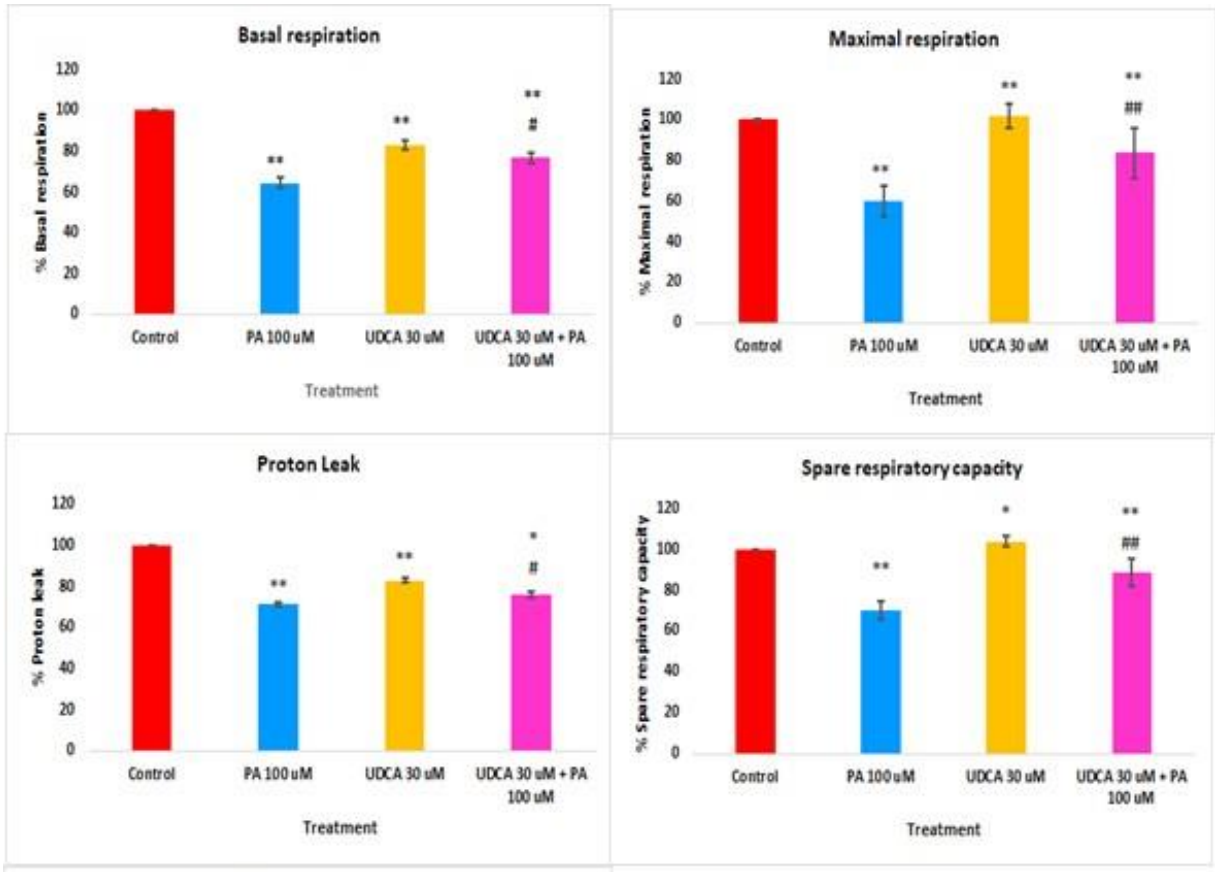


Figure 3.12. Effect of UDCA and PA treatment on oxygen utilization in VL17A cells treated for 24h.

Oxygen consumption rate was measured by the following parameters; basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity. The results are presented as mean ± SEM (n = 4 determinations of 5 replicates per treatment). * P, 0.05, ** P < 0.001 as compared to the control. # P < 0.05, ## P < 0.001 as compared to PA.

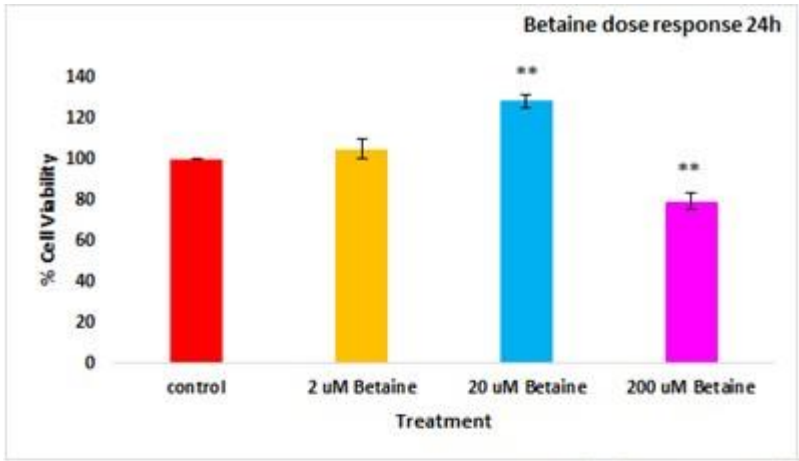
3.4. Effect of betaine on VL17A cells

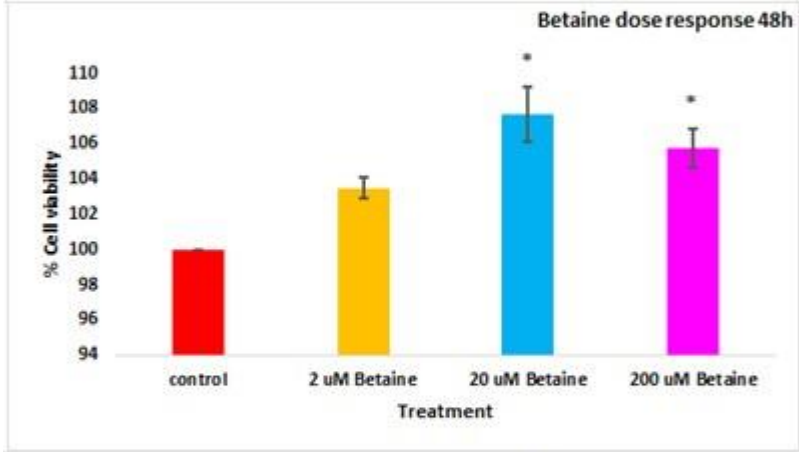
As aforementioned in Section 1.1., the incidence of NAFLD is increasing rapidly and thus far no putative therapeutic approach exists. It is vital to establish alternative therapies to lessen the burden of the condition on the healthcare systems worldwide. Betaine, a methyl donor is a naturally occurring dietary antioxidant that can also be synthesized by choline and has recognised beneficial effects on liver metabolism. The following experiments analysed the effect of betaine in a time and dose dependent response on cell viability and lipid accumulation in VL17A cells.

3.4.1. Effect of betaine on the viability of VL17A cells

Cell viability of VL17A exposed to a range of concentrations of betaine (2 μM, 20 μM and 200 μM) was examined by the MTT assay at 24, 48 and 72 hours. Data showed a significant increase in cell viability at 20 μM of betaine at 24 h (+28%, $p < 0.001$; Figure 3.13).

However, there was a less significant effect of 20 μM betaine at 48 h (+8%, $p < 0.05$) and 72 h (+6%, $p < 0.05$) time points. Furthermore, at 200 μM of betaine there was a profound decrease in cell viability at 24 h (-21%, $p < 0.001$) and 72 h (-26%, $p < 0.001$).





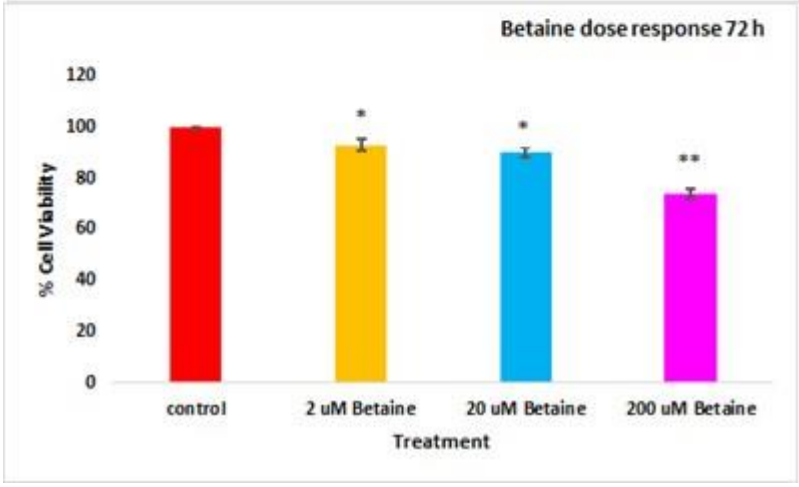


Figure 3.13. Effect of betaine on the viability of VL17A cells

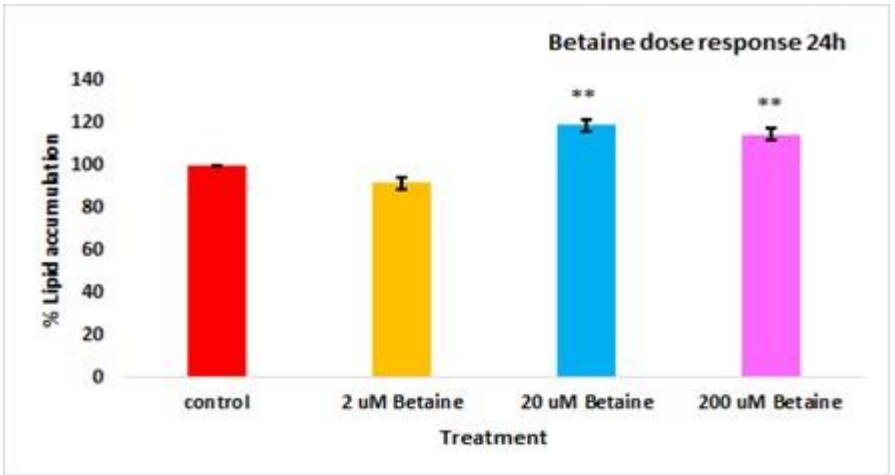
VL17A cells (25 x 10^3 cells/well) were seeded onto 96-well plates followed by betaine treatment. Cell viability was assessed after betaine exposure at 24h, 48 h and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.001$.

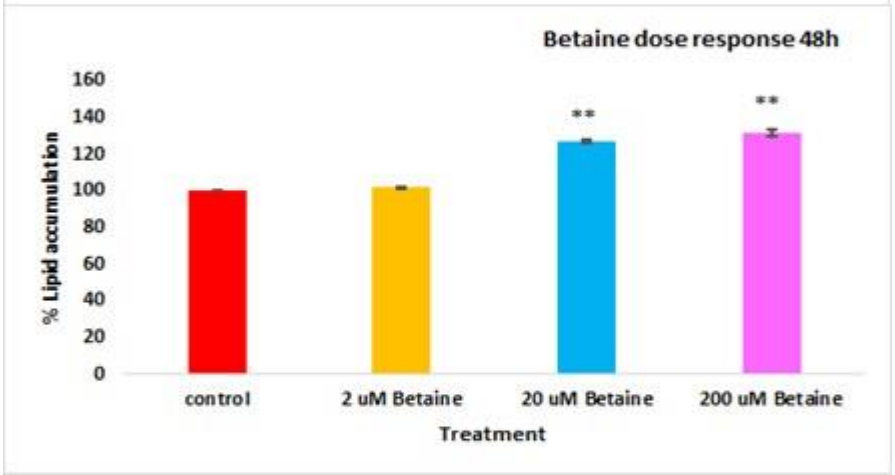
3.4.2. Effect of betaine on steatosis in VL17A cells

To assess the hepatoprotective effects of betaine, VL17A cells were treated with different concentrations of betaine (2 µM, 20 µM and 200 µM) for 24 h, 48 h and 72 h.

Cells treated with 2 µM betaine at all time points showed a less significant steatogenic effect (24h, -8%, $p < 0.05$; 48 h, +2%; 72 h, -7%, $p < 0.05$) in lipid accumulation compared to control (Figure 3.14). On the other hand, 20 µM betaine treatment had a more significant effect on steatosis in VL17A cells. For example, at 24 h data showed lipid accumulation increases by 19%, ($p < 0.001$) which is further amplified at 48 h by 27%, ($p < 0.001$) compared to control. Interestingly at 72 h, the increase in lipid accumulation was not observed.

Additionally, data showed with 200 µM betaine there was an increase in lipid accumulation at 24 h (+15%, $p < 0.001$) and at 48 h (+31%, $p < 0.001$) compared to control. However, at 72 h there was a profound decrease in lipid accumulation at 200 µM (-26%, $p < 0.001$) from control, a pattern observed for all concentrations at 72 h.





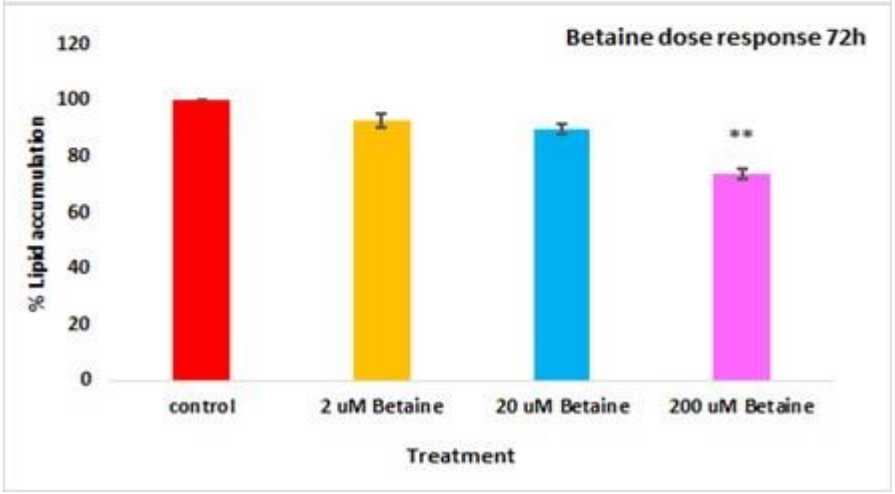


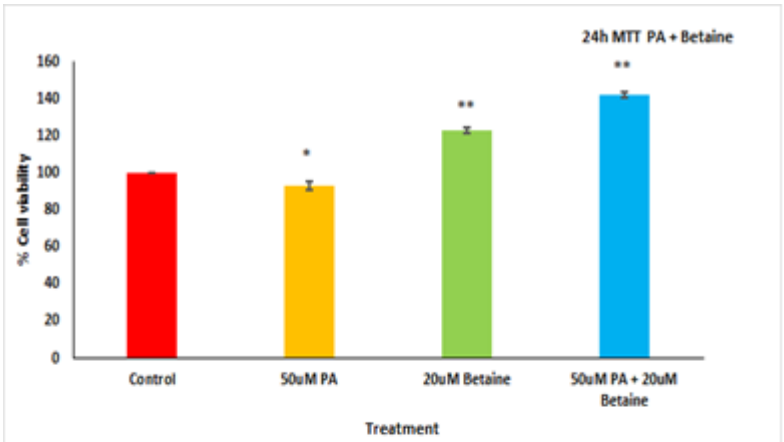
Figure 3.14. Effect of betaine dose response on steatosis in VL17A cells.

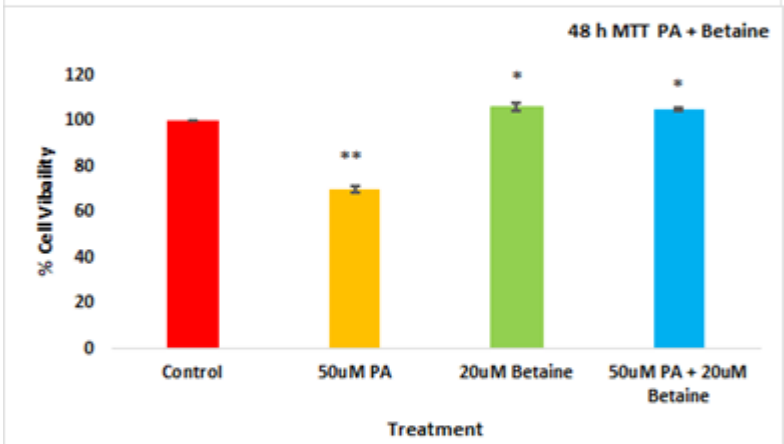
VL17A cells (50 x 10^3 cells/well) were seeded onto 96-well plates followed by betaine treatment. Lipid accumulation was assessed after betaine exposure at 24h, 48 h and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). ** $p < 0.001$ as compared to control.

