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Fumonisin B1 increases the NAD-dependant deacetylase SIRT1 to disrupt cholesterol flux in galactose supplemented HepG2 cells

By

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Declaration

I, Mr **Theolan Adimulam**, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party. Where a colleague has indeed prepared a thesis based on related work essentially derived from the same project, this must be stated here, accompanied by the name, the degree for which submitted, the University, the year submitted (or in preparation) and a concise description of the work covered by that thesis such that the examiner can be assured that a single body of work is not being used to justify more than one degree.

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List of abbreviations

ABC	ATP-binding cassette
ABCA1	ATP-binding cassette A1
ABCG	ATP-binding cassette G
AFB_1	Aflatoxin B_1
apopA-I	Apolipoprotein A-I
apoB	Apolipoprotein B
apoE	Apolipoprotein E
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCM	Complete culture media
cDNA	Complementary DNA
CoA	Coenzyme A
DMEMD	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
ds	Double stranded
EGF	Epidermal growth factor
EMEM	Eagle's Essential Minimal Media
ETC	Electron transport chain

FB_1	Fumonisin B_1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCC	Hepatocellular carcinoma
HepG2	Hepatocellular carcinoma cell
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HMGCR	3-hydroxy-3-methyl-glutaryl coenzyme A reductase
HRP	Horse radish peroxidase
IC_{50}	Half maximal inhibitory concentration
IDOL	Inducible degrader of LDLR
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LXR	Liver X receptor
mTORC	Mammalian target of rapamycin complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFDM	Non-fat dry milk
NPC1L1	Niemann-Pick type C1-like 1
NPC2	NPC intracellular cholesterol transported 2
NTD	Neuronal tube defects
OH	Hydroxyl
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin kexin 9
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
RBD	Relative band density
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SREBP-1	Sterol regulatory element-binding protein-1 relative band density
SREBP-2	Sterol regulatory element-binding protein-2
ss	Single stranded
TCA	Tricarboxylic acid
TMED	Tetramethyl ethylenediamine
TTBS	Tween 20-Tris buffered saline
VLDV	Very-low-density lipoproteins

Abstract

The *Fusarium* species are major fungal contaminants of maize, that produce an extremely toxic mycotoxin called Fumonisin B_1 (FB_1). Fumonisin B_1 is a known causative agent of animal-related mycotoxicosis and is implicated in cancer initiation in humans (epidemiological) and animals (experimental). The mechanisms of tumorigenesis have implicated oxidative stress, incomplete apoptosis, and chromatin instability as major contributing factors. While the canonical mechanism of FB_1 induced ceramide dysregulation and oxidative stress are the established drivers of cancer initiation, accumulating evidence indicates that changes in cholesterol metabolism may further enhance the tumorigenic potential of FB_1. The cholesterolemic effect of FB_1 has been shown in experimental models yet molecular evidence elucidating the disturbed cholesterol flux remains vague. This study investigates the molecular mechanism that regulate the effect of FB_1 on cholesterol homeostasis, by investigating the role of cholesterol flux proteins and their regulation at the transcriptional and post translational level, in galactose supplemented HepG2 liver cells.

Cancer cells like the liver derived HepG2 cell line, differ from normal tissues with respect to their excessive use of aerobic glycolysis, to satisfy the high energy demand. The proven method of circumventing the Crabtree effect exhibited by cancer cells, is to replace glucose with galactose in the culture media, forcing cells to activate the mitochondria and rely on oxidative phosphorylation rather than glycolysis for ATP.

SIRT1 is a NAD-dependent deacetylase, that responds to changes in nutrient availability, to induce regulatory effects on cellular metabolism. The toxicity of FB_1 was determined (6hr incubation; IC_{50} = 25µM) on metabolic output, cholesterol regulatory transcription factors and key cholesterol flux proteins using the spectrophotometric MTT assay, ATP luciferase assay, qPCR (*SIRT1, SREBP-1C, LXR, LDLR, PCSK9* and *ABCA1)* and western blots (SIRT1, SREBP-1C, LXR, LDLR, PCSK9 and ABCA1).

FB_1 induced differential HepG2 cell viability and metabolic output. There was no effect on cell viability for the glucose-supplemented media, while a decrease in cell viability was observed for the galactose-supplement media. Concurrent with the decrease in cell viability of the galactose-supplementation, a decrease in HepG2 ATP output was observed ($p=0.0135$). FB_1 did not compromise membrane integrity, despite the decrease in cell viability. The expression of the SIRT1 gene ($p=0.0004$) and protein ($p=0.0005$) was significantly increased by FB_1. The expression of the SREBP-1c gene ($p=0.0050$) was increased while protein expression ($p=0.0063$) was decreased. The role of LXR and PCSK9 in the regulation of LDLR was further highlighted.

FB_1 increased the expressions of LXR ($p=0.0003$) and LDLR ($p=0.0004$ and $p=0.0049$ respectively) genes and proteins but decreased the gene ($p=0.0017$) and protein ($p=0.0018$) expression of PCSK9.

The data provides evidence that SIRT1 reduces the expression of PCSK9 and deacetylates LXR to prevent degradation of LDLR, resulting in dysregulated cholesterol flux in liver cells. The disruption of cholesterol homeostasis by FB_1 is beginning to shift away from established ceramide synthase inhibition, changing the perspective to shed light on the diseases caused by dysregulated cholesterol metabolism such as cancer initiation and promotion.

CHAPTER 1: INTRODUCTION

Mycotoxins are fungal secondary metabolites that adversely affect humans, animals and plants and their contamination of foods and animal feeds presents a significant problem to food security (Cardwell, Desjardins et al. 2001, Bhat and Vasanthi 2003). Fumonisin B_1 (FB_1) is a group 2B carcinogenic mycotoxin, mainly produced by *Fusarium verticilliodies* and *Fusarium proliferatum* which commonly infect maize and other agricultural products including rice, sorghum, wheat bran and soybean meal (Stockmann-Juvala and Savolainen 2008, Zain 2011). FB_1 predominates in 70% of the total FBs naturally occurring in infected food and feed samples (Kamle, Mahato et al. 2019). Thus, it comes as no surprise that Fumonisins have been associated with elevated human oesophageal cancer in Africa, America and Asia (Zain 2011, Alizadeh, Rohandel et al. 2012, Xue, Tang et al. 2019). A study showed a correlation between the consumption of Fumonisin contaminated maize, and human oesophageal cancer in South Africa (Marasas, Riley et al. 2004). The outcomes of FB_1-induced toxicity includes; oxidative stress, cytotoxicity, apoptosis, immunotoxic effects and epigenetic dysregulation (Stockmann-Juvala and Savolainen 2008). The canonical mechanism of FB_1 toxicity is based on its structural similarity to cellular sphingolipids and subsequent competitive inhibition of the enzyme ceramide synthase resulting in an accumulation of sphingosine, sphinganine and its derivatives (Merrill, Van Echten et al. 1993, Soriano, Gonzalez et al. 2005, Zitomer, Mitchell et al. 2009).

A major portion of ingested FB_1 is distributed to the liver, and focus has been on FB_1 induced hepatotoxicity and liver cancer. FB_1-mediated tumorigenesis has been attributed to chromatin instability, via FB_1 induced hypomethylation of global DNA (Chuturgoon, Phulukdaree et al. 2014) and induced oxidative stress in HepG2 cells (Gelderblom, Abel et al. 2001, Arumugam, Pillay et al. 2019).

Interestingly, FB_1 has complex activity on cholesterol metabolism. In the liver and plasma *in vivo*, FB_1 increases cholesterol levels, while *in vitro* studies have shown a decrease in free cholesterol (Gelderblom, Smuts et al. 1996, Gelderblom, Smuts et al. 1997) . In normal conditions, the liver is the primary site for cholesterol biosynthesis, storage, and also the site of cholesterol excretion and metabolism (Nemes, Åberg et al. 2016). Cancer cells exhibit lipid and cholesterol reliance, which is met by increasing the uptake of dietary lipids and lipoproteins or via over activation of lipogenesis and cholesterol synthesis, a feature considered to be a hallmark of cancer progression (Beloribi-Djefaflia, Vasseur et al. 2016). Cholesterol is a major membrane component, that offers stability, however cholesterol also functions in the cytoplasm, where it is stored in lipid droplets, which from the main lipid stores in eukaryotes (Maxfield and Wüstner 2002, Martin and Parton 2006). Recent studies have highlighted the possibility of liposomal cholesterol activating mammalian target of rapamycin complex 1 (mTORC1) via the SLC38A9-Neimann-Pick C1 signalling complex (Castellano, Thelen et al. 2017). The activation of mTORC1 increases cell proliferation, invasion and metastasis of cancer cells (Kim, Cook et al. 2017). Despite being a major component for membrane integrity, cholesterol is also a component of lipid rafts, whose structure and function are dependent on the composition of cholesterol and phospholipids (Yan, Qu et al. 2014). Lipid rafts facilitate signal transduction of oncogenic signals, thus alterations in cholesterol level may impair the integrity of lipid rafts, leading to detrimental outcomes (George and Wu 2012).

The low-density lipoprotein receptor (LDLR) pathway is a negative feedback system, that plays a key role in the regulation of plasma and intracellular cholesterol homeostasis. The LDLR is a

transmembrane protein, responsible for cellular lipid uptake, and cholesterol transfer (Zhang, Ma et al. 2016). Mature LDLR presents on the cell surface where it mediates the internalisation and degradation of plasma LDL-cholesterol (Zhang, Ma et al. 2016). The expression of LDLR is tightly regulated both transcriptionally, post-transcriptionally and post-translationally.

Proprotein convertase subtilisin kexin 9 (PCSK9) is also a key player in cholesterol metabolism, due to its regulatory role on LDLR (Peterson, Fong et al. 2008). The PCSK9 binds to LDLR on the cell membrane, internalises it, and then directs LDLR to the lysosome for degradation (Zhang, Ma et al. 2016). In this way, PCSK9 exhibits posttranslational regulation of LDLR. Proprotein convertase subtilisin kexin 9 itself, is also subject to regulation. *In vivo* and *in vitro* activation of Sirtuin 1 (SIRT1), lowered plasma PCSK9 secretion in murine hepatocytes and mice (Miranda, van Tits et al. 2014).

Sirtuin 1 is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase, that influences glucose and lipid metabolism. It has been demonstrated that SIRT1 regulates proteins and genes involved in lipid metabolism. To inhibit lipid synthesis and fat storage, SIRT1 down-regulates both sterol regulatory element-binding protein-1 (SREBP-1) and SREBP-2 (Ye, Li et al. 2017). Sirtuin 1 is also a positive regulator of liver X receptor (LXR). The liver X receptors are key transcriptional regulators pf cholesterol metabolism. LXRα and LXRβ are sterol-dependent nuclear receptors which are activated in response to excess cellular cholesterol (Zelcer and Tontonoz 2006).

SIRT1 deacetylates LXR, resulting in cholesterol efflux from cells (Ye, Li et al. 2017). It has been demonstrated that LXR decreases LDLR-dependent cholesterol uptake via LXR-IDOL (inducible degrader of the LDLR) pathway (Zelcer, Hong et al. 2009). Liver X receptor is responsible for inducing IDOL, which then catalyses the ubiquitination of LDLR, tagging it degradation (Zelcer, Hong et al. 2009).

The effect of FB_1 on cholesterol flux is vague, while its mechanisms of tumorigenesis and the canonical action on ceramide are well studied. In this study, to gain a better understanding of FB_1 on cholesterol homeostasis, the change in expression profiles of proteins involved in cholesterol transport, were investigated in hepatocellular carcinoma (HepG2) cells supplemented with galactose media. The phenomenon described as the Crabtree effect, demonstrates how cancer cells rely on aerobic glycolysis, while still retaining the function of oxidative phosphorylation (Zheng 2012). A proven intervention of circumventing this phenomenon is to replace glucose with galactose in the cell culture media, as demonstrated in several studies (Aguer, Gambarotta et al. 2011, Shiratori, Furuichi et al. 2019, Orlicka-Płocka, Gurda-Wozna et al. 2020).

1.1 Aim:

To investigate the effect of FB_1 on SIRT1, LXR and SREBP-1c and associated proteins in cholesterol flux, in oxidatively poised liver HepG2 cells.

1.2. Hypothesis:

FB_1 disrupts cholesterol flux in an LXR dependent manner in HepG2 cells supplemented with galactose media.

1.3 Objectives:

To determine the following parameters induced by FB_1 in HepG2 cells supplemented with galactose media:

Metabolic changes

Cell membrane integrity

Expression profiles of key proteins involved in cholesterol flux using western blots.

Expression of *SIRT1*, transcription factors; *LXR* and *SREBP1* and their targets; *LDLR*, *PCSK9* and *ABCA1* using qPCR.

CHAPTER 2: LITERATURE REVIEW

2.1 Mycotoxins

2.1.1 A brief history

The availability of safe and edible food is crucial for sustaining human and animal life. Food shortages are concerning, thus grew the need to store and preserve food for extended periods of time. With this came the need for specialized packaging for storage and transport. These factors increased the susceptibility of food sources to adverse weather conditions and the exposure to different microorganisms. This leads to food spoilage and contamination with different microorganisms, including fungi and the mycotoxins they produce (Ahmed Adam, Tabana et al. 2017). The 1960s outbreak of the Turkey X disease, initiated concern over mycotoxins, where approximately 100 000 turkey poults died after consuming aflatoxin contaminated peanut meal (Peraica, Radić et al. 1999, Bennett and Klich 2003). This led to the discovery of a large number of mycotoxins, from a wide array of fungal genera, which are implicated in several outbreaks and disease (Peraica, Radić et al. 1999, Ahmed Adam, Tabana et al. 2017).

2.1.2 Where do they come from and what do they do?

The most common producers of mycotoxins are the fungal genera *Aspergillus*, *Penicillium* and *Fusarium* (Yiannikouris and Jouany 2002). For growth and metabolism, fungal species absorb nutrients of low molecular weights, and as a result of enzymatic degradation of macromolecules, produce secondary metabolites known as mycotoxins (Moss 1991). These secondary metabolites are not directly required for growth of the fungal species (Fox and Howlett 2008) however, they are associated with providing security and protecting the fungus against viral, protozoan and bacterial infections (Zain, Awaad et al. 2014). The term mycotoxin is used to describe pharmacologically active fungal secondary metabolites, characterized as vertebrate toxicity (Bennett and Klich 2003), that are known to cause chronic and acute effects in humans and animals.

Mycotoxins are extensively produced in cereal grains and animal feed during and after harvests (Yiannikouris and Jouany 2002). Ecological conditions with high temperatures, heightened humidity and rainfall, such as tropical climates, provide favourable conditions for mycotoxin formation (Bhat and Vasanthi 2003). Therefore, the severity of contamination is dependent on environmental factors and storage practices (Coulombe Jr 1993). The contamination of food and feeds with mycotoxins is of global concern, as the ingestion of mycotoxins leads to toxicity (Zain 2011). Aflatoxin, ochratoxins, trichothecenes and Fumonisin, are some of the major food contaminant mycotoxins, which exhibit nephrotoxicity, hepatotoxicity and carcinogenicity in humans and animals (Bennett and Klich 2003). Diseases as a result of fungal-derived toxins in human and animals has risen extensively (Enyiukwu, Ononuju et al. 2018), owing to the increased population and reliance on food that does not meet best practice regulations (Bhat and Vasanthi 2003).

Human exposure to mycotoxins occurs through the ingestion of contaminated grain products and animal products containing secondary metabolites (Zain 2011). Inhalation and dermal contact have also been described in an occupational setting, from airborne dust, spores and hyphae fragments (Pal, Gizaw et al. 2015, Viegas, Viegas et al. 2018). The extent of exposure can be

described as acute or chronic effects, that are associated with disease progression (Coulombe Jr 1993). Acute toxicity can be described as a rapid exposure to a toxin over a short period, with a distinct toxic response, while chronic toxicity is due to exposure to toxin over a longer period, in smaller doses (Bennett and Klich 2003). Exposure to mycotoxins leads to the occurrence of diseases known as mycotoxicosis, expressed as blood toxicity, food poisoning and cancer (Zain, Awaad et al. 2014, Ahmed Adam, Tabana et al. 2017).

2.1.3 Biological effects of mycotoxins

Mycotoxins are ranked higher than synthetic contaminants, plant toxins, food additives and pesticide residues, as the most important chronic dietary risk factors (Bennett and Klich 2003). A general mechanism is not associated to mycotoxins due to their diverse chemical structures and extensive variety of biological effects (Kiessling 1986). The biological effects of mycotoxins are characterized by their ability to alter cellular processes, which range from DNA and protein biosynthesis, to metabolism (Kiessling 1986). The interaction of mycotoxins with the DNA template, and disturbed transcription and translation, interfere with protein synthesis, thus exhibit aberrant enzyme activity and regulation (Kiessling 1986).

Among the mycotoxins, aflatoxin B_1 (AFB_1) is the most carcinogenic, it can penetrate the cell membrane and bind to DNA to attain stability forming DNA adducts (Woo, Egner et al. 2011). The liposolubility of AFB_1 facilitates its absorption in to the cell, where it is metabolized by cytochrome P450 in the liver, to a highly unstable epoxide that binds to DNA or a protein molecule (Ahmed Adam, Tabana et al. 2017). The binding of the metabolite to DNA causes GC to TA transversion mutations, directly affecting the p53 tumour suppressor gene and subsequently cell cycle (Baertschi, Raney et al. 1988). Therefore it comes as no surprise that AFB_1 is the most potent hepatocarcinogen in mammals and listed as a Group 1 carcinogen (Muhammad, Sun et al. 2017). In the intestine, exposure to AFB_1 caused intestinal lesions via the disruption of intestinal barrier, cell proliferation, cell apoptosis, and immune system (Liew and Mohd-Redzwan 2018).

