

Unravelling the role of AKR1D1 in human hepatocytes

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by

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is rapidly becoming one of the leading indications for liver transplantation worldwide. The cellular processes and molecular targets that govern disease development and progression remain to be fully defined and currently there are no licensed treatments. Steroid 5 β -reductase (AKR1D1) is highly expressed in human liver where it inactivates steroid hormones and catalyses a fundamental step in bile acid synthesis. I have hypothesised that AKR1D1 plays a crucial regulatory role in hepatic metabolic homeostasis.

In human hepatoma cell lines, genetic manipulation of AKR1D1 altered primary bile acid biosynthesis and steroid hormone action. Furthermore, gene silencing of AKR1D1 increased hepatocyte triglyceride accumulation through increased *de novo* lipogenesis and decreased β -oxidation, fueling hepatocyte inflammation as well as increasing glycogen synthesis. I have shown that AKR1D1 has a potent ability to regulate the metabolic phenotype of human hepatocytes suggesting a crucial role in the pathophysiology of NAFLD.

To mum and dad

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Abbreviations

11β-HSD1	11 β -hydroxysteroid dehydrogenase type one
11β-HSD2	11 β -hydroxysteroid dehydrogenase type two
17β-HSD	17 β -hydroxysteroid dehydrogenase
17OHP	17 α -hydroxyprogesterone
3β-HSD	3 β -hydroxysteroid dehydrogenase
5αR1	Steroid 5 α -reductase 1
5αR2	Steroid 5 α -reductase 2
5β-THE	5 β -tetrahydrocortisone
5β-THF	5 β -tetrahydrocortisol
ABCA	ATP-binding cassette transporter A1
ACC	Acetyl-CoA carboxylase
ACTH	Adrenocorticotropin hormone
AKR	Aldo-keto reductase family
AKR1D1	Aldo-keto reductase family 1 member d1
ALT	Alanine-aminotransferase
AME	Apparent mineralocorticoid excess
AMPK	AMP-kinase
AR	Androgen receptor
AST	Aspartate-aminotransferase
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BA	Bile acid
BMI	Body mass index
CA	Cholic acid
cAMP	Cyclic adenosine monophosphate
CDCA	Chenodeoxycholic acid
cDNA	Complementary DNA
CETP	Cholesteryl ester transfer protein
ChREBP	Carbohydrate response element binding protein
CPT1	Carnitine palmitoyl-CoA transferase I

CRH	Corticotropin-releasing hormone
Ct	Cycle threshold
CT	Computed tomography
CVD	Cardiovascular disease
CYP11A1	Cytochrome P450scc
CYP11B1	11 β -hydroxylase
CYP11B2	Aldosterone synthase
CYP7A1	7 α -hydroxylase
CYP7B1	Non-specific 7 α -hydroxylase
CYP8B1	Sterol 12 α -hydroxylase
DBD	DNA-binding domain
DCA	Deoxycholic acid
DGAT	Diacylglycerol acyltransferase
DNL	<i>De novo</i> lipogenesis
ER	Endoplasmatic reticulum
FADH2	Flavin adenine dinucleotide reduced
FAS	Fatty acid synthase
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
FXR	Farnesoid X Receptor
FXRE	Farnesoid X receptor element
G6Pase	Glucose-6-phosphatase
GCK	Glucokinase
GLUT	Glucose transporter
GnRH	Gonadotropin-releasing hormone
GPAT	Glycerol phosphate acyltransferase
GR	Glucocorticoid Receptor
GWAs	Genome wide association studies
GWLs	Genome-wide linkage studies
HDL	High-density lipoprotein
HEK	Human embryonic kindey

HPA	Hypothalamic-pituitary-adrenal
HSPs	Heat shock proteins
IBAMP	Intestinal bile-acid binding protein
IDL	Intermediate-density lipoprotein
IDL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
JNK	c-Jun N-terminal kinase
L-PK	Liver-type pyruvate kinase
LAL	Liver-associated lymphocytes
LBD	Ligand-binding domain
LCA	Lithocholic acid
LDL	Low-density-lipoprotein
LEP	Leptin
LEPR	Leptin receptor
LH	Luteinizing hormone
LHR-1	Liver receptor homolog type 1
LPL	Lipoprotein lipase
LXR	Liver X receptor
MC2R	Melanocortin type-2 receptor
MC4R	Melanocortin type-4 receptor
MR	Mineralocorticoid Receptor
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCEP	National Cholesterol Education Program
NEFA	Non-esterified fatty acid
NF-κB	Nuclear factor-κB
NHS	National Health System
NK	Natural killer
NPY	Neuropeptide Y

NSAID	Non-steroidal anti-inflammatory drugs
PA	Primary aldosteronism
PC1	Pro-hormone convertase 1
pCMB	4-Chloromercuribenzoic acid
PCOS	Polycystic ovarian syndrome
PEP	Phosphoenolpyruvate
PFK-1	Phosphofructokinase-1
PLTP	Phospholipid transfer protein
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PVN	Hypothalamic paraventricular nucleus
PXR	Pregnane X Receptor
RANTES	Chemokine ligand 5
RXR	Retinoid X Receptor
SCD	Stearoyl-CoA desaturase
SHP	Small heterodimer partner
siRNA	small interference RNA
SRD5A1	Steroid 5 α -reductase 1 gene
SRD5A2	Steroid 5 α -reductase 2 gene
SREBF1	Sterol regulatory element-binding protein 1 (gene)
SREBP-1c	Sterol regulatory element-binding protein 1
StAR	Steroidogenic acute regulatory protein
SULT2A1	Sulfotransferase Family 2A Member 1
TAG	Triacylglycerol (triglyceride)
TGR5	G α s protein-coupled receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis alpha
TZDs	Thiazolidinediones
UCP	Uncoupling protein
UDCA	Ursodeoxycholic acid
VLDL	Very low-density lipoprotein
WHO	World Health Organisation
ZF	Zona fasciculata
ZG	Zona glomerulosa

ZR

Zona reticularis

Chapter 1 General Introduction

1.1. General background

The global burden of metabolic disease, including obesity, type 2 diabetes mellitus (T2DM) and its hepatic manifestation, non-alcoholic fatty liver disease (NAFLD), continues to escalate. NAFLD is common, with an unselected population prevalence of approximately 30%, rising to over 80% in obese patients and those with T2DM. It is a spectrum of disease ranging from simple steatosis through to inflammation (non-alcoholic steatohepatitis, NASH) and eventual scarring, fibrosis and cirrhosis with a significant risk of development of hepatocellular carcinoma (HCC) alongside increased cardiovascular mortality (Ekstedt et al. 2015).

Steroid hormones, including glucocorticoids and androgens are potent regulators of metabolic phenotype and have been implicated in driving hepatic lipid accumulation. Glucocorticoid excess, androgen deficiency in men, and excess in women are all associated with NAFLD (Hazlehurst & Tomlinson 2013). Steroid hormone action is regulated at a pre-receptor level within key target tissues, including the liver, by a series of enzymes that can either enhance their clearance or augment their action. As examples, the isozymes of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) interconvert cortisol (the active major circulating glucocorticoid) with inactive cortisone; 11 β -HSD2 inactivates cortisol to cortisone (largely in mineralocorticoid sensitive tissues such as the kidney), whilst 11 β -HSD1 regenerates cortisol from cortisone and amplifies local glucocorticoid action. In parallel, steroid 5 α -reductases (SRD5A1 and SRD5A2) both inactivate glucocorticoids, and simultaneously generate the most potent androgen, dihydrotestosterone (5 α -DHT) from less active testosterone. Genetic and pharmacological manipulation of both 11 β -HSD1 and 5 α -reductase activity has been demonstrated to have a profound impact upon metabolic phenotype including the

development of hepatic steatosis (Dowman et al. 2013; Hazlehurst et al. 2016; Hermanowski-Vosatka et al. 2005; Masuzaki et al. 2001; Morton et al. 2001).

Steroid 5 β -reductase (AKR1D1) also has an important pre-receptor role in the regulation of steroid hormone action. AKR1D1 is highly expressed in the liver where it generates all 5 β -reduced dihydrosteroid metabolites for C19-C27 steroids, including glucocorticoids, mineralocorticoids and androgens (Chen & Penning 2014). Both cortisol and cortisone are metabolised to 5 β -dihydrocortisol and 5 β -dihydrocortisone respectively by AKR1D1, and are then subsequently converted, in a non-rate limiting step, to their tetrahydro-metabolites (tetrahydrocortisol, 5 β -THF and tetrahydrocortisone, 5 β -THE) by 3 α -hydroxysteroid dehydrogenases (AKR1C1-AKR1C4) which are also members of the AKR superfamily (Penning et al. 2000). AKR1D1 also inactivates progesterone and testosterone to 5 β -dihydroprogesterone and 5 β -DHT, respectively. However, in addition to its role in steroid hormone clearance, it has a crucial function in bile acid synthesis. Bile acids are now established as potent signalling molecules that regulate metabolic phenotype acting through previously orphan nuclear receptors including the farnesoid-X-receptor (FXR). Oxysterols, including bile acid intermediary substrates, are also able to activate the liver-X receptor α (LXR α) (Janowski et al. 1996). FXR and LXR α are both nuclear hormone receptors and their activation has been implicated in the pathogenesis of NAFLD. FXR deletion results in hepatic steatosis, insulin resistance and hyperglycaemia with evidence suggesting decreased expression in NAFLD (Cipriani et al. 2010; Yang et al. 2010). FXR agonism has already shown early promise as a therapeutic strategy in NAFLD (Neuschwander-Tetri et al. 2015). In contrast, LXR α expression is increased in NAFLD (Janowski et al. 1996) and activation drives lipid accumulation whilst suppressing the local inflammatory response (Ahn et al.