3.4.3 Effect of betaine co-treatment with palmitic acid (PA) on cell viability

VL17A cells were exposed to 20 µM betaine with 50 µM palmitic acid for 24 h, 48 h and 72 h to assess the hepatoprotective effects of betaine. The data showed PA exhibits cytotoxic effects on VL17A cells as cell viability decreases by 7% ($p < 0.05$) at 24 h. Although when co-incubated with betaine, cytotoxicity exhibited by PA is attenuated as there is a 42% increase in cell viability at 24 h ($p < 0.001$) from control (Figure 3.15).

Similarly, at 48 h cell viability significantly decreased further with PA treatment alone (-30%, $p < 0.001$) and betaine had a less significant effect at a longer incubation time with PA as cell viability increased by 5% from control. Lastly, betaine did not exhibit hepatoprotective properties at 72 h when co-treated with PA as cell viability decreases by 9% ($p < 0.05$) from control.





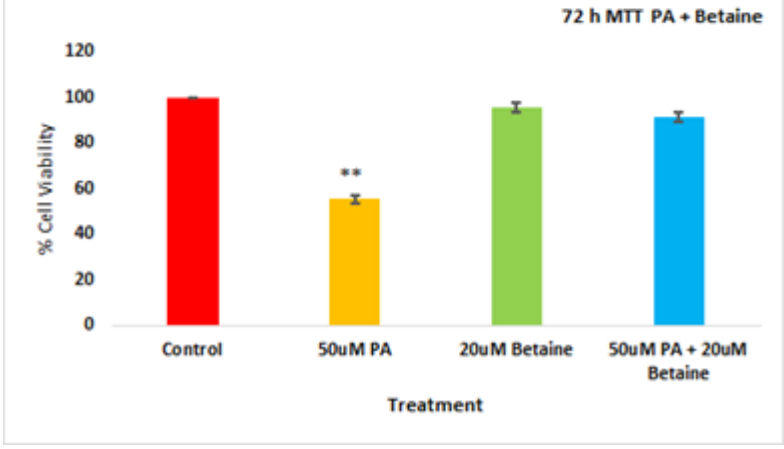


Figure 3.15. Effect of betaine co-treatment with palmitic acid (PA) on cell viability

VL17A cells (25 x 10^3 cells/well) were seeded onto 96-well plates followed by betaine and PA treatment. Cell viability was assessed after betaine and PA exposure at 24h, 48 h and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.001$ as compared to control.

3.4.4. Effect of betaine co-treatment with palmitic acid (PA) on steatosis

To evaluate the lipoprotective activity of betaine, VL17A cells were co-treated with 20 µM betaine and 50 µM PA for 24 h, 48 h and 72 h and lipid accumulation was measured by the Oil red O assay. Data showed at 24 h, PA exhibits significant lipotoxicity as lipid accumulation increases by 18% ($p < 0.001$) when compared to control VL17A cells.

However, at 24 h betaine displays lipoprotective properties as there is no significant change in lipid accumulation from control (Figure 3.16). In addition to this, at 48 h betaine co-incubated with PA did attenuate lipid accumulation as a slight decrease of 4% was observed in the VL17A cells in comparison to PA treated cells. Correspondingly, at 72 h results showed a further decrease in lipid accumulation in the VL17A cells co-treated with betaine and PA (-8%, $p < 0.05$).

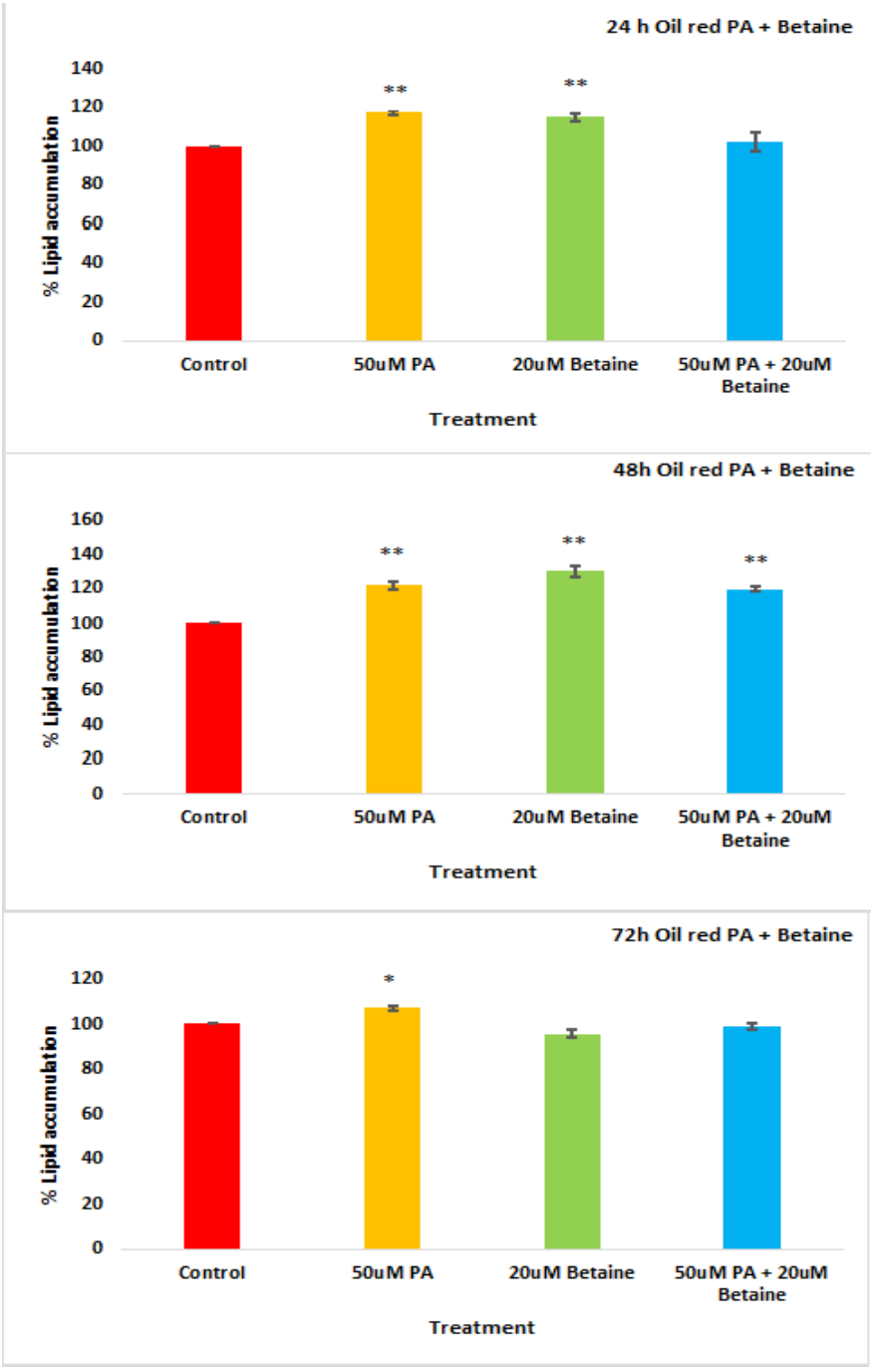


Figure 3.16. Effect of betaine co-treatment with palmitic acid (PA) on steatosis.

VL17A cells (50 x 10^3 cells/well) were seeded onto 96-well plates followed by betaine and PA treatment. Lipid accumulation was assessed after betaine and PA exposure at 24h, 48 h and 72 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.001$ as compared to control.

3.4.5. Effect of betaine co-treatment with palmitic acid (PA) on ROS production

As aforementioned, an increase in oxidative stress induced by excessive fatty acids (palmitic acid) in hepatocytes leads to the production of ROS in NAFLD. In the following experiments ROS levels were measured and the anti-oxidative properties of betaine (20 µM) were assessed when co-incubated with palmitic acid (50 µM, 150 µM and 300 µM) at 30 minutes, 1 h and 3 h.

At 30 minutes, data showed betaine did not exhibit a significant effect on ROS levels when co-incubated with palmitic acid at all concentrations from control. For example, 20 µM betaine and 50 µM PA there was no change in ROS levels. Also, with 20 µM betaine and 150 µM the greatest decrease in ROS levels (-6%) was observed when compared to control and lastly, at 20 µM and 300 µM treatment intracellular ROS levels decreased by 4% (Figure 3.17).

In comparison to 30 minutes, at 1 h betaine demonstrated hepatoprotective effects when co-incubated with all concentrations of PA. Data showed when cells were co-treated with 20 µM betaine, as the concentration of PA increased, the intracellular ROS levels decreased (20 µM betaine + 50 µM PA, -16%, $p < 0.001$; 20 µM betaine + 150 µM PA, - 23%, $p < 0.001$; and 20 µM betaine + 300 µM PA, -27%, $p < 0.001$) from control. Similarly, at 3 h intracellular ROS levels decreased with increasing PA concentration. This effect was most predominant at 3 h where a 33% decrease in ROS levels was observed with 20 µM betaine and 300 µM PA co-treatment.

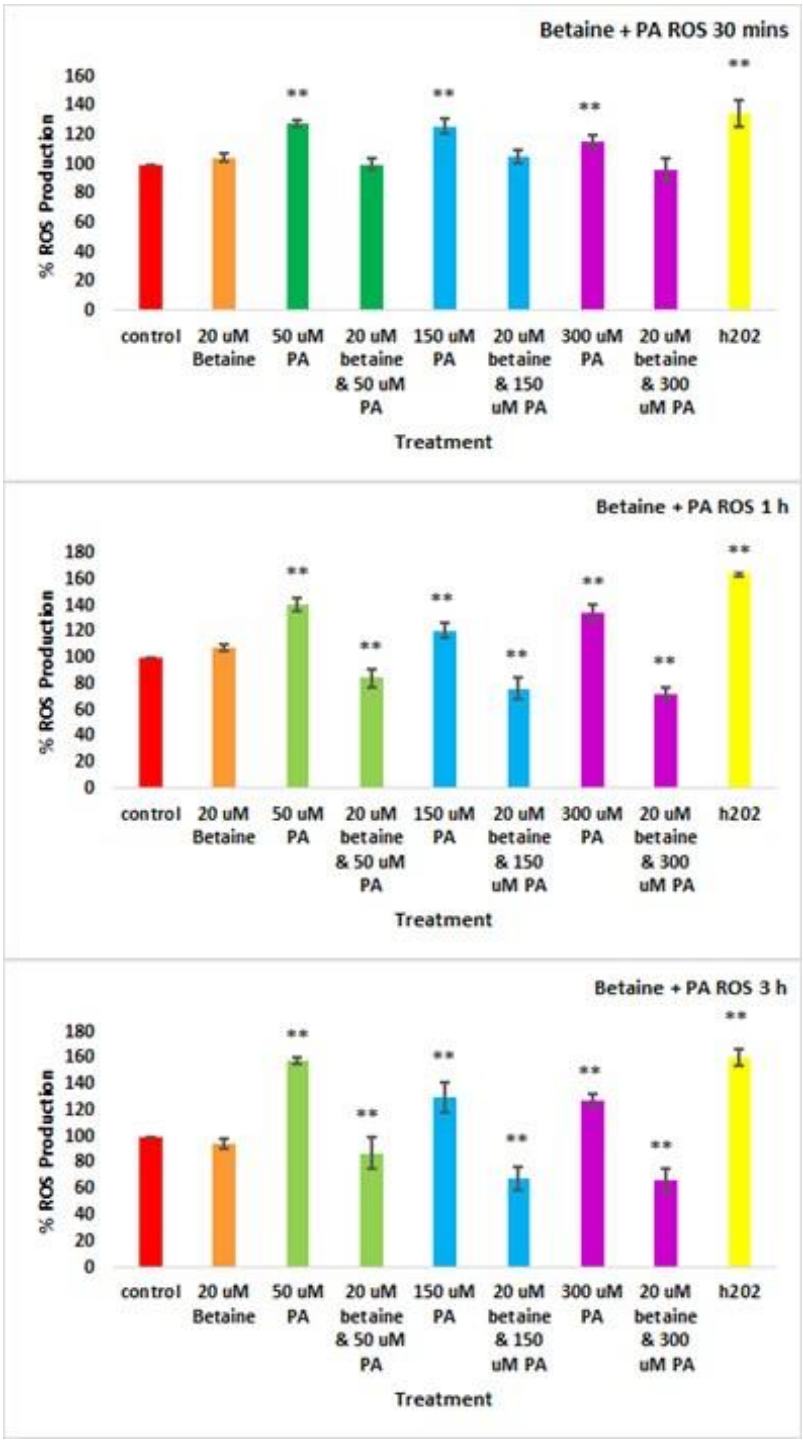


Figure 3.17. Effect of betaine and palmitic acid (PA) on ROS production

VL17A cells (15 x 10^3 cells/well) were seeded onto 96-well plates followed by betaine treatment. ROS levels were assessed after betaine and PA exposure at 30 mins, 1 h and 3 h. The results are expressed as percentage of control and represented mean ± SEM (n=8). **p < 0.001.

3.4.6. Effect of palmitic acid (PA) and betaine on oxygen consumption rate (OCR).

As aforementioned in section 3.3.5. a feature of NAFLD is dysfunctional hepatic mitochondrial activity. The effect of PA and betaine on oxygen consumption rate (OCR) was analysed using the Seahorse XF24 analyser after treating VL17A cells for 24 h.

Basal respiration was analysed and data showed PA treatment led to a marked decrease of 18% ($p < 0.001$) as compared to control. However, UDCA treatment had a less significant effect on basal respiration with a decrease of 2% as compared to control. Additionally, co-treatment of PA and UDCA improved basal respiration by 5% in comparison to PA treated VL17A cells (Figure 3.18).

ATP production and the proton leak were evaluated and showed a similar pattern for all treatments. For instance, PA treatment led to a great decrease for both parameters (-19%, $p < 0.001$ and -23%, $p < 0.001$ respectively) as compared to control. Betaine demonstrated a beneficial effect on both ATP production and proton leak (+9%, $p < 0.001$ and +14%, $p < 0.001$ respectively). Furthermore, co-treatment of PA and betaine increased ATP production and proton leak in comparison to PA treated cells. ATP production increased by 10% ($p < 0.001$) and the proton leak by 13% ($p < 0.001$) (Figure 3.18).

PA treatment led to a marked decrease of 37% ($p < 0.001$) in maximal respiration and a 19% ($p < 0.001$) decrease in spare respiratory capacity as compared to the control. On the other hand, betaine demonstrated an improved maximal respiration (+9%, $p < 0.001$) and spare respiratory capacity (+2%) as compared to control. Interestingly, co-treatment of PA and betaine results showed a great increase in maximal respiration by 26% ($p < 0.001$) when compared to PA treatment. Similarly, spare respiratory capacity had a slight increase by 3% from PA (Figure 3.18).

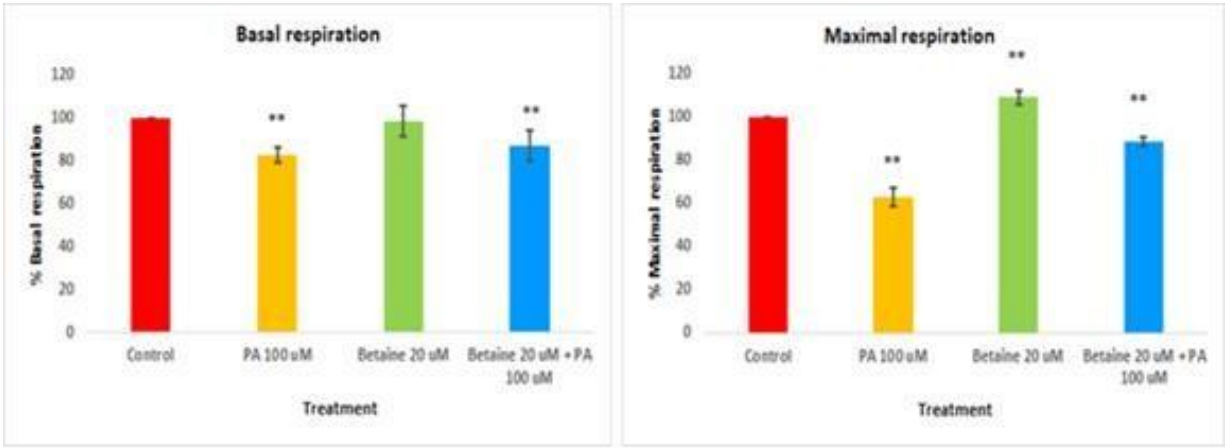


Figure 3.18. Effect of betaine and PA on oxygen consumption in VL17A cells treated for 24 h. Oxygen consumption rate was measured by the following parameters; basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity. The results are presented as mean ± SEM (n=3 determinations of 5 replicates of each treatment). * $P < 0.05$, ** $P < 0.001$ as compared to the control.