Ochratoxin A is a nephrotoxin with potent liver toxicity, teratogenicity, carcinogenic and immune suppressant in humans and animals (Kuiper-Goodman and Scott 1989). It has been shown to inhibit mitochondrial adenosine triphosphate (ATP) production, stimulate lipid peroxidation (Arya, Shergill et al. 2005), lead to the formation of DNA adducts, apoptosis and oxidative stress (Kőszegi and Poór 2016).

Trichothecene mycotoxins have inhibitory effects on eukaryotic cells where they inhibit protein, DNA and RNA synthesis, mitochondrial function and cell membranes (Rocha, Ansari et al. 2005).

2.2 The Fumonisins

Fumonisins are produced by the *Fusarium verticilliodies* species which commonly infect maize and associated agricultural products, throughout the world (Stockmann-Juvala and Savolainen 2008). This strain was first isolated in 1970 from a batch of mouldy maize, and implicated in leukoencephalomalacia in horses in South Africa (Marasas 2001). Maize and maize-based products (Figure 2.1) are most frequently contaminated by this fungus, however garlic bulbs, onion powder, nuts and soybeans are also contaminated with Fumonisin (Seefelder, Gossmann et al. 2002, Boonzaaijer, Van Osenbruggen et al. 2008, Liu, Liu et al. 2008, Aoyama, Nakajima et al. 2010). Fumonisins are sub-divided into four groups: Serial A, B, C and P (Dutton 1996). Fumonisin B_1 is considered the most toxic of the groups (Marasas 1996), and represents between

70% to 80% of the total Fumonisin content in naturally contaminated foods and feed (Krska, Welzig et al. 2007).

Figure 2.1: Fusarium contaminated maize (A) and wheat (B)(Dutton 1996, Bergstrom 2014).

2.3 Fumonisin B_1

2.3.1 Chemical Structure

Fumonisins are polar water-soluble compounds with long chain structures (Figure 2.2). The structure of FB_1 is representative of a diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-14,15-dihydrooxyecosane, in which the hydroxyl (OH) groups on carbon 14 and 15 are esterified to form tricarboxylic acid (TCA). As opposed to the cyclic nature of other mycotoxins, FB_1 has a polyketide-derived backbone which facilitates the attachment of TCA, methyl, OH and amino groups (Figure 2.2) (Stockmann-Juvala and Savolainen 2008).

The primary amine group in FB_1 shares a significant resemblance to sphingolipid precursors, sphinganine and sphingosine. This affords FB_1 the ability to disrupt sphingolipid metabolism. It has been shown that the acetylation of the amino group in FB_1 inhibits its toxicity and disruption of sphingolipid metabolism (Stockmann-Juvala and Savolainen 2008).

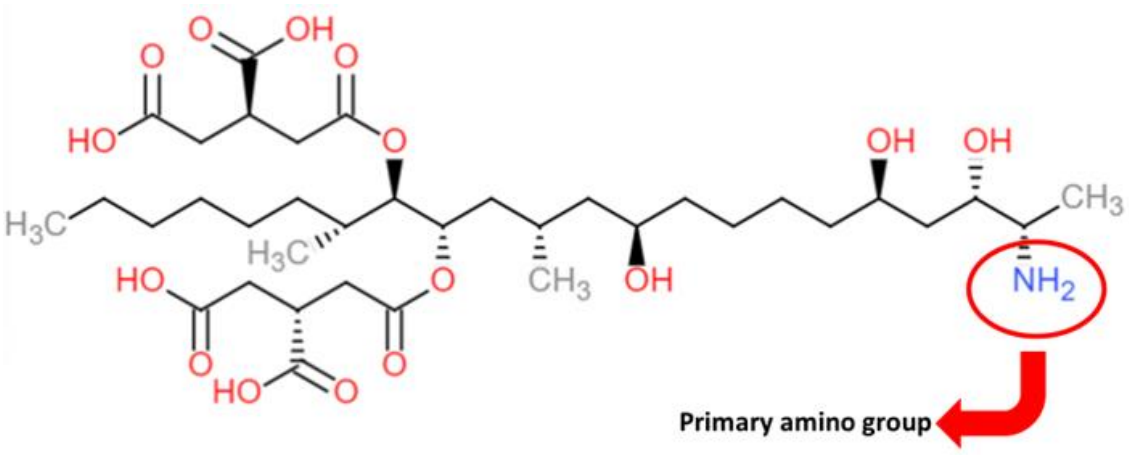

Figure 2.2: Chemical structure of FB_1 (prepared by author).

2.3.3 Toxicity

2.3.3.1 Toxicity in animals

Fumonisin B_1 has been reported to induce many animal diseases, due to the contamination of their feed. It is regarded as the most hazardous mycotoxin causing animal ill-health and associated economic loss. The liver and kidney are the foremost targets of FB_1 in most animal species. The pathogenic effects include equine leukoencephalomalacia (Marasas, Naude et al. 1976), porcine pulmonary oedema syndrome (Kriek, Kellerman et al. 1981), hepatic tumour in rats (Gelderblom and Snyman 1991), acute and fatal nephrotoxicity and hepatotoxicity in lambs (Edrington, Kamps-Holtzapple et al. 1995). Toxic responses including decreased body weight gain, increased mortality, size reduction of bursa of *Fabricius*, thymus and spleen, myocardial degeneration, myocardial haemorrhage, alterations in homeostatic mechanisms and necrosis of hepatocytes, have all been observed in chickens, ducklings and turkeys (Lumsangkul, Chiang et al. 2019).

2.3.3.2 Toxicity in Humans

Humans are continually exposed to low doses of FB_1, the daily tolerable intake is 2µg (WHO, 2002), however the consumption can range from 12 to 140µg/person per day, with peaks at 2500µg/person where maize forms part of dietary staples (WHO 2012).

The consumption of FB_1 contaminated maize by pregnant woman, was associated with the onset of neuronal tube defects (NTD) in offspring. Folate deficiency is a causative factor of NTD. The alterations in sphingolipid content of cell membranes as a result of FB_1, disrupts the folate receptor and prevents the uptake of folate, leading to NTD (Sadler, Merrill et al. 2002).

Human epidemiological studies in South Africa, Italy and China highlighted the relationship between oesophageal cancer and the intake of maize grains contaminated with FB_1 (Liew and Mohd-Redzwan 2018). FB_1-induced oxidative stress by increased reactive oxygen species (ROS) production and increased the antioxidant defences in osteoblastic cells (Khan, Phulukdaree et al. 2018). The exposure of FB_1 to HepG2 cells, induced oxidative stress and initiated the Nrf2-regulated transcription of antioxidants (Arumugam, Pillay et al. 2019).

The relation between FB_1 and growth impairment in children has also been described (Kimanya, De Meulenaer et al. 2010, Shirima, Kimanya et al. 2015, Chen, Mitchell et al. 2018). Among children in Tanzania, the relationship between exposure to FB_1 and the impact on growth was confirmed based on urinary biomarker levels of Fumonisin (Shirima, Kimanya et al. 2015). These growth impairments were attributed to the contamination of breast milk and cow's milk, by Fumonisins (Polychronaki, West et al. 2007, Magoha, Kimanya et al. 2014).

The consumption of FB_1 is also associated to acute mycotoxicosis. In 27 villages in India, residents experienced an outbreak of diarrhoea and abdominal pain, after it was found that they consumed rain damaged mouldy maize and sorghum, which exhibited higher levels of FB_1 (Stockmann-Juvala and Savolainen 2008).

2.3.2 Mechanism of action

2.3.2.1 Canonical mechanism

Due to the structural resemblance of FB_1 to sphingoid bases, the main action of the toxin is to disrupt sphingolipid metabolism (Heidtmann-Bemvenuti, Mendes et al. 2011). Eukaryotic cells are abundant in sphingolipids as they are major constituents of membranes, lipoproteins, and lipid rich structures. Sphingolipids maintain the structure of membranes, like cholesterol, and modulate receptor activity (Merrill Jr, Schmeiz et al. 1997). Thus it comes as no surprise that FB_1 induces lipid peroxidation that alters the cell membrane, causing apoptotic cell death (Soriano, Gonzalez et al. 2005).

The synthesis of sphingolipids occurs *de novo* within the endoplasmic reticulum. The enzyme serine palmitoyl transferase initiates sphingolipid synthesis via the catalysation of the condensation of serine and palmitoyl coenzyme A (palmitoyl CoA) to yield 3-ketosphinganine. This is then reduced to sphinganine in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent manner (Futerman and Riezman 2005). There are two fates of sphinganine; it can undergo phosphorylation to form sphinganine-1-phosphate or, acylation by ceramide synthase to form dihydroceramide. Another major function of ceramide synthase is for the recycling of sphingosine to ceramide via the sphingolipid degradation pathway (Šegvić and Pepeljnjak 2001).

Due to the structural similarity of the aminopentol backbone between FB_1 and sphingoid bases, ceramide synthase identifies FB_1 as a substrate, thus there exists competition for the binding site in ceramide synthase. The enzyme is also inhibited by the tricarboxylic group of FB_1, which obstructs the fatty acyl-CoA binding site on ceramide synthase (Wang, Norred et al. 1991). These combined effects on the enzyme, impede ceramide biosynthesis, disrupting the formation of essential sphingolipids. This leads to an accumulation of sphingosine and sphinganine and disturbed sphingosine recirculation (Figure 2.3). As concentrations of sphingoid bases increases, they become cytotoxic, initiate cell injury and exhibit pro-apoptotic activity (Voss and Riley 2013).

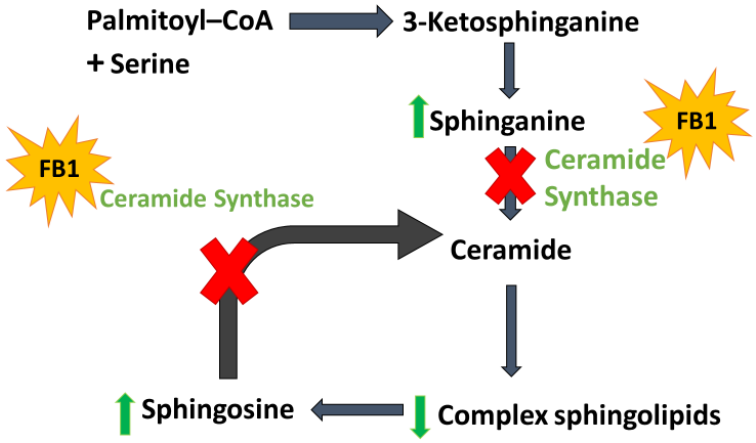

Figure 2.3: The resultant disruption of sphingolipid metabolism by FB_1 (prepared by author).

2.3.2.2 Emerging mechanisms

Fumonisin B_1 also has the potential to inhibit protein synthesis and DNA synthesis, at higher concentrations. Fumonisin B_1 decreases the activities of DNA methyltransferases and induces global DNA hypomethylation and histone demethylation, resulting in chromatin instability (Chuturgoon, Phulukdaree et al. 2014).

Recently it was discovered that FB_1 induced oxidative DNA damage, via the downregulation of expression, of tumour suppressor phosphatase and tensin homolog (PTEN) via miR-30c (Arumugam, Ghazi et al. 2020). PTEN is a key maintenance factor of genomic stability and DNA repair, thus responds to DNA damage by inhibiting the phosphoinositide 3-kinase/protein kinase B signalling cascade, preventing the inhibitory phosphorylation of checkpoint kinase 1 (Ming and He 2012). This prevents the repair of FB_1 induced oxidative DNA lesions, which contributes to the cytotoxicity and carcinogenicity (Arumugam, Ghazi et al. 2020).

2.4 The Liver

2.4.1 Liver functions and structure

The liver is a vital organ in the body that is responsible for a multitude of functions that enable metabolism, immunity, digestion, detoxification, vitamin storage and excretion (Kalra and Tuma 2018). The liver is the storage centre for fat-soluble vitamins and facilitates cholesterol homeostasis. The metabolic and synthetic functions of the liver include carbohydrate, lipid, and protein metabolism (Campbell 2006). It also manages the synthesis of plasma proteins, mainly albumin, binding globulins, protein C, protein S and clotting factors of the intrinsic and extrinsic pathways (Miller, Bly et al. 1951).

The excretion of exogeneous compounds such as xenobiotics and endogenous substances like bilirubin take place from the liver. But before they can be excreted, exogenous chemicals are metabolized. There are two types of enzymatic manipulations that occur to an exogenous compound, depending on its structure, that are classified under the general headings of Phase I and Phase II reactions (Grant 1991). Phase I reactions include oxidation, reduction, and hydrolysis by multiple enzymes classes, to either introduce or expose functional groups (often hydroxyl groups) to facilitate its excretion by increasing its hydrophobicity. Phase II reactions are conjugative or synthetic, with the aim of adding water -soluble side groups to increase the excretability of the chemical. These biotransformation's are aimed for detoxification however, inactive compounds (prodrugs) can be consequently converted to active metabolites, by attaining altered structures, that can influence its pharmacological specificity (Grant 1991). Consequently, the liver is exposed to significant concentrations of chemicals, that can result in liver dysfunction, cellular injury, and organ failure.

It also secrets bile, a fluid that aids in the absorption and digestion of lipids (Kalra and Tuma 2018). The immunological ability of the liver is achieved by hepatic macrophages known as Kupffer cells (Fenton 2001).

The liver comprises several cell types of different embryological origin and specified function. It includes hepatocytes, biliary epithelial cells, stellate cells, Kupfer cells and liver sinusoidal endothelial cells (Trefts, Gannon et al. 2017). Hepatocytes make up most of the liver volume and are highly metabolic in nature (Figure 2.4). They are rich in smooth and rough endoplasmic reticulum, essential for xenobiotic metabolism and protein synthesis respectively (Gu and

Manautou 2012). The liver is also rich in mitochondria forming an integrated hub of carbohydrate, lipid, and protein metabolism, required for hepatocyte survival and death.

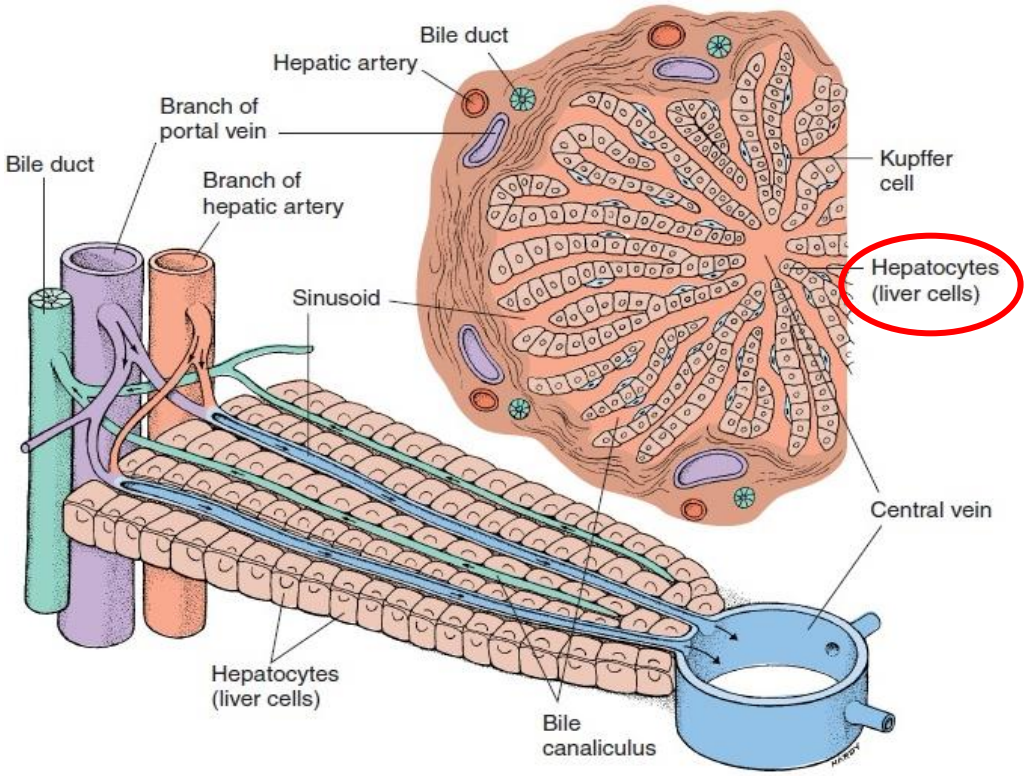

Figure 2.4: Structure of the liver (Premkumar 2004).

2.4.2 Use of the HepG2 cell line

The primary culture of hepatocytes is a profound model for investigating the effect of substances on human liver cells (Snopov, Teryukova et al. 2017). The hepatocellular carcinoma cell line is of particular use in liver toxicity studies and drug-induced mitochondrial toxicity, due to their similar function and metabolic profile to intact hepatocytes (Kamalian, Chadwick et al. 2015, Ramirez, Strigun et al. 2018). These are nontumorigenic cells with high proliferative rates and epithelial-like morphology that accomplish differentiated hepatic function (Donato, Tolosa et al. 2015). These cells can adapt their phenotype, in response to nutrient availability. This is attributed to HepG2 cells being the most characterized cells in terms of signalling pathways and transcriptional response (Lai, Forde et al. 2018). It is for these reasons that the HepG2 cell line is employed in this study, to determine the effect of FB_1 on cholesterol homeostasis in galactose supplemented media.