2014; Ito et al. 2015). In rodent models, LXR antagonism attenuates high fat diet induced hepatic steatosis (Sim et al. 2014), but clinical translational data are lacking.

AKR1D1 therefore sits at the interface between two fundamentally important biological pathways (steroid hormone metabolism and bile acid synthesis) both of which are potent regulators of metabolic phenotype. The putative role of AKR1D1 in this regard is almost entirely unexplored; a single study utilizing the measurement of urinary steroid hormone metabolites has demonstrated an association between increased 5 β -reductase activity and hepatic steatosis (Westerbacka et al. 2003). More recently, levels of 7- α -hydroxy-4-cholesten-3-one, the bile acid precursor substrate for AKR1D1, have been shown to be elevated in patients with NAFLD (Mouzaki et al. 2016).

1.2. Obesity epidemic

The prevalence of obesity continues to escalate and more than 1.9 billion adults worldwide are now classified as overweight or obese (Ahmad & Edwards 2016). Childhood obesity is also increasing at an alarming rate and, according to the World Health Organisation (WHO), 41 million children under the age of 5 were classified as being overweight or obese in 2014. Obesity was recognised as a disease by WHO in 1975 (James 2008). Overweight and obesity are defined based on a body mass index (BMI) from 25 to 29.9kg/m² and higher than 30kg/m², respectively (Mitchell et al. 2011).

The prevalence of obesity has been recorded in both developing and developed countries, but with different sex patterns (Figure 1.1-1); in addition, the absolute rates are higher in individuals with low income and education levels (Molarius et al. 2000).

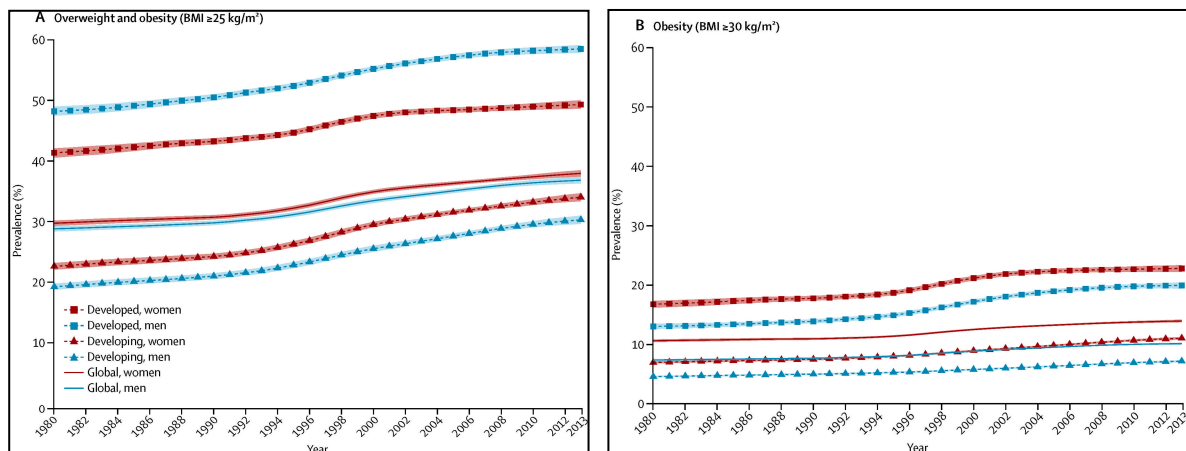


Figure 1.2-1 Age-standardised prevalence of overweight and obesity and obesity alone, ages ≥ 20 years, by sex, 1980–2013, taken from Ng et al. (2014).

Overweight and obesity increase the risk of many chronic diseases. Recent studies have demonstrated increased all-cause mortality in overweight and obese individuals and obesity is now linked to several comorbidities, including cardiovascular disease (CVD), type 2 diabetes

mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), and many forms of cancer (Berrington de Gonzalez et al. 2010; Shea et al. 2012).

Overweight and obesity bring an economic burden on health services and on the wider economy. In 2006-7, overweight and obesity cost the National Health Service (NHS) more than £5 billion (Scarborough et al. 2011). Moreover, in 2007, a report from the UK's Office for Science Foresight Programme estimated that, if obesity rates continued to escalate it would add another £5.5 billion in medical costs by 2050 (Wang et al. 2011) (Figure 1.1-2).

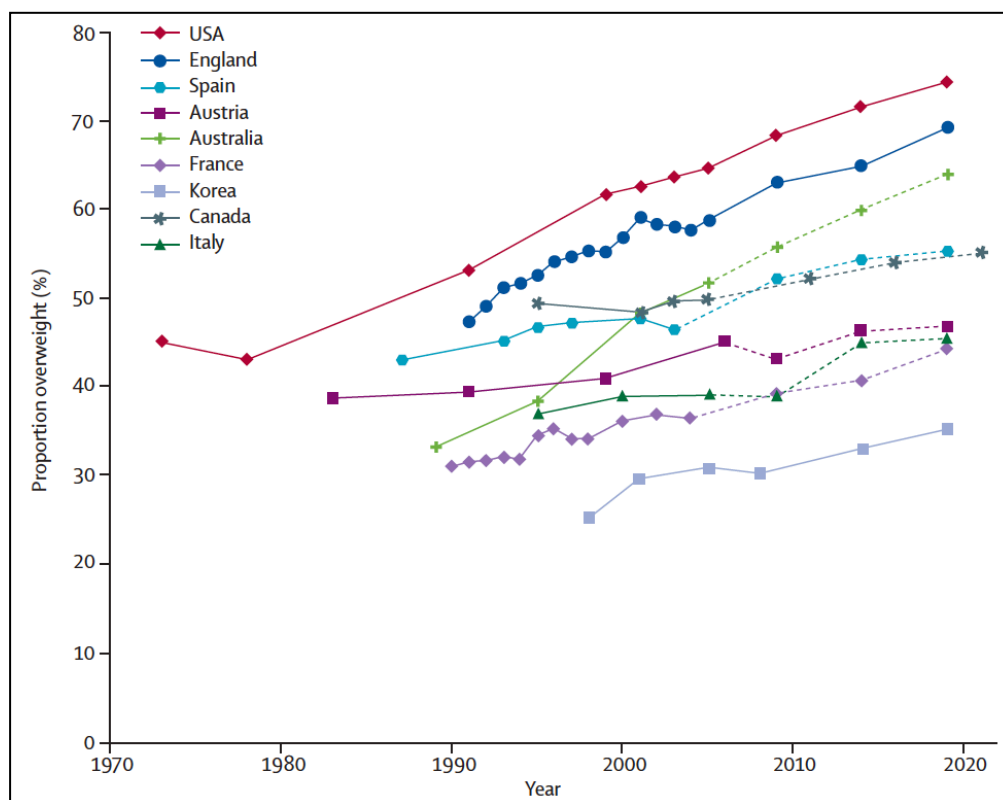


Figure 1.2-2 Past and projected prevalence of overweight ($BMI \geq 25 \text{ kg/m}^2$), taken from Wang et al. (2011).

1.2.1. Genetic causes of obesity

Obesity is a complex and multifactorial condition characterised by energy imbalance and increased weight gain. Although lifestyle is widely known to contribute to obesity, genetic factors also play a crucial role in the development of obese phenotype. Over the years, numerous studies have been focused on the genetic background of obesity and most of the genes implicated in the appearance of the obese phenotype are associated with food/energy intake, energy expenditure, and genes involved in adipogenesis (O’Rahilly & Farooqi 2008; Shawky & Sadik 2012).

Since 1940, when Hetherington and Ranson first suggested that the hypothalamus was involved in the regulation of energy metabolism (Hetherington & Ranson 1940), it has been generally accepted that signalling molecules involved in the central nervous system play an important role in food intake and regulate energy balance. For example, leptin and its receptors (LEP, LEPR), which are expressed in adipocytes and regulate fat metabolism and energy expenditure, are amongst these genes; leptin deficiency has been identified in children with severe early-onset obesity and increased hyperphagia (Sadaf Farooqi 2005). Mutations in pro-opiomelanocortin (POMC) gene, which leads to adrenocorticotrophic hormone (ACTH) deficiency have also been associated with hyperphagia and early-onset obesity (Sadaf Farooqi 2005). In addition, more than 70 mutations in melanocortin 4 receptor (MC4R) gene have been linked to early-onset obesity (Kublaoui & Zinn 2006; Lubrano-Bertheliet et al. 2006). Other genes involved in central nervous system pathways are pro-hormone convertase 1 (PC1) gene, neuropeptide Y (NPY) gene and the ghrelin receptor and mutations in all these genes have been directly or indirectly, through other receptors, implicated in early-onset obesity, lipid metabolism and obesity development during puberty (Sadaf Farooqi 2005; van Rossum et al. 2006; Holst & Schwartz 2006).

In addition to genes implicated in food and energy intake, mutations in genes associated with energy expenditure have also been identified. The $\beta 1$ - , $\beta 2$ - and $\beta 3$ - adrenoceptor genes play a critical role in lipolysis and adipocyte metabolism and their mutations have been linked to hypertension, obesity, impaired lipolytic activity and increased lipid accumulation within adipocytes (Marti & Martinez 2006; Park et al. 2005; Li et al. 2006; Linne et al. 2005). Uncoupling proteins (UCPs), which regulate energy metabolism in the mitochondria, have also been suggested to contribute in obesity, as mutations in UCP variants have been associated with increased obesity risk (Ochoa et al. 2004).