3.5. Effect of 2-[4-(5-chlorobenzothiazothiazol-2-yl) phenoxy]-2-methyl-propionic acid (MHY908) on VL17A cells.

As discussed in chapter 1, the progression of NAFLD is multifactorial and is characterised by stages such as inflammation and insulin resistance to name a few. Peroxisome proliferator activated receptors (PPAR) α/ γ have been known to attenuate the symptoms of NAFLD.

PPARα plays a vital role in fatty acid metabolism by upregulating the expression of genes involved in mitochondrial fatty acid and peroxisome fatty acid oxidation. Activation of PPARα prevents and decreases hepatic fat storage and thus alleviates lipotoxicity in the liver. Therefore, these agonists pose as potential therapeutic agents in treating NAFLD patients and to prevent progression to more severe pathologies.

To date, there are no studies on the effect of MHY908, a dual PPARα/γ agonist on VL17A cells. Peroxisome proliferator activated receptors (PPAR) are ligand-activated transcriptional factors that play a crucial role in lipid homeostasis and tightly regulate lipogenesis in the liver to prevent lipotoxicity and cell death The following series of experiments analysed the effect of MHY908 at 24 h on cell viability, lipid accumulation, ROS production and mitochondrial function in VL17A cells.

3.5.1. Effect of MHY908 on cell viability in VL17A cells

VL17A cells were exposed to a range of MHY908 concentrations (1 µM, 5 µM and 20 µM) for 24 h and cell viability was measured by the MTT assay. Treatment with 1 µM MHY908, showed a significantly pronounced increase in cell viability by 20% ($p < 0.001$) from control (Figure 3.19). On the other hand, VL17A cells incubated with 20 µM MHY908 exhibited the greatest cytotoxic effects as cell viability decreased significantly by 33% ($p < 0.001$).

3.5.2. Effect of MHY908 on steatosis in VL17A cells

To evaluate the lipoprotective properties of MHY908, VL17A cells were treated with a range of concentrations (1 µM, 5 µM and 20 µM) for 24 h and lipid accumulation was measured by the Oil red O assay.

Cells treated with 1 µM MHY908 exhibited lipoprotective effects compared to the higher doses of MHY908 as lipid accumulation decreased by 5% ($p < 0.05$) compared to control (Figure 3.20).

Furthermore, treatment with 5 µM did not demonstrate hepatoprotective effects as lipid accumulation had the greatest increase of 26% ($p < 0.001$). Interestingly, 20 µM treatment of MHY908 did show a slight increase in lipid accumulation (+9%, $p < 0.05$) compared to control but was less significant than the 5 µM dose.

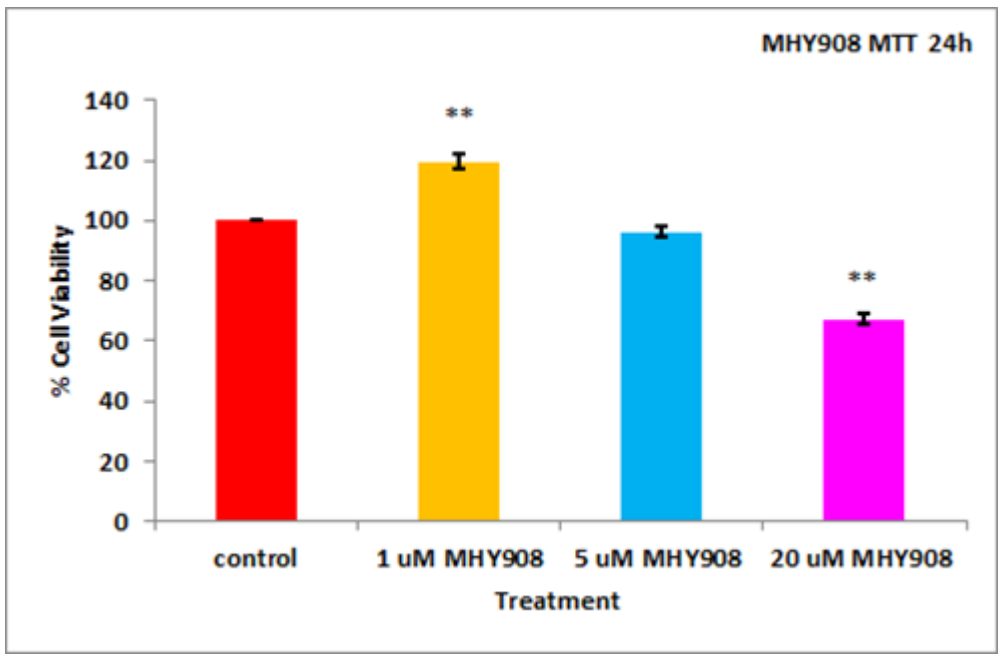


Figure 3.19. Effect of MHY908 on cell viability in VL17A cells.

VL17A cells (25 x 10^3 cells/well) were seeded onto 96-well plates followed by MHY908 treatment. Cell viability was assessed after MHY908 exposure at 24 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). ** $P < 0.001$ as compared to control.

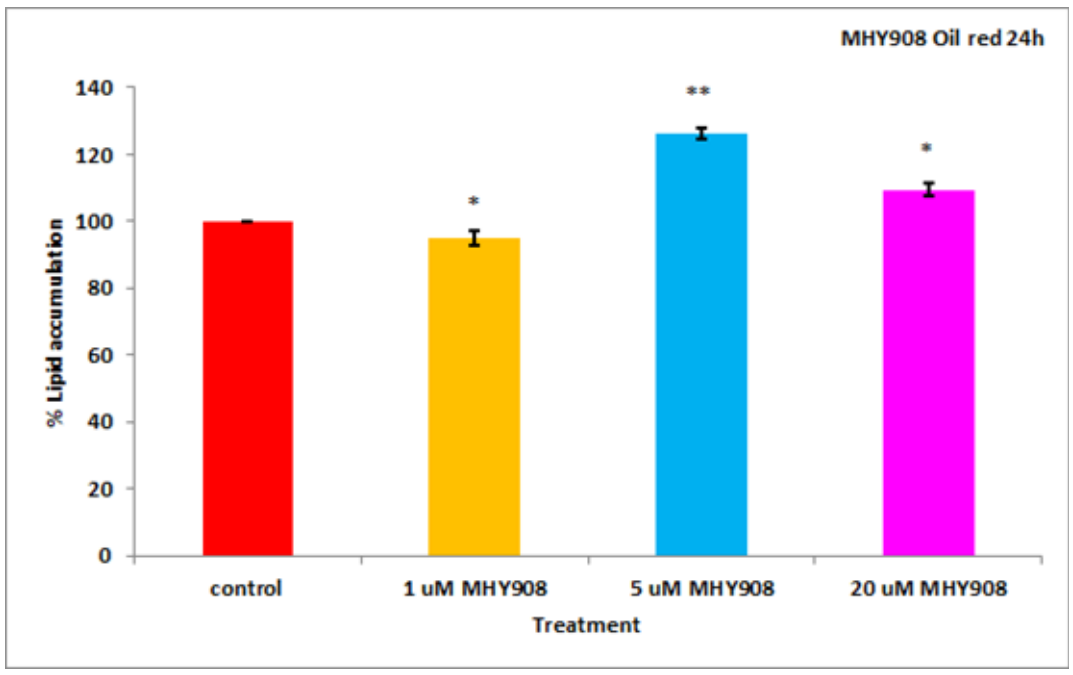


Figure 3.20. Effect of MHY908 on steatosis in VL17A cells

VL17A cells (50 x 10^3 cells/well) were seeded onto 96-well plates followed by MHY908 treatment. Lipid accumulation was assessed after MHY908 exposure at 24h. The results are expressed as percentage of control and represented mean ± SEM (n=3). * $P < 0.05$, ** $P < 0.001$ as compared to control.

3.5.3. Effect of MHY908 on ROS production in VL17A cells

To study the effect of MHY908 to reduce oxidative stress in VL17A cells, cells were exposed to a range of concentrations (1 µM, 5 µM and 20 µM) for 1 hour followed by the ROS assay. Data showed the lowest dose of 1 µM MHY908 treatment had minimal effect on ROS production (3% decrease), whereas an elevation in ROS production was parallel to the higher doses of MHY908. For example, 5 µM MHY908 increased by 8% ($p < 0.05$) and 20 µM increased by 25% ($p < 0.001$) from control (Figure 3.21).

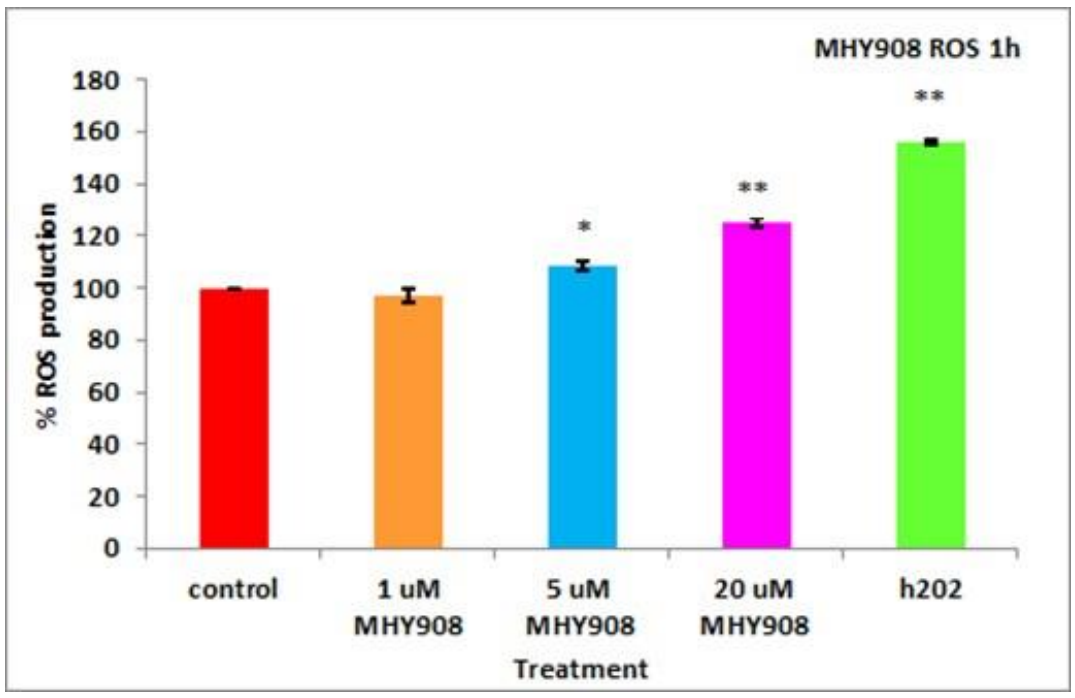


Figure 3.21. Effect of MHY908 on ROS production in VL17A cells

VL17A cells (15 x 10^3 cells/well) were seeded onto 96-well plates followed by MHY908 treatment. ROS levels were assessed after MHY908 exposure at 1 h. The results are expressed as percentage of control and represented mean ± SEM (n=4). * $P < 0.05$, $P < 0.001$ compared to control.

3.5.4. Effect of co-treatment of MHY908 and palmitic acid (PA) on VL17A cells

To determine whether MHY908 lower hepatic steatosis without exhibiting overt cytotoxicity in VL17A cells; in the following series of experiments cells were incubated with MHY908 and palmitic acid. From the dose response results 50 µM PA concentration was used in subsequent experiments.

3.5.5. Effect of co-treatment of MHY908 and PA on cell viability

VL17A cells were treated with 50 µM and a range of concentrations of MHY908 (1 µM, 5 µM and 20 µM) for 24 h.

Results show 1 µM MHY908 and 50 µM PA co-treatment exhibited hepatoprotective effects in the cells as there was a significant increase in cell viability (+ 26%, $p < 0.001$) (Figure 3.22).

However, higher concentrations of MHY908 exhibited cytotoxic effects as it led to a profound decrease in cell viability. In particular, 20 µM exhibited the most cytotoxic effect as cell viability decreased significantly by 53% ($p < 0.001$) from control (Figure 3.22).

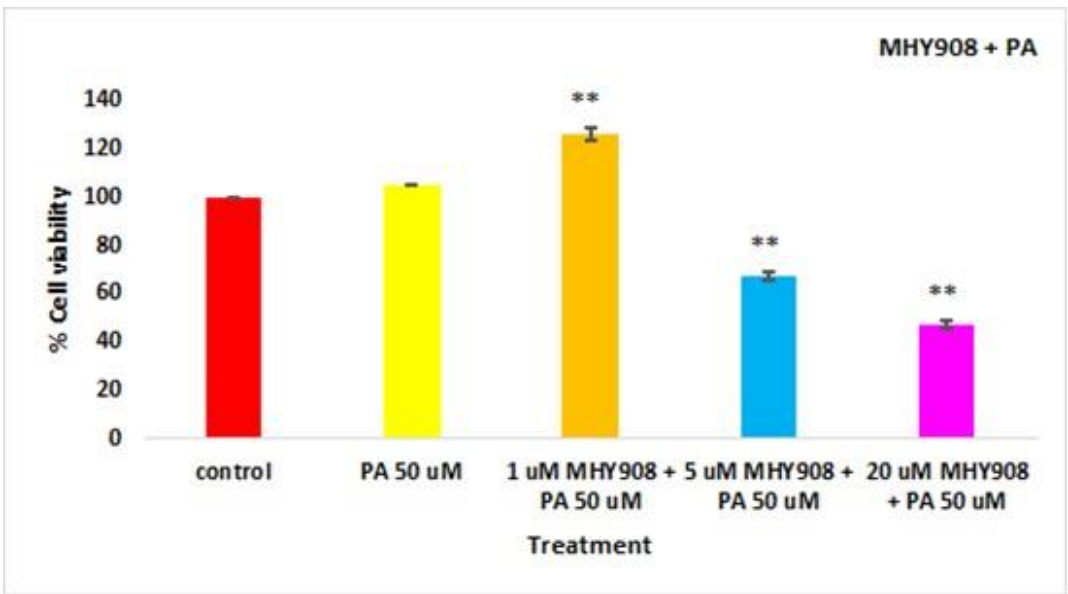


Figure 3.22. Effect of co-treatment MHY908 and PA on cell viability

VL17A cells (25 x 10^3 cells/well) were seeded onto 96-well plates followed by MHY908 treatment. Cell viability was assessed after MHY908 and PA exposure at 24 h. The results are expressed as percentage of control and represented mean ± SEM (n=3). ** $p < 0.001$.

3.5.6. Effect of co-treatment of MHY908 and PA on steatosis

To evaluate the lipoprotective effects of MHY908, cells were co-treated with 50 µM PA and MHY908 (1 µM, 5 µM and 20 µM) and lipid accumulation was measured by the Oil red O assay. Data showed co-treatment of PA with 1 µM MHY908 reduced lipid levels significantly by 13% ($p < 0.001$) from control and thus 1 µM MHY908 exhibited lipoprotective effect in comparison to the higher doses (Figure 3.23). For example, 20 µM MHY908 with 50 µM PA exhibited the greatest lipotoxic effects as lipid accumulation levels increased by 18% ($p < 0.001$) from control.

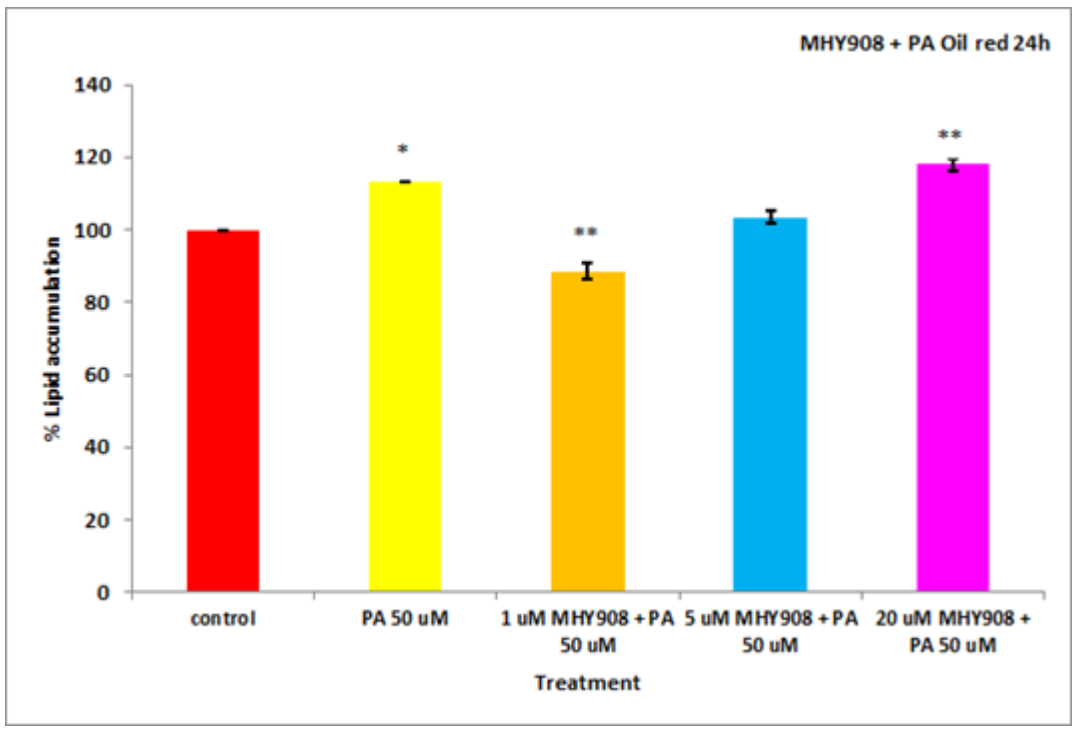


Figure 3.23. Effect of co-treatment of MHY908 and PA on steatosis

VL17A cells (50 x 10^3 cells/well) were seeded onto 96-well plates followed by MHY908 and PA treatment. Lipid accumulation was assessed after MHY908 and PA exposure at 24h. The results are expressed as percentage of control and represented mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.001$ compared to control.