2.5 Galactose media

Cancer cells differ from normal cells with respect to their energy metabolism as they take up glucose and glutamine excessively for aerobic glycolysis, while still retaining the function of oxidative phosphorylation (OXPHOS) (Zheng 2012). Glycolysis is less energy efficient, yielding

less ATP than OXPHOS, cancer cells rely on this due to the speed of ATP generation, which is suited to the energy demands of cancer cells for rapid proliferation (Zheng 2012). This phenomenon is described as the Crabtree effect, that relies on glucose-induced inhibition of cell respiration and thus on OXPHOS (Orlicka-Płocka, Gurda-Wozna et al. 2020). The proven method of circumventing this phenomenon is to replace glucose with galactose in the culture media. Several studies have demonstrated the efficacy of this approach to activate mitochondria and force cells to rely on OXPHOS for ATP production, as demonstrated in Figure 2.5 (Aguer, Gambarotta et al. 2011, Shiratori, Furuichi et al. 2019, Orlicka-Płocka, Gurda-Wozna et al. 2020).

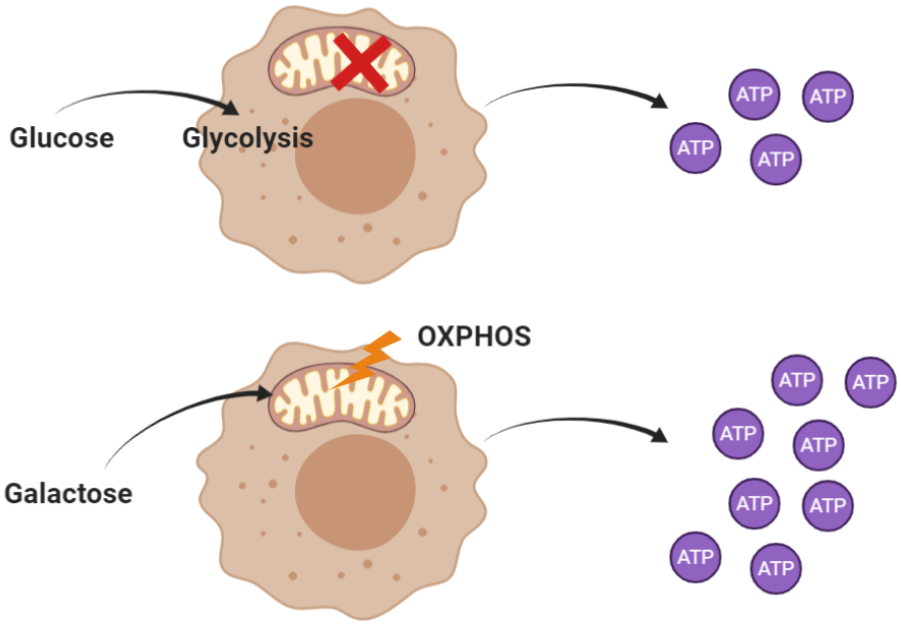

Figure 2.5: Galactose induces OXPHOS in cancer cells to produce ATP efficiently (prepared by author).

2.6 Mechanisms and regulation of cholesterol homeostasis

The liver is the leading metabolic organ that maintains the body's cholesterol and bile acid homeostasis. These molecules are steroid molecules that exert essential roles for biological functions. Cholesterol is an essential component of cell membranes and plays an important role in the make-up of lipid bilayers, as it is necessary for membrane biogenesis and cell proliferation (Maxfield and van Meer 2010, Silvente-Poirot and Poirot 2012). It is also a precursor for the biosynthesis of steroid hormones, lipid-soluble vitamins, and bile acids (Russell 1999, Russell 2003).

The cellular levels of cholesterol are determined by the interaction between *de novo* biosynthesis, uptake, export and storage (Figure 2.6) (Luo, Yang et al. 2020). Cholesterol synthesis begins from Acetyl-CoA and involves intense action of more than 20 enzymes localized in the membrane of the endoplasmic reticulum. Dietary sources also account for cholesterol and are absorbed by Niemann-Pick type C1-like 1 (NPC1L1) protein in enterocytes in the intestine (Altmann, Davis et al. 2004). The dietary cholesterol is then released as chylomicrons, from which cholesterol can be taken up by the liver. The liver is then responsible for the delivery of endogenously synthesized and exogenous cholesterol to the bloodstream as very-low-density lipoproteins (VLDLs). Once processed in the bloodstream, VLDLs give rise to low-density lipoprotein/s (LDL/s), which are

taken up by cells via LDL receptor-mediated endocytosis (Brown and Goldstein 2009). Vascular and non-vascular mechanism transport cholesterol to various organelles with in the cell, to satisfy its specific functions (Maxfield and van Meer 2010).

Cholesterol biosynthesis is energetically expensive as it demands Acetyl-CoA, ATP, oxygen and reducing intermediates (NADPH/NADH). The cholesterol biosynthetic pathway is mediated by SREBP2 as it is a transcriptional regulator of the rate-limiting enzymes,: 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR) and squalene monooxygenase (Maxfield and van Meer 2010). However, cholesterol flux remains the focus of this study as reviewed below.

2.6.1 Regulation of cholesterol uptake

Apart from biosynthesis, cholesterol homeostasis is maintained by the diet via cholesterol uptake from the blood. Two mechanisms of cholesterol uptake are explained, the first being cholesterol uptake from the intestinal lumen via the NPC1L1 and secondly LDLR mediated uptake of LDL cholesterol from the blood, both through a clathrin-dependent pathway (Luo, Yang et al. 2020). Within polarized cells, like hepatocytes, LDLR is found on the basolateral membrane to facilitate its function, while NPC1L1 is on the opposite side to acquire unesterified cholesterol from the intestine lumen.

2.6.1.1 LDLR-mediated LDL endocytosis

The LDL-receptor is principle for the uptake of cholesterol by peripheral cells. It is cell surface glyco-protein with five structural and functionally detailed domains (Jeon and Blacklow 2005). LDLR contains an N-terminal apolipoprotein B (apoB) and apolipoprotein E (apoE) binding domain, an epidermal growth factor (EGF) domain, a six-bladed β-propeller and a third EGF-like repeat, an O-linked oligosaccharide-rich domain, a transmembrane domain and a short C-terminal tail (Jeon and Blacklow 2005).

SREBP2 is a transcriptional factor of LDLR, and once synthesized the 120kDa precursor undergoes glycosylation to form a mature 160kDa protein located on the plasma membrane (Lopez, Socarrás et al. 2007, Wijers, Kuivenhoven et al. 2015).

Circulating cholesterol is captured by surface LDLR via the extracellular ligand binding domain, forming the LDLR-LDL complex, and recruits endocytic adaptors to enable the incorporation of LDL into clathrin-coated vesicles. This leads to LDLR pinching off the plasma membrane and entering the endocytic pathway (Luo, Yang et al. 2020). Within the acidic environment of the endosome, LDLR is subjected to conformational changes to facilitate the release of LDL (Rudenko, Henry et al. 2002). LDLR then has the ability to return to the cell surface via the endosomal recycling complex for additional endocytosis, or it can interact with PCSK9 which directs LDLR to lysosomal degradation (Zhang, Ma et al. 2016, Luo, Yang et al. 2020). At the plasma membrane, LDLR can also be targeted for lysosomal degradation in a process that is regulated by the E3 ligase, namely the inducible degrader of the LDL receptor (IDOL) (Zelcer, Hong et al. 2009).

The process involved in IDOL-induced degradation of LDLR follows a complex ubiquitination of LDLR allowing for lysosomal degradation (Luo, Yang et al. 2020). Several factors are implicated in regulating this event, however the most prominent is LXR. LXRs bind to the IDOL promotor and upregulate its expression, and in accordance, the activation of the LXR-IDOL

system using synthetic LXR agonists, decreased LDLR levels and reduced LDL uptake (Zelcer, Hong et al. 2009, Hong, Marshall et al. 2014).

2.6.1.2 PCSK9-induced degradation of LDLR and its regulation

PCSK9 belongs to the secretory serine protease family as one of nine proprotein convertases. Structurally it contains a prodomain, a catalytic domain and a specialized C-terminal domain that is rich in both Cysteine and Histidine residues (Seidah and Prat 2012). The prodomain of a newly synthesized precursor, serves as the N-terminal domain that is self-cleaved in the endoplasmic reticulum, and non-covalently binds to the rest of the protein, consequently blocking protease activity. PCSK9 is modified by glycosylation, phosphorylation and sulfation, until it is finally released into the extracellular environment where it binds to LDLR and other proteins (Lagace 2014).

A portion of the catalytic domain on PCSK9 interacts with LDLR on the epidermal growth factor (EGF) repeat, forming the PCSK9-LDLR complex (Kwon, Lagace et al. 2008). The complex is internalized via clathrin-coated pits and processed to endosomes, where the C-terminal of PCSK9 is stimulated to interact with the ligand binding domain of LDLR, within an acidic environment (Tveten, Holla et al. 2012). The interaction increases the binding strength of the PCSK9-LDLR complex, preventing LDLR from achieving the recycling conformation, ultimately leading to lysosomal degradation (Zhang, Garuti et al. 2008, Lagace 2014). In HepG2 cells, PCSK9 can direct LDLR in the trans-Golgi towards lysosomal degradation (Li, Dong et al. 2009).

SREBP2 upregulates PCSK9 along with other transcription factors and post-transcriptional regulators.

2.6.2 Regulation of cholesterol efflux

Excess cholesterol that cannot be catabolized is stored as cholesteryl esters in lipid droplets or disposed of out of the cell (Luo, Yang et al. 2020). The ATP-binding cassette (ABC) transporter superfamily are responsible for cholesterol efflux in a cell specific manner. ABC subfamily A member 1 (ABCA1) and ABC subfamily G (ABCG) mediate cholesterol efflux by macrophages, while ABCG5 and ABCG8 are present in hepatocytes and enterocytes (Luo, Yang et al. 2020). However, ABCA1 is expressed throughout the body and studies have highlighted the role of ABCA1 in hepatocytes (Bashore, Liu et al. 2019, Sasaki, Komatsu et al. 2019).

2.6.2.1 ABCA1-mediated cholesterol efflux

ABCA1 is a transport protein that has two tandem repeats of the membrane-spanning domains, each comprising of six transmembrane segments and a glycosylated extracellular domain (Luo, Yang et al. 2020). Lipid-free apolipoprotein A-I (apoA-I) is the primary acceptor for cholesterol efflux via ABCA1 (Rosenson, Brewer Jr et al. 2012). ABCA1 can flip or directly transport phospholipids across the lipid bilayer by lateral recruitment from the inner membrane (Qian, Zhao et al. 2017). ABCA1 mediates cholesterol efflux to apoA-I via three controversial mechanisms. The first suggestion is that ABCA1 can interact with apoA-I, when cholesterol and phospholipids are bound in the extracellular domain, allowing for transfer of the lipids to apoA-I (Nagata, Nakada et al. 2013, Ishigami, Ogasawara et al. 2018). The second view is that ABCA1 induces the formation of an activated microdomain, by promoting phospholipid transport, that protrudes from the cell surface to facilitate apoA-1 binding (Phillips 2018). ABCA1 the unfolds the N-terminus, allowing apoA-1 to be inserted into the membrane, initiating micro-solubilization of the

lipid bilayer, allowing for the efflux of cholesterol and phospholipids. The third view is that ABCA1 and apoA-1 undergo clathrin-dependent endocytosis from the cell surface to lysosomes, allowing apoA-1 to receive LDL cholesterol through ABCA1 directly from NPC intracellular cholesterol transported 2 (NPC2) (Boadu, Nelson et al. 2012).

Cholesterol export is regulated at the transcriptional level by LXRs and RXR (Costet, Luo et al. 2000). LXR targets the ABCA1 and ABCG1 genes to promote the efflux of cellular cholesterol in an attempt to maintain sterol homeostasis (Repa, Turley et al. 2000).

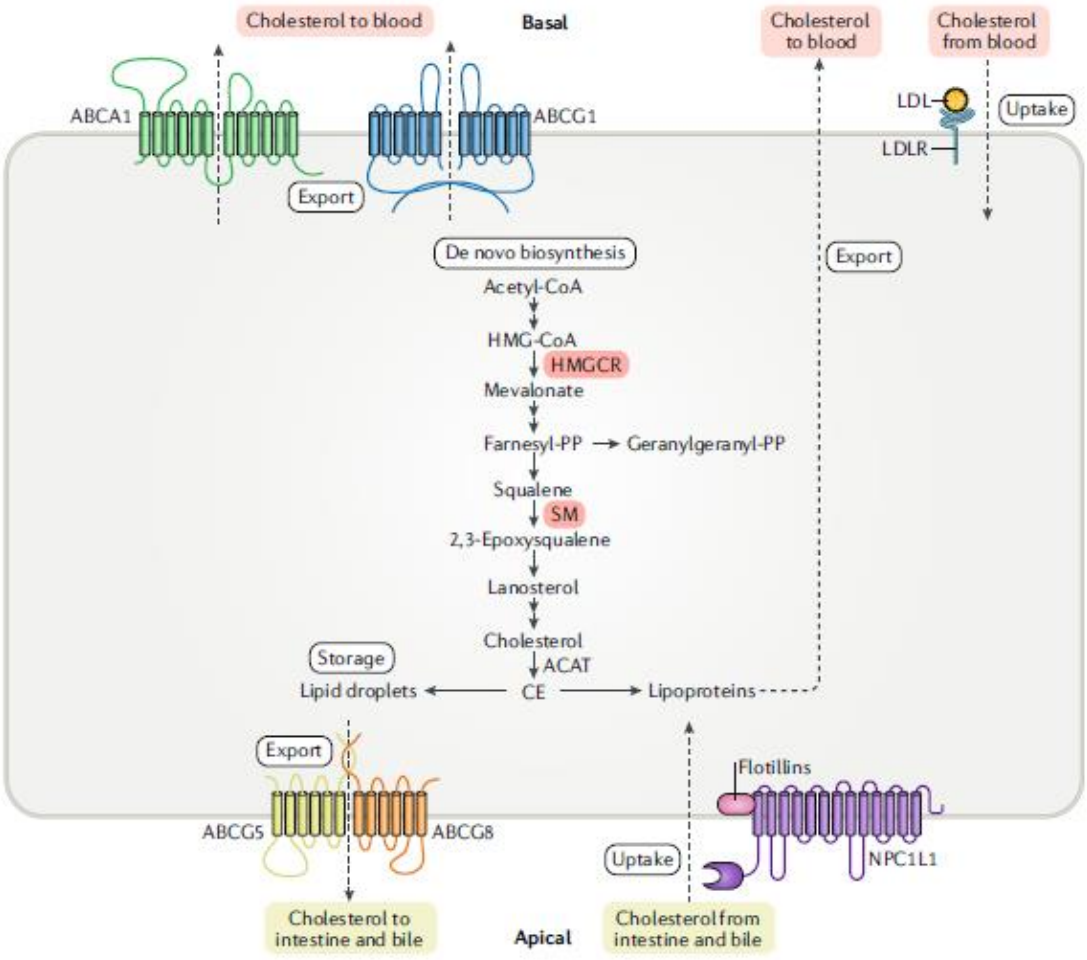

Figure 2.6: Major pathways of cholesterol metabolism and flux (Luo, Yang et al. 2020).

2.7 The role of cholesterol in cancer development

Irrespective of their critical functions in the body, excesses of cholesterol and bile acids lead to human diseases in the form of cardiovascular disease, hepatobiliary disease and liver cancer (Kemper, Choi et al. 2013). In addition the disturbance of the homeostatic state is known to be associated with cardiovascular disease attributed to dietary and lifestyle habits (Hu, La Vecchia et al. 2012).

The role of cholesterol in carcinogenesis has increased, with clinical evidence that suggest that changes in cholesterol metabolism is involved in the onset of cancer (Silvente-Poirot and Poirot 2012). Cholesterol can activate oncogenic signalling, due to it being a cell membrane component

can close relation to membrane receptors (Ding, Zhang et al. 2019). It has been reported that cholesterol can activate the oncogenic Hedgehog signalling pathway, a well-known cancer associated signalling pathway, by binding the smoothened receptor (Huang, Nedelcu et al. 2016). The activation of this pathway is associated with cell differentiation, cell proliferation and tumour formation (Hooper and Scott 2005).

In conjunction to the cell membrane, cholesterol also has functions in the cytoplasm. It was shown that lysosomal cholesterol could lead to the activation of mTORC1, which is also associated with increased cell proliferation, invasion and metastasis (Kim, Cook et al. 2017).

Apart from being a key component for membrane integrity, cholesterol is also an important component of lipid rafts. Lipid rafts are small specialized lipid domains within the cell membrane, whose structure and function are reliant on the composition of cholesterol and phospholipids (Yan, Qu et al. 2014). Lipid rafts facilitate signal transduction for oncogenic signalling pathways, and alterations in cholesterol level impair the structural integrity of lipid rafts, leading to the activation or inhibition of death receptor proteins, protein kinases and calcium channels (George and Wu 2012).

Changes in cholesterol metabolism during cancer development occur during cholesterol synthesis and cholesterol flux. Therefore, it is imperative that these processes are tightly regulated, to evade disease states.

2.8 The role of SIRT1 in lipid regulation and cancer

There are seven sirtuin proteins in mammals (SIRT1-7) that are ubiquitously expressed and comprise of a common catalytic core domain but also have specialized features in their respective cellular localizations, enzymatic functions and target proteins (Kemper, Choi et al. 2013). The sirtuins are of particular interest due to their diverse roles in various physiological ad pathological events including life-span extension, neurodegeneration, age-related disorders, obesity, heart disease, inflammation, and cancer.