Obesity can be divided into three forms based upon genetic and phenotypic characteristics: monogenic syndromic obesity, monogenic non-syndromic obesity and polygenic obesity (Albuquerque et al. 2015). The monogenic forms affect only 5-10% of the population and are rare (Farooqi & Rahilly 2005; González-Jiménez et al. 2012); however, they have been highly informative in understanding the molecular pathogenesis of human obesity. Polygenic obesity, also referred to as common obesity, is the most prevalent form of obesity in humans and changes in several genes significantly contribute to its prevalence (Rankinen et al. 2006). Environmental factors, including physical activity and diet, also contribute to polygenic obesity, and in combination with the abundance of genetic variants within different individuals, makes its study far more complex (Hinney et al. 2010). However, genome-wide linkage studies (GWLs), candidate gene association and genome wide association (GWA) studies have successfully identified about 127 sites in the human genome related to the development of obesity, enhancing our knowledge in both monogenic and polygenic obesity (Alonso et al. 2015).

1.2.2. Obesity and metabolic syndrome

The term metabolic syndrome is used to describe a cluster of conditions characterised by metabolic abnormalities and associated with the increased risk of heart disease, stroke and T2DM. Since 1947, when the first symptoms were first described during observational studies (Vague 1956), several definitions for the metabolic syndrome have been introduced, but the criteria to unify the diagnosis have been devising due to heterogeneity (Martin et al. 2015). At present, the most widely accepted criteria for the research and clinical diagnosis of metabolic syndrome have been introduced by the National Cholesterol Education Program (NCEP) and requires the presence of 3 of the 5 following factors: abdominal obesity (a waist circumference greater than 102cm for men and 88 cm for women), elevated triglycerides ($\geq 150\text{mg/dL}$), reduced high density lipoprotein cholesterol ($< 40\text{mg/dL}$ for men, $< 50\text{mg/dL}$ for women), elevated blood pressure ($\geq 130/85\text{mmHg}$) and impaired fasting glucose ($\geq 110\text{mg/dL}$) (National Cholesterol Education Program (NCEP) Expert Panel 2002).

Obesity is a common feature of the metabolic syndrome. The prevalence of the metabolic syndrome is higher in obese than in non-obese patients, however, there are also lean individuals who develop the syndrome, while some obese individuals do not (Reaven 1988). Amongst all organs, the liver plays a central role in the development of metabolic syndrome, as it is the site of production of fasting plasma glucose and triglyceride, and patients with the metabolic syndrome are frequently characterised by increased triglyceride accumulation and hepatic insulin resistance (IR) (Kotronen et al. 2007).

1.3. Liver

The liver is the largest solid organ and the largest gland in humans, accounting of approximately 2% to 3% of the weight of an average adult. It is also the major metabolic visceral organ in the human body and performs over 500 different functions (Naruse et al. 2007) including bile and albumin production and secretion, foreign and metabolic poison detoxification and metabolism of lipids, carbohydrates and vitamins (Sherlock 1978). It lies in the right upper quadrant of the abdominal cavity, under the right hemidiaphragm, and consists of two main lobes, a large right and a smaller left lobe, with the latter being no more than a quarter or one fifth the size of the right lobe (Campbell 2006a; Abdel-Misih & Bloomston 2010). On the inferior face of the right lobe sits the gallbladder, a thin-walled sac with an average 8cm length and 4cm diameter in adults, where bile is concentrated before it is released into the small intestine (Meilstrup et al. 1991).

On the inferior surface is the porta hepatis, a deep fissure through which the portal vein, the hepatic artery and the hepatic duct pass into the liver. The liver receives, on average, 1.5 litres of blood per minute (25-30% of cardiac output). Blood inflow occurs through the hepatic artery and the portal vein, with the latter providing 70-75% of the blood inflow, while the hepatic artery supplies the remainder. Blood from both sources flows through hepatic sinusoids, in which oxygen and nutrient exchange alter its biochemical composition, with a significant decrease in oxygen saturation. The deoxygenated blood is then transferred through the hepatic veins to the inferior vena cava to enter, then, the right atrium of the heart (Mahadevan 2014; Ellis 2011; Campbell 2006a)

1.3.1. Liver histology and physiology

The liver is comprised of parenchymal (hepatocytes) and non-parenchymal cells (Kupffer cells, sinusoidal endothelial cells, hepatic stellate cells and liver-associated cells), with hepatocytes occupying approximately 80% of cellular liver mass. The non-parenchymal cells, although making up 40% of the total number of liver cells, contribute only 6.5% to the liver volume (Kmieć 2001).

A typical hepatocyte is cuboidal in structure and has a volume of $3.4 \times 10^{-9} \text{cm}^3$ (Lodish et al. 2008). It consists of an eosinophilic cytoplasm including a round nucleus, numerous mitochondria, free ribosomes and large amounts of rough endoplasmic reticulum (ER), all of which are important for the metabolic functions of the liver. Glycogen and lipid are also stored in the cytoplasm and can be found in the form of granules or droplets.

Hepatocytes are arranged into hexagonal lobules, each one of which has a portal space at each corner with branches of the portal vein, the hepatic artery and a bile duct (portal triad). Within a lobule, hepatocytes are organized radially in cords and the spaces between the cords form the sinusoids. The sinusoids are vascular channels from which blood is drained by the portal vein and the hepatic artery in the periphery of the lobule. They have a discontinuous endothelial cell lining (endothelial cells) with large gaps between cells, enabling communication between the lumen of the sinusoid and the hepatocyte (Figure 1.2-1). The endothelial cells have no basement membrane and thus are separated from the hepatocytes by the space of Disse, which is crucial for hepatic lymphatic drainage. Between two sinusoids, there is one layer of hepatocytes. In the middle of each lobule there is a central vein, which transfers the blood from the sinusoids, through the interlobular vein, to the hepatic vein. Within each lobule there is also an oxygen gradient, with the cells on the periphery being more highly oxygenated than those closer to the central vein (Campbell 2006a).

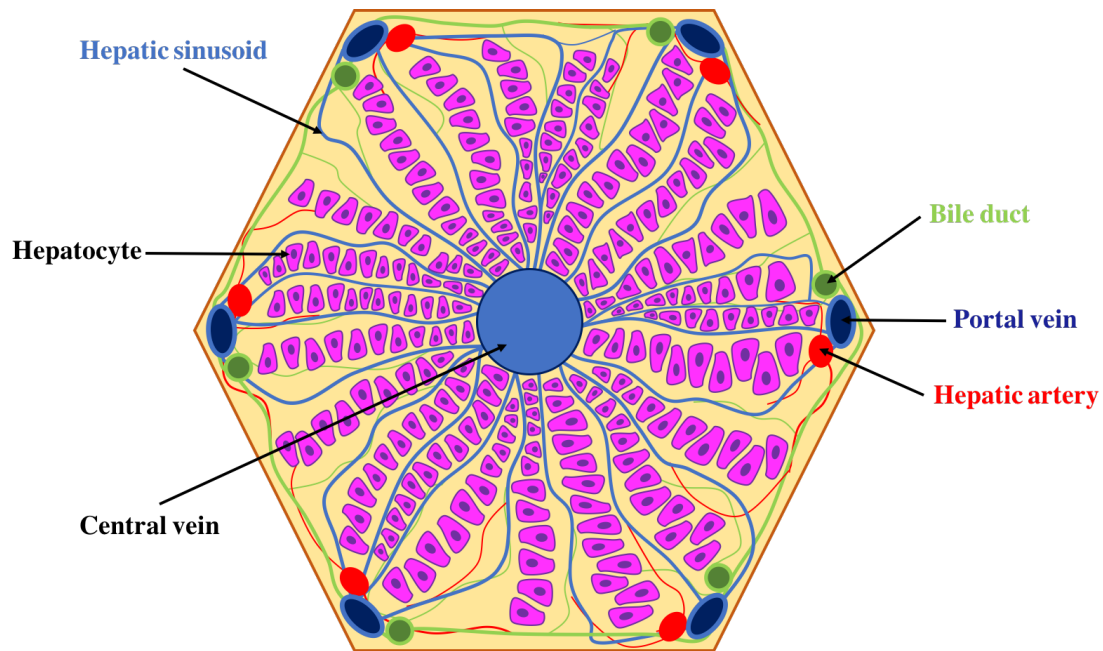


Figure 1.3-1 Typical hepatic lobule structure. Hepatocytes are arranged into the lobule, which has a portal space at each corner with branches of the portal vein, the hepatic artery and a bile duct (portal triad). Within a lobule, hepatocytes are organized radially in cords and the spaces between the cords form the sinusoids.

In addition to endothelial cells, sinusoids are also lined by Kupffer cells, which are a specialized macrophage population and, unlike hepatocytes, they are irregular in shape. They rest on a reticulin layer and form cytoplasmic extensions that are responsible for their phagocytic function (Krishna 2013). Liver-associated lymphocytes (LAL) are also present in sinusoids as well as in the space of Disse and demonstrate natural killer (NK) properties (Winnock et al. 1995). Stellate cells (Ito cells), which belong to the myofibroblast family, can also be found within the space of Disse and control sinusoidal blood flow, fibrogenesis and vitamin A storage (Saxena et al. 1999; Wisse et al. 1996)

In addition to the “classical” hexagonal hepatic lobule model, other models have also been proposed, to better represent hepatocyte heterogeneity on both a structural and functional level. The portal lobule, a triangular block of liver parenchyma with the portal triad in the centre, has been suggested. This triangular block is supplied by circumlobular branches of

one interlobular vein and is used to describe the way in which bile acids are secreted by the liver parenchyma into the bile duct of the central portal triad.