3.5.7. Effect of co-treatment of MHY908 and PA on ROS production

To elucidate the effect of MHY908 and PA co-treatment on ROS levels, cells were co-incubated with 50 µM PA and MHY908 (1 µM, 5 µM and 20 µM) for 1 hour. ROS levels decreased slightly with 1 µM MHY908 and 50 µM PA treatment (-3 %) however, MHY908 did not display hepatoprotective effects as ROS levels increased at the higher doses of 5 µM MHY908 and 50 µM PA (+12%, $p < 0.05$) and 20 µM MHY908 and 50 µM PA (+39%, $p < 0.001$) from control (Figure 3.24).

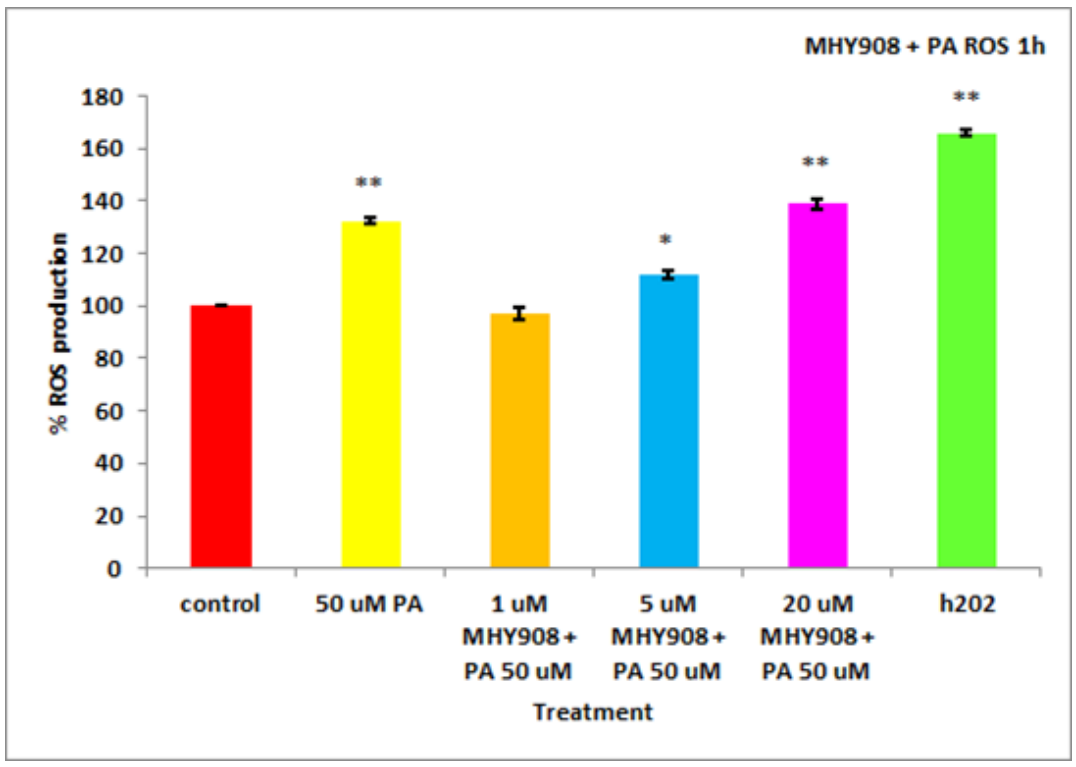


Figure 3.24. Effect of co-treatment of MHY908 and PA on ROS production

VL17A cells (15 x 10^3 cells/well) were seeded onto 96-well plates followed by MHY908 and PA co-treatment. ROS levels were assessed after MHY908 and PA exposure at 1 h. The results are expressed as percentage of control and represented mean ± SEM (n=4). * $P < 0.05$, $P < 0.001$.

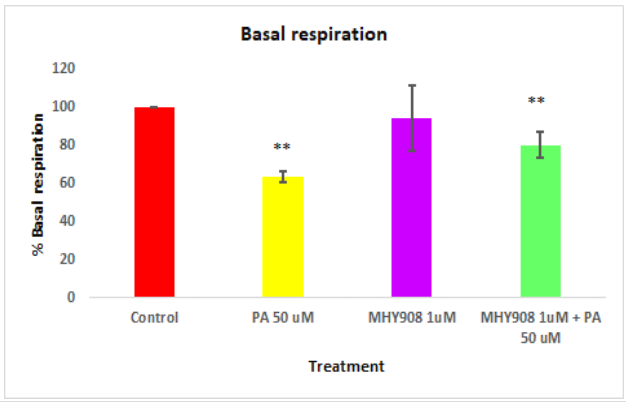
3.5.8. Effect of palmitic acid (PA) and MHY908 on oxygen consumption rate (OCR).

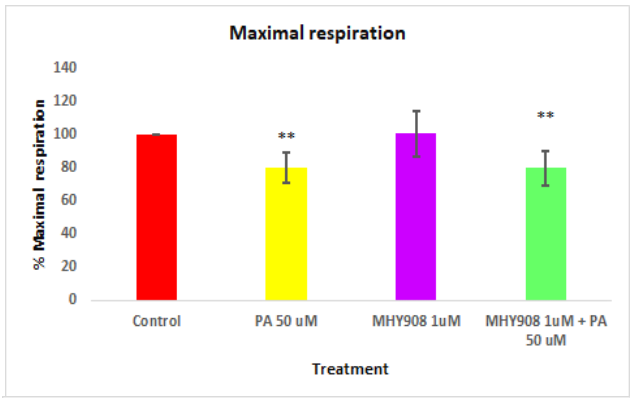
The mitochondrial activity of PA and MHY908 treated cells were analysed as described in sections 3.3.5 and 3.47 using the Seahorse XF24 analyser to measure several parameters.

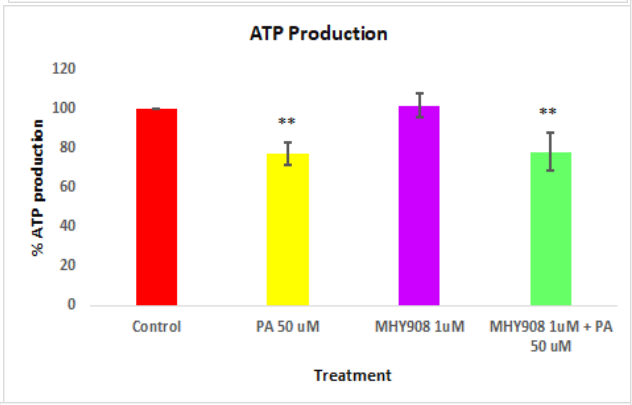
Basal respiration results showed PA treatment had a detrimental effect as there was a 37% ($p < 0.001$) decrease as compared to control. Similarly, MHY908 treatment had a slight decrease in basal respiration (-6%) as compared to the control. On the other hand, PA and MHY908 co-treated cells showed an increase of 17% ($p < 0.001$) in basal respiration as compared to PA treatment (Figure 3.25).

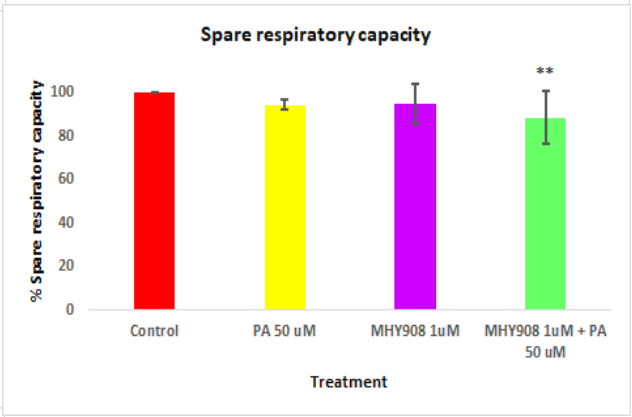
ATP production was also measured and again PA showed a decrease of 23% ($p < 0.001$) from the control. MHY908 treatment showed a slight 2% increase from control however, co-treatment with PA and MHY908 showed a less significant effect as compared to PA (+1%) treatment only.

Furthermore, the proton leak for PA co-treated with MHY908 did not exhibit lipoprotective effects as there was a 5% decrease as compared to PA control. Interestingly, MHY908 treatment increased the proton leak by 7% ($p < 0.05$) as compared to the control and PA again showed a significant decline in proton leak (-21%, $p < 0.001$) (Figure 3.25). Maximal respiration results showed VL17A cells treated with PA led to a significant decrease (-20%, $p < 0.001$) as compared to the control and interestingly MHY908 co-treated with PA did not show any significant effect on basal respiration. Spare respiratory capacity showed a decrease for all treatments as compared to control.









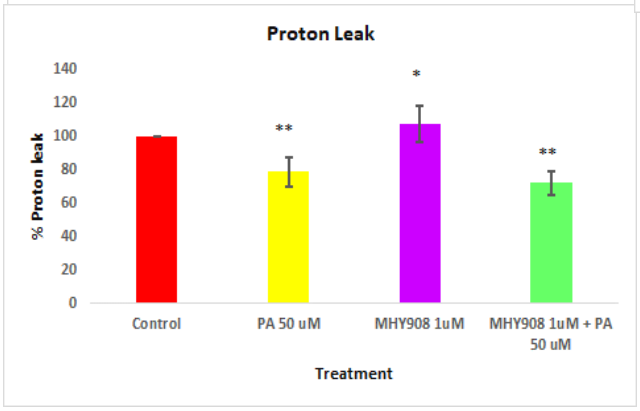


Figure 3.25. Effect of palmitic acid (PA) and MHY908 on oxygen consumption rate (OCR). Oxygen consumption rate was measured by the following parameters; basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity. The results are presented as mean ± SEM (n=3 determinations of 5 replicates of each treatment). * $P < 0.05$, ** $P < 0.001$ as compared to the control.

Chapter 4

Discussion

4. Discussion

The manifestation of excess intracellular lipid accumulation in hepatocytes is known as steatosis and is the key hallmark of non-alcoholic fatty liver disease (NAFLD). As aforementioned in Chapter 1 (1.3.1.), the pathogenesis of NAFLD remains poorly understood. However, it has been established that disturbed lipid metabolism results in steatosis in the liver commonly referred to as the 'first hit' in NAFLD progression.

A greater understanding of how fatty acids contribute to NAFLD development has crucial implications since the prevalence of NAFLD (which is associated with cardiovascular disease, the metabolic syndrome, diet and obesity) is rapidly increasing. In fact, over the last 20 years worldwide there has been a substantial shift in dietary habits with an influence of a high fat Western diet. As a result, the global prevalence of NAFLD has doubled since 1980 and is estimated at 25% of the population (Younossi et al., 2016). Thus, identifying key transcriptional factors/ genes in the regulatory network that governs hepatic lipid metabolism is a vital step towards development of treatments for NAFLD.

Furthermore, there is increasing evidence supporting the role of alternative therapies such as, bile acid activated FXR in promoting lipid oxidation, antioxidant properties of betaine and a PPARα/γ dual agonist MHY908 as potential treatments for NAFLD.

The current study aimed to characterise the effect of the saturated fatty acid, palmitic acid (PA) on lipotoxicity, liver steatosis and oxidative stress in VL17A (HepG2) cells to ascertain if it could be used as a novel *in vitro* model of NAFLD. A further aim was to determine the effect of alternative therapies such as bile acids (CDCA and UDCA), betaine and MHY908 on the prevention of lipotoxicity, lipid accumulation, oxidative stress and mitochondrial function in VL17A cells to shed new light on the molecular mechanisms involved in NAFLD.

4.1. Effect of Palmitic acid (PA): Lipotoxicity and steatosis

In this study, VL17A cells which over- express the enzymes CYP2E1 and ADH derived from a HepG2 line of liver cells could be used as an *in vitro* model for NAFLD, dose and time-dependant experiments were carried out with saturated fatty acid palmitic acid (PA).

Palmitic acid is the most abundant circulating free fatty acid (FFA) (Fujita et al., 2009). The principal source of these FFAs are from adipose tissue lipolysis and dietary fatty acids, for example; PA is

found abundantly in many food sources such as palm oil, dairy and meat (Machado et al., 2016). A study by Feng et al (2017) supports this notion as FFA profiles of 66 healthy subjects (PA levels = 432 µg/ml) and 55 obese subjects (PA levels = 496 µg/ml) were evaluated in this study and both groups showed PA as the highest circulating FFA in comparison to other fatty acids such as oleic acid, myristic acid, linolenic acid to name a few (Feng et al., 2017). Similar findings were reported by Almeida et al (2002), where palmitic acid concentration was raised in serum FFAs of NASH subjects. Therefore, to understand the effects of fatty acids such as PA and its role it plays in the development of NAFLD is integral. Additionally, further studies have reported not only palmitic acid but also the monosaturated fatty acid oleic acid are high circulating FFAs in NAFLD patients and promote inflammation and fibrosis in the liver (Gambino et al., 2016).

Ideally for an *in vitro* model of NAFLD, it is essential the intracellular fat content should be comparable to that found in human steatosis but with negligible lipotoxicity. To generate this level of lipid accumulation without overt cytotoxicity a dose response with a range of PA concentrations (10 µM – 300 µM) was employed.

Hepatic cytotoxicity and steatosis exhibited by PA were studied at 24, 48 and 72 h time points (Figure 3.1 and 3.2). Cell viability decreased concomitantly with increasing PA concentration, particularly at 200 µM – 300 µM (Figure 3.1). These findings are in line with a study by Zhang et al (2012) where 250 µM treatment of PA in H4IIE rat liver cells significantly increased cell death. Similarly, the results are also consistent with a study by Oh et al., al (2012) where 250 µM PA and a higher concentration of 500 µM PA were used than in this study also induced high levels of lipotoxicity and oxidative stress on SK-Hep-1 cells at 24 h (Oh et al.,2012).

Furthermore, results showed lipid accumulation increased significantly at higher concentrations of PA (Figure 3.2). This finding is in relatively good agreement with a study conducted by Nissar et al (2015). *In vitro* study of three principal liver cell lines including HepG2, Hep3B and Huh7 cells were lipid-overloaded by 0.1 mM, 0.25 mM and 0.5mM PA in a PA concentration depend experiment. Results showed all cell lines developed fat droplets in the hepatocytes in accordance with this study in the VL17A cells.

Nissar et al., further validated these findings in an *in vivo* mouse model where C57BL/6J mice were fed a high cholesterol (2%) and high fat diet (12% saturated fatty acid) (HC/HF) for 16 weeks. HC/HF

mice presented with elevated levels of serum triglycerides, cholesterol and insulin compared to control mice. Moreover, histopathological analysis of mouse liver presented with micro and macro- vesicular steatosis, inflammation and hepatic ballooning and confirmed the effects of PA induced lipotoxicity in NAFLD. A further study supports this as Wistar rats fed a high fat diet with saturated fatty acids correlated with steatosis and endoplasmic reticulum stress (Wang et al., 2006).

A possible mechanism of the PA induced lipotoxicity exhibited in this study may be the activation of the lipogenic enzyme Stearoyl-CoA Desaturase-1 (SCD1). SCD1 is expressed abundantly in the liver and converts saturated fatty acids such as PA to monosaturated fatty acids. Therefore, it can be expected that SCD1 is a key player in regulating hepatic lipid oxidation. These monosaturated fatty acids are vital structural parts of the cellular membrane phospholipid and act as substrates in the production of complex triglycerides which contributes to the increasing levels of hepatic lipid accumulation as seen in this study at 300 µM PA at 24 h (Matsui et al., 2012).

Furthermore, Nissar et al., found 0.5 mM PA treatment led to a 2 fold increase in SCD1 expression which promotes the increased flux of free fatty acids in the hepatocytes leading to the progression of steatosis as seen in this study. The exact mechanism of PA induced toxicity and elevated lipid accumulation is unclear, but it may be due to increased intracellular *de novo* lipogenesis in NAFLD.