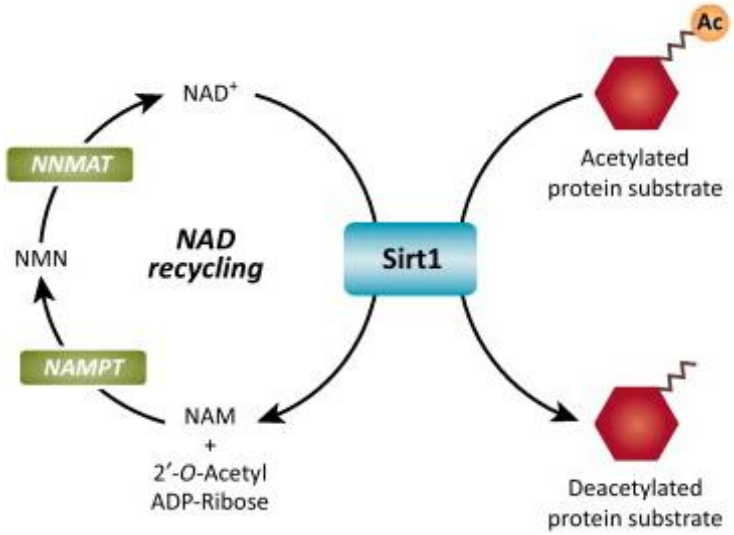

Figure 2.7: SIRT1 NAD-dependent deacetylase activity (Revollo and Li 2013).

SIRT1 is a NAD-dependent deacetylase (Figure 2.7) that plays extensive roles in cellular metabolism and stress response by regulating the activity of transcription factors and cofactors by

protein deacetylation (Ponugoti, Kim et al. 2010). SIRT1 is primarily localized in the nucleus and has a role in genomic stability, telomere maintenance and cell survival (Chen, Zhang et al. 2011). It is responsible for the removal acetyl groups from the ε-amino group of lysine residues of histone and non-histone protein targets thus altering their function (Haberland, Montgomery et al. 2009).

2.8.1 SIRT1 lipid and cholesterol regulation

Studies have shown that SIRT1 inhibits hepatic lipogenesis by deacetylating SREBP-1c, a known key lipogenic activator, during a fasted state (Ponugoti, Kim et al. 2010). SREBPs are transcription factors that bind to DNA, specifically involved in the regulation of cellular lipid and cholesterol levels (Brown and Goldstein 1997). SREBPs exist as three isoforms (SREBP-1c, -1a and 2) encoded by two genes (Horton, Bashmakov et al. 1998). SREBP-2 is synthesized by a separate gene, while SREBP-1c and -1a are synthesized by the same gene. SREBP-1c is abundantly expressed in adult liver, and in a fed state, binds to and activates its lipogenic target genes, such as fatty acids synthase, Acetyl CoA carboxylase, stearyl CoA desaturase and interestingly its own gene SREBP-1c, resulting in increased fatty acid synthesis (Repa, Liang et al. 2000). Biochemical proteomic analysis and mechanistic study highlighted that SREBP-1c is acetylated by p300 and deacetylated by SIRT1 at Lys-289 and Lys-309 resulting in downregulated SREBP-1c transcriptional activity by promoting ubiquitination and proteasomal degradation (Ponugoti, Kim et al. 2010, Walker, Yang et al. 2010).

SIRT1 also plays a critical role in cholesterol metabolism by deacetylating, and inducing the activity of oxysterol receptors, LXRs (LXRα and LXRβ) (Kemper, Choi et al. 2013). Therefore SIRT1-mediated deacetylation of LXRs can lead to reverse cholesterol transport and hepatic lipogenesis (Kemper, Choi et al. 2013). Both LXR and SREBP-1c contribute to the transcriptional activation of the SREBP-1c gene (Yoshikawa, Shimano et al. 2001), thus termination of SREBP-1c activity by SIRT1-mediated deacetylation may allow SIRT1 to exhibit beneficial effects on cholesterol metabolism (Kemper, Choi et al. 2013).

2.8.2 The role of SIRT1 in liver cancer

The role of SIRT1 in cancers remains controversial since they could act as either a tumour suppressor or tumour promoter dependent on a host of factors: the cellular context, its targets in signalling pathways and specific cancers (Lin and Fang 2013).

SIRT1 is regarded as a tumour promoter due to its increased expression in cancer cell types and its role in the inactivation of tumour suppressor proteins and DNA damage repair (Choi, Bae et al. 2011). The same group found that the expression of SIRT1 was significantly higher in hepatocellular carcinoma (HCC) tissues, as compared to non-tumour tissues. Another study showed that SIRT1 is overexpressed in HCC and that it plays an oncogenic role via the enhancement of cell proliferation and resistance to chemotherapy (Chen, Jeng et al. 2012).

2.9 The impact of FB_1 on cholesterol

Previously it was shown that FB_1 can translocate from a water environment to a lipid phase containing cholesterol and/or bile salts, and FB_1 demonstrated the ability to bind to cholesterol (Mahfoud, Maresca et al. 2002). This provides reason for the interest in the toxin and its effect on humans. As such, the effect of FB_1 on cholesterol homeostasis has been poorly addressed. Therefore, this study aims to determine the effect of FB_1 on cholesterol homeostasis, by examining the major proteins central to cholesterol flux. This will offer an understanding of the

effect of the mycotoxin on cholesterol flux and suggest an alternate dimension to understanding its toxicity.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Fumonisin B_1 (FB_1), isolated from *Fusarium moniliforme*, was obtained from Sigma-Aldrich (St Louis, MO, USA). The human hepatocellular carcinoma (HepG2) cell line was attained from Highveld Biologicals (Johannesburg, South Africa). The cell culture reagents and supplements were purchased from Lonza BioWhittaker (Basel, Switzerland). Western Blot reagents were purchased from Bio-Rad (Hercules, CA, USA) and anti-bodies were procured from Abcam (Cambridge, UK). All other reagents were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

3.2 Cell culture

The liver is considered the centre of metabolism and it is responsible for detoxification of harmful xenobiotics. FB_1 has been reported to cause hepatocarcinogenicity and disrupt lipid metabolism. The liver plays a central role in lipid metabolism. Collectively these reasons warrant the use of the HepG2 cell line in this study.

3.2.1 Cell culture conditions

The HepG2 cells were cultured in monolayer (10^6 cells per $25cm^3$ culture flask) using complete culture media [CCM: Eagle's Essential Minimal Media (EMEM) supplemented with: 10% foetal calf serum, 1% penstrepfungizone and 1% L-glutamine] at 37°C in a humidified incubator. After every 48 hours, cells were washed with 0.1M phosphate buffered saline (PBS) and reconstituted with CCM (5ml).

For treatments HepG2 cells were exposed to galactose media [Gibco Dulbecco's Modified Eagle Medium (DMEM) supplemented with: 10mM galactose, 6mM L-glutamine, 5mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 10% foetal calf serum, 1mM sodium pyruvate, and 1% penstrepfungizone].

3.2.2 Preparation of Fumonisin B_1

A 5mM stock of FB_1 was prepared by dissolving 2mg of FB_1 in 0.1M PBS (554.2µl).

3.2.3 Cell preparation for assays

HepG2 cells were allowed to reach 80% confluency in $25cm^3$ flasks before treatments. Cells were washed with 0.1M PBS and culture media was replaced with galactose media. After 1hr the galactose media was removed, and cells were washed, to allow for the phenotypic change to induce oxidative phosphorylation. A concentration of 25µM was obtained from cell viability assays and used in all subsequent assays supplemented with galactose media. Two controls were prepared, an untreated control containing CCM and galactose media, respectively.

After the 6hr incubation, cells were detached using trypsinization and gentle agitation; the Trypan Blue exclusion method of cell counting was utilized to determine cell viability and cell number as per assay requirements. The galactose control was employed for statistical comparison against FB_1 treatment; all experiments were performed as three independent experiments and in triplicate for each assay.

Maize contains approximately 72% starch, 10% protein, and 4% fat, thus supplying an energy density of 365 Kcal/100g. It can be processed into a variety of food products and has a low production cost, thus along with the high consumption of maize flour, particularly where micronutrient deficiencies are common public health concerns, make this food a part of the staple diet (Ranum, Peña-Rosas et al. 2014). In African countries such as Zambia, Malawi, Zimbabwe, South Africa and Kenya, many people eat maize meal twice or three times a day (FIhlani 2019). Therefore, a 6hr treatment was decided upon to resemble a single exposure profile, and satisfies the half-life of the toxin (Martinez-Larranaga, Anadon et al. 1999).

3.3 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

3.3.1 Principle

The MTT assay is a colorimetric test commonly used to determine the cytotoxicity of a compound at variable concentrations, but also a method to measure cell viability. The assay directly measures mitochondrial activity as the enzyme mitochondrial dehydrogenase converts the MTT salt into an insoluble formazan salt, illustrated by a yellow to purple colour change (Figure. 3.1). It is postulated that viable cells have constant mitochondrial activity, allowing the assay to accurately distinguish cell viability.

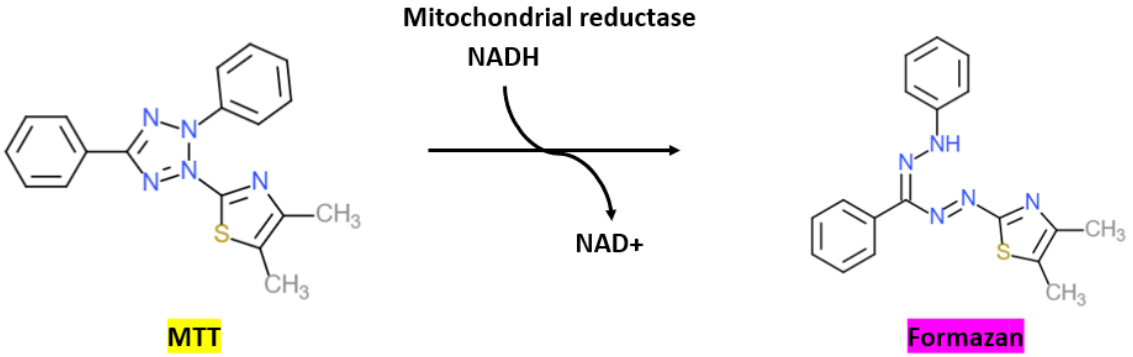

Figure 3.1: The structures and conversion of MTT salt to Formazan via mitochondrial reductase as a measure of cell viability (prepared by author).

The concentration of solubilized formazan is measured by a spectrophotometer at 570nm with a reference wavelength of 690nm. A decrease viability is indicative of growth inhibition, while toxin sensitivity is expressed at a concentration that indicates 50% growth inhibition (IC_{50}) (van Meerloo, Kaspers et al. 2011).

3.3.2 Protocol

The cell viability of HepG2 cells in FB_1, supplemented with galactose media, was determined using the MTT assay. Approximately 20,000 cells/well (triplicate) were seeded overnight in a 96-well microtitre plate (37°C, 5% CO_2). Then cells were washed with 0.1M PBS and supplemented with galactose media (200µl) only for 1hr. Thereafter, cells were washed again with PBS and treated with variable concentrations of FB_1 (0-500µM - 200µl) for 6hrs. Control cells contained CCM only (200µl). Following the incubation, treatments were removed, and cells were washed with PBS. The cells were then incubated with MTT salt (20µl; 5mg/ml in 0.1M PBS) and CCM (100µl) for 4hrs. The MTT solution was removed and replaced with dimethyl sulphoxide (DMSO) (100µl per well) and incubated for 1hr. The optical density was measured using a spectrophotometer (BioTek uQuant, Winooski, VT, USA) at a wavelength of 570nm with a

reference wavelength of 690nm. The percentage cell viability was calculated by standardizing control cells to 100% and then comparing this relative to FB_1 treated cells. Results were expressed as log concentration versus percentage cell viability using GraphPad Prism v5.0 software.

3.4 ATP luciferase assay

3.4.1 Principle

The major energy molecule of the cell is adenosine triphosphate (ATP), its production is vital for cellular process. To satisfy the demand for energy, the mitochondria produces ATP via the electron transport chain (ETC) which is coupled to OXPHOS in addition to substrate level phosphorylation. Cancer cells depend on glycolysis to generate ATP rather than OXPHOS, even when oxygen and functional mitochondria are available. Suppressing glycolysis by galactose supplementation, upregulates mitochondrial function and subsequently forces cells to rely on OXPHOS to obtain ATP, which is more energy efficient (Zheng 2012, Shiratori, Furuichi et al. 2019).

The CellTire Glo™ (Promega) assay was used to determine ATP concentration. The assay relies on bioluminescence to measure ATP levels in cells, based on the luciferase reaction. Luciferin is mono-oxygenated to oxy-luciferin in the presence of Mg^{2+}, O_2 and ATP. This reaction produces energy in the form of luminescence, which is directly proportional to the concentration of ATP in the cells (Figure 3.2). The light can be measured with a luminometer.

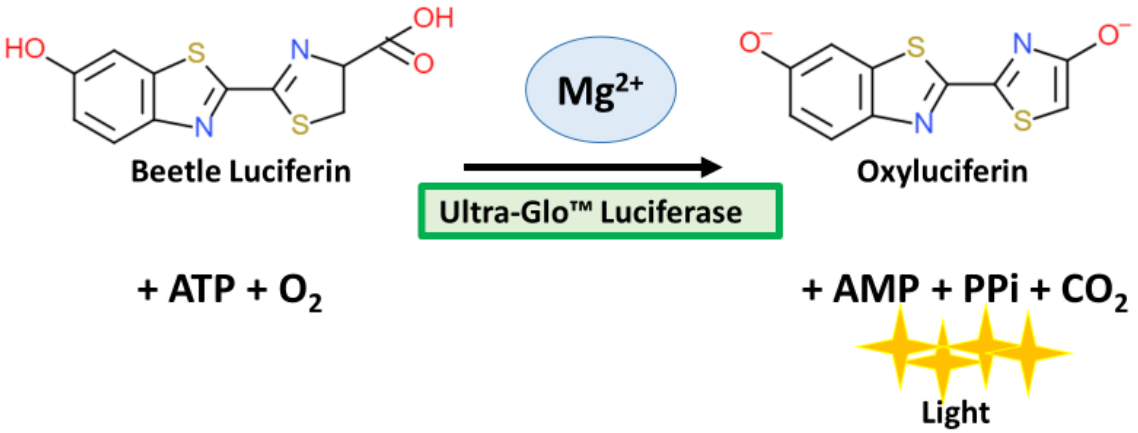

Figure 3.2: The structures and chemical conversion of luciferin to oxyluciferin involved in the CellTire Glo™ assay to quantify cellular ATP concentration (prepared by author).

3.4.2 Protocol

The CellTire Glo™ assay was used to determine the amount of ATP in FB_1 treated HepG2 cells, supplemented with galactose. Approximately 20,000 cells were aliquoted into a white opaque microtitre plate (triplicate), followed by 25µl CellTire Glo™ reagent (Promega, Madison, USA) and then incubated the in dark (30min, RT), to allows the reaction to propagate. The luminescent signal was measured by employing a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The relative luminescent signal was used to determine intracellular ATP concentration and then expressed as relative light units (RLU).

3.5 Lactate dehydrogenase (LDH) assay

3.5.1 Principle

Cell membrane integrity can be determined by measuring the activity of cytoplasmic enzymes, released by damaged cells. The cytoplasmic enzyme, LDH, is present in all cells and is released into cell culture supernatant, when cell membrane integrity is compromised. The amount of LDH activity correlates to the degree of membrane damage.

The LDH enzyme activity is measured via a two-step enzymatic test. The first step involves the reduction of NAD^+ to $NADH/H^+$ by the conversion of lactate to pyruvate, catalysed by LDH. The second step, the diaphorase enzyme transfers H/H^+ for the $NADH/H^+$ species, to the tetrazolium salt INT, forming a formazan product (Figure 3.3). The amount of formazan product can be measured spectrophotometrically and correlated to the activity of LDH.

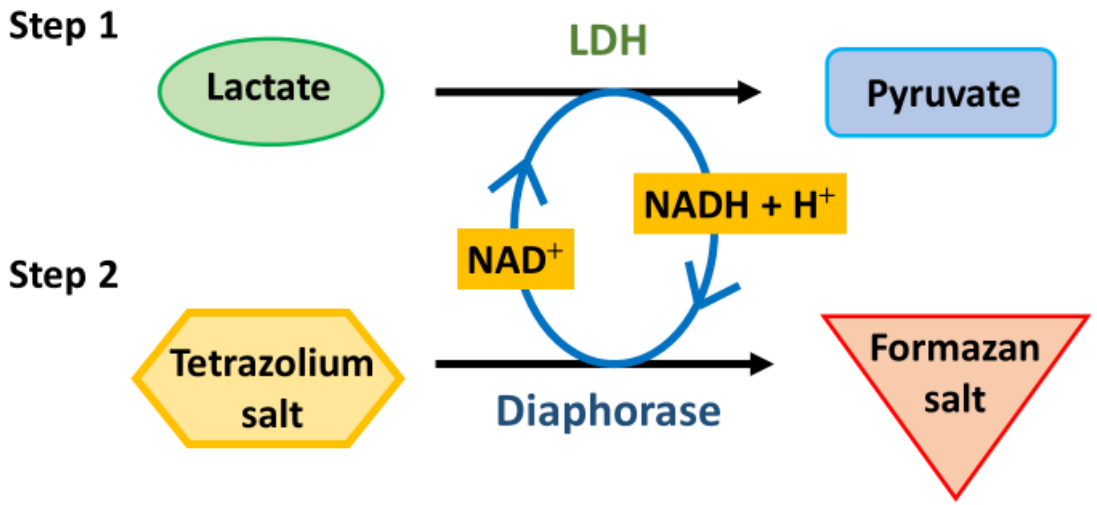

Figure 3.3: Reactions of the LDH membrane integrity assay (prepared by author).