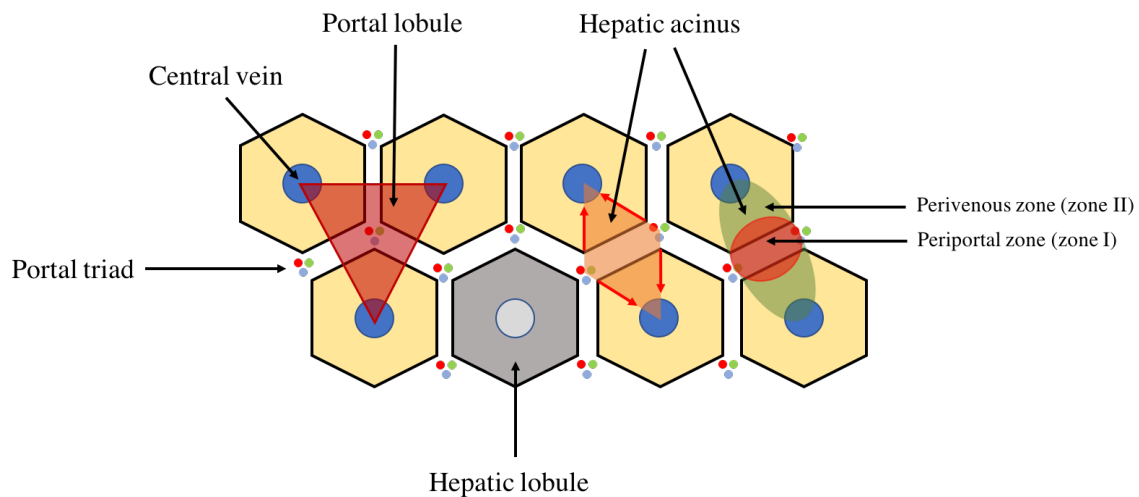


Figure 1.3-2 Structural and functional organisation of liver parenchyma. Three basic models have been suggested, the hepatic lobule, portal lobule and the liver acinus model.

Functionally, the “acinus” model has been considered the best model to describe blood flow within the liver and can be visualised by a line connecting two portal triads and extends outwards to the two adjacent central veins. Based on this model, two zones have also been proposed, one around the portal triads (periportal zone), and a second one around the central vein (perivenous zone). These two zones form the “metabolic zonation” pattern, as the different metabolic functions of the liver are based on oxygen gradient and, thus, can be found in one or the other zone (Kietzmann 2017) (Figure 1.2-2). In a recent study by Halpern et al. 2017, it was demonstrated that around 50% of over 7,000 expressed liver genes are significantly zoned in mouse liver, thus revealing 9 different zones instead of the traditional 3 zone nomenclature. In particular, their analysis revealed three distinct populations consisting of Kupffer cells, endothelial cells and hepatocytes, with hepatocytes demonstrating highly heterogeneous expression patterns. Interestingly, genes

involved in bile acid biosynthesis exhibited a spatial order that matched their position in the classic bile acid pathway. However, *akr1d1* expression did not reveal any zonal distribution and that may reflect the diversity of its roles. Moreover, this study was restricted to the mouse, therefore, potential differences between human and mouse liver cannot be excluded (Felmlee et al. 2017).

1.3.2. Metabolic functions of the liver

The liver plays a key role in the metabolic control of the body regulating carbohydrate, fat and protein metabolism (Mitra & Metcalf 2009). It also serves secretory and excretory functions, including secretion of bile which, subsequently, helps in the excretion of both endogenous substances (e.g. bilirubin, steroid hormones, calcium) and exogenous substances (e.g. antibiotics and drug metabolites) (Campbell 2006b). The principle focus of this thesis is carbohydrate and lipid metabolism.

1.3.2.1. Carbohydrate metabolism in the liver

Glucose is the central molecule in carbohydrate metabolism and the liver plays a crucial role in the maintenance of blood glucose concentrations within the normal range. This is achieved through the activity of two ongoing pathways that are tightly regulated and either synthesise glucose from acetyl-CoA, coming from amino acid and fatty acid oxidation, or break down glycogen (Postic et al. 2004).

1.3.2.1.1. Glycogenesis

Glycogenesis is the process by which glycogen is synthesised from glucose and is activated during fasting, following the Cori Cycle. It is controlled by insulin, which activates the process in response to high glucose levels during the fed state. During this process, glucose is converted to glucose-6-phosphate by glucokinase or hexokinase, which is then converted to glucose-1-phosphate by the action of phosphoglucomutase. Glucose-1-phosphate is converted into uracil-diphosphate glucose (UDP-glucose) by UDP-glucose pyrophosphorylase. UDP-glucose is finally converted to glycogen through the action of glycogen synthase, which is the rate-limiting step of glycogenesis.

1.3.2.1.2. Gluconeogenesis

Gluconeogenesis refers to the *de novo* synthesis of glucose from non-carbohydrate precursors, including lactate, amino acids, propionate, lactate and glycerol, and is activated under fasting conditions when blood glucose concentrations are low. The gluconeogenesis pathway consists of eleven enzyme-catalysed reactions which are controlled by insulin and glucagon and, under stress conditions, cortisol and catecholamines. During gluconeogenesis ATP, which is primarily derived from fatty acid oxidation, is consumed. It begins in the mitochondria where oxaloacetate is formed through pyruvate carboxylation by pyruvate carboxylase. Oxaloacetate is then converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK). Following oxaloacetate formation, a series of reactions, that are the same as reversed glycolysis, occur and fructose-6-phosphate is formed from fructose 1,6-bisphosphate by fructose-1,6-biphosphatase; this is the rate-limiting step of gluconeogenesis. Fructose-6-phosphate is subsequently converted to glucose-6-phosphate by phosphohexose isomerase. The final reaction of gluconeogenesis occurs in the endoplasmatic reticulum (ER) where glucose-6-phosphate is converted to glucose by glucose-6-phosphatase (G6Pase) and glucose is then released out of the cells (Figure 1.2-3).

1.3.2.1.3. Glycolysis

Glycolysis refers to the metabolic pathway that converts glucose into pyruvate and generates free energy in the form of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) molecules. In addition to pyruvate, glycolytic intermediates are also used as substrates to synthesise lipids, amino acids and other important molecules that are additionally oxidised to produce ATP (Rui 2014). It is an oxygen independent metabolic pathway consisting of ten enzyme-catalysed reactions. The rate-limiting steps for this pathway include the conversion of glucose into glucose-6-phosphate by glucokinase (GCK);

the conversion of fructose-6-bisphosphate into fructose-1,6-bisphosphate by phosphofructokinase-1 (PFK-1); and conversion of phosphoenolpyruvate (PEP) into pyruvate by liver-type pyruvate kinase (L-PK) (Han et al. 2016). The expression levels and activity of these enzymes are decreased in the fasted state and increased post-prandially (Kim et al. 2010). Glycolysis is not only controlled by the key regulatory enzymes, but is also regulated by a transcriptional mechanism that is activated during feeding conditions and involves sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP) activity (Dentin et al. 2005) (Figure 1.2-3).

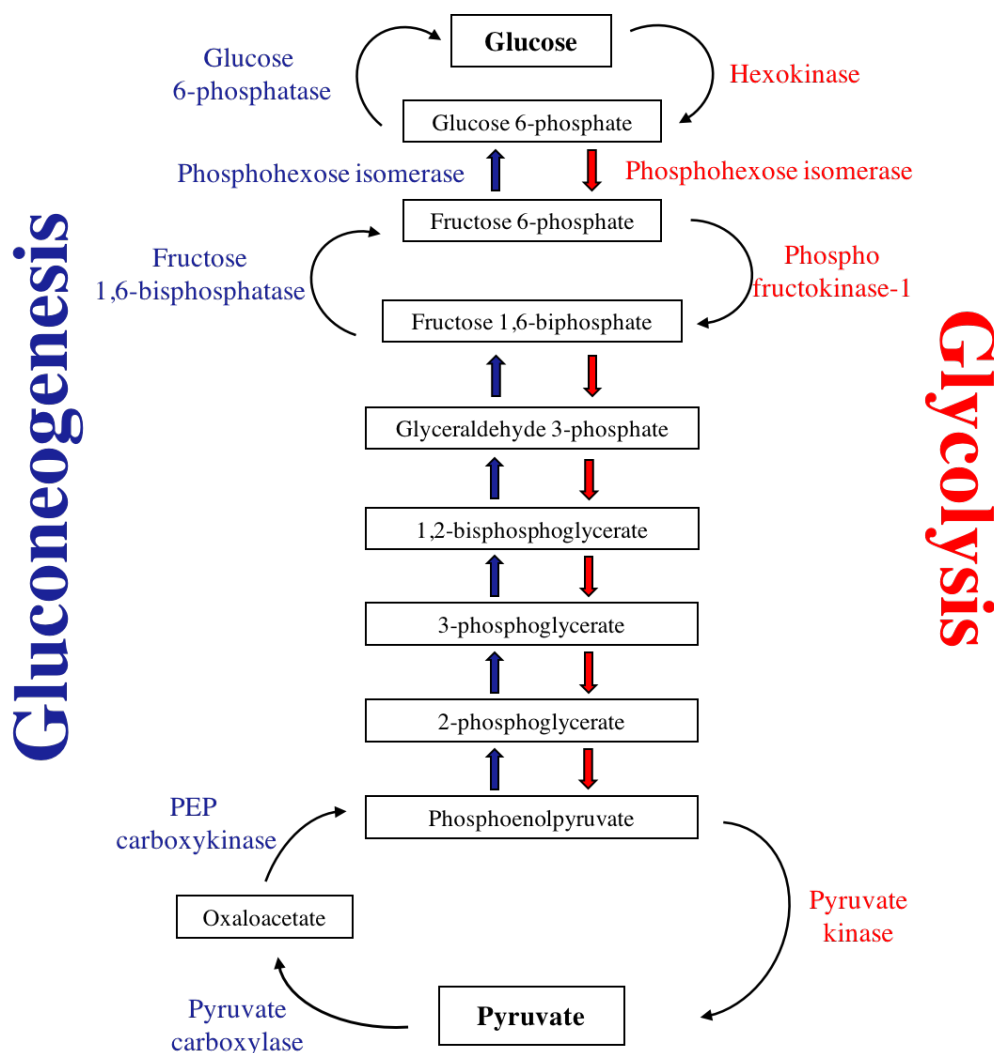


Figure 1.3-3 Gluconeogenesis pathway with key molecules and enzymes. Many steps are the opposite of those found in the glycolysis.