De novo lipogenesis is initiated to remove excess free fatty acids (FFAs), therefore triglycerides (TGs) are synthesised which are then stored as lipid droplets in the hepatocytes or secreted into the blood as very-low-density- lipoprotein (VLDL) (Berlanga et al., 2012). The rate of *de novo* lipogenesis is regulated primarily by transcriptional factors such as sterol regulatory element binding protein 1c (SREBP1c).

In NAFLD, SREBP1c expression is increased as it binds to its lipogenic enzymes such as fatty acid synthase (FAS) and stimulates lipogenesis and therefore excess fatty acids accumulate in the hepatocyte which leads to cytotoxicity and cell death as seen in this study when VL17A cells were treated with high concentrations of PA, cell viability decreased significantly over longer incubation times. This was confirmed in a study of HepG2 cells treated with 0.5 mM PA treatment where a 1.5 fold increase in SREBP1 expression was observed (Nissar et al., 2015).

Simultaneously in NAFLD, the expression of PPARα, a transcriptional factor responsible for fatty acid oxidation and decreasing hepatic fat storage is inactive. These mechanisms lead to elevated intracellular levels of TG within hepatocytes which consequently exhibit lipotoxicity and eventually leading to cell death (Berlanga et al., 2012).

4.1.1. Effect of PA on reactive oxygen species (ROS) production

It is known continuous deposition of free fatty acids in the hepatocytes are an assault to normal mitochondrial function and consequently leads to lipotoxicity and cell death in NAFLD. Subsequent to mitochondrial damage, leakage of electrons at complexes I and III in the electron transport chain (ETC) occur. Complexes I and III are considered as major sites of superoxide and therefore generate ROS (Simoes et al., 2018), Masarone et al., 2018). Substrates which prompt electron flow or obstruct these complexes will in theory amplify ROS production and leakage in the mitochondria. This is consistent with the findings in this study where higher concentrations of PA (300µM) in VL17A cells promoted ROS production significantly at all time points (Figure 3.3).

The source of this ROS production may be attributable to an increase in the permeability of the inner mitochondrial membrane and therefore disturbs the membrane potential as electrons flow freely through the ETC (Massarone et al., 2018). As a result, this leads to impaired mitochondrial function and a surge in ROS production as seen with 300 µM PA treatment. Furthermore, another cause can be due to an increased β-oxidation rate in response to lipid accumulation with PA occurring in the hepatocyte. Consequently, this causes an increase in the reducing agents flavin adenine dinucleotide (FADH2) and nicotinamide adenine dinucleotide (NADH) which stimulate complex I and III of the ETC which then generates ROS (Simoes et al., 2018).

PA induced ROS production has been widely reported and supports the findings in this current study. Tedoro et al (2008) studied a choline-deficient mouse model and found after consuming a high fat diet for 16 weeks, mouse mitochondria presented with altered ETC complexes, a loss in membrane potential and reduced ATP synthesis. (Tedoro et al., 2008). Similarly, palmitic acid treatment in HepG2 cells led to a surge in ROS and therefore developed an oxidative burden in the hepatocytes (Nissar et al., 2015). Further to this, an imbalance in redox initiated the down-regulation of the

antioxidant response and released inflammatory cytokine IL-6 which is characterised as the ‘second hit’ in NAFLD (Nissar et al., 2015).

Interestingly, at lower PA concentrations (10 µM – 30 µM) ROS levels were elevated but had a less significant effect at 48 h. Again, this may be associated to an increased fatty oxidation which leads to the electron leakage in the ETC but to a lesser extent in comparison to higher PA concentrations. As a result, this leads to a reaction between oxygen and water which generates ROS (Grattagliano et al., 2012). Usually, this process is mediated by cytochrome C oxidase which combines oxygen and protons to form water. The loss of cytochrome C oxidase in the mitochondria produces three- fold more hydrogen peroxide (H2O2) an impaired function of complex I of the ETC (Kushnareva et al., 2002). This was reflected in the results in this study where ROS production significantly increased for all time points (Figure 3.3).

Furthermore, incomplete β-oxidation leads to the aggregation of lipotoxic intermediates such as malondialdehyde which damage the cell membrane. Lower levels of ROS at low PA concentrations may also be due to less β-oxidation occurring as there is less lipid accumulation in VL17A cells as seen in Figure 3.2. Therefore, as PA is a saturated fatty acid more energy is required to break the C-C bond in comparison to unsaturated fatty acids and fewer reducing agents are produced.

Garcia-Ruiz et al (2015) reported HepG2 cells treated with 200 µM PA led to the decreased expression of mitochondrial DNA and promoted oxidative damage in the cells after 24 h. This agrees with our study where at 200 µM ROS levels increased significantly which is a marker of oxidative stress. Similarly, generation of ROS has been reported in rat hepatoma cell line H411EC3 when exposed to oleic acid and PA and can be credited to the inhibition of ETC complexes I and III by the fatty acids (Nakamura et al., 2009).

Studies have also demonstrated the mitochondrial enzyme CYP2E1, is a direct source of ROS and plays a key role in the development of NAFLD. An increase in ROS production as seen in VL17A cells when treated with higher concentrations of PA can be attributable to the CYP2E1 enzyme as the cell line VL17A cells over-express this enzyme. Additionally, high PA concentrations can stimulate CYP2E1 as the enzyme is induced by high fat diet or alcohol which promotes a surge in ROS and reactive nitrogen species. As a result, mitochondria become dysfunctional as it stimulates the peroxidation of the inner mitochondrial membrane as well as components of the ETC.

4.1.2. Effect of PA on VL17A cells

In summary, increasing the incubation time and concentration of PA led to increases in lipid accumulation, cytotoxicity and mitochondrial damage in VL17A cells at all time points but was most significant at 300 µM. These findings suggest that PA may induce mitochondrial damage via the CYP2E1 enzyme or as a result of the disturbance of the ETC by losing membrane potential as the permeability of the inner mitochondria membrane is compromised as a response to the lipid accumulation in the hepatocytes and results in ROS production. It can be concluded the VL17A cells induced by PA is a suitable model for NAFLD and the subsequent studies carried out employed 50 µM and 100 µM treatments.

4.2. Bile acids as a potential therapy for NAFLD

As aforementioned in Chapter 1, Section 1.6., bile acids are synthesised in the liver and play a key role in the synthetic and regulatory metabolic pathways. They act as indispensable signalling molecules for lipid, carbohydrate and cholesterol regulation and co-ordinate these biological processes.

Furthermore, bile acids are endogenous ligands for the FXR receptor and once activated it plays a major role in lipid and carbohydrate metabolism. FXR activation reduces fibrosis and inflammation in the liver and is of interest to develop a putative therapeutic approach for the treatment of NAFLD.

Here in this study, VL17A cells were treated with the primary bile acid chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) and the effects on steatosis, cytotoxicity, ROS production and mitochondrial function were analysed.

4.2.1. Effect of bile acids on cell viability, steatosis and SREBP1 expression

VL17A cells were treated with either CDCA or UDCA alone or co-incubated with PA in dose and time-dependant experiments to assess the effect on cytotoxicity and to determine whether lipid accumulation is attenuated.

Firstly, the role of CDCA will be discussed as CDCA is a primary bile acid synthesised by the classical pathway in bile acid homeostasis. It has been established that CDCA is the most potent FXR ligand and therefore the effect of CDCA in VL17A cells was assessed in this study (Fiorucci et al., 2018).

Also, CDCA has been widely used to treat liver conditions such as primary biliary cholestasis and gallstones and therefore may be beneficial for the treatment of NAFLD (Portincasa et al., 2012).

VL17A cells were treated with CDCA for 24, 48 and 72 h respectively. CDCA exhibited greater cytotoxicity and lipid accumulation at higher doses (100 µM and 300 µM) in comparison to UDCA treatment and did not exhibit hepatoprotective effects (Figure 3.4 and Figure 3.5). This finding is consistent with a study conducted on male Sprague-Dawley rat hepatocytes. CDCA treatment with 250 µM induced hepatocellular injury and led to a significant drop of 23% in cell viability along with an 86% depletion of cellular ATP production (Spivey et al., 1993).

The lack of hepatoprotective properties of CDCA in comparison to UDCA in this study may be attributable to the chemical structure of the compounds as the toxicity and protection of bile acids is related to hydrophobicity (Thomas et al., 2008). UDCA possesses two hydroxy groups (-OH) with α and β- orientation at positions 3 and 7 of the cholane ring structure (Perez and Briz, 2009). The C-7 β-orientation confers the compound a greater hydrophilicity than CDCA. Therefore, CDCA is more hydrophobic and has the ability to interact and disrupt lipid membranes. As a result, this could lead to cellular dysfunction which consequently increases the risk of hepatocellular injury characterised by steatosis as observed in this study with VL17A cells.

Several clinical studies support the results in this study and have reported elevated CDCA levels in NAFLD and NASH patients. Kalhan et al. (2011), analysed the plasma profile of NAFLD and NASH patients in a metabolomic analysis. Cholic acid (CA) levels were raised 4 fold and CDCA levels were elevated 2 fold in NASH patients in addition to elevated AST and ALT which are markers for liver damage. Similarly, an *in vivo* mouse model fed a high fat cholesterol diet (HFC) for 2 weeks showed total hepatic bile acids were significantly raised. The levels of CDCA continuously increased for the whole treatment and positively correlated with steatosis and elevated serum ALT levels. The results confirmed that bile acid homeostasis is disturbed in HFC diet induced NAFLD (Jia et al., 2014).

Furthermore, CDCA co-incubated with PA (Figure 3.6) further promoted lipid accumulation and cytotoxicity at all concentrations and time points in VL17A cells. The lipid accumulation in VL17A cells was validated by the accumulation of triglycerides where there was a 9% increase with CDCA and PA co-treated cells (Figure 3.8). A possible explanation for this increase in bile acid levels could be the failure of the vital feedback regulation of bile acids regulated by FXR (Chow et al., 2017). Amplified

bile acid levels lead to bile acid overload and subsequently induces cytotoxicity by the activation of inflammatory pathways and oxidative stress (Legry et al., 2017).

In humans, CDCA accounts for the most predominant bile acid in the bile acid pool. Under normal physiological conditions the total bile acid pool levels are tightly controlled and maintained by the CYP7A1 enzyme and is repressed by FXR to inhibit bile acid synthesis (Zhang and Deng, 2018). The repression of CYP7A1 is facilitated by the small heterodimer partner (SHP) which then blocks the activity of liver receptor homolog (LRH-1) to inhibit bile acid synthesis (Yuan and Bambha, 2015). This is a vital mechanism to protect the hepatocytes from bile-acid induced toxicity.

A study by Jiao et al. (2017) reported serum concentrations of bile acids were increased in NAFLD patients and the mRNA expression levels of CYP7A1 were significantly higher in NAFLD patients. Similar findings were observed in a study of 113 morbidly obese patient's liver biopsies and serum samples where again mRNA levels of CYP7A1 were elevated and therefore bile acid levels were also raised which corresponded with the NAFLD activity score (Bechmann et al., 2013). Although this study did not analyse the protein or gene expression of the CYP7A1 gene in VL17A cells, in accordance with the findings from other studies it indicates the negative feedback mechanism of CDCA was altered which led to bile acid overload induced toxicity in the cells and lipid accumulation.

In contrast to CDCA, UDCA treatment led to improvements in both cell viability and lipid accumulation in VL17A cells when treated on its own and when co-incubated with PA (Figure 3.5, 3.6 and 3.7). The lipid accumulation was validated by the enzymatic measurement of triglyceride in the VL17A cells where a 27% decrease was observed (Figure 3.8). There are several *in vitro* and *in vivo* studies that support the hepatoprotective effects exhibited by UDCA in the liver as determined in this study.

Tsuchida et al. (2012) evaluated the effect of UDCA on a diabetic mouse model with exacerbated hepatic steatosis. Mice were fed a high fat diet and were orally administered with 50, 150 or 450 mg UDCA for 2 weeks. Results showed UDCA treatment led to a marked decrease in hepatic triglyceride levels as seen in this study with VL17A cells. Another study consistent with these findings was a 12 month randomised control trial where 126 subjects were given a high dose of UDCA (28-35 mg/kg). findings showed treatment with high dose UDCA was safe, improved ALT levels and decreased liver fibrosis (Ratziu et al., 2011).

A possible mechanism to explain the action of UDCA in this study to lower steatosis and improve cell viability in the VL17A cells could be attributable to the FXR regulation of the FGF19 gene in humans. In the intestine, as a response to bile acid overload, FXR is activated and prompts the transcription of FGF19 in the ileum. It then enters the circulation where FGF19 binds to its receptor FGFR4 and initiates the downregulation of CYP7A1 and therefore inhibits bile acid synthesis. Again, this FXR-FGF19-CYP7A1 pathway is tightly regulated as a hepatoprotective mechanism.

Furthermore, VL17A cells treated with UDCA lowered hepatic SREBP1 expression (Figure 3.9). SREBP1c is well recognised as the principal transcriptional factor in lipid metabolism and regulates the genes involved in lipogenesis. Similarly, FXR has demonstrated its impact on lipid metabolism (Xu et al., 2014). To prevent excess fat accumulation in the hepatocyte a marker of NAFLD, FXR activation inhibits the expression of SREBP1c via SHP and its target enzymes including fatty acid synthase (FAS) and acetyl-coA carboxylase (ACC) and therefore fatty acid synthesis is suppressed (Xu et al., 2014). Evidence for this FXR-SHP-SREBP1c pathway was demonstrated by Trauner et al. (2010) in an FXR null mice model. The mice developed hepatic steatosis and hypertriglyceridemia. Moreover, in NAFLD patient's FXR expression and mRNA was decreased whereas the expression of SREBP1c was elevated which was supported by an increase in triglyceride synthesis (Yang et al., 2010). Overall, FXR activation inhibits lipid accumulation in the liver and thus protects the liver from lipotoxicity.

4.2.2. Effect of bile acids on ROS and oxygen consumption rate

CDCA exhibited cytotoxic effects and had a significant increase in ROS production at all time points (Figure 3.9). On the other hand, UDCA exhibited lipoprotective effects at 1 h as ROS levels decreased.

It has been established; overt bile acid accumulation primes the hepatocytes to cellular injury and oxidative stress which plays a fundamental role in the pathogenesis of NAFLD. The main site of oxidative stress induced by bile acids is the hepatic mitochondria where increased lipid peroxidation also occurs. This was demonstrated by Sokol et al. (1993) in an *in vitro* study where rat hepatocytes were incubated with 100- 200 µM/L bile acids. CDCA treatment led to a 40% decrease in cell viability and correlated with lipid peroxidation as a result of bile acid toxicity (Sokol et al., 1993).

In relation to this study, the increase in ROS presented by CDCA treatment may be attributable to the hydrophobicity of the bile acid. In hepatic mitochondria, hydrophobic bile acids decrease the activity of complexes I, III and IV of the electron transport chain (Perez and Briz, 2009). Furthermore, the bile acids decrease respiration in complex III and is perhaps caused by the inhibitory action of bile acids on the phosphorylation system, though the mechanism remains unclear.

As a response to the diminished respiration in complex III, respiration is induced by bile acids in complex IV. This is caused by the increased permeability of the mitochondria to protons and this uncoupling allows bile acids to destabilise the mitochondrial membrane structure and therefore decrease ATP production (Sokol et al., 1995). Subsequently, this bile acid induced hepatotoxicity leads to the production of intracellular ROS in the mitochondria. Overall, the findings show CDCA did not exhibit lipoprotective effects when co-incubated with PA or alone thus it is not a suitable treatment for NAFLD as oxidative stress is increased.

Furthermore, this study shows in comparison to PA treatment, UDCA led to a decrease in ROS production as well as exhibiting a beneficial effect on mitochondrial function in VL17A cells by measuring ROS levels as well as oxygen consumption rate (Figure 3.11).

As discussed above in Section 4.1.1., research has established PA treatment leads to the mitochondrial dysfunction induced by free fatty acid accumulation in the hepatocytes in both rodents and humans. The current study assessed mitochondrial impairment by measuring the oxygen consumption rate. PA treatment (100 µM) led to an impaired mitochondrial function as there was a decline in mitochondrial respiration accompanied with a significant decrease in ATP production. Furthermore, an increased proton leak from mitochondria was observed and is a parameter for mitochondrial damage. Overall, the mechanism responsible for this mitochondrial dysfunction is caused by PA induced cytotoxicity. PA overload induces the opening of the mitochondrial permeability transition pores (MPTP) which leads to electron leakage in the ETC. Therefore, this results in mitochondrial swelling and is accompanied with the uncoupling of oxidative phosphorylation due to the inhibition of the ETC complexes I and III. This reduces the mitochondria membrane potential and leads to ATP deletion as demonstrated in this study where ATP generation was depleted by 32% in VL17A cells. Furthermore, the loss of the electron carrier cytochrome c in the

respiratory chain is lost which leads to the build of reducing equivalents NADH and FADH which as a result generate ROS.