3.5.2 Protocol

To measure cell membrane integrity, the extracellular LDH detection kit (Roche) was employed. Supernatant (100µl) was aliquoted into a 96-well microtitre plate (triplicate). Thereafter, substrate mixture (100µl) containing catalyst and dye solution (diaphorase/ NAD^+ and INT/sodium lactate) was added to the samples and incubated (20min, RT). The optical density of the formazan product was measured using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA) at 500nm. The results were expressed as mean optical density.

3.6 Protein Isolation, quantification, and standardization

3.6.1 Principle

Protein isolation was mandatory for western blotting. Protein isolation was carried out on ice to prevent protein degradation, using both mechanical (cell scraper) and chemical lysis (Cytobuster) techniques. The crude protein was quantified and then standardized to ascertain whether enough protein was available, to carry out the assays, and to make distinguishable comparisons between samples (Huang, Long et al. 2010).

The bicinchoninic acid (BCA) assay was employed to determine protein concentration. The reaction is dependent on two chemical reactions, under alkaline conditions. Firstly, the biuret reaction involves the reduction of cupric ions (Cu^{2+}) into cuprous ions (Cu^{+}) via the formation of peptide bonds (Figure 3.4). The second reaction involves the chelation of Cu^{+} with two molecules of BCA, producing a powerful purple chromophore, which absorbs light at a maximum of 562nm. The intensity of the signal produced is directly proportional to the concentration of the protein; it is proportional to the reduction of Cu^{2+}.

The concentrations of the protein samples are determined using a standard curve. Briefly a set of bovine serum albumin (BSA) standards are prepared, and their absorbances are used to plot the standard curve (Huang, Long et al. 2010).

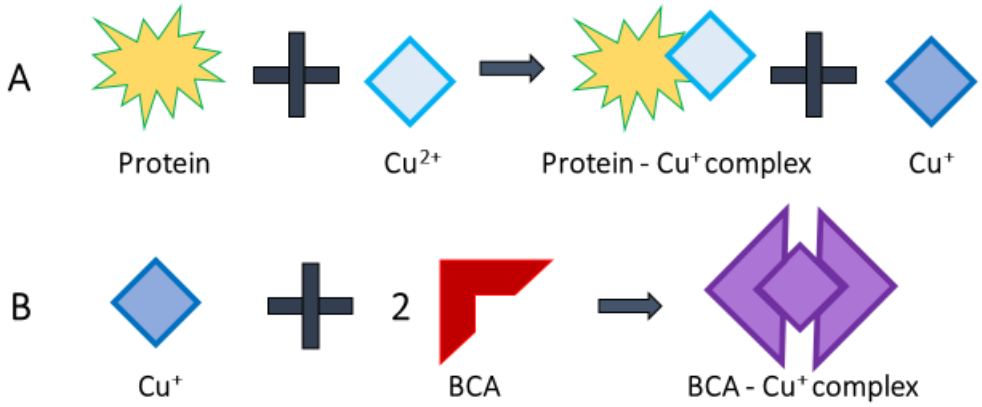

Figure 3.4: A summary of the BCA assay for protein quantification. Reaction A illustrates the reduction of Cu^{2+}, to form free Cu^{+}, while reaction B illustrates the chelation of two BCA molecules to Cu^{+} (prepared by author).

3.6.2 Protocol

Crude protein was extracted using Cytobuster™ (200µl) (Novagen, USA) which was supplemented with protease and phosphatase inhibitors (Roche, 05892791001 and 04906837001, respectively) to maintain the integrity of the protein samples.

The cells were incubated on ice with Cytobuster™ for 10min, followed by mechanical lysing and finally decanted into 2ml micro-centrifuge tubes. Cell lysates were centrifuged (13,000xg, 10min, 4°C) to yield crude protein, which was then quantified using the BCA assay.

A set of BSA standards (0 – 1 mg/ml) were prepared, and 25µl of the samples (in duplicate) and the standards (in triplicate) were dispensed into a 96-well microtiter plate. The BCA working solution (196µl BCA: 4µl $CuSO_4$ per well) was also dispensed into each well. The plate was then incubated in the dark for 30min at 37°C. The optical density of the samples and standards was determined using a spectrophotometer (BioTek uQuant, Winooski, VT, USA). A standard curve was constructed and employed to determine the protein concentrations of the samples. Once quantified, sample proteins were standardized to 1.0mg/ml with Cytobuster.

3.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting

3.7.1 Principle

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting are advantageous techniques, applied for the expression profiles of target proteins. Briefly, it involves the separation of proteins on the basis of molecular size, followed by the transfer of these proteins onto a membrane, and lastly detection of a target protein via immunoblotting (Kurien and Scofield 2006).

3.7.1.1 Sample Preparation

Prior to separation via SDS-PAGE, standardized proteins are boiled in Laemmli buffer. The components of the buffer are key factors that allow for optimum electrophoresis (Table 3.1). Boiling the samples provides a high temperature for the denaturation of proteins and subsequent breakage of disulphide bonds (Mahmood and Yang 2012).

Table 3.1: The components of Laemmli buffer and their functions.

Component	Function
Glycerol	Contributes to the density of the sample, allowing the samples to settle more efficiently at the bottom of the wells of the gel.
Sodium dodecyl sulphate (SDS)	Causes the proteins to denature, acquire a homogenous negative charge and linearization.
B-mercaptoethanol	Breaks down disulphide bonds by reduction, allowing for protein unfolding and linearity.
Bromophenol Blue	Allows for the tracking of samples during electrophoresis.
Tris-HCl	Used as a buffer and maintains pH of the proteins during electrophoresis.

3.7.1.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Following sample preparation, samples were separated based on their respective molecular weights using SDS-PAGE. In an electric field, particles with a uniform charge are drawn towards an electrode with the opposing charge. By denaturing the protein samples using Laemmli buffer, the charge of the proteins is unified (negatively charged), therefore the samples are drawn towards the positive electrode. The Laemmli buffer ensures that the proteins adopt their primary state, which allows for separation to occur based on their respective molecular weights only, and not their shape or charge (Mahmood and Yang 2012).

For the proteins to separate based on their size, they migrate through polyacrylamide gels. The gels are polymers formed by acrylamide and N, N'-methylene bis-acrylamide which together, form cross-links, that impede the migration of the proteins. Low percentage gels have fewer cross-links, thus have larger pores for larger molecules to pass through. Equally, high percentage gels have much smaller pores on accord of more cross-linking.

Two types of gels are employed in SDS-PAGE: resolving and stacking gels (Figure 3.5). The resolving gel, which has a higher content of polyacrylamide as compared to the stacking gel, has smaller pores, due to more cross-links. Allowing for better separation of proteins, based on their respective sizes. The stacking gel has a lower polyacrylamide content, thus has larger pores, allowing for poor separation of proteins. This ensured that the proteins all began separating at the same point, for a fair and equal separation (Mahmood and Yang 2012).

The rate of migration is therefore dependent on the gel percentage, size of the protein, shape, and charge. Larger proteins remain closer to the origin, while smaller proteins migrate through the crosslinks and can be found lower on the gel, the same can be said in terms of the shape of the proteins. Molecular markers are employed to enable determination of the respective sizes of target proteins.

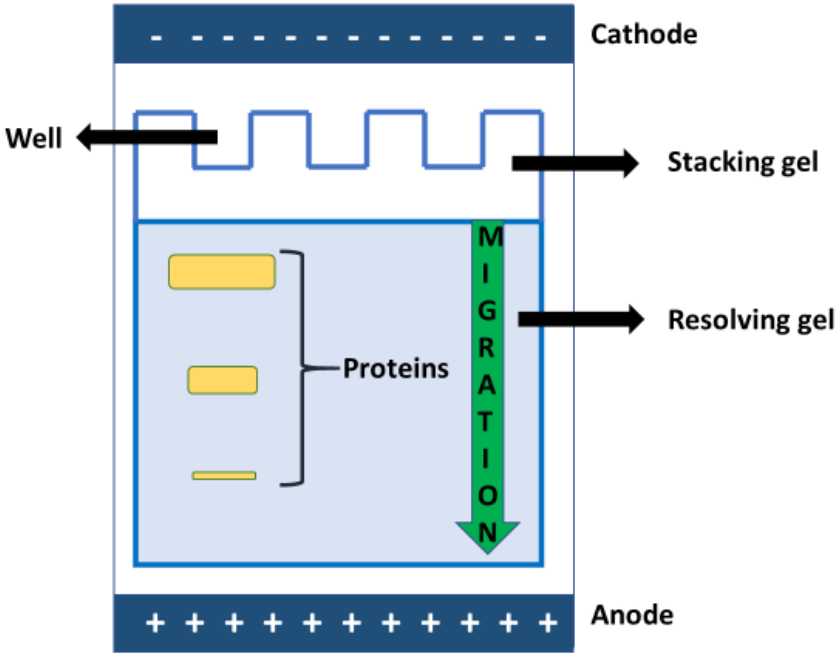

Figure 3.5: A simplified illustration of the migration of proteins, based on size, through the two gel layers (prepared by author).

3.7.1.3 Western blotting

Western blotting allows for the transfer of separated proteins from the polyacrylamide gel, to a nitrocellulose membrane. The nitrocellulose provides a solid support for the proteins. The polyacrylamide gel and membrane are sandwiched between two fibre pads and plate electrodes (Figure 3.6). The sandwich allows for uniform transfer of separated proteins. The gel and membrane sandwich are exposed to an electric field perpendicularly, driving the negatively charged proteins off the gel and onto the nitrocellulose membrane (Kurien and Scofield 2006).

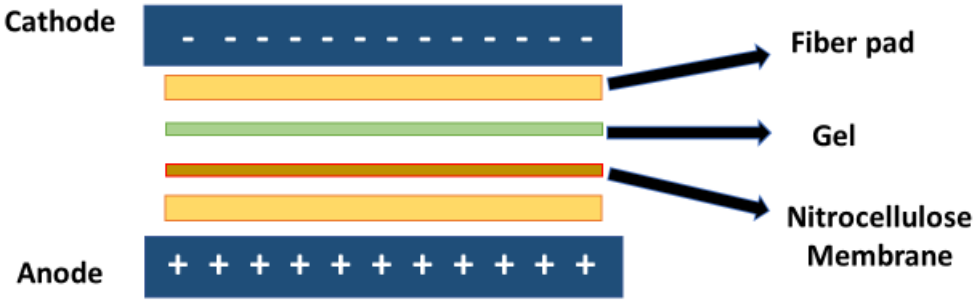

Figure 3.6: The configuration of the polyacrylamide gel and nitrocellulose membrane between fibre pads, sandwich, for western blotting (prepared by author).

Once proteins are transferred, target proteins are detected using specific antibodies. Before antibodies are used, the membrane was incubated with BSA or non-fat dry milk (NFDM). This is a blocking step that minimizes non-specific binding of antibody to the membrane, reducing any background (Gavini and Parameshwaran 2020). Antibodies are species specific. Primary antibody binds to the target protein on the nitrocellulose membrane, and the secondary antibody attaches to the primary antibody-protein complex. The secondary antibody is conjugated to the enzyme horse radish peroxidase (HRP), which produces a signal when the primary and secondary antibodies bind (Figure 3.7). A chemiluminescent detection reagent (luminol) which is oxidized by HRP, is applied to enhance the signal (Kurien and Scofield 2006). The intensity of the emanated light signal is directly proportional to the expression of the target protein (Gavini and Parameshwaran 2020).

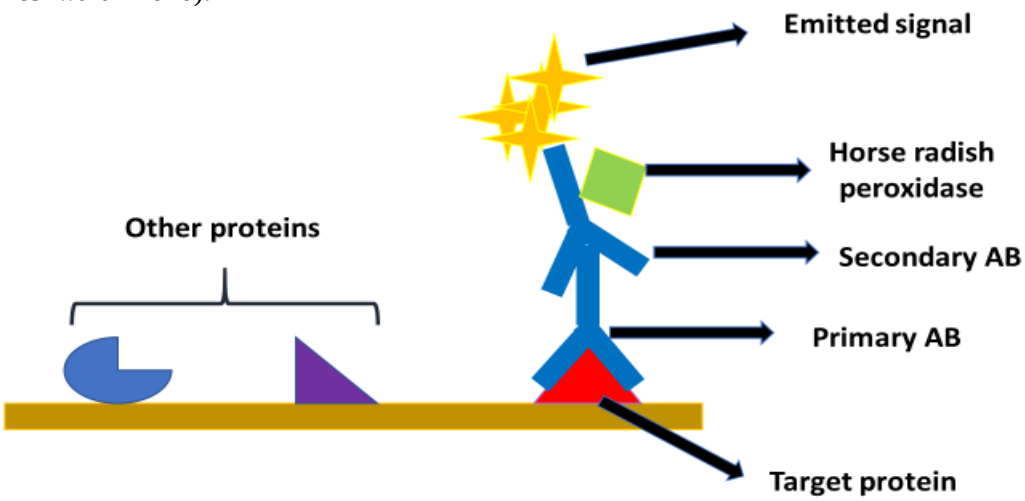

Figure 3.7: The signal emitted after successful immunoblotting (prepared by author).

3.7.1.4 Detection

The emanated light signals are detected using a high-power camera-based imaging system. By comparing the protein bands to that of the molecular weight marker, the target protein can be distinguished between other bands. To normalize the target protein expression, a housekeeping protein is employed, allowing for accurate protein level determination. The housekeeping protein, usually β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is constitutively expressed and is used to correct for loading error and protein concentration variation (Gavini and Parameshwaran 2020).

3.7.2 Protocol

3.7.2.1 Sample Preparation

Standardized proteins were boiled in Laemmli buffer [dH_2O, 0.5M Tris-HCl (pH6.8), glycerol, 10% SDS, β-mercaptoethanol, and 1% bromophenol blue] for 5min in a water bath at 100°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Polyacrylamide gels for SDS-PAGE were prepared using the Mini-PROTEAN Tetra Cell casting frame (Bio-Rad). A 12% resolving gel [dH_2O, 30% Bis-acrylamide, Tris (1.5M, pH8.8), 10% SDS, 80% glycerol, 10% Ammonium persulphate (APS), and Tetramethyl ethylenediamine (TEMED)] was prepared and allowed to set for 2min. Then, a 4% stacking gel [dH_2O, 30% Bis-acrylamide, Tris (0.5M, pH6.8), 10% SDS, 80%, APS, and TEMED] was set on top of the resolving gel. For the formation of wells, a 1cm plastic comb was positioned between the glass plates, and the gel was allowed to set (40min).

Thereafter, the gel cassettes were moved into the electrode assembly and positioned inside the electrode tank (Mini-PROTEAN Tetra Cell System, Bio-Rad). Before the combs were removed, running buffer (25mM Tris, 192mM glycine and 0,1% SDS) was poured into the tank. Protein samples (25µl) and the molecular weight marker (5µl) (Precision Plus Protein All Blue Standards, catalogue no. #161-0373, Bio-Rad) were then loaded into wells. The electrophoresis tank was topped with running buffer and electrophoresed [150 volts (V), 1hr) using a Bio-Rad compact power supply. The assembly was kept on ice.

3.7.2.2 Western blotting

Nitrocellulose membranes, fibre pads and electrophoresed gels were equilibrated in transfer buffer (25mM Tris, 191.8mM glycine and 20% methanol), for 10mins. Thereafter, the gel sandwich was prepared (Figure 3.6), and placed between the two electrodes of the transfer equipment. The separated proteins were then electro-transferred onto the nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo Transfer System (30min, 20V).

Once transferred, the membranes were blocked with blocking solution. Either 5% BSA or 5% NFDM in Tween 20-Tris buffered saline (TTBS: 150mM NaCl, 3mM KCl, 25mM Tris, 0.05% Tween 20, dH_2O, pH7.5), was used, and gently shaken [1hr, Room temperature (RT)]. Membranes were immunoblotted with primary antibody (Table 3.2), overnight (4°C). After incubation, the membranes were equilibrated to RT, the primary antibody was discarded, and membranes were washed using TTBS (5 times, 10min). Thereafter, membranes were probed with HRP-conjugated secondary antibody (Table 3.2) while gently shaken (2hr, RT). Membranes were washed with TTBS (5 times, 10min) and target protein bands were visualized and captured using the iBright Western Blot Imaging Systems (ThermoFisher).

Once proteins were detected, membranes were quenched with 5% H_2O_2 for 30min, and incubated in 5% BSA (1hr, RT). Membranes were washed three times in TTBS and probed with HRP-conjugated anti-β-actin. Protein expressions were determined using the iBright Analysis Software (ThermoFisher) and expressed as relative band density (RBD). Target protein expression was normalized against β-actin.