1.3.2.1.4. Glycogenolysis

Glycogenolysis is the process by which glycogen is broken down into glucose molecules to generate energy in the form of ATP. This pathway is activated in response to low glucose circulating levels during the fasted state and is regulated by the enzyme glycogen phosphorylase. Glycogen phosphorylase catalyses the reaction of glucose-1-phosphate generation by removing a glucose residue from the non-reducing end of a glycogen chain. Subsequently, glucose-1-phosphate is converted into glucose-6-phosphate by phosphoglucomutase and is then incorporated into the glycolysis pathway (Han et al. 2016).

Glycogen phosphorylase is principally regulated by phosphorylation and is active when phosphorylated at its serine 14 residue through a cascade mechanism of the catabolic hormones epinephrine and glucagon (Han et al. 2016). Catabolic hormone secretion is decreased during feeding conditions, thus inactivating the phosphorylation cascade, leading to inactivation of glycogen phosphorylase. In addition, with the parallel increase in insulin secretion, protein phosphatase 1 (PP1) is activated, leading to dephosphorylation of glycogen phosphorylase and a consequent decrease in glycogenolysis.

1.3.2.2. Lipid metabolism in the liver

In addition to carbohydrate metabolism, the liver plays a central role in the control of lipid metabolism through several pathways, including glycolysis, *de novo* lipogenesis, lipolysis and fatty acid oxidation. These pathways are tightly regulated and inter-dependent and regulate fat accumulation.

1.3.2.2.1. Lipogenesis

Lipogenesis involves both *de novo* lipogenesis (DNL), where non-lipid precursors, most commonly glucose, are used as substrates for *de novo* formation of fatty acids, and the subsequent synthesis of triacylglycerol (TAG) from pre-formed fatty acids; however, DNL and TAG synthesis are separate pathways.

Once glucose enters the cells, it is hydrolysed into pyruvate through the glycolytic pathway and pyruvate transfers to the mitochondria to generate acetyl-CoA. As coenzyme A from acetyl-CoA cannot pass through the mitochondrial membrane, acetyl-CoA is converted to citrate through the tricarboxylic acid (TCA) cycle and then exits the mitochondrion. The translocation of citrate from the mitochondria to the cytoplasm takes place only when mitochondrial acetyl-CoA levels are high; once in the cytoplasm, citrate is converted back into acetyl-CoA by the enzyme ATP-citrate lysase, and oxaloacetate (OAA) (Ameer et al. 2014). The second step of *de novo* fatty synthesis involves the conversion of cytosolic acetyl-CoA to malonyl-CoA by the enzyme acetyl-CoA carboxylase 1 (ACC1), which is the rate-limiting step of the pathway. ACC2, another isoform of ACC is also produced by the mitochondria and, in contrast to ACC1, it predominantly regulates fatty acid oxidation. Indeed, although both ACC isoforms mediate the conversion of acetyl-CoA to malonyl-CoA, the malonyl-CoA generated by ACC2 has been shown to inhibit carnitine palmitoyl-CoA transferase I (CPT1) activity, with a subsequent decrease in mitochondrial fatty acid oxidation (Zhao et al. 2010). The malonyl-CoA produced by ACC1 is converted through a multi-step reaction to palmitate, a 16-carbon fatty acid, by the activity of the enzyme fatty acid synthase (FAS) (Ruderman et al. 2003). On a transcriptional level, both FAS and ACC expression are controlled by SREBP-1c and the glucose-dependent transcription factor ChREBP (Dentin et al. 2005). In the ER, palmitate can be elongated by fatty acyl-CoA

elongase (Elovl) to generate long-carbon fatty acids (LCFAs) (>16 carbon-chain) or desaturated by stearoyl-CoA desaturase (SCD) to generate palmitoleate, a monounsaturated fatty acid (MUFA).

Fatty acids produced by *de novo* lipogenesis are saturated and can be converted to MUFAs by SCD, which is the rate-limiting step of the reaction (Dobrzyn et al. 2004). SCD products, particularly oleate, have been shown to regulate liver glucose and lipid metabolism in mice (Miyazaki et al. 2007). Three fatty acids esterified to a glycerol molecule form a TAG molecule and the reaction is performed by two different enzymes: diacylglycerol acyltransferase (DGAT) and glycerol phosphate acyltransferase (GPAT) (Figure 1.2-4).

Two isoforms of DGAT (DGAT1 and DGAT2) have been identified; DGAT1 is widely expressed in skeletal muscle, skin, intestine and testis, with lower levels in adipose tissue and liver (Cases et al. 1998). DGAT2 is similarly widely expressed but with increased levels in liver and fat cells (Cases et al. 2001). Ultimately, upon formation within the hepatocytes, TAG molecules are packaged with cholesterol esters, cholesterol, phospholipid and proteins to produce very low-density lipoproteins (VLDLs), and then exported in the bloodstream to be used by other tissues (Harvey & Ferrier 2011). In addition to VLDL, the liver also forms high-density lipoprotein (HDL). Lipoproteins are classified based on their size, mobility, density and their relative content of TAG, cholesterol and protein; the major five plasma lipoprotein classes are: chylomicron (produced in enterocytes in humans), VLDL, HDL, low-density-lipoprotein (LDL) and intermediate-density lipoprotein (IDL) (Cox & Garcia-palmieri 1990).

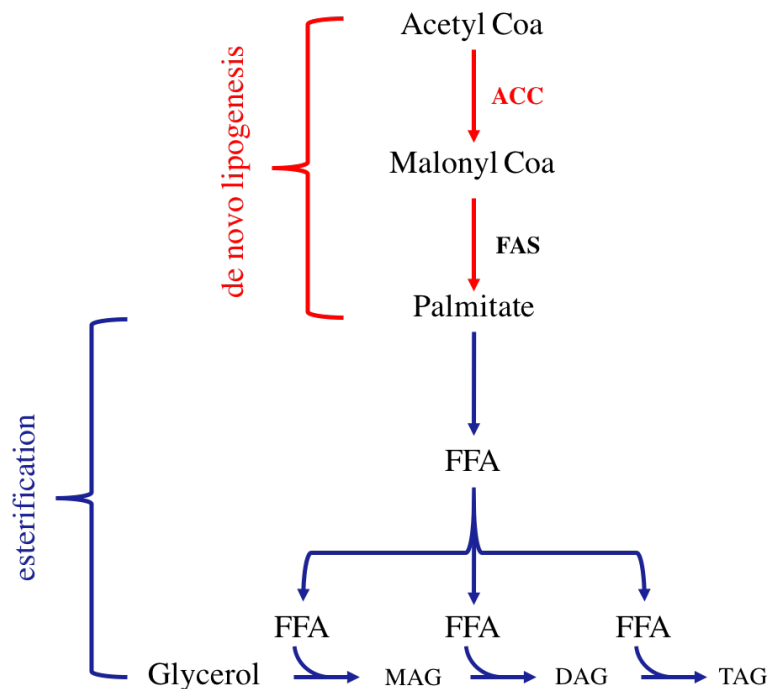


Figure 1.3-4 De novo lipogenesis and triglyceride synthesis. Once glucose enters the cells, it is converted is hydrolysed into pyruvate through the glycolysis pathway and pyruvate transfers to the mitochondria to generate acetyl-CoA. Acetyl-CoA is converted to malonyl-CoA by the rate limiting enzyme acetyl-CoA carboxylase (ACC). The malonyl-CoA produced by ACC is converted through a multi-step reaction to palmitate, a 16-carbon fatty acid, by the activity of the enzyme fatty acid synthase (FAS). Free fatty acids esterified to a glycerol molecule and produce monoglyceride (MAG), diglyceride (DAG) and triglyceride (TAG).

1.3.2.2.2. Fatty acid uptake

The liver receives fatty acids from different sources. In the fasting state, the liver gets most of its energy from fatty acids, in the form of non-esterified fatty acids (NEFAs), after the hydrolysis of TAG within adipose tissue (Hodson & Frayn 2011).

In the fed state, bile acids that are synthesised within the hepatocytes are secreted to promote intestinal lipid absorption and subsequent lipid droplet hydrolysis. Hydrolysed lipids are then packed into lipoprotein particles in the enterocytes and bypass the liver, entering the blood circulation (plasma lipoproteins). Nascent chylomicrons are formed after lipid hydrolysis in the enterocytes and consist of TAG (85–92%), phospholipids (6–12%),

cholesterol (1–3%), and proteins (1–2%) (Hussain et al. 2000). During circulation, nascent chylomicron composition changes through the removal of two apoproteins (apoA-I and apoA-IV) and the addition of apoE and apoC-II. While apoC-II is recognized by adipocytes for further processing, apoE in the chylomicron remnants is recognized by the hepatocyte LDL receptor, the LDL receptor-related protein (LPR) and scavenger receptor B-1 and chylomicrons are absorbed and metabolised within hepatocytes (Bechmann et al. 2012). Within hepatocytes, chylomicron remnants are cleared through the lysosomal degradation pathway and their glycerol, fatty acid, cholesterol, amino acid and phosphate residues are released and recycled to form new VLDLs (Bechmann et al. 2012). In addition, in the fed state, the liver receives NEFAs that have been also released after chylomicron metabolism by lipoprotein lipase (LPL) within adipose tissue. These fatty acids are sometimes called “spillover” fatty acids and are released in the blood circulation, absorbed by hepatocytes via transporters [e.g. fatty acid transport protein (FATP), fatty acid translocase (FAT), CD36] or diffusion and produce TAG and cholesterol esters. Some NEFAs also translocate to the nucleus and interact with transcription factors to induce *de novo* lipogenesis. NEFAs are also used as substrate to form phospholipids, which are an essential component of cell membranes, and lipid droplet, VLDL and bile particle formation (Rui 2014).