In comparison, UDCA showed a beneficial effect on all parameters of oxygen consumption rate in the mitochondria. Mitochondrial respiration improved in VL17A cells accompanied with an increase in ATP production. A possible mechanism for this is UDCA possess protective effects against the lipotoxicity caused by fatty acids such as PA. A mechanism of this protection shown by UDCA was determined in a study by Botla et al. (1995) where UDCA treatment prevented MPTP formation. Therefore, in this study the mitochondrial membrane potential remains stable as there is no electron leakage occurring and as a result ATP production is improved by UDCA treatment in comparison to PA treatment. A study by Serviddio et al. (2004) is consistent with the findings in this study where UDCA prevented mitochondrial oxidative stress in a Wistar rat model. Rats were administered with 2.5g/kg UDCA for 28 days and results showed control group rats had a 3.5 fold increase in lipid peroxidation compared to the UDCA group (Serviddio et al., 2004).

4.2.3. Bile acids Summary

In summary, CDCA did not exhibit hepatoprotective effects in VL17A cells and this finding is consistent with *in vitro* and *in vivo* studies where lipid accumulation was not attenuated. Furthermore, high levels of toxicity were exhibited and as a result led to significant decreases in cell viability as well as mitochondrial dysfunction characterised by elevated ROS production. On the other hand, studies on the beneficial effects of UDCA remain divergent as a large randomised controlled trial that enrolled 166 NASH patients were administered with a dose of 13-15 mg/kg/day UDCA did not show changes in the degree of steatosis, inflammation or fibrosis compared to the placebo group (Lindor et al., 2004). Although from our study UDCA presented with promising results for the treatment of NAFLD as the hepatic steatosis was reduced in VL17A cells and showed improvement in mitochondrial function

4.3. Betaine as a potential therapy for NAFLD

Alternative therapeutic options are urgently required to tackle the mounting global burden of NAFLD and its associated conditions such as obesity and type II diabetes. There are encouraging results for antioxidant therapies in attenuating lipid accumulation in the liver. Betaine (trimethylglycine), a

methyl donor derived from the oxidation of dietary sources of choline may be a potentially effective treatment for NAFLD (Abdelmalek et al., 2009).

In this study, VL17A cells were treated with betaine (20 µM) on its own or co-incubated with PA to assess the effect on cell viability, steatosis and mitochondrial function.

Betaine exhibited hepatoprotective effects on VL17A cells when incubated with PA at 24h, 48 h and 72h. There was no change in lipid accumulation levels at 24 h from control but at 48 h and 72 h decreases in lipid accumulation was noted. These findings are consistent with an *in vitro* study where HepG2 cells were incubated with 100 µM PA to induce steatosis. Subsequently, HepG2 cells were supplemented with 2 mM betaine and results showed a significant decrease in lipid accumulation (Zhang et al., 2018).

Attenuation of lipid accumulation was also reported in Kunming mice, fed either a high fat diet or normal chow diet for 13 weeks and treated with betaine. Findings showed hepatic triglyceride levels decreased in high fat diet fed mice. A decrease in liver damage markers ALT and AST was also reported (Du et al., 2018).

The mechanism of betaine to reduce hepatic lipid levels is associated with the increased stimulation of hepatic AMP- activated protein kinase (AMPK). The gene expression of AMPK was raised significantly in mice fed a high fat diet with betaine compared to control group (Xu et al., 2015). AMPK plays a key role in hepatic lipid and glucose homeostasis by controlling the expression of the enzymes Acetyl-CoA-carboxylase (ACC) and fatty acid synthase (FAS). A gene expression study on steatogenic HepG2 cells supplemented with betaine showed betaine inhibited the expression of the genes SREBP1 and SCD1 (Zhang et al., 2018). These genes are involved in *de novo* lipogenesis and therefore it can be implicated betaine down-regulates the expression of these genes and hence reduces fatty acid accumulation in the hepatocytes.

Furthermore, in this study betaine exhibited anti-oxidative properties as the mitochondrial dysfunction induced by PA in VL17A cells was alleviated. Results showed ROS levels decreased significantly at 1 h and more predominantly at 3 h. This finding agrees with a study by Heidari et al (2018) where 10 and 50 mg/kg betaine supplementation in Sprague-Dawley rats improved hepatic injury markers of oxidative stress compared to bile duct ligare (BDL) rats. ROS levels decreased and

total anti-oxidant capacity significantly increased in the higher dose of betaine treatment (Heidari et al., 2018).

Also, this study shed light on the anti-oxidative properties of betaine as all mitochondrial activity parameters were improved by betaine in comparison to PA (Figure 3.16). For example, ATP production increased by 10% in betaine + PA treated VL17A cells. This was supported by an *in vitro* study where presence of mitochondria was detected by intracellular staining in HepG2 cells supplemented with 2 mM betaine and as a result increased ATP levels and mitochondria content. This was further validated by measuring the quantitative mtDNA levels (Zhang et al., 2018). Heiderai et al., also reported increases decreases in ROS generation, lipid peroxidation and liver glutathione all liver tissue markers of oxidative stress (Heidari et al.,2018). As betaine is abundantly found in the liver, it can be proposed that the antioxidative activity of betaine provides hepatoprotective effects and deters mitochondrial dysfunction.

A possible mechanism highlighted by other studies is betaine stimulates and increase in S-Adenosyl methionine (SAM) in the hepatocytes, the major methyl donor, and reduces levels of both homocysteine and S-adenosylhomocysteine (SAH). Betaine lowers circulating levels of homocysteine by facilitating its conversion to methionine, which also lowers SAH. Betaine is the only alternate source of methyl groups for the conversion of homocysteine to methionine. Furthermore, betaine can also substitute for SAM as a methyl donor for the direct methylation of phosphatidylethanolamine. This pathway serves as an alternative route of phosphatidylcholine formation. From these functions, betaine has derived its beneficial effects on metabolism and lessens defects in the methylation pathway caused by deficiencies or impaired function of the folate cycle (Kathivrel et al., 2010).

SAM plays an integral role in the reconstruction of impaired biological targets such as membrane phospholipids and is necessary for lipid synthesis. Thus, it can be concluded betaine exhibits hepatoprotective effects associated with SAM production in the liver and in sustaining the function of mitochondria. Additionally, SAM is known as a free radical scavenger that promotes cellular antioxidant defence mechanism and cytoprotection exhibited by betaine could be credited to SAM in the liver.

4.4. MHY908 as a potential therapy for NAFLD

In this study, the previous two therapies; bile acids and betaine showed promising results in an *in vitro* model of steatosis and for the treatment of NAFLD. However, research work from other groups are conflicting and therefore this is still a pressing need to find alternative treatments.

It is well recognised that defective lipid homeostasis plays a key role in the pathogenesis of NAFLD and therefore therapeutic approaches should target the lipogenic pathway. Peroxisome proliferator activated receptors (PPAR) are ligand-activated transcriptional factors that play a crucial role in lipid homeostasis and tightly regulate lipogenesis in the liver to prevent lipotoxicity and cell death (Wang et al., 2017).

PPAR consists of three different isotypes, PPARα, PPARβ/δ and PPARγ and all exhibit different tissue expression but they all bind lipids and govern lipid metabolism in the liver (Wahli and Michalik, 2012). PPAR α/γ dual agonists are emerging as promising therapeutic agents for NAFLD as they alleviate symptoms associated with dysregulated lipogenesis such as steatosis, inflammation and fibrosis in the liver (Pawlak et al., 2014). Furthermore, in regard to the PPAR dual agonists, it is known the transcriptional activity of PPARα induces lipid consumption by increasing β-oxidation and ameliorates steatosis in hepatocytes (Montagner et al., 2015). This was demonstrated in PPARα knockout mice where severe hepatic steatosis and increases in cholesterol and liver damage markers were observed and therefore highlights the important role of PPARα to maintain lipid homeostasis (Montagner et al., 2015). However, PPARγ stimulates adipogenesis in response to lipid deposition in adipocytes to reduce toxicity (Lehrke and Lazar, 2005).

In this study, the effect of the PPARα/γ 2-[4-(5-chlorobenzothiazothiazol-2-yl)phenoxy]-2-methyl-propionic acid (MHY908) and PA on VL17A cells was investigated in steatosis, cell viability, ROS and mitochondrial function. Currently there is very limited literature and research work available on the effect of MHY908 and in the treatment of NAFLD.

MHY908 treatment (1 µM) significantly attenuated cytotoxicity and lipid accumulation when co-incubated with PA (50 µM) in VL17A cells (Figure 3.20 and 3.21). Also, the effect of MHY908 is concentration dependant as higher concentrations of MHY908 (5 µM and 20 µM) did not display hepatoprotective effects and further promoted steatosis and cytotoxicity.

Park et al. (2013) reported similar findings in a diabetic mouse model (db/db). Mice were fed lidium, calorie restricted diet or administered with MHY908 (1 mg or 3 mg/kg/day) mixed in food for 4 weeks. They found MHY908 treatment elevated the transcriptional activity of PPAR α/γ in AC2F liver cells. Moreover, AC2F cells showed a decrease in serum triglycerides, glucose and insulin levels. Another study on aged rats administered with 3 mg/kg/day MHY908 showed a marked decrease in lipid accumulation as seen in this study in VL17A cells (Park et al., 2015).

A possible hepatoprotective mechanism for MHY908 may be attributable to the increase in transcriptional activity of PPARα which then increases fatty acid β-oxidation in response to lipid overload in the hepatocytes. Under normal conditions, fatty acid β-oxidation occurs in the mitochondria to produce acetyl-coA which can be merged into the tricarboxylic acid cycle for full oxidation (Koo., 2013). This process is facilitated by the activity of carnitine palmitoyltransferase I (CPT1) where fatty acyl-coAs are transported across the mitochondrial membrane.

Park et al (2013) reported the elevated expression of CPT-1 in ACF2 liver cells in response to MHY908 treatment (Park et al., 2013). Furthermore, CPT1 converts fatty acyl-coAs to fatty acyl- cartinines to allow them to translocate from the outer mitochondrial membrane into the intermembrane space where β-oxidation occurs (Koo., 2013). Therefore, it can be assumed that PPAR α/γ enhances β-oxidation as a response to PA overload in the VL17A cells and therefore lipid accumulation and lipotoxicity are reduced.

VL17A cells treated with 1 µM MHY908 showed a significant decrease in ROS generation and improvements in mitochondrial respiration rates compared to PA treatment (Figure 3.21 and 3.22). This corresponds with the finding in a diabetic mouse study where ROS levels and leptin levels decreased (Park et al., 2013). Leptin is a marker of endoplasmic reticulum stress (ER) and reflects insulin resistance. Park et al., reported diabetic mice had impaired leptin receptors making them more susceptible to hepatotoxic injury. Prolonged over-nutrition as seen in obese and NAFLD patients increase fat accumulation in the hepatocytes stimulated by insulin. MHY908 down-regulated leptin levels in diabetic mice (Park et al., 2013).

Additionally, Park et al (2013) reported adiponectin levels in diabetic mice were reduced (Park et al., 2013). Adiponectin is a specific PPARγ adipokine which increases lipid metabolism and insulin signalling in the liver. Therefore, MHY908 exhibited PPAR γ agonistic effects in this study.

In summary, MHY908 demonstrated PPAR α agonistic effects on VL17A cells where steatosis was reduced in the hepatocytes and is consistent with *in vivo* and *in vitro* studies and therefore poses as a suitable treatment for NAFLD. However, the agonistic activity of PPAR γ in this study remains inconclusive as markers of adipogenesis were not studied. Furthermore, the mechanism of action of MHY908 on mitochondrial function remains unclear.

4.5. The novelty of the study

Firstly, the novel cell line VL17A (a human hepatoma cell line, HepG2) was used for the first time to study the effect of the saturated fatty acid PA to investigate the molecular mechanisms involved in the development of NAFLD and further utilised in hepatoprotective studies with the bile acids UDCA and CDCA, betaine and MHY908.

The main finding in this study was the characterisation of the lipogenic transcription factor SREBP1c mechanism involved in NAFLD development following co-treatment of PA and UDCA in VL17A cells. Data showed UDCA had a hepatoprotective effect as SREBP1c levels decreased as compared to when treated with PA only.

Furthermore, in this study the mitochondrial function was measured in real-time using Seahorse mito-stress assays in VL17A cells using the following treatments, PA, UDCA, betaine and MHY908 for the first time. It can be concluded from the data that PA does induce lipotoxicity, mitochondrial dysfunction and prompts NAFLD development. UDCA and MHY908 improved mitochondrial function and therefore could be potential therapies to treat and prevent NAFLD.

4.6. Limitations of the study

In this study, the lack of protein and gene expression data of the main transcriptional factors such as SREBP1c, ChREBP, PPAR α which are involved in lipogenesis and the development of NAFLD is the main limitation.

Protein and gene expression experiments were not carried out due to the lack of time attributable to numerous laboratory issues beyond my control. There was continuous episoidic fungal contamination for 9-12 months in the communal tissue culture facility. This compromised the sterile environment required for cell work and therefore had an adverse effect on the research conducted.

Chapter 5

Conclusion and Future work

5.1. Conclusion

The manifestation of excess intracellular lipid accumulation in hepatocytes is known as steatosis and is the key hallmark of non-alcoholic fatty liver disease (NAFLD). In the disease progression of NAFLD, steatosis can prime the liver to more severe pathologies including, non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Therefore, a greater understanding of how fatty acids contribute to NAFLD development has crucial implications since the prevalence of NAFLD is rapidly increasing. Thus, identifying key transcriptional factors/ genes in the regulatory network that governs hepatic lipid metabolism is a vital step towards development of treatments for NAFLD.

Furthermore, there is increasing evidence supporting the role of alternative therapies such as, bile acid activated FXR in promoting lipid oxidation, antioxidant properties of betaine and a PPARα/γ dual agonist MHY908 as potential treatments for NAFLD.

The current study aimed to characterise the effect of the saturated fatty acid, palmitic acid (PA) on lipotoxicity, liver steatosis and oxidative stress in VL17A (HepG2) cells to ascertain if it could be used as a novel *in vitro* model of NAFLD. A further aim was to determine the effect of alternative therapies such as bile acids (CDCA and UDCA), betaine and MHY908 on the prevention of lipotoxicity, lipid accumulation, oxidative stress and mitochondrial function in VL17A cells to shed new light on the molecular mechanisms involved in NAFLD.

The effect of Palmitic acid (PA)

It was determined that VL17A cells treated with the saturated fatty acid, palmitic acid (PA) induced lipid accumulation in the hepatocytes and therefore was a suitable *in vitro* model of NAFLD.

Also, it was established the lipid accumulation and cytotoxicity exhibited by PA was time and dose dependant and was more prevalent in higher incubation time and concentrations of PA treatment. Particularly, lipid accumulation increased between 150 µM and 300 µM PA dose and led to significant decreases in cell viability at 72 h time point. The exact mechanism of PA induced toxicity and elevated lipid accumulation is unclear, but it may be due to increased intracellular *de novo* lipogenesis in NAFLD. It is likely the expression of the transcriptional factor SREBP1 increases as it binds to its lipogenic gene fatty acid synthase (FAS) stimulated by the increase in fatty acid PA and consequently stimulates lipogenesis. Consequently, this leads to lipid accumulation in hepatocytes and cytotoxicity.

Lastly, the ROS experiment revealed higher doses of PA (200 µM – 300 µM) exhibited toxicity and led to the generation of ROS in VL17A cells. The impaired mitochondrial function may be attributable to the leakage of electrons at complexes I and III of the ETC. Consequently, this increases the permeability of the mitochondrial membrane and disrupts the membrane potential. As a result, this leads to the generation of ROS.

In summary, for a suitable *in vitro* model of NAFLD it is essential the concentration of PA leads to the development of steatosis without expressing overt cytotoxicity therefore in subsequent experiments 50 µM and 100 µM PA treatment was employed.

The effect of bile acids

In this study it was determined the primary bile acid chenodeoxycholic acid (CDCA) did not display hepatoprotective properties when co-incubated with PA in VL17A cells. Cell viability decreased significantly at all time points and lipid accumulation was not attenuated. A possible explanation for this is the chemical structure of CDCA as it is a more hydrophobic bile and could disrupt the lipid membrane which leads to hepatocellular injury.

Additionally, it is known bile acid synthesis is tightly regulated by a negative feedback system involving FXR and the rate limiting enzyme CYP7A1. The lack of improvement in lipid accumulation by CDCA co-incubated with PA may be caused by the failure of FXR to repress the expression of CYP7A1 to inhibit bile acid synthesis. Therefore, this leads to lipid overload in the hepatocytes. Furthermore, it was found ROS levels were not improved at any time point.