Table 3.2: Target protein antibodies and dilutions used in western blotting

	Antibody	Dilution	Catalogue no.
Primary Antibodies	Rabbit Anti-LXR alpha	1:1000 in 5% BSA	ab106464 (Abcam)
	Mouse Anti-ABCA1	1:1000 in 5% BSA	ab66217 (Abcam)
	Rabbit Anti-SREBP1	1:1000 in 5% BSA	ab28481 (Abcam)
	Rabbit Anti-LDLR	1:1000 in 5% BSA	ab52818 (Abcam)
	Rabbit Anti-SIRT1	1:1000 in 5% BSA	ab32441 (Abcam)
	Rabbit Anti-PCSK9	1:1000 in 5% BSA	ab125251 (Abcam)
Secondary Antibody	Goat Anti-Rabbit IgG HRP	1:10,000	#7074S (Cell Signalling Technology)
	Rabbit Anti-Mouse IgG HRP	1:10,000	ab6728 (Abcam)
Housekeeping Antibody	Anti-β-actin	1: 5000 in 5% BSA	A3854 (Sigma Aldrich)

3.8 Real Time Quantitative Polymerase Chain Reaction

3.8.1 Principle

The conventional polymerase chain reaction (PCR) is a simple tool used for the rapid amplification of specific gene sequences, from template bands. Thermostable *Taq* polymerase drives the synthesis of complementary DNA (cDNA) strands by the addition of deoxynucleoside triphosphates (dNTPs) to the 3'end of oligonucleoside primers. These primers are complementary to the DNA sequence that flank the target gene (Pestana, Belak et al. 2010). For a successful PCR to occur, the following components are essential:

- DNA template – comprises the target sequence.
- Forward and Reverse primers – allows for the binding to the 3'ends of the forward and reverse stands of the target sequence.
- *Taq* polymerase – serves as a catalyst for the synthesis of new strands of DNA, that are complementary to the target sequence.
- Deoxynuceloside triphosphates (dNTPs) – provide the building blocks utilized in the synthesis of new strands of DNA.
- $MgCl_2$ – a stabilizing agent for DNA and ensures optimal functioning of *Taq* polymerase.
- Buffer system – maintains ideal pH for the PCR reaction to occur.

The PCR reaction is completed in a thermocycler where it undergoes continuous cycling of three steps at distinct temperatures. These three steps make up one cycle of a PCR reaction (Figure 3.8). The three steps include:

1. Denaturation – initiated at 95°C allowing the H bonds between double stranded (ds) DNA to break, yielding single stranded (ss) DNA.
2. Annealing – the temperature is lowered to 55°C and the primers hybridize to complementary sequences, flanking the 3' end of the target sequence.

3. Extension – the temperature is the raised to 72°C, allowing optimal activity of *Taq* polymerase, which attaches dNTPs to the annealed primers, forming a copy of the DNA target gene.

This cycle is repeated between 30 to 40 times to establish acceptable amplification (Arya, Shergill et al. 2005).

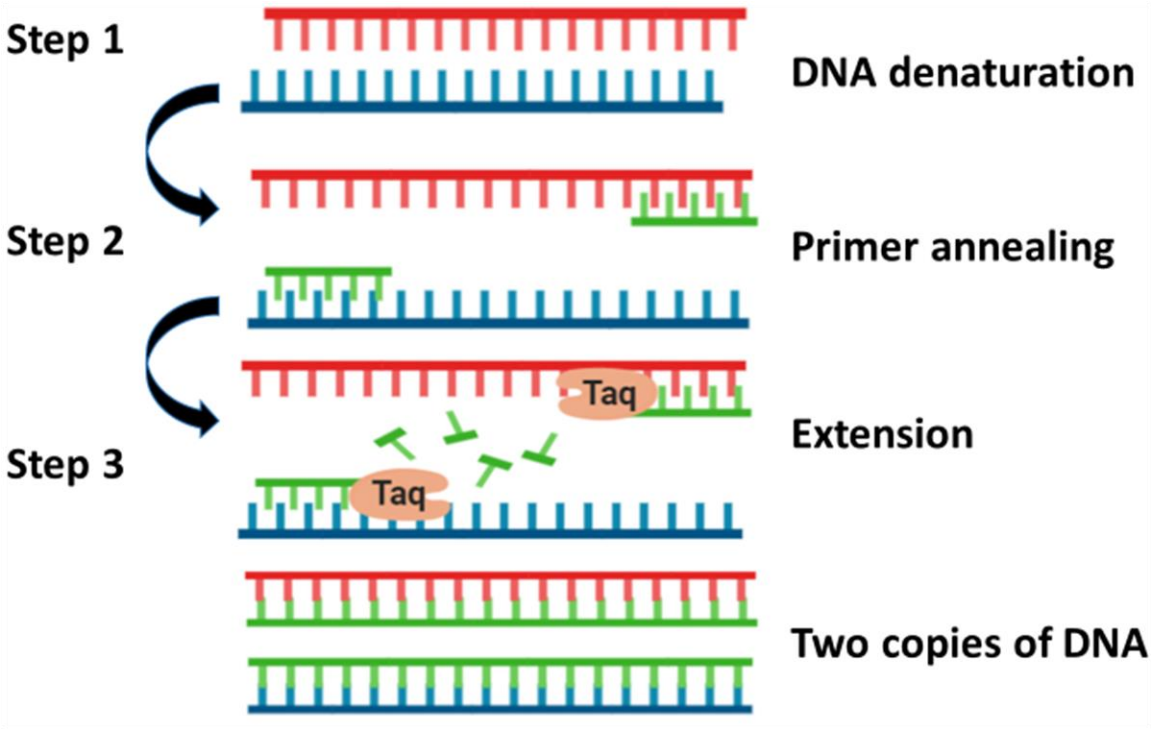

Figure 3.8: The three steps of a single PCR cycle progressing up to DNA amplification (prepared by author).

Unlike conventional PCR, quantitative PCR (qPCR) allows for the quantification of PCR amplicons generated during each cycle of the PCR process (Pestana, Belak et al. 2010). Amplicons can be detected using SYBR® Green 1, which is a DNA-intercalating dye. The dye fluoresces intensely when it binds to the minor groves of dsDNA. Therefore, the intensity of the emitted fluorescence is directly proportional to the amount of dsDNA present (Arya, Shergill et al. 2005).

The expression of a housekeeping gene is analysed together with the gene of interest, in the samples. The amount of target DNA is thus reported relative to the amount of the housekeeping gene. Housekeeping genes are generally expressed at constant levels at all stages of development and their expression profiles remain relatively constant across samples (Arya, Shergill et al. 2005). This allows for the normalization of target gene expression. Quantification of gene expression is determined, by comparing the gene expressions of the target and housekeeping genes, employing the Livak and Schmittgen method. The data is expressed in terms of fold change ($2^{-\Delta\Delta Ct}$) (Schmittgen and Livak 2008).

3.8.2 Protocol

3.8.2.1 Ribonucleic acid isolation

Treatments were removed and cells were incubated with 500µl of Qiazol (Qaigen, Hilden, Germany) and 500µl 0.1M PBS (5min, RT). The cells were then mechanically lysed and transferred into 1.5ml Eppendorf's and stored overnight in Qiazol (-80°C). Thereafter, cells were thawed and 100µl of chloroform was added, then centrifuged (2,000xg, 15min, 4°C). Supernatant was collected and 250µl of isopropanol was added, samples were the stored overnight (-80°C). Samples were thawed and centrifuged (2,000xg, 20min, 4°C), the supernatants were discarded, and the resulting pellet was washed with 500µl ethanol (75%) and centrifuged (7,400xg, 15 min, 4°C). The pellet was allowed to air dry, then resuspended in nuclease free water (15µl) and placed on ice. The amount of ribonucleic acid (RNA) was then quantified using the Nanodrop2000 spectrophotometer (ThermoFisher Scientific), the A260/A280 ratios were used to evaluate RNA integrity. The RNA concentration of samples was then standardized to 1000ng/µl.

3.8.2.2 Complementary deoxyribonucleic acid synthesis

Complementary DNA was synthesized from crude RNA standards, using the iScript™ cDNA synthesis Kit (Bio-Rad, catalogue no. 107-8890) as per manufacturer's instructions. A reaction mix was prepared for each sample (Table 3.3)

Table 3.3: cDNA synthesis mixture for a single sample

Reagent	1 x reaction volume (µl)
5X iScript reaction mixture	2
iScript reverse transcriptase	0.5
Nuclease free water	1
Standardized RNA template	2

The samples were them incubated in a thermocycler (GeneAmp® PCR System 9700, Applied Biosciences, California, USA) for 40min (5min at 25°C, 30min at 42°C, and lastly 5min at 85°C). The cDNA was then stored (-20°C).

3.8.2.3 Quantitative Polymerase Chain Reaction

Gene expression of target genes was determined using the iQ™ SYBR® Green PCR kit and CFX Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA), as per manufacturer's instructions. The mRNA expressions of *ABCA1*, *LXR*, *LDLR* and *PCSK9* were investigated using forward and reverse primers (Table 3.5). The housekeeping gene, *GAPDH* was also determined as normalization factor. A reaction master mix was prepared to evaluate gene expressions in triplicate (Table 3.4).

Table 3.4: Reaction mix for a single qPCR

Reagent	1 x reaction volume (µl)
SYBR® Green	5
Forward primer	1
Reverse primer	1
Nuclease free water	2
cDNA template	1

The CFX Touch™ Real Time PCR Detection System was used to amplify the samples. As per qPCR conditions, the initial denaturation occurred at 95°C (4min), followed by 37 denaturation cycles [95°C, 15 seconds (sec)]; then annealing (40sec, temperatures as per Table 3.5) and extension (72°C, 30sec). The plate was then read.

To determine changes in relative mRNA expression, the method described by Livak and Schmittgen was applied. The fold change in mRNA expression is expressed as $2^{-\Delta\Delta Ct}$. The expression of target genes was normalized against the expression of the housekeeping gene (*GAPDH*), which amplified simultaneously, under the same conditions.

Table 3.5: The primer sequences and annealing temperatures and target genes.

Gene	Annealing Temperature (°C)	Primer	Sequence (5'→ 3')
ABCA1	57	Forward Reverse	GGAAGAACAGTCATTGGGACAC GCTACAAACCCTTTTAGCCAGT
SIRT1	57	Forward Reverse	AGGACATCGAGGAACTACCTG GATCTTCCAGATCCTCAAGCG
LXR	58	Forward Reverse	CCTTCAGAACCCACAGAGATCC ACGCTGCATAGCTCGTTCC
LDLR	62	Forward Reverse	CCCCGCAGATCAAACCCCCACC AGACCCCCAGGCAAAGGACACGA
PCSK9	62	Forward Reverse	CCAAGATCCTGCATGTCTTCC AACTTCAAGGCCAGCTCCAG
SREBP1	58	Forward Reverse	ACAGTGACTTCCCTGGCCTAT GCATGGACGGGTACATCTTCAA
GAPDH	-	Forward Reverse	TCCACCACCCTGTTGCTGTA ACCACAGTCCATGCCATCAC

3.9 Total cholesterol

Total cholesterol in supernatants was determined by Quest Africa Diagnostics (a commercial pathology laboratory, Durban, South Africa).

3.10 Statistical analysis

Microsoft Excel 2019 and GraphPad Prism version 5.0 (GraphPad Software Inc., California) were employed to perform statistical analysis. The unpaired t-test. All results were presented as the mean ± standard deviation unless otherwise stated. A value of $p<0.05$ was considered statistically significant.

CHAPTER 4: RESULTS

4.1 HepG2 cells express differential metabolic activity when exposed to FB_1 in glucose and galactose media

Cell viability and metabolic activity of cells can be estimated quantitatively via exposure to tetrazolium salts. This provides viability and metabolic distinction, between glucose and galactose metabolism in FB_1 treated HepG2 cells.

The MTT assay displays contrasting relations to cell viability and media type, in FB_1 treated cells. There is a general decrease in percent cell viability for the galactose media, while no significant change in percent cell viability is observed for the glucose medium (Figure 4.1). After 6 hrs, cell viability of the galactose medium was significantly decreased (25µM FB_1), accounting for a 50% decrease in cell viability. Thus, treatment with FB_1 supplemented by galactose was found to be acutely toxic with increased mycotoxin concentrations (0-250µM FB_1), resulting in a decrease in cell viability and metabolic activity.

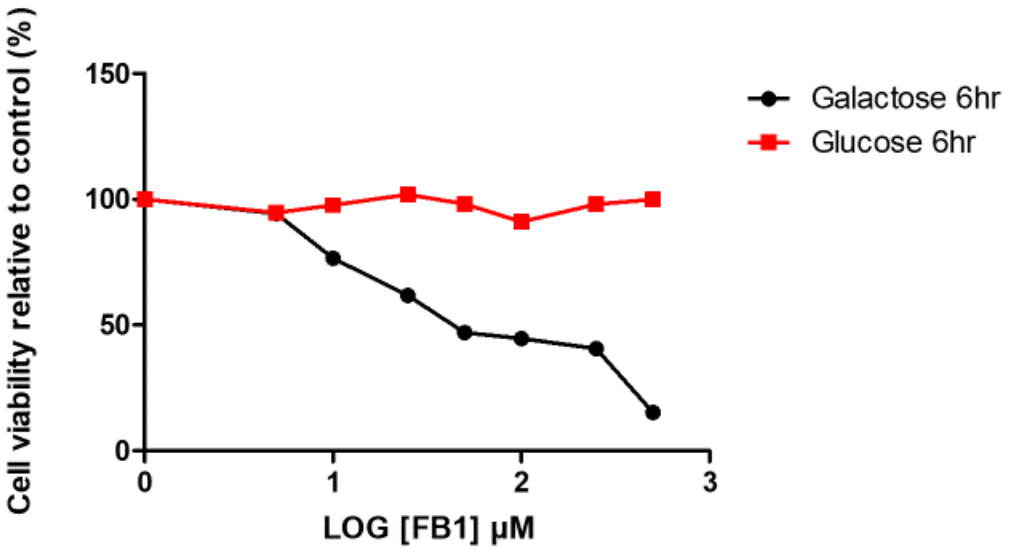

Figure 4.1: Results of MTT assay for HepG2 cells treated with FB_1 in glucose and galactose supplemented media.

4.2 HepG2 cells exhibit a decrease in metabolic activity however, without membrane disruption following FB_1 exposure

ATP is the energy currency of the cell, through determination of the level of ATP, the effect of FB_1 on metabolic output can be uncovered. The ATP concentrations of FB_1 treated cells was determined using the Cell Glo luminometry assay. In accordance with the decrease in viability (Figure 4.1), the metabolic output of cells decreased significantly in galactose media (Figure 4.2).

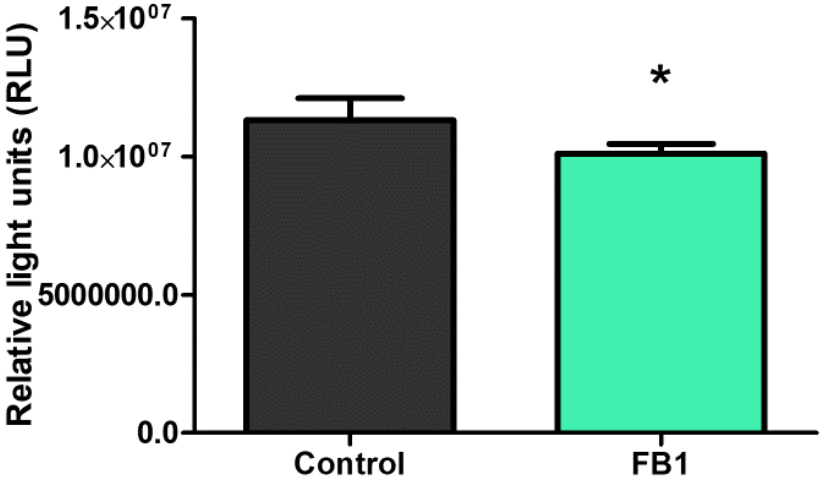

Figure 4.2: A significant decrease in ATP concentration following 6hr treatment of FB_1 on HepG2 cells. **p<0.01.

As cholesterol is a major component of the cell membrane, the effect of FB_1 on membrane integrity was considered. Fumonisin B_1 did not jeopardise membrane integrity, as there was no significant change in detection of LDH leakage from cells, when compared to the control (Figure 4.3).

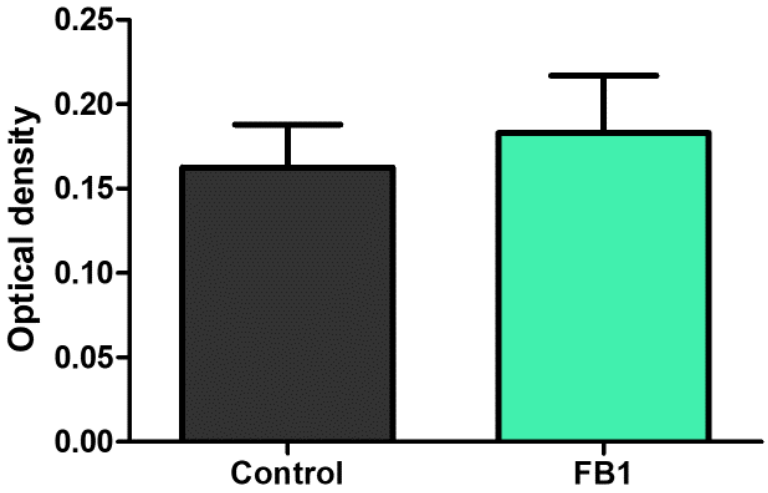

Figure 4.3: FB_1 caused no significant membrane damage following 6hr treatment of FB_1 on HepG2 cells.

4.3 A minor change in cholesterol was demonstrated in HepG2 cells

In order to survey the effect of FB_1 on cholesterol flux, total cholesterol of cell supernatants was evaluated. Surprisingly, no significant change in total cholesterol was observed in FB_1 treated cells (Figure 4.4).