1.3.2.2.3. Lipolysis

Lipolysis is the biochemical pathway responsible for the breakdown of lipids and involves the hydrolysis of TAG into glycerol and NEFAs, which are subsequently used as energy substrates, precursors for lipid and membrane synthesis or cell signalling mediators (Lass et al. 2011). Hepatic lipolysis is controlled by liver lipases that are identified in lysosomal, microsomal, cytosolic and plasma membrane cell fractions. Hepatic lipase is the main lipolytic enzyme, it is predominantly synthesised by hepatocytes and is localised at the

surface of liver sinusoidal capillaries (Perret et al. 2002). Hepatic lipase hydrolyses both TAG and phospholipids of plasma lipoproteins, especially HDLs (Cheung 2003). Mutations in the hepatic lipase gene are associated with elevated plasma TAG and phospholipid levels, IDL accumulation, TG-enriched LDL and HDL and premature atherosclerosis (Hegele et al. 1993). Another type of liver lipase, the lysosomal lipase is also present at hepatocytes and is capable of hydrolysing TAG, diacylglycerols and cholesterol esters through receptor-mediated endocytosis. Patients with Wolman's disease, a rare genetic disorder, suffer from lysosomal lipase deficiency and demonstrate intralysosomal TAG accumulation (Young & Patrick 1970).

1.3.2.2.4. Fatty acid oxidation

Fatty acid oxidation (β -oxidation) is the biochemical pathway by which fatty acid molecules are broken down to acetyl-CoA, NADH and FADH₂ (Houten & Wanders 2010). β -oxidation occurs in the mitochondria, peroxisomes and microsomes and is the major pathway of catabolism providing the liver with energy when energy levels are low. In addition to energy for hepatocytes, mitochondrial β -oxidation also generates ketone bodies (β -hydroxybutyrate, acetoacetate and acetone) which are released into the bloodstream and used as energy substrates for extrahepatic tissues during fasting conditions (Rui 2014). In the fasted state, long chain acyl groups in the cytoplasm are translocated by carnitine palmitoyltransferase I (CPT1) to the mitochondrial matrix to regenerate free CoA. This is the rate-limiting step of β -oxidation. β -oxidation is a quick and effective reaction, and one molecule of palmitate generates up to 129 ATP molecules (Lehninger et al. 2008). Acetyl-CoA can be further processed through the TCA cycle to generate more ATP molecules. As mentioned in section 1.2.2.2.1, CPT1 activity can be inhibited by ACC2 through malonyl-CoA production; when this occurs, the entry of long chain acyl groups into the mitochondria is prevented. In

addition, ACC2 activity is negatively regulated by AMPK (an AMP kinase which phosphorylates ACC1 and affects ACC1 activity) contributing to the increase of β -oxidation.

Peroxisome proliferator-activated receptor alpha (PPAR α), a nuclear receptor family member, also regulates and promotes β -oxidation in both the mitochondria and peroxisomes within the liver (Kersten et al. 1999). Deletion of PPAR α has been associated with impaired hepatic β -oxidation in the fasted state, hypoglycaemia, hypoketonemia and hepatic steatosis (Kersten et al. 1999; Leone et al. 1999). PPAR α also appears to be activated by FAS products in the liver (Chakravarthy et al. 2009; Chakravarthy et al. 2005) and multiple factors can control β -oxidation through PPAR α activation, including the hepatic fibroblast growth factor 21 (FGF21) and glucagon (Longuet et al. 2008; Badman et al. 2007).

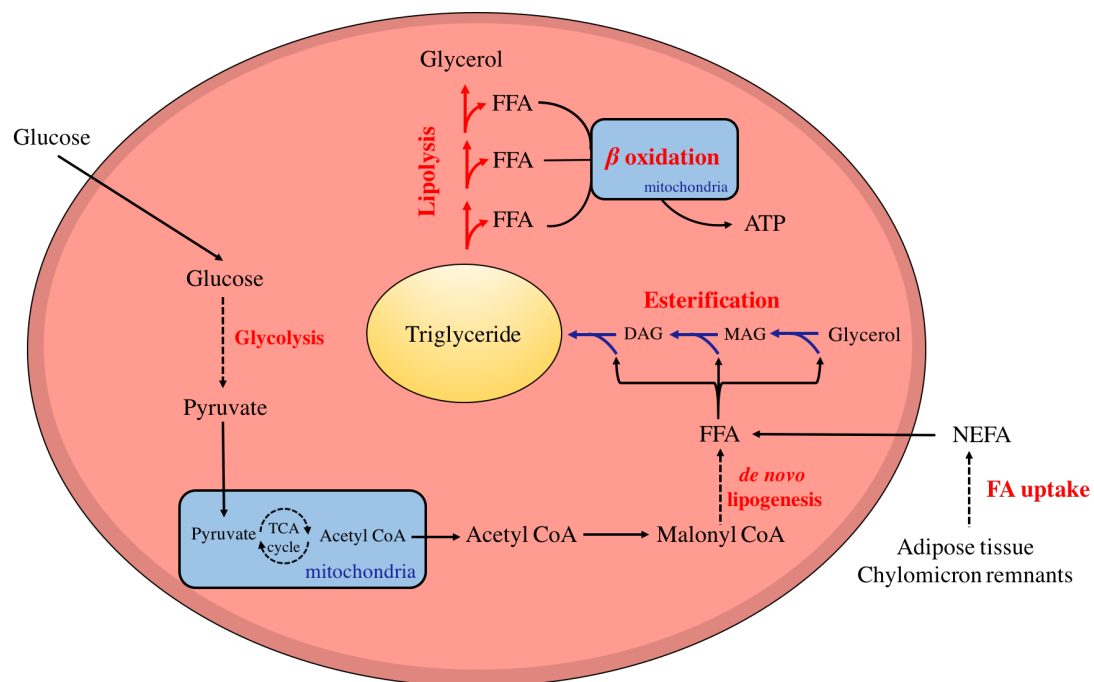


Figure 1.3-5 Pathways of lipid metabolism in the fed state. (NEFA, non-esterified fatty acids; FFA, free fatty acids; ATP, adenosine triphosphate)

1.3.3. Non-alcoholic fatty liver disease (NAFLD)

Diseases of the liver are increasing in prevalence. Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disease, characterised by the absence of excess alcohol consumption, ranging from simple steatosis (TAG accumulation in >5% of hepatocytes) to steatohepatitis (NASH) (TAG accumulation plus inflammation), progressing to fibrosis and finally to cirrhosis and hepatocellular carcinoma (Trauner et al. 2010). It is considered to be the hepatic manifestation of metabolic disease, as it has been tightly associated with central abdominal obesity, T2DM and other features of the metabolic syndrome, including IR and dyslipidaemia (Hardy et al. 2016).

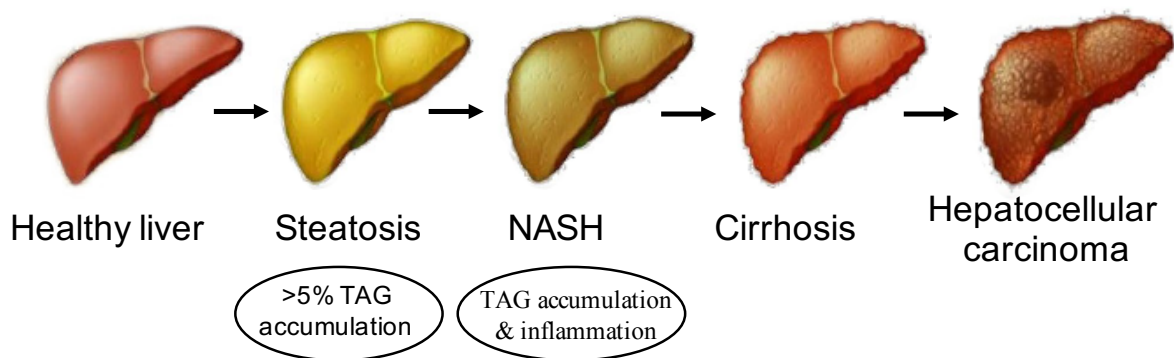


Figure 1.3-6 The spectrum of NAFLD, showing the stages starting from simple lipid accumulation (steatosis), to steatosis with inflammation (NASH), then cirrhosis and finally hepatocellular carcinoma.

NAFLD is one of the most common hepatic disorders and is rapidly becoming the major form of chronic liver disease in the western world (de Alwis & Day 2008). In the United States, NAFLD represents over 75% of the chronic liver disease patients (Younossi et al. 2011). In addition, it is projected to be the leading cause of liver failure and the most common indication for liver transplantation within a decade (Baumeister et al. 2008; Wong et al. 2015). NAFLD is associated with increased CVD and has the capacity to drive the

progression of arteriosclerosis (Than & Newsome 2015; Brea & Puzo 2013). NAFLD is not limited to adults; in children, there is an estimated 7.6% and a 34.2% prevalence in general population and clinical obese population studies, respectively, with a consistently higher prevalence in male than in female children/adolescents (Anderson et al. 2015).