On the other hand, VL17A cells treated with ursodeoxycholic acid (UDCA) exhibited hepatoprotective effects as cell viability improvement was most obvious with 30 µM + 50 µM PA at 24 h timepoint.

Lipid accumulation was also ameliorated and was validated by measuring the enzymatic triglyceride content in VL17A cells. A possible molecular mechanism that may have been implicated is the FXR-FGF19-CYP7A1 pathway. Bile acids such as UDCA increase the expression of FXR which in turn activates its transcriptional gene FGF19. FGF19 binds to its receptor FGFR4 and as a result downregulates the expression of CYP7A1. This leads to the inhibition of bile acid synthesis and is a hepatoprotective mechanism.

Furthermore, it was found UDCA treatment improved mitochondria function and reduced ROS generation in VL17A cells. A possible mechanism for this is UDCA prevented the formation of mitochondria permeability transition pores thus there was no electron leakage. As a result, the mitochondrial membrane remained stable and ROS were not generated.

In summary, treatment with UDCA showed beneficial effects on attenuating lipid accumulation and cytotoxicity when co-incubated with PA in VL17A cells. In comparison, treatment with CDCA did not show lipoprotective effects in VL17A cells and is not a suitable treatment for NAFLD.

The effect of Betaine

This study on the anti-oxidative properties of betaine showed promising results for the treatment of NAFLD. It was revealed 20 µM betaine treatment with PA showed hepatoprotective effects and particularly at 24 h cell viability improved. Similarly, lipid accumulation was also decreased at 48 h and 72 h. A possible mechanism outlined is betaine is associated with the AMPK gene which plays a key role in hepatic lipid and glucose homeostasis by controlling the expression of the enzymes Acetyl-CoA-carboxylase (ACC) and fatty acid synthase (FAS). These genes are involved in *de novo* lipogenesis and therefore it can be implicated betaine down-regulates the expression of these genes and hence reduces fatty acid accumulation in the hepatocytes.

In addition to this, it was established betaine treatment led to a decrease in ROS formation and improved mitochondrial function. Betaine stimulates and increase in S-Adenosyl methionine (SAM) in the hepatocytes, the major methyl donor, and reduces levels of both homocysteine and S-adenosylhomocysteine (SAH). Betaine lowers circulating levels of homocysteine by facilitating its conversion to methionine, which also lowers SAH. Betaine is the only alternate source of methyl groups for the conversion of homocysteine to methionine. Therefore, it can be concluded betaine exhibits hepatoprotective effects associated with SAM production in the liver and in sustaining the function of mitochondria.

The effect of MHY908

in this study it was determined 1 µM MHY908 treatment reflects PPARα agonistic effects in VL17A cells as lipid accumulation was decreased. A possible mechanism of action of MHY908 is PPAR α is

stimulated by fat overload in the hepatocyte. As a result, mitochondrial β-oxidation is initiated to inhibit fatty acid synthesis and therefore is vital maintain lipid homeostasis in the liver. Furthermore, it was found MHY908 lowered ROS levels in VL17A cells. However, the mechanism behind this is yet to be elucidated as there is limited literature on the effects of MHY908 in liver cells.

5.2. Future work

It has been demonstrated that saturated fatty acid PA caused a great decrease in cell viability concomitantly with an increase in lipid accumulation. PA also exhibited cytotoxic effects and led to mitochondrial dysfunction. Whilst this shows the effect of saturated fatty acids, further investigations are required to gain a better understanding of the lipogenic pathways involved in the development of steatosis also commonly referred to as the ‘first hit’ in NAFLD. Future work should evaluate the protein expression by western blotting and gene expression by PCR of the following transcriptional factors/ genes; SREBP1 and SHP. These studies would provide an insight into the lipogenic pathways and the role of fatty acids in NAFLD.

With reference to the effect of bile acids, it was deduced that UDCA treatment led to improvements in all markers of NAFLD such as cell viability, lipid accumulation and mitochondrial function. There is accumulating evidence of betaine with vitamin E treatment in alleviating NAFLD symptoms. Future work should involve treating NAFLD with betaine in conjunction with other anti-oxidants to produce a more potent therapeutic agent to tackle the growing burden of fatty liver disease. Furthermore, studying the protein expression by western blot of CYP7A1 the rate limiting enzyme and FXR would provide an insight into the molecular mechanisms involved in the role of bile acids in NAFLD.

In regard to betaine treatment it was established betaine displayed anti-oxidative properties in VL17A cells. All markers of hepatocellular damage that were measured showed an improvement. Future work should aim to evaluate these fin

Lastly, MHY908 a PPARα/γ dual agonist showed beneficial effects across all markers of steatosis induced toxicity. Mitochondrial function remained stable with MHY908 and cell viability was also improved. However, the mechanism/ pathway for the action of MHY908 remains unclear and needs further investigation. Furthermore, future work should measure leptin levels as a marker of

endoplasmic reticulum stress in PA induced steatotic VL17A cells. The activity and effect of PPARγ were not evaluated in this study. Protein expression of the transcriptional factor PPAR α/γ by western blotting would be vital to gain a better understanding of the mechanism of action and expression of MHY908.

References

Abdelmalek, M.F., Sanderson, S.O., Angulo, P., Soldevila, C., Liu ,.C, Peter, J., Keach, J., Cave, M., Chen, T., McClain, C.J., Lindor, K.D. (2009). Betaine for nonalcoholic fatty liver disease: results of a randomized placebo-controlled trial.
Hepatology. 50 (6), 1818-1826.

Abu-Shanab A, Quigley EM. (2010).The role of the gut microbiota in nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol. 7(12):691–701.

Adorini, L., Pruzanski, M., Shapiro, D. (2012). Farnesoid X receptor targeting to treat nonalcoholic steatohepatitis. Drug Discov Today. 17:988-97.

Aithal, G.P., Thomas, J.A., Kaye, P.V., Lawson, A., Ryder, S.D., Spendlove, .I, Austin, A.S., Freeman, J.G., Morgan, L., Webber, J. (2008). Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. Gastroenterology.135(4):1176-84.

Aharoni-Simon, M., Hann-Obercyger, M., Pen, S., Madar, Z., Tirosh, O. (2011). Fatty liver is associated with impaired
activity of PPARγ-coactivator 1α (PGC1α) and mitochondrial biogenesis in mice. Lab invest. 91 (7), 1018-28.

Ananthanarayanan , M., Balasubramanian ,V.N., Makishima , M., Mangelsdorf , J.D., Suchy, J.F. (2001). Human bile salt export pump (BSEP) promoter is transactivated by the farnesoid X receptor/bile acid receptor (FXR/BAR). J. Biol. Chem. 27 : 28857 – 28865.

Anstee,Q.M., Day,C.P. (2013). Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis.. Nat Rev Gastroenterol Hepatol. 10 (6), 330-44.

Auger,C., Alhasawi, A., Contavadoo, M., Appanna,D.V. (2015). Dysfunctional mitochondrial bioenergetics and the pathogenesis of hepatic disorders. Front Cell Dev Biol.. 3 (40), 1-11.

Baran, B., Akyuz, F. (2014). Non-alcoholic fatty liver disease: What has changed in the treatment since the beginning?.World J Gastroenterol. 20 (39), 14219–14229.

Bechmann, L.P., Kocabayoglu, P., Sowa,J.P., Sydor, S., Best, J., Schlattjan, M. (2013). Free fatty acids repress small heterodimer partner (SHP) activation and adiponectin counteracts bile acid-induced liver injury in superobese patients with nonalcoholic steatohepatitis. Hepatology.(57):1394-1406.

Berlanga, A., Guiu-Jurado, E., Porras, AJ., Auguet, T. (2014). Molecular pathways in non-alcoholic fatty liver disease. Clinical and experimental gastroenetrology. 4 (7), 221- 239.

Bernstein,C.,Holubec, H.,Bhattacharyya,K.A., Nguyen,H.,Payne,M.C, Zaitlin,B., Bernstein, H. (2011). Carcinogenicity of deoxycholate, a secondary bile acid. Archives of Toxicology. 85 (8), 863-871.

Belfort R et al. (2006). A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. N Engl J Med. 355(22):2297–307.

Boettcher, E., Csako, G., Pucino, F., Wesley, R., Loomba, R. (2012). Meta-analysis: pioglitazone improves liver histology and fibrosis in patients with non-alcoholic steatohepatitis. Aliment Pharmacol Ther. 35:66–75.

Boyer,J.L.(1980).New concepts of mechanisms of hepatocyte bile formation. Physiol Rev.60:303–326. Caldwell,S., Argo, C. (2010). The natural history of non-alcoholic fatty liver disease. Dig Dis. 28 (1), 162-168.

Carr,M.R., Reid, E.A. (2015). Synthetic FXR Agonist GW4064 Prevents Diet-induced Hepatic Steatosis and Insulin Resistance. Pharm Res. 17 (16), 1-16.

Cariou B, van Harmelen K, Duran-Sandoval D, van Dijk TH, Grefhorst A, Abdelkarim M, Caron S, Torpier G, Fruchart J,Gonzalez FJ, Kuipers F, Staels B (2006) The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. J Biol Chem. 281:11039–11049.

Cipriani, S., Mencarelli, A., Palladino, G., et al. (2010). FXR activation reverses insulin resistance and lipid abnormalities and protects against liver steatosis in Zucker (fa/fa) obese rats. J Lipid Res. 51:771-84.

Claudel, T., Zollner, G., Wagner, M., Trauner, M. (2011). Role of nuclear receptors for bile acid metabolism, bile secretion, cholestasis, and gallstone disease. Biochim Biophys Acta.1812 (8,)67–878.

Chiang, L.Y.J. (2009). Bile acids: regulation of synthesis. Journal of lipid research. 50 (1),1955-1966.

Chitturi, S., Farrell, G.C., Hashimoto, E., Saibara, T., Lau, G.K., Sollano, J.D. (2007). Non-alcoholic fatty liver disease in the Asia-Pacific region: definitions and overview of proposed guidelines.. J Gastroenterol Hepatol. 22 (6), 778-87.

Childs, S., Yeh, R.L., Georges, E., Ling, V. (1995). Identification of a sister gene to P- glycoprotein. Cancer Res.55:2029– 2034.

Chow, D.M., Lee, Y., Guoc, L.G. (2015). The Role of Bile Acids in Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. Mol Aspects Med. 56(1):34-44.

Dawson , P. A. , M. Hubbert , J. Haywood , A. L. Craddock , N. Zerangue , W. V. Christian, and N. Ballatori . (2005).The heteromeric organic solute transporter alpha-beta, Ostalpha- Ostbeta, is an ileal basolateral bile acid transporter. J.Biol. Chem. 280 : 6960 – 6968.

Durazzo, M., Belci, P., Collo, A., Grisoglio, E., Bo, S.(2012). Focus on Therapeutic Strategies of Nonalcoholic Fatty Liver Disease. International Journal of Hepatology.(1), 1-9.

Duvnjak, M., Tomasic, V., Gomercic, M., Smircic, Duvnjak, L., Barsic, N., Lerotic, I. (2009). Therapy of nonalcoholic fatty liver disease: current status. J Physiol Pharmacol. 60 (7), 57-66.

Einer,C., Hohenester,S.,Wimmer,R., Wottke,L., Artmann,R., Schulz,S., Gosmann,C., Simmons,C.,, Leitzinger,C., Eberhagen,C., Borchard,S.,ne Schmitt, S., Hauck,S., Toerne,V.C., Jastroch,M., Walheim,E., Rust, C.,Gerbes,L.A., Popper,B., Mayr,M., Vollmar, A., Zischka,H. (2017). Data on chow, liver tissue and mitochondrial fatty acid compositions as well as mitochondrial proteome changes after feeding mice a western diet for 6–24 weeks. Data Brief.15(1), 163-169.

Elsharkawy AM, Mann DA. (2007).Nuclear factor-kappaB and the hepatic inflammation-fibrosis-cancer axis. Hepatology.46(2): 590–7.

Fiorucci, S., Bioagioli,M., Zampella, A., Distrutti, E., (2018). Bile Acids Activated Receptors Regulate Innate Immunity. Front Immunol. 13(9): 1-17.

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

Ghaemi,A.,Taleban,A.F.,Hekmatdoost,A.,Rafiei,A.,Amiri,Z.,Homnyafour,R.,Fakheri,H. (2013). How Much Weight Loss is Effective on Nonalcoholic Fatty Liver Disease?. Hepat.Mon. 13 (12), 13-20.

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.

Hofmann, A.F. (2004). Detoxification of lithocholic acid, a toxic bile acid: relevance to drug hepatotoxicity. Drug Metab Rev. 36:703–722.

Hofmann, A. F., L. R. Hagey. (2008). Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics.Cell. Mol. Life Sci. 65 : 2461 – 2483.

Hylemon, B.P., Zhou, H., Pandak, M.W., Ren, S.,Gil, G., Dent, P. (2012). Bile acids as regulatory molecules. Journal of lipid research. 50:1509-1520.

Jia,X., Suzuki, Y., Naito, H., Yetti, H., Kitamori, K., Hayashi, Y.(2014). A possible role of chenodeoxycholic acid and glycine-conjugated bile acids in fibrotic steatohepatitis in a dietary rat model. Digestive Diseases and Sciences. 59 (1):1490-1501.

Kalhan, S.C., Guo, L., Edmison, J., Dasarathy, S., McCullough, A.J., Hanson, R.W., Milburn, M. (2011). Plasma metabolomic profile in nonalcoholic fatty liver disease. Metabolism. 60 (3), 404-13.

Kathirvel,E., Morgan,K., Nandgiri,G., Sandoval,B., Caudill,M., Bottiglieri,T., French,S.W., Morgan,T. (2010). Betaine improves nonalcoholic fatty liver and associated hepatic insulin resistance: a potential mechanism for hepatoprotection by betaine. Am J Physiol Gastrointest Liver Physiol.299: G1068–G1077.

Kim I, Morimura K, Shah Y, Yang Q, Ward JM, Gonzalez FJ. (2007). Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice. Carcinogenesis. 28:940–6.

Konstantinova, S.V., Tell, G.S., Vollset, S.E., Nygård, O., Bleie, Ø., Ueland, P.M. (2008). Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women.. J Nutr. 138 (5), 914-20.

Kodama, Y., Brenner, D.A. (2008). c-Jun N-Terminal Kinase Signaling in the Pathogenesis of Nonalcoholic Fatty Liver Disease: Multiple Roles in Multiple Steps. Hepatology. 49 (1), 6-8.

Koo, S. (2013). Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis. Clin Mol Hepatol. 19(3): 210–215.

Kucera, O., Cervinkova. (2014). Experimental models of non-alcoholic fatty liver disease in rats.. World J Gastroenterol.. 20 (26), 8364-76.

Langhi C, Le May C, Kourimate S, Caron S, Staels B, Krempf M, Costet P, Cariou B. (2008). Activation of the farnesoid X receptor represses PCSK9 expression in human hepatocytes. FEBS Lett. 582:949–955.

Legry, V., Francque,S., Haas,T.j., Verrijken,A., Caron, S., Talavera, O., Vonghia,L., Dirink,E., Verhaegen,A., Kouach, M., Lestavel, S., Lefebvre,P., Gaal, L., Tailleux,A., Paumelle,R., Staels,B. (2017). Bile Acid Alterations Are Associated With Insulin Resistance, but Not With NASH, in Obese Subjects. J of Clin Endo Met. 102(10):3783-3794

Lehrke M, Lazar MA (2005) The many faces of PPARgamma. Cell 123: 993–999.

Li, Y., Jadhav, K., Zhang, Y. (2013). Bile acid receptors in non-alcoholic fatty liver disease. Biochemical Pharmacology.(86)1517–1524.

Lindor, K.D., Kowdley, K.V., Heathcote, E.J., Harrison, M.E., Jorgensen, R., Angulo, P., Lymp, J.F., Burgart, L., Colin, P.(2004). Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: results of a randomized trial. Hepatology. 39 (3):770-8

Liu,Y., Binz,J., Numerick,J.M., Dennis,S., Luo,G., Desai,B., MacKenzie,I.K., Mansfield,A.T., Kliewer,A.S., Goodwin,B., Jones,A.S. (2003). Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. J. Clin. Invest. 112 (1),1678–1687.

Lomanaco, R., Sunny, N.E., Bril, F., Cusi, K. (2013). Nonalcoholic Fatty Liver Disease: Current Issues and Novel Treatment Approaches. Drugs. 73 (1), 1-14.

Lonardo, A., Ballestri, S., Marchesini, G., Angulo, P., Loria, P. (2015). Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. Dig Liver Dis. 47 (3), 181-90.

Ma Y, Huang Y, Yan L, Gao M, Liu D. (2013). Synthetic FXR agonist GW4064 prevents diet-

induced hepatic steatosis and insulin resistance. Pharm Res.30:1447–1457.