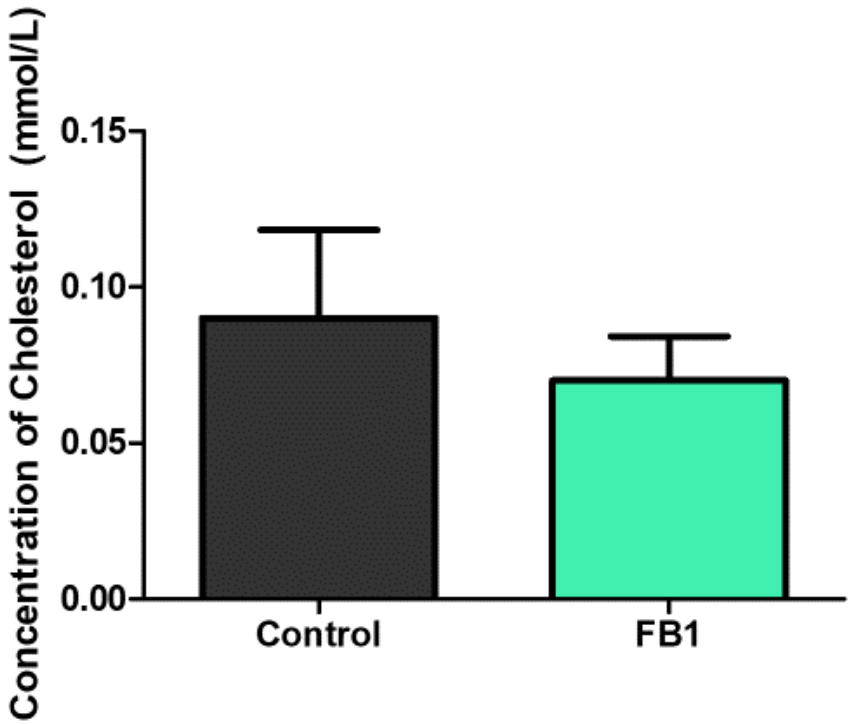

Figure 4.4: Result of laboratory determination of total cholesterol reveal no significant change of total cholesterol in HepG2 cells exposed to FB_1.

4.4 FB_1 increased SIRT1 expression to regulate the key lipogenic activator SREBP1

The SIRT1 deacetylase has a critical role in cellular metabolism and stress responses by modulating the activity of transcription factors and cofactors by protein deacetylation. SIRT1 is an established regulator of lipid/cholesterol homeostasis and is known to deacetylate SREBP1 and positively regulates LXR (Li, Zhang et al. 2007, Ponugoti, Kim et al. 2010). Accordingly, the gene and protein expressions of SIRT1 and SREBP were determined, the controllers of expression of fatty acid and triglyceride synthesis.

The test concentration of FB_1 increased SIRT1 gene (Figure 4.5A) and protein (Figure 4.5B) expression considerably in comparison to the control. Meanwhile a significant increase in SREBP1 gene (4.5C) expression was observed, but a significant decrease in protein expression was discovered (4.5D).

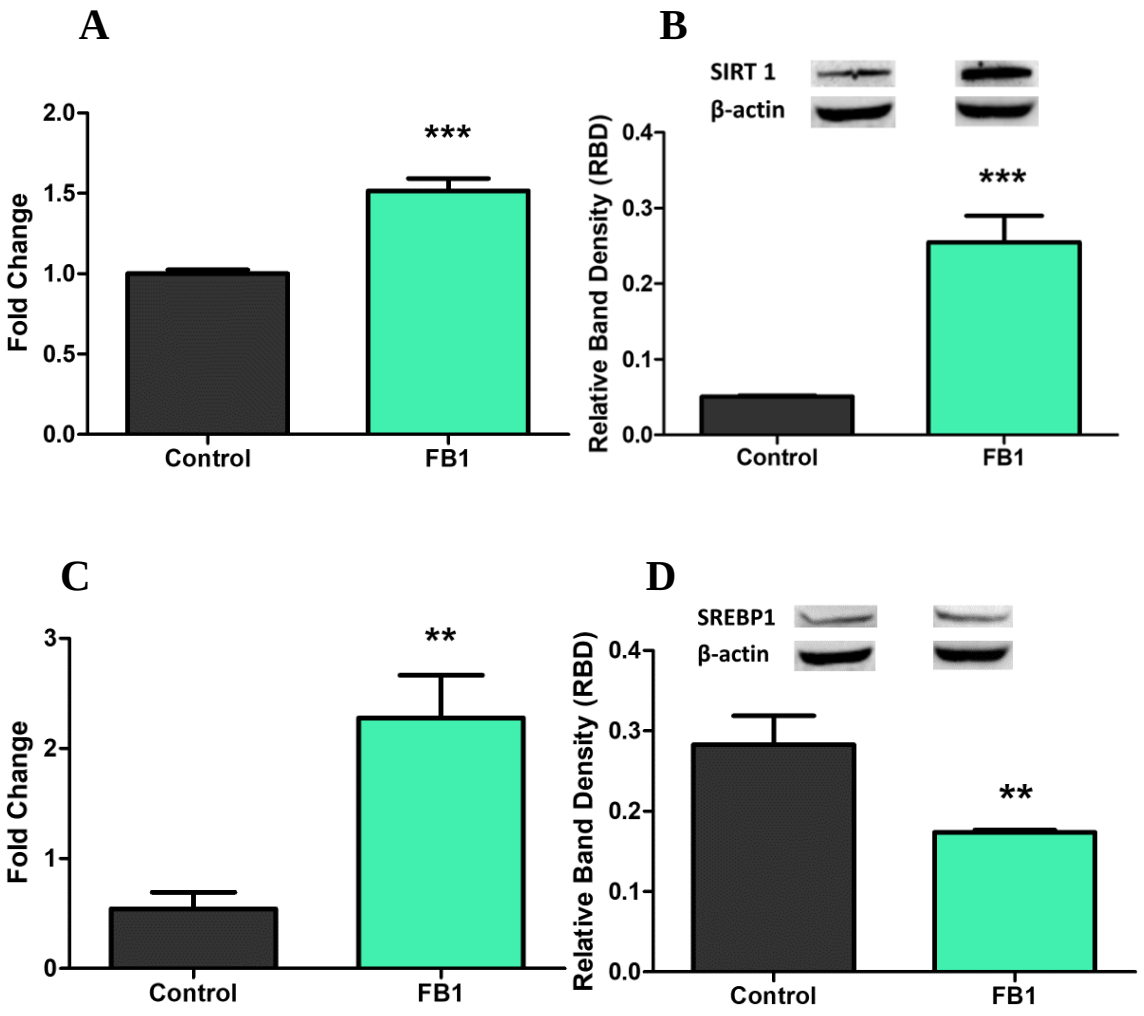

Figure 4.5: In HepG2 cells FB_1 increased the gene (A) and protein (B) expression of SIRT1 but increased SREBP1 gene expression and decreased SREBP1 protein expression **p<0.01, ***p<0.001.

4.5 FB_1 increases expression of lipogenic transcription factor LXR and decreases the expression of PCSK9

LXR is also a chief transcriptional regulator of cholesterol metabolism and homeostasis while, PCSK9 is a regulator of LDLR. Both LXR and PCSK9 are major regulatory proteins involved in cholesterol metabolism and homeostasis, that are regulated by SIRT1. Intrinsically the influence of FB_1 on their expressions was explored.

FB_1 significantly increased the gene and protein expressions of LXR (4.6A and 4.6B), whereas a significant decrease in expression was observed for PCSK9 (4.6C and 4.6D).

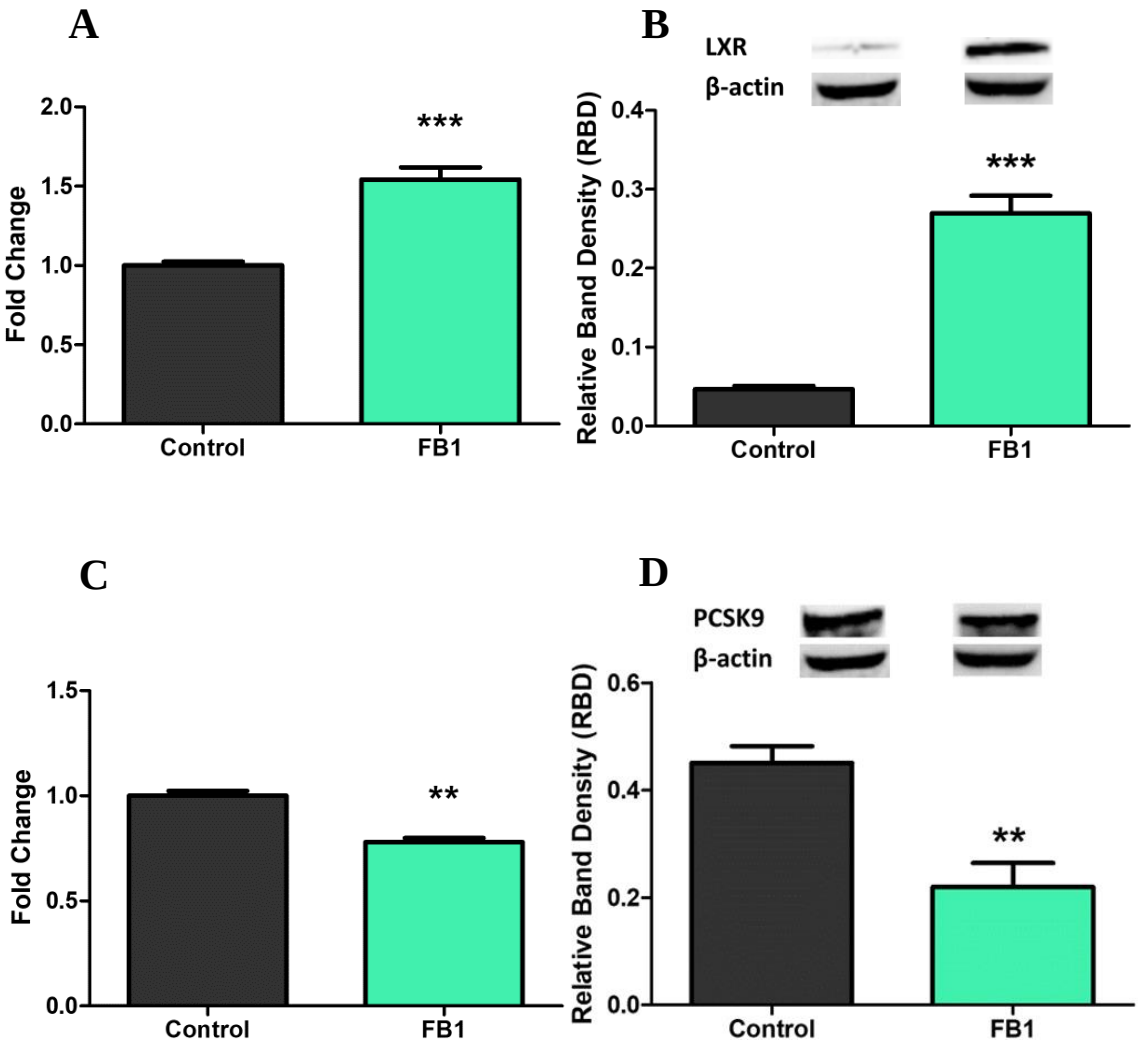

Figure 4.6: FB_1 regulates cholesterol cellular flux in a PCSK9 dependent manner in HepG2 cells by decreasing its expression (A and B) irrespective of elevated expression of its transcriptional activator (C and D). **p<0.01, ***p<0.0001.

4.6 FB_1 impacts cholesterol flux in HepG2 cells

To ascertain the effect of FB_1 on cholesterol flux, the expression profiles of cholesterol influx and efflux proteins were considered. FB_1 increased gene and protein expression of LDLR significantly (Figure 4.7A and 4.7B) in comparison to the control. A minor decrease in ABCA1 gene expression was observed for the FB_1 treatment (Figure 4.7C), while a significant increase in protein expression (Figure 4.7D) was noted in contrast to the control.

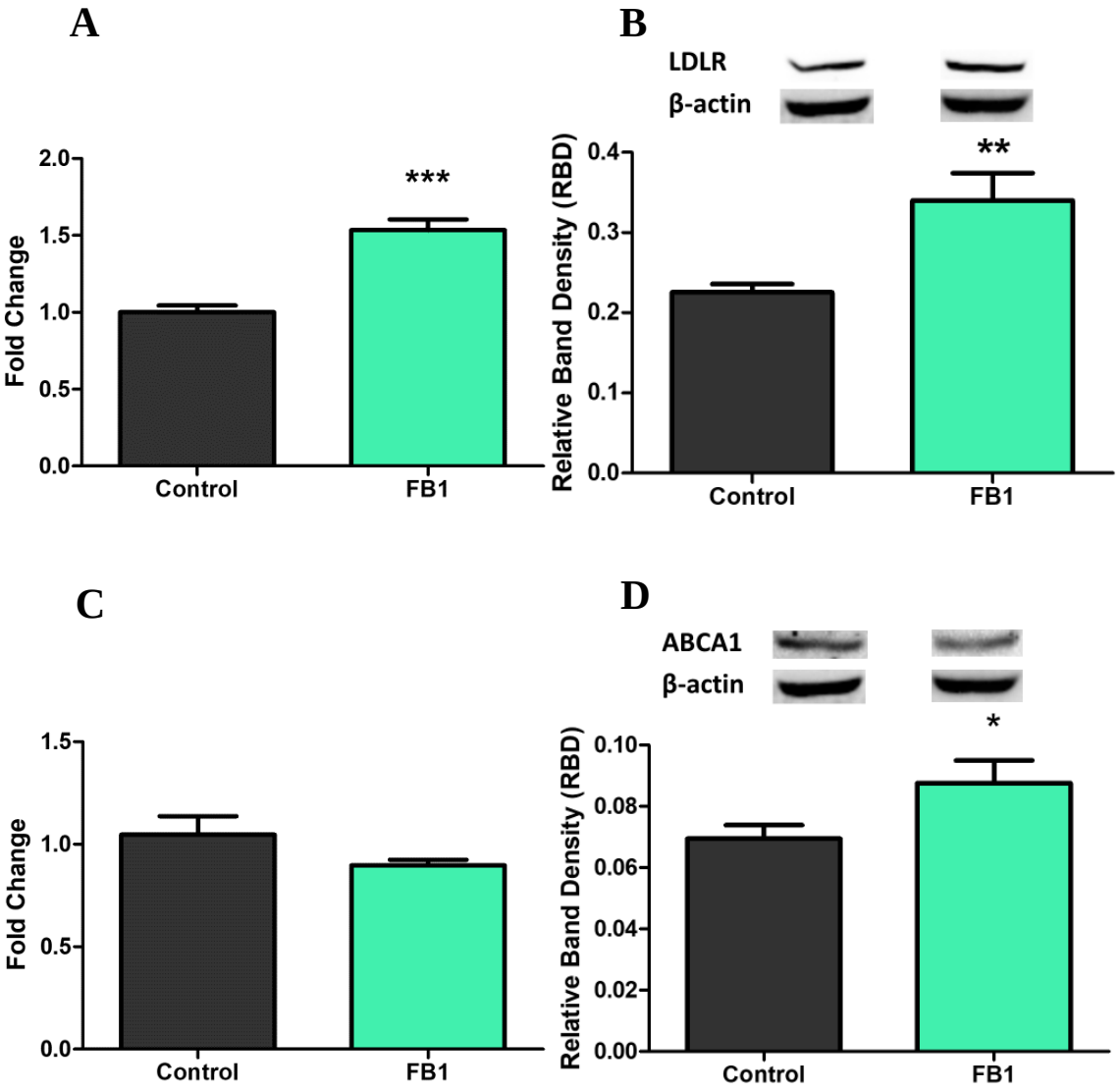

Figure 4.7: In HepG2 cells FB_1 stimulates lipid uptake (B) but also promotes cholesterol efflux (D). *p<0.5, **<0.01, ***p<0.001.

CHAPTER 5: DISCUSSION

Mycotoxins have attracted worldwide attention owing to their negative impacts on human health and economic losses. However, the downsides of economic calamity do not exceed the harm to human life. The physiological consequence of FB_1 toxicity is attributed to dysregulated lipid metabolism and lipidomic profiles which can disrupt membrane integrity and lipid raft formation (Merrill Jr, Schmeiz et al. 1997, Riedel, Abel et al. 2016, Burger, Abel et al. 2018). It is known that FB_1 inhibits ceramide synthase with a consequential accumulation of sphinganine, sphingosine and their 1-phosphate derivatives, while depleting complex sphingolipids (Merrill Jr, Schmeiz et al. 1997). In addition, *in vivo* and *in vitro* studies in rat liver and primary hepatocyte models show that FB_1 increased circulating cholesterol levels (Riedel, Abel et al. 2016, Burger, Abel et al. 2018). In the present study the effect of FB_1 on SIRT1 is established and evidence is provided to suggest that FB_1 disrupts cholesterol homeostasis in a cholesterol flux dependent manner, in the HepG2 liver cell line.

The liver is the central metabolic organ and is an important modulator of cholesterol homeostasis (Takeyama, Uehara et al. 2017). The HepG2 cell line is the ideal model for investigating the effect of xenobiotics on the human liver and have the ability to adapt their phenotype in response to nutrient availability (Snopov, Teryukova et al. 2017, Lai, Forde et al. 2018). The MTT assay illustrates that HepG2 cells exposed to FB_1 supplemented with galactose, is more cytotoxic as compared to HepG2 cells exposed to FB_1 in glucose media, after 6hrs. These findings advocate that there is a stark contrast in HepG2 metabolic response to FB_1, between glucose and galactose media. Thus, the decrease in metabolic activity as highlighted by the decrease in cell viability, supports the phenotypic adaptation of HepG2 cells.

An IC_{50} value of 25µM for FB_1 (6hrs) was obtained for HepG2 cells supplemented with galactose media, from the MTT assay. This IC_{50} is much lower than the 200µM reported previously following a 24hr treatment (Chuturgoon, Phulukdaree et al. 2014, Arumugam, Ghazi et al. 2020). This difference may be due to the different treatment times, and the growth medium (glucose vs galactose). It was also noted that glucose offered protection against FB_1 toxicity in swine and rats (Liu, Lu et al. 2001, Fernandez-Surumay, Osweiler et al. 2004). As such, the galactose media was used in subsequent assays, as this forced the cells to adopt aerobic metabolism. This adjustment in metabolism mimics the metabolic profile of normal cellular metabolism (Aguer, Gambarotta et al. 2011).

In addition to the decreased cell viability (Figure 4.1), the metabolic output of HepG2 cells declined significantly, as illustrated by a decreased cellular ATP output (Figure 4.2). This result is predictable, as with decreased cell viability, a decrease in ATP would be expected. In this study HepG2 cells were stimulated to divert from glycolysis, to rely on OXPHOS to provide energy, by replacing glucose media with galactose (Aguer, Gambarotta et al. 2011, Shiratori, Furuichi et al. 2019, Orlicka-Płocka, Gurda-Wozna et al. 2020). Glycolysis is less energy efficient, yielding less energy than OXPHOS. However, this alone does not explain the decrease in cellular ATP level. It is therefore speculated, that the decrease in ATP level is due to FB_1. In rat primary astrocytes and human neuroblastoma, FB_1 was found to inhibit mitochondrial complex I, resulting in a decreased rate of mitochondrial and cellular respiration (Domijan and Abramov 2011). Previously it was suggested that glucose deprivation and mitochondrial complex I inhibition, synergize and induce cancer cell death (Palorini, Simonetto et al. 2013). Therefore, it is presumed that the decrease in ATP level, is due to the effect of FB_1.

Cell death is a result of activation of intrinsic cell death programs or passive disruption of membrane integrity, by external factors (Zhang, Chen et al. 2018). The LDH leakage assay is particularly sensitive to chemicals that directly damage cellular membranes (Ivanova and Uhlig 2008). The data herein indicate that membrane integrity was not compromised after the exposure to FB_1 for 6hrs as evidenced by no significant release of LDH (Figure 4.3). These contrasting data of decreased cell viability, decreased ATP output and no disruption of membrane integrity, suggests that FB_1- induced loss of membrane integrity is dependent on factors such as cell/tissue type, concentration, and duration of exposure.

The role of cholesterol in cancer development has increased, clinical evidence suggests that changes in cholesterol metabolism is associated with cancer (Silvente-Poirot and Poirot 2012), and that cholesterol can activate oncogenic signalling (Ding, Zhang et al. 2019). Changes in cholesterol metabolism during cancer, occur during cholesterol synthesis and cholesterol flux. Previously it was reported that FB_1 decreased the level of free cholesterol within primary rat hepatocytes (Gelderblom, Smuts et al. 1996), as a result of a decreased level of sphingomyelin in cell membranes, which has an influence on cholesterol synthesis and metabolism (Gupta and Rudney 1991) .The determination of total cholesterol in cell supernatants suggest that FB_1, has no significant change in cholesterol flux, evidenced by no significant change in cholesterol level (Figure 4.4). However, a decrease in cholesterol levels were observed, and can be attributed to minor changes in cholesterol flux proteins, over a short FB_1 exposure. Another reason may be that the laboratory method for total cholesterol determination, is specialized for blood samples and not optimized for cell supernatants.

Various studies have highlighted the role of SIRT1 as a tumour promoter, with evidence of increased expression in hepatocellular carcinoma (Chen, Jeng et al. 2012, Lin and Fang 2013). In addition, SIRT1 has a major role in cholesterol regulation, due to its effect on SREBPs and LXRs (Ponugoti, Kim et al. 2010, Kemper, Choi et al. 2013). The data presented in this study highlights the possible role of SIRT1 regulatory activity on SREBP-1c, LXR and PCSK9 in FB_1 treated HepG2 cells, supplemented by galactose media, for 6hrs.

The expression of SIRT1 is dependent on the levels of ATP, with ATP negatively regulating SIRT1 activity (Kang, Oka et al. 2017). QPCR and Western blot analysis of SIRT1 exhibit significant increases in both gene and protein expressions (Figure 4.5A and 4.5B) with a decrease in ATP levels (Figure 4.2). This is the first observation on the effect of FB_1 on SIRT1, despite the influence of ATP. Concurrently, differential gene and protein expression of SREBP-1c was noted (Figure 4.5C and 4.5D), in FB_1 treated HepG2 cells. The decrease in SREBP-1c protein expression is explained by the deacetylase activity of SIRT1. The deacetylation of SREBP-1c by SIRT1 at Lys-289 and Lys-309 (Ponugoti, Kim et al. 2010), inhibits SREBP-1c activity by decreasing its stability and its association to its lipogenic target gene promoters. However, this observation does not coincide with the observed increased gene expression. This is attributed to the vast regulation of SREBP-1c at the transcriptional level (Eberlé, Hegarty et al. 2004). The expression of SREBP-1c can be up-regulated by insulin signalling due to carbohydrate uptake (Azzout-Marniche, BÉCARD et al. 2000), or via LXR-mediated induction, which can manage fatty acid and cholesterol homeostasis (Repa, Liang et al. 2000, Schultz, Tu et al. 2000) or by autoactivation (Amemiya-Kudo, Shimano et al. 2000). Therefore, the increase in SREBP-1c gene expression can be explained further, by activity of LXR.

It is known that LXRs play a critical role in cholesterol metabolism, and are targeted by SIRT1 for deacetylation, promoting the activity of LXR (Feige and Auwerx 2007). Both LXR and SREBP-1c contribute to the transcriptional activation of the SREBP-1c gene (Yoshikawa, Shimano et al. 2001), therefore the significant increase in LXR expressions (Figure 4.6A and 4.6B), can account for the increase in *SREBP-1c* despite the low protein expression of SREBP-1c. LXR can act as a cholesterol sensor, and induce SREBP-1c for the generation of fatty acids for the formation of cholesterol esters (Tontonoz and Mangelsdorf 2003).

LXR and PSCK9 further regulate the expression of cholesterol flux proteins. The transcription of LDLR is regulated by a pathway involving LXR, which maintains cholesterol homeostasis through upregulation of the IDOL, consequently activating the ubiquitination and degradation of LDLR (Zelcer, Hong et al. 2009). Despite the increase in LXR expression, there is no decrease in LDLR gene and protein expression (Figure 4.7A and 4.7B). This suggest that the inhibitory effect of LXR on the LDLR pathway, through the transcriptional induction of IDOL, is not active. However, this study does not consider the effect of IDOL on LDLR. Thus, the attention was turned to PCSK9, the induced degrader of LDLR (Li, Dong et al. 2009). PCSK9 is a protein involved in the post-translational degradation of LDLR, thereby restricting its recycling to the cell membrane (Athavale, Chouhan et al. 2018). This is supported by the data, which highlights that gene and protein expression of PCSK9 had decreased significantly, in HepG2 cells treated with FB_1 (Figure 4.6C and 4.6D).

These contrasting data suggests that the deacetylation of LXR by SIRT1, prevents the degradation of LDLR via the IDOL pathway, and suppresses the activity of PCSK9. This suggestion is supported by the evidence that SIRT1 suppresses the PCSK9 gene, reducing PSCK9 secretion, resulting in increased LDLR expression (Miranda, van Tits et al. 2014). This is further supported by the increased SIRT1 expression. Taken together, these findings suggest that FB_1 influences cholesterol up-take in a SIRT1 dependent manner.

To further evaluate the effect of FB_1 on cholesterol flux, the expression of ABCA1, the transport protein responsible for cholesterol efflux was determined (Bashore, Liu et al. 2019). The ABCA1 gene is targeted by LXR at the transcriptional level, to promote cholesterol efflux (Repa, Turley et al. 2000). The gene expression of ABCA1 exhibits a slight decrease, while the protein expression is significantly increased (Figure 4.7C and 4.7D). Although the protein expression of ABCA1 is significantly increased, there total cholesterol of the FB_1 treatment is lower than the control. It is speculated that the protein is not specialized for cholesterol efflux in the HepG2 cell line, but rather macrophages. The ABC transporter superfamily are responsible for cholesterol efflux in a cell specific manner, with ABCA1 mediated cholesterol efflux in macrophages and ABCG5 in hepatocytes (Luo, Yang et al. 2020). Thus, FB_1 increases the expression of ABCA1 cholesterol efflux protein, but has no apparent effect on cholesterol efflux in HepG2 cells.

The cancer promoting properties of FB_1 (Gelderblom, Abel et al. 2001) have recently been linked to changes in lipid raft composition and altered cholesterol profiles (Burger, Abel et al. 2018). Our data provides the molecular basis for carcinogenic events observed in FB_1 toxicity by dysregulating the circuitry involved in cholesterol metabolism, particularly with respect to lipid influx and cholesterol efflux from liver cells.

A summary of the effect of FB_1 on cholesterol flux and its possible role in carcinogenicity in HepG2 cells is represented in Figure 5.1.

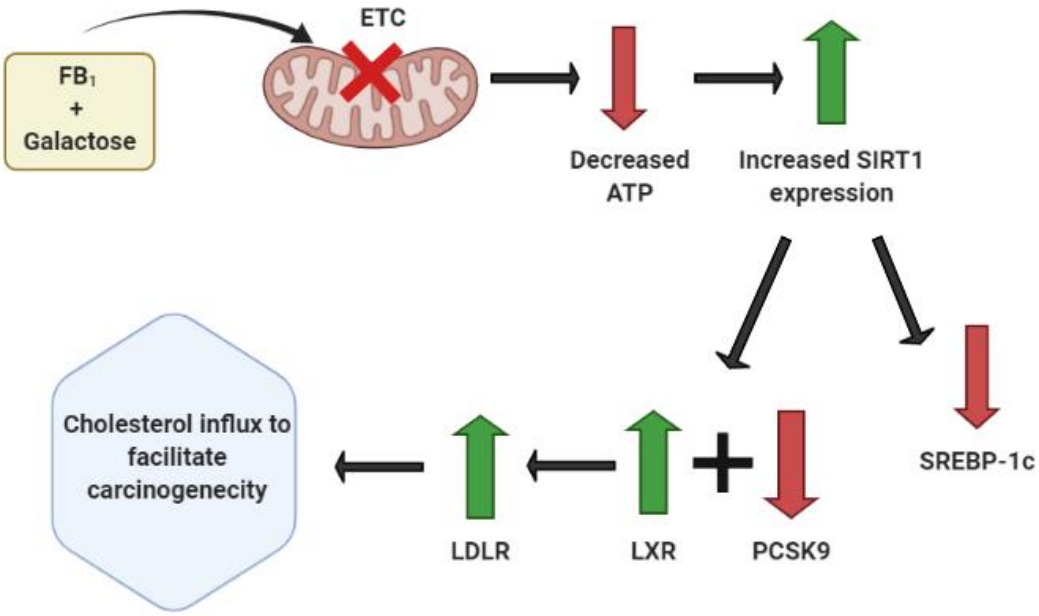

Figure 5.1: Summarised account of events. FB_1 in galactose media inhibits the ETC, resulting in decreased ATP leading to increased SIRT1 activity. This subsequently results in decreased SREBP-1c and PCSK9 expression, but increased LXR expression. As a result, the expression of LDLR in increased, leading to cholesterol influx, promoting carcinogenicity (prepared by author).

5.1 Limitations of study

This study employed an *in vitro* liver model to assess the effect on FB_1 exposure, on cholesterol homeostasis. *In vitro* studies do allow for more detailed analysis compared to *in vivo* studies however, a short coming of the employed model is that the cells are removed from their natural environment, thus eliminating interactions and mechanisms that would otherwise be available. To overcome this shortfall, the use of an *in vivo* mouse model in addition to a primary hepatocyte cell model may provide a better understanding of how cells will respond to FB_1, with respect to cholesterol homeostasis.

Due to the COVID-19 pandemic, a total cholesterol kit and ABCG5 protein antibodies could not be sourced and had to resort to alternative methods.

CHAPTER 6: CONCLUSIONS

6.1 Conclusions

With the large availability of food, a substantial part of life is spent in the postprandial state, thus allowing dietary toxins to direct cellular phenotypes and induce a state of metabolic toxicity. For the first time, the relationship between SIRT1 and FB_1 is characterized. In the context of this study, SIRT1 is induced by FB_1 in galactose supplemented HepG2 cells, leading to disturbed cholesterol flux. We further show that these effects are dependent on the LXR transcription factor and PCSK9. These results reiterate the toxicological relevance of FB_1 in the dysregulation of cholesterol metabolism and underlines the potential of this toxin in promoting changes in cholesterol metabolism and related disorders, such as cancer.

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APPENDICIES

Appendix 1: Raw data for MTT assay (6hrs)

Table 1: Raw data used to determine the IC_{50} value using the cell viability (MTT) assay.

FB_1 concentration (µM)	Log FB_1 concentration	Average absorbance		Cell viability (%)	
		Glucose media	Galactose media	Glucose media	Galactose media
0	0,000	1,26	0,42	100,00	100,00
5	0,699	1,19	0,40	94,72	94,27
10	1,000	1,23	0,32	97,74	76,61
25	1,398	1,28	0,26	101,88	61,81
50	1,699	1,23	0,20	98,17	47,10
100	2,000	1,14	0,19	91,03	44,71
250	2,398	1,23	0,17	98,04	40,73
500	2,699	1,25	0,06	99,95	15,35

Appendix 2: Standard curve for Protein standardisation

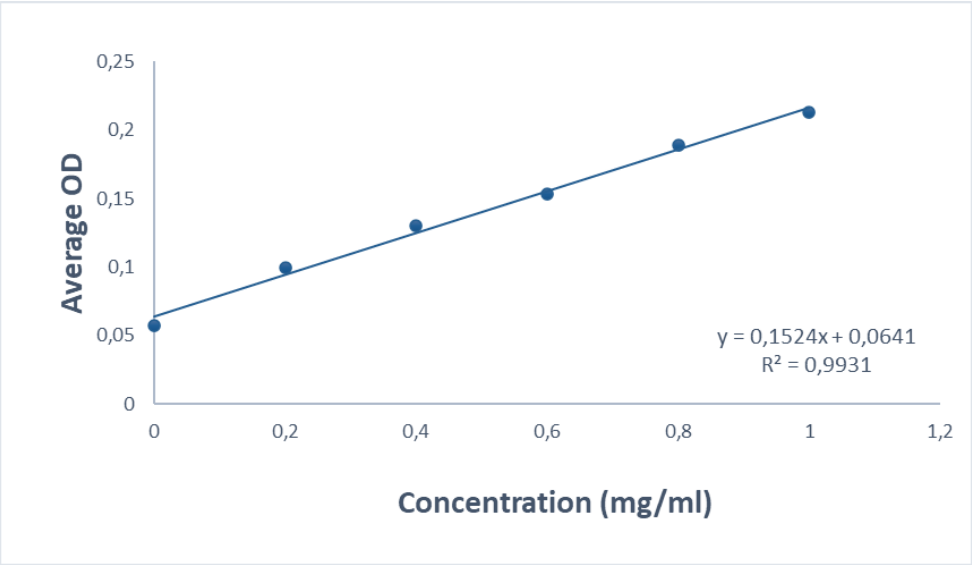

Figure 1: Standard curve of concentrations of bovine serum albumin versus optical density to determine sample protein concentrations using bicinchoninic acid assay.

Appendix 3: Full Western blot images

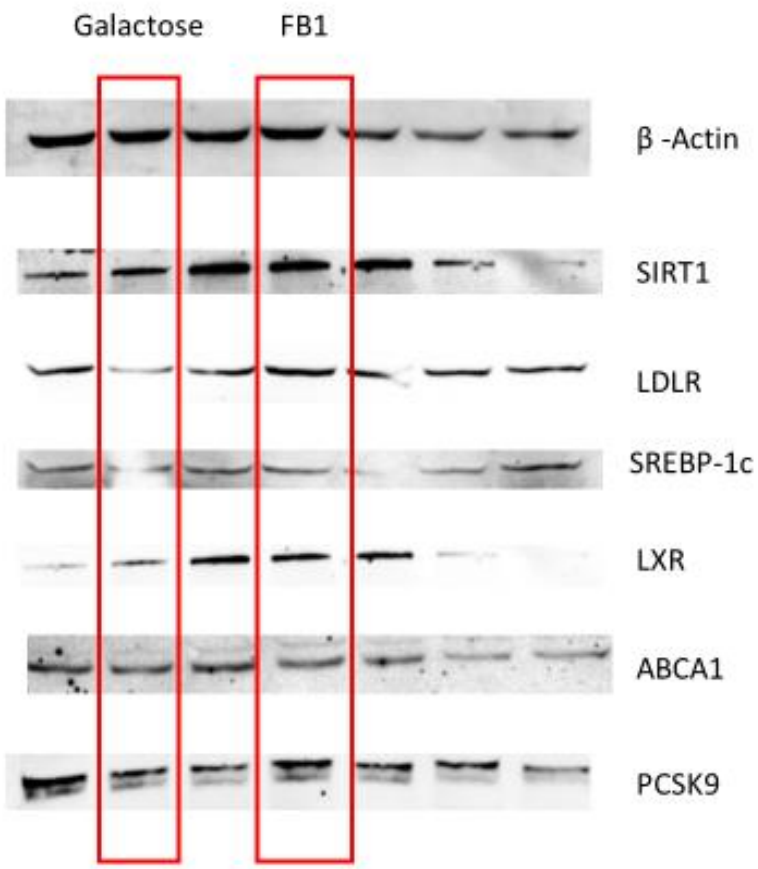

Figure 1: Complete western blot images in no particular order