The initial events in NAFLD are associated with IR and obesity and both conditions lead to increased fatty acid flux to the liver. In addition, altered hepatic processing of dietary fats, increased DNL or impaired hepatocyte lipid export and oxidation result in increased TAG production and accumulation in the cytoplasm of hepatocytes (Green & Hodson 2014). DNL appears to be a key factor in the progression of NAFLD as it has been shown to be activated even in fasting periods, with a parallel increase in the expression of lipogenic transcription factors (e.g. SREBP-1c), contributing 26% to TAG synthesis in individuals with NAFLD (Donnelly et al. 2005; Diraison et al. 2003; Lambert et al. 2014). Hepatic steatosis can in some but not all individuals progress to NASH with inflammation, as increased free fatty acid circulation and TAG accumulation lead to lipotoxicity and endoplasmatic reticulum (ER) stress. This model has been best described as the “two-hit” hypothesis model; the first hit reflects the TAG accumulation and free fatty acid flux within hepatocytes, followed by the second hit of lipid peroxidation, mitochondrial dysfunction and inflammation resulting in hepatocellular damage and fibrosis (Day et al. 1998). Importantly, the inflammatory response during NASH is regulated by induced cytokine release by Kupffer cells and pattern recognition receptors (e.g. toll like receptors - TLR4) via the nuclear factor- κ B (NF- κ B) and the c-Jun N-terminal kinase (JNK) signalling pathways (Hirosumi et al. 2002; Cai et al. 2005). When these processes persist, NASH and fibrosis can progress and patients develop cirrhosis and hepatocellular carcinoma. More recently, GWAs have revealed that NAFLD is also linked to genetic polymorphisms and mutations on genes associated with increased fat

content, cholesterol biosynthesis and glucose metabolism (e.g. PNPLA3, FDFT-1, GCKR) (Dongiovanni et al. 2013; Speliotes et al. 2011; Chalasani et al. 2010; Santoro et al. 2012).

NAFLD is commonly asymptomatic, but sometimes patients with fatty liver or mild NASH complain about fatigue or epigastric discomfort (Dietrich & Hellerbrand 2014). A number of methods to diagnose and stage NAFLD have been introduced; measurement of serum Aspartate-aminotransferase (AST) and Alanine-aminotransferase (ALT) concentrations, serum cytokeratin-18, tumour necrosis factor-alpha (TNF α), interleukin-6 (IL-6), chemokines MCP-1, RANTES and FGF21 as biomarkers, as well as ultrasonography, computed tomography (CT) and Magnetic Resonance Imaging (MRI) (Dyson et al. 2014; Festi et al. 2013; Brea & Puzo 2013; Shen et al. 2012; Banerjee et al. 2014; Kwok et al. 2014). However, although invasive, liver biopsy remains the gold-standard method for the diagnosis and staging of the disease (Adams & Feldstein 2011).

Currently, lifestyle intervention remains the corner stone of the treatment of NAFLD. 5-10% of weight loss can result in significant increase in insulin sensitivity, reduced inflammation, serum ALT and hepatic fat content (Magkos 2010; Suzuki et al. 2005; Bacchi et al. 2013). Weight loss as the result of bariatric surgery has also been shown to have beneficial effects in insulin sensitivity, thus improving the metabolic health of NAFLD patients (Hassanian et al. 2014). In addition, considering the importance of IR in the pathogenesis of the disease, insulin sensitizers such as metformin and thiazolidinediones (TZDs) have been widely used in clinical studies for the treatment of NAFLD (Chang et al. 2013); however, the beneficial effects of these drugs on the treatment of NAFLD have not been consistent (Musso et al. 2012; Li et al. 2013; Sanyal et al. 2010).

1.4. Steroid hormones; glucocorticoids, androgens and mineralocorticoids

Steroid hormones are synthesised in the adrenal glands and the gonads and play a crucial role in the development, differentiation and homeostasis in humans (Simons 2008). All steroid hormones are synthesised from cholesterol and hydroxylation of the side chain at the C20 and C22 positions, with cleavage of the side chain by the rate-limiting cholesterol side-chain cleavage enzyme (cytochrome P450_{scc}, CYP11A1) in the mitochondria leads to the production of pregnenolone, which is a common precursor steroid for all steroid hormones (Ruiz-Cortes 2012). The transfer of cholesterol within the mitochondria is regulated by the steroidogenic acute regulatory protein (StAR), which is the rate-limiting enzyme in steroid synthesis (Stocco 2001; Miller & Auchus 2011). All steroid hormones are fat-soluble molecules and via the blood circulation they can pass through the cell membrane and bind to cytoplasmic or nuclear steroid hormone receptors. They all share a basic structure of three cyclohexane rings and one cyclopentane ring (Figure 1.3-1).

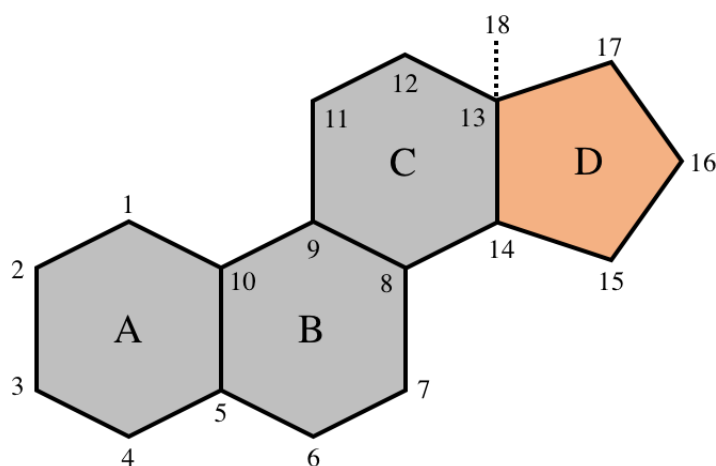


Figure 1.4-1 Standard structure of adrenal steroids. The number represent carbon atoms and the letters define the four rings.

However, differences in the number of carbon atoms and the side groups in the basic ring structure result in differences in their biological activity. Based on this, there are five major classes of steroid hormones, summarised in figure 1.3-2.

As mentioned in section 1.1, AKR1D1 is involved in steroid hormone metabolism where it inactivates glucocorticoids, mineralocorticoids and androgens and these steroid groups will be reviewed in the following paragraphs. AKR1D1 also inactivates progestogens, however, as there is little evidence of this group to play any role in carbohydrate and lipid metabolism, it will not be discussed.

Group	Number of carbon atoms	Tissue of origin
Oestrogen	C18	Placenta, Gonads, Adrenal cortex, Adipose Tissue, Breast,
Androgen	C19	Gonads, Adrenal
Progestogen	C21	Gonads, Adrenal
Mineralocorticoid	C21	Adrenal cortex
Glucocorticoid	C21	Adrenal cortex

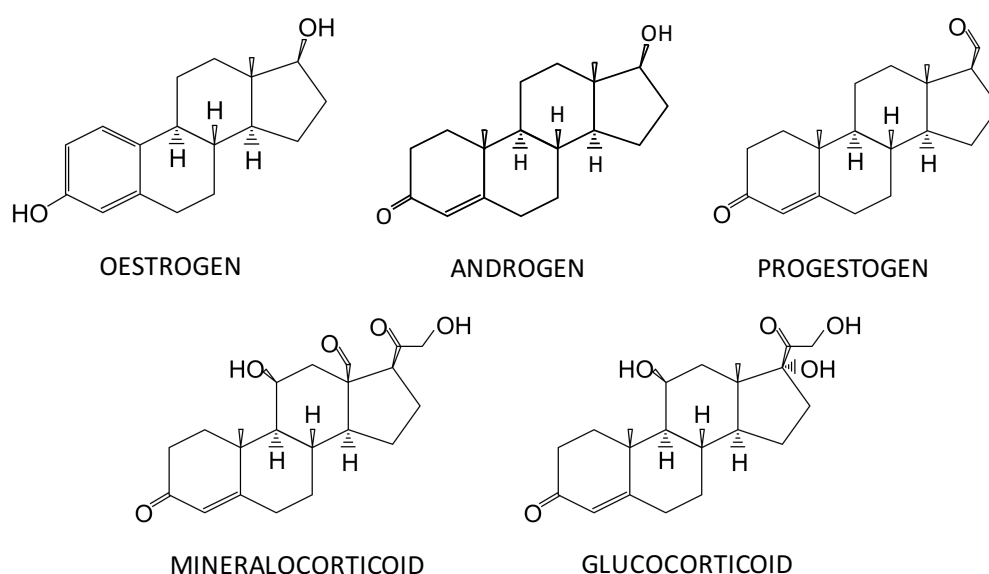


Figure 1.4-2 Five major groups of adrenal steroids: oestrogen, androgen, progestogen, mineralocorticoid and glucocorticoid.

1.4.1. Adrenal steroidogenesis

The adrenal glands are endocrine organs found above the kidneys and consist of an outer cortex and an inner medulla. The outer cortex is responsible for steroid hormone generation and is divided into three zones: zona glomerulosa (ZG), the zona fasciculata (ZF) and the zona reticularis (ZR) (Pawlina 2015). All the steroid groups defined in figure 1.3-2 are synthesised from cholesterol in the cortex of the adrenal glands. Mineralocorticoids are synthesised in the ZG, glucocorticoids in the ZF and androgens in the ZR.

Three enzymes with sequential action are required for mineralocorticoid synthesis: 3β -hydroxysteroid dehydrogenase type 2 (3β -HSD2), 21-hydroxylase (CYP21A2, P450c21) and aldosterone synthase (CYP11B2, P450c11AS). The ZG is the only zone for aldosterone synthesis as it expresses CYP11B2, which catalyses the final three steps of aldosterone synthesis; in contrast, it expresses low levels of 17α -hydroxylase/ $17,20$ -lyase (CYP17A1, P450c17), an enzyme that catalyses 17-OH-pregnenolone synthesis towards cortisol and androgen production (Turcu & Auchus 2015).

Glucocorticoid synthesis is regulated by adrenocorticotropin (ACTH) activity. The first step in glucocorticoid synthesis involves the hydroxylation of pregnenolone and progesterone by CYP17A1 (P450c17) towards 17α -hydroxypregnenolone and, to a much lesser degree, 17α -hydroxyprogesterone (17OHP), respectively. Subsequently, 17α -hydroxysteroids are converted to 11-deoxycortisol by 3β -HSD and 21-hydroxylase (CYP21A2, P450c21) activity. Lastly, 11 β -hydroxylase (CYP11B1, P450c11) catalyses the hydroxylation of 11-deoxycortisol, completing glucocorticoid synthesis (cortisol in man and most mammals, corticosterone in rodents and lower vertebrates) (Turcu & Auchus 2015).

As mentioned above, adrenal androgen synthesis occurs in the ZR and leads to the production of dehydroepiandrosterone (DHEA) and its enzyme making sulfate (DHEAS) through the

activity of CYP17A1. CYP17A1 is expressed in both the ZF and ZR, however its 17,20-lyase reaction, which is important for adrenal androgen synthesis, is dependent on the cofactor cytochrome *b*₅ (CYB5A) and is present only in the ZR (Turcu & Auchus 2015) (Figure 1.3-3).

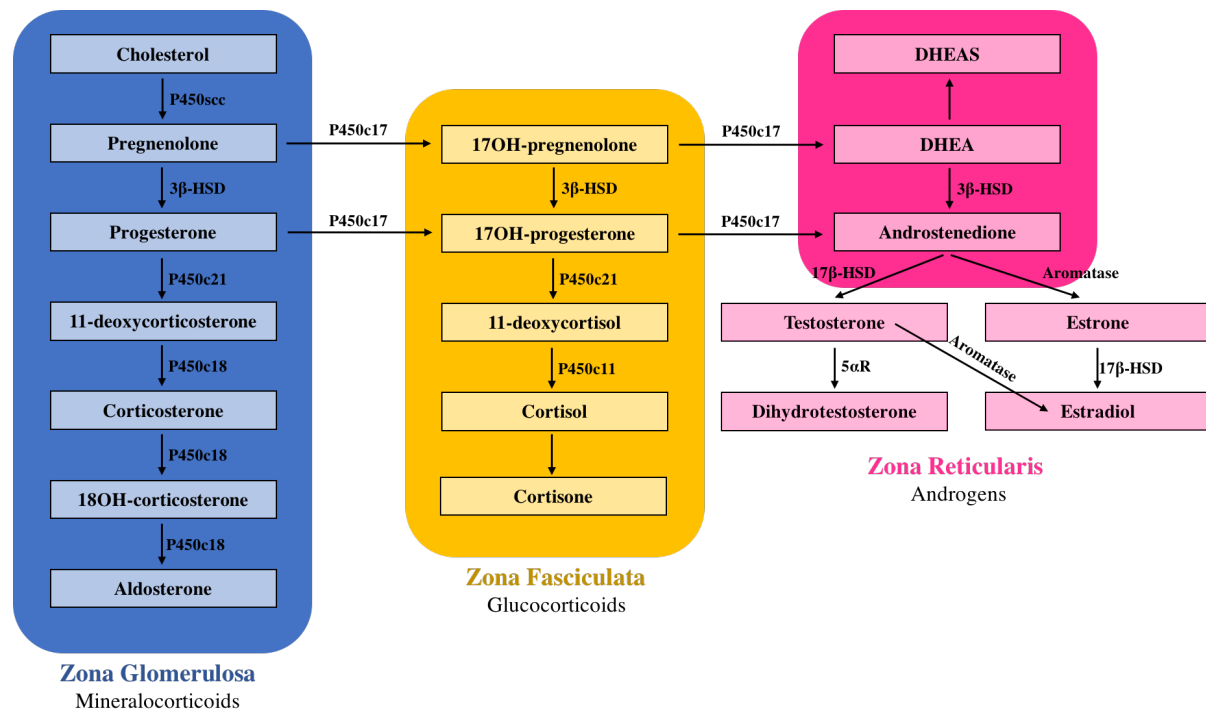


Figure 1.4-3 The major enzymatic reactions in the pathways of steroid biosynthesis.

1.4.1.1. Glucocorticoids

1.4.1.1.1. The hypothalamic-pituitary-adrenal axis

Glucocorticoid synthesis is under the control of hypothalamic-pituitary-adrenal (HPA) axis activity. The signalling cascade is initiated by the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) into the pituitary portal circulation (Antoni 1986; Rivier & Vale 1983; Gillies et al. 1982). Subsequently, the release of CRH and vasopressin increases the secretion of ACTH from the anterior pituitary through their binding on CHR receptor 1

(CHRP1) and AVP 1B receptor (V1bR), respectively (Antoni et al. 1984; Chalmers et al. 1995). ACTH is then released into the bloodstream and reaches the adrenal glands where it binds to the melanocortin type-2 receptor (MC2R) (Mountjoy et al. 1994). Upon activation of the MC2R by ACTH, the receptor undergoes conformation changes which stimulate activation of the enzyme adenylyl cyclase with subsequent increase in adenosine monophosphate (cAMP) intracellular levels (Hanukoglu et al. 1990). ACTH regulates cortisol secretion by both rapid and long-term mechanisms. The rapid effects occur within minutes and involve the delivery of cholesterol to the mitochondria as well as lipoprotein uptake in the cells of the adrenal cortex, to increase intracellular cholesterol levels. The long-term effects are observed over several hours and include the stimulation of transcription of genes involved in steroid synthesis, including CYP11A1, steroid 11 β -hydroxylase, and their associated electron transfer proteins (Hanukoglu et al. 1990). In addition, ACTH enhances the transcription of mitochondrial genes involved in mitochondrial oxidative phosphorylation systems (Raikhinstein & Hanukoglu 1993).

Cortisol synthesis feeds back upon the HPA axis negatively at the level of the hypothalamus and pituitary by controlling CRH neuronal activity and by inhibiting CRH expression and secretion from the PVN (Sawchenko 1987; Plotsky et al. 1986). Through this negative feedback mechanism, both ACTH and cortisol levels are tightly maintained (Figure 1.3-4). Moreover, cortisol secretion follows a circadian rhythm, with high levels early in the morning and low during sleep. This diurnal release pattern is regulated by the circadian clock, an endogenous biological timekeeper whose action prepares the organism against daily environment changes.

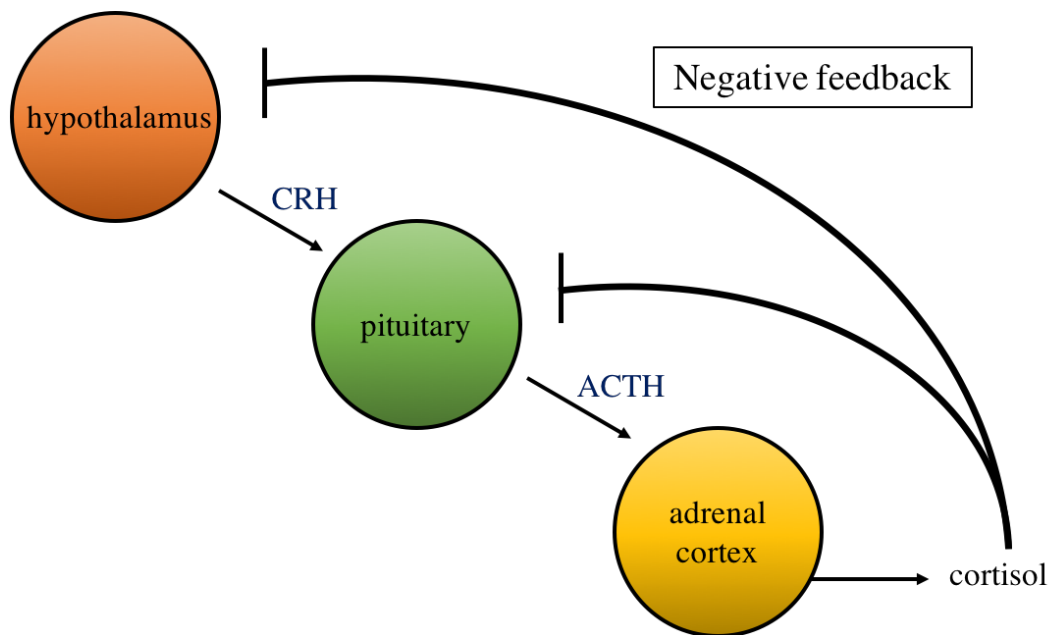


Figure 1.4-4 Schematic of the HPA axis (CRH, corticotropin-releasing hormone; ACTH, adrenocorticotrophic hormone)

1.4.1.1.2. Glucocorticoid action

Glucocorticoids act through the mineralocorticoid receptor (type 1, MR) and the glucocorticoid receptor (GR α and GR β isoforms) to exert their effects. Although these two ligand-dependent nuclear receptors share considerable structural and functional homology, the GR is widely expressed in most tissues (including adipose tissue and liver), whilst the MR is predominantly expressed in the kidney and the brain. Cortisol has a higher binding affinity for the MR than the GR, however, it does not bind to the MR in the mineralocorticoid target tissues due to increased local inactivation of cortisol to cortisone, which has weak or no affinity for the MR, by the enzyme 11 β -hydroxysteroid dehydrogenase 2 (11 β -HSD2) (Nobel et al. 2001; Edwards et al. 1988).

The GR gene consists of 9 exons and is located on chromosome 5. Several GR splicing variants have been identified, two of which are highly homologous and have been extensively