Makishima , M. , Okamoto ,Y.A., Repa, J.J., Tu ,H., Learned,M.R., Luk ,A., Hull ,V.M., Lustig, D.K.,Mangelsdorf, J.D., Shan, B. (1999) . Identification of a nuclear receptor for bile acids. Science. 284: 1362 – 1365.

Masarone, M., Rosato, V.,Dallio,M., Gerarda,A., Aglitti,A.,Loguierico,C., Federico,A., Persico,M. (2018). Role of Oxidative Stress in Pathophysiology of Nonalcoholic Fatty Liver Disease. Hindawi. 2018 (1), 1-14.

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.

Mavrogiannaki,N.A., Migdalis,N.I. (2013). Nonalcoholic Fatty Liver Disease, Diabetes Mellitus and Cardiovascular Disease: Newer Data. international journal of endrocinology. 13 (1), 1-13.

Mazuy,C., Helleboid,A., Staels,B., Philippe Lefebvre. (2014). Nuclear bile acid signaling through the farnesoid X receptor. Cell. Mol. Life Sci.. 72 (9), 1631-50.

Mello,T., Materozzi, M., Galli, A. (2016). PPARs and Mitochondrial Metabolism: From NAFLD to HCC. Hindawi. 2016 (1), 1-18.

Mencarelli, A., Renga, B., Migliorati, M., Cipriani, S., Distrutti, E., Santucci, L., Fiorucci, S. (2009). The bile acid sensor farnesoid X receptor is a modulator of liver immunity in a rodent model of acute hepatitis. Journal of immunology. 183 (10), 6657-66.

Mi, L.Z., Devarakonda, S., Harp, J.M., Han, Q., Pellicciari, R., Willson, T.M., Khorasanizadeh, S., Rastinejad, F. (2003). Structural basis for bile acid binding and activation of the nuclear receptor FXR. Mol Cell. 11 (4), 1093-100.

McNear, S., Harrison, S.A. (2009). Current status of therapy in nonalcoholic fatty liver disease. Therapeutic Advances in Gastroenterology. 2 (1), 29-43.

Moon, Y. (2017). The SCAP/SREBP Pathway: A Mediator of Hepatic Steatosis. Endocrinol Metab . 32 (1), 6-10.

Moslehi, A., Hamidi-Zad, Z. (2018). Role of SREBPs in Liver Diseases: A Mini-review. J Clin Transl Hepatol. 6 (3), 332-328.

Mudaliar, S., Henry, R.R., Sanyal, A.J., Morrow, L., Marschall, H.U., Kipnes, M., Adorini, L., Sciacca, C.I., Clopton, P., Castelloe, E., Dillon, P., Pruzanski, M., Shapiro, D. (2013). Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease.. Gastroenterology. 145 (3), 574- 82.

Muller,A.F., Sturla,J.S. (2019). Human in vitro models of nonalcoholic fatty liver disease. Science direct. 16 (1), 9-16.

Neuschwander-Tetri, B,A., Loomba, R., Sanyal, A.J., et al. (2014). Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet

Nissar,A., Sharma,L., Tasduq,A.S. (2015). Palmitic acid induced lipotoxicity is associated with altered lipid metabolism, enhanced CYP 450 2E1 and intracellular calcium mediated ER stress in human hepatoma cells. . Toxicology research. 1 (1), 1-32.

Nassir,F., Ibdah,J.A. (2014). Role of mitochondria in nonalcoholic fatty liver disease. Int J Mol Sci. 15 (5), 8713-42.

Ogawa,Y., Imajo,K., Honda,Y., Kessoku,T., Tomeno,W., Kato,S., Fujita,K., Yoneda,M., Saito,S., Saigusa,Y., Hyogo, H., Sumida,Y., Itoh, Y., Eguchi,K., Yamanaka,T., Wada,K., Nakajima. (2018). Palmitate-induced lipotoxicity is crucial for the pathogenesis of nonalcoholic fatty liver disease in cooperation with gut-derived endotoxin. Scientific Reports, 8: 1-8.

Onis, M., Blossner, M., Borghi, E. (2010). Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. American Journal of Clinical Nutrition. 92(1), 1257-64.

Parks, D.J., Blanchard, S.G., Bledsoe, R.K., Chandra, G., Consler, T.G., Kliewer, S.A., Stimmel, J.B., Willson, T.M., Zavacki, A.M., Moore, D.D., et al. (1999). Bile acids: natural ligands for an orphan nuclear receptor. Science. 284:1365– 1368.

Park, H.M., Park.Y.J., Lee, J.H., Kim,H.D.,Jeong,H.,Park,C., Chun,P.,Moon,R.H.,Chung,Y.H. (2013). Potent Anti-Diabetic Effects of MHY908, a Newly Synthesized PPAR α/γ Dual Agonist in db/db Mice. Plos one. 8 (11), 1-9.

Pandak , W. M. , Ren ,S., Marques ,D., Hall ,E., Redford ,K., Mallonee ,D., Bodhan , P.,Heuman ,D.,Gil ,G., P. Hylemon . (2002) .Transport of cholesterol into mitochondria is rate-limiting for bile acid synthesis via the alternative pathway in primary rat hepatocytes. J. Biol. Chem. 277 : 48158 – 48164 .

Pérez,C.M., Del,H. P., Martín, M.A., Rubio, J.C., Martín, A., Castellano, G., Colina, F., Arenas, J., Solis-Herruz,J.M. (2003). Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. Hepatology. 38 (4), 999-1007.

Pellicciari, R., Fiorucci, S., Camaioni, E., Clerici, C., Costantino, G., Maloney, P.R., Morelli, A., Parks, D.J., Willson, T.M. (2002). 6alpha-ethyl-chenodeoxycholic acid (6-ECDCA), a potent and selective FXR agonist endowed with anticholestatic activity.. J Med Chem. 45 (17), 3569-72.

Pettinelli, P., Videla, L.A. (2011). Up-regulation of PPAR-gamma mRNA expression in the liver of obese patients: an additional reinforcing lipogenic mechanism to SREBP-1c induction. J Clin

Endocrinol Metab. 96 (5), 2010-2029.

Perez, J.M., Briz, O. (2009). Bile acid induced cell injury and protection. World J Gastroenterol. 15 (14): 1677-1689.

Porez, G., Prawitt, J., Gross, B., Staels,B. (2012).Bile acid receptors as targets for the treatment of dyslipidemia and cardiovascular disease: Tematic Review Series: New Lipid and Lipoprotein Targets for the Treatment of Cardiometabolic Diseases. J Lipid Res. 53:1723–1737.

Portincasa,P., Cialou,A., Bonfrate,L., Wang, H.Q.D. (2012). Therapy of gallstone disease: What it was, what it is, what it will be. World J Gastrointest Pharmacol Ther. 3 (2), 7-20.

Quan, X.F., Wang, Y., Ma, X.F., Liang, Y., Tian, W.X., Ma, Q.Y., Jiang, H.Z., Zhao, Y.X. (2012). a-Mangostin induces apoptosis and suppresses differentiation of 3T3-L1 cells via inhibiting fatty acid synthase. Plos One. 1, 37-44.

Raszeja-Wyszomirska, J., Lawniczak, M., Marlicz, W., Miezyńska-Kurtycz, J., Milkiewicz, P. (2008). Non-alcoholic fatty liver disease--new view. Pol Merkur Lekarski. 24 (144), 568-71.

Ratziu, V., Pienar, L. (2011). Pharmacological therapy for non-alcoholic steatohepatitis: How efficient are thiazolidinediones?. Hepatology Research. 41 (1), 687–695.

Rinella, M., Charlton,M. (2016). The globalization of nonalcoholic fatty liver disease: Prevalence and impact on world health. Hepatology. 64 (1), 19-22.

Romeo, S., Kozlitina, J., Xing, C., Pertsemlidis, A., Cox, D., Pennacchio, L.A., Boerwinkle, E., Cohen, J.C., Hobbs, H.H. (2008). Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease.. Nat Genet. 40 (12), 1461- 5.

Rui,L. (2014). Energy metabolism in the liver. Compr Physiol. 4 (1), 177-97.

Russell, D.W. (2003). The enzymes, regulation, and genetics of bile acid synthesis. Annu. Rev. Biochem. 72 (1), 137-174.

García-Ruiz, I., Solís-Muñoz, P., Fernández-Moreira, D., Muñoz-Yagüe, T., Solís-Herruzo, J.A. (2015). In vitro treatment of HepG2 cells with saturated fatty acids reproduces mitochondrial dysfunction found in nonalcoholic steatohepatitis.. Dis Model Mech. . 8 (2), 183-91.

Russell,D.W., Setchell, K.D. (1992). Bile acid biosynthesis. Biochemistry. 31 (20), 4737-49.

Sanders,B.W.F., Griffin, L.J. (2016). De novo lipogenesis in the liver in health and disease: more than just a shunting yard for glucose. Bio Rev Camb Philos Soc. 91 (2), 452-468.

Sanyal, A.J., Chalasani, N., Kowdley, K.V., McCullough, A., Diehl, A.M., Bass, N.M., Neuschwander-Tetri, B.A., Lavine, J.E., Tonascia, J., Unalp, A., Van, N.M., Clark, J., Brunt, E.M., Kleiner, D.E., Hoofnagle, J.H., Robuck, P.R. (2010). Pioglitazone, vitamin E, or placebo for

nonalcoholic steatohepatitis.The New England Journal of Medicine. 362 (18), 1675-85.

Saremi, A., Arora, R. (2010).Vitamin E and cardiovascular disease. Am J Ther.17(3):56–65.

Serviddio,G., Bellanti,F., Tamborra,R.,Rollo,T.,. (2008). Uncoupling protein-2 (UCP2) induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis (NASH) liver to ischaemia-reperfusion injury. Gut. 57 (1), 957-965.

Sharma, A.M., Staels, B. (2007). Review: Peroxisome proliferator-activated receptor gamma and adipose tissue-- understanding obesity-related changes in regulation of lipid and glucose metabolism. J Clin Endocrinol Metab. 92:386– 395.

Shimomura, I., Bashmakov, Y., Horton, J.D. (1999). Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. J Biol Chem. 274 (42), 30028-32.

Sokol, R.J., Devereaux, M., Khandwala, R., O'Brien, K.(1993). Evidence for involvement of oxygen free radicals in bile acid toxicity to isolated rat hepatocytes.Hepatology. 17 (5): 869-81.

Softic, S., Gupta, K.M., Wang,G., Fujisaka,S., O'neill,T.B.,Rao,N.T., Willoughby, J., Harbison, C., Fitzgerald,K.,ILkaevya,O., Newgard,B.C.,Cohen,E.D.,Kahn,R.C. (2017). Divergent effects of glucose and fructose on hepatic lipogenesis and insulin signaling. J Clin Invest. 127 (11), 4059-4074.

Sokol, R.J., Dahl, R., Devereaux, M.W., Yerushalmi, B., Kobak, G.E., Gumpricht, E. (2005). Human hepatic mitochondria generate reactive oxygen species and undergo the permeability transition in response to hydrophobic bile acids.J Pediatr Gastroenterol. 41(2):235-43.

Sinal CJ et al. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell.2000;102(6):731–44.

Simões, I.C.M., Fontes, A., Pinton, P., Zischka, H., Wieckowski, M. (2018). Mitochondria in non-alcoholic fatty liver disease.. Int J Biochem Cell Biol. 95 (1), 93-99.

Song , K-H. , T. Li , E. Owsley , S. Strom , and J. Y. L. Chiang . (2009).Bile acids activate fi broblast growth factor 19 signaling in human hepatocytes to inhibit cholesterol hydroxylase gene expression.Hepatology . 49 : 297 – 305.

Spivey, J.R., Bronk, S.F., Gores, G.J, (1993). Glycochenodeoxycholate-induced lethal hepatocellular injury in rat hepatocytes. Role of ATP depletion and cytosolic free calcium.92(1):17-24.

Staudinger , J. L. , Goodwin ,B., Jones ,A.S., Hawkins-Brown ,D., Mackenzie ,I.K., LaTour ,A., Liu ,Y., Klaassen ,D.C., Brown ,K.K, Reinhard, J. (2001).The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc. Natl. Acad. Sci.USA . 98 :3369 – 3374

.

Stayrook, R.K., Bramlett, S.K., Savkur, S.R., et al.. (2005). “Regulation of carbohydrate metabolism by the farnesoid X receptor,” Endocrinology. 146(3): 984–991.

Strautnieks, S,S., Kagalwalla, A.F., Tanner, M.S., Knisely, A.S., Bull, L., Freimer, N., Kocoshis, S.A., Gardiner, R.M., Thompson, R.J. (1997)Identification of a locus for progressive familial intrahepatic cholestasis PFIC2 on chromosome 2q24. Am J Hum Genet.61:630–633.

Takashi,Y., Fukusato,T. (2014). Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol. 20 (42), 15539–15548.

Tanaka, N., Matsubara, T., Krausz, K.W., Patterson, A.D., Gonzalez, F.J.(2012). Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis.Hepatology. 56(1):118-29.

Tedoro,S.J.,Rolo,P.A., Duarte,V.F.,Simoes,M.A.,Palmeira,M.C. (2008). Differential alterations in mitochondrial function induced by a choline-deficient diet: understanding fatty liver disease progression. Mitochondrion. 8 (5), 367-376.

Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J., Schoonjans, K. (2008). Trageting bile acid signalling for metabolic diseases. Nat Rev Drug Disc. 7(8):678-93.

Trauner, M., Claudel, T., Fickert, P., Moustafa, T., Wagner, M. (2010) Bile acids as regulators of hepatic lipid and glucose metabolism.Dig Dis. 28(1):220-4.

Vignozzi, L., Morelli, A., Filippi, S., Comeglio, P., Chavalmane, A.K., Marchetta, M., Toce, M., Yehiely-Cohen, R., Vannelli, G.B., Adorini, L., Maggi, M. (2011). Farnesoid X receptor activation improves erectile function in animal models of metabolic syndrome and diabetes.. Journal of sexual medicine. 8 (1), 57-77.

Vlahcevic , Z. R., Heuman, D.M., Hylemon, B.P. (1996). Physiology and pathophysiology of enterohepatic circulation of bile acids. In Hepatology: A Textbook of Liver Disease. D. Zakim and T. D. Boyer, editors. W. B. Sanders Co., Philadelphia, PA. 376–417.

Wahli. W., Michalik, L.(2012). PPARs at the crossroads of lipid signaling and inflammation. Trends Endocrinol Metab. 23:351–63.

Wei, Y., Rector, R.S., Thyfault, J.P., Ibdah, J.A. (2008). Nonalcoholic fatty liver disease and mitochondrial dysfunction. World J Gastroenterol. 14 (2), 193-99.

Weltman,D.M., Farrell, C.G., Liddle, C. (1996). Increased Hepatocyte CYP2E1 Expression in a Rat Nutritional Model of Hepatic Steatosis With Inflammation. Gastroenterology. 111 (1), 1645-1653.

Xiang,Z., Chen,Y., Ma,K., Zheng,L., Ming,Y., Jin, X.(2013). The role of Ursodeoxycholic

acid in non-alcoholic steatohepatitis: a systematic review. Gastero. 13(140).

Xiong, X., Wang, X., Lu, Y., Wang, E., Zhang, Z., Yang, J., Zhang, H., Li, X. (2014). Hepatic steatosis exacerbated by endoplasmic reticulum stress-mediated downregulation of FXR in aging mice. J Hepatol. 60 (4), 847-854.

Xu, J., Li,Z., Zhang,L., Ji,G. (2014). Recent insights into farnesoid X receptor in non-alcoholic fatty liver disease. World J Gastroenterology. 20(37): 13493-13500.

Yang, Z.X., Shen, W., Sun, H. (2010). Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease. Hepatol Int. 4:741–748.

Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J, et al.(2004)/ Bile acids regulate gluconeogenic gene expression via small heterodimer partnermediated repression of hepatocyte nuclear factor 4 and Foxo1. J Biol Chem. 279:23158–65.

Younossi,Z.M., Koenig, A.B., Abdelatif, D., Fazel, Y., Henry, L., Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes.. Hepatology. 64 (1), 73-84.

Yuan, L., Bambha,K. (2015). Bile acid receptors and nonalcoholic fatty liver disease. 7(28): 2811-2818.

Zhang,Y., Lee, F.Y., Barrera, G., et al. (2006). Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. Proc Natl Acad Sci U S A.103:1006-11.

Online resources

European Association for the study of Liver disease (EASL, 2016).

https://easl.eu/publication/the-management-of-non-alcoholic-fatty-liver-disease-nafld/. Accessed: June 2019.

Boundless.com

https://www.boundless.com/biology/textbooks/boundless-biology2061 textbook/cellular-respiration-7/oxidative-phosphorylation-76/electron-transport2062 chain-362-11588/images/fig-ch07_04_01/

Accessed: June 2019)

Cancer Research UK

https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/liver-cancer#heading-Zero

Accessed: May 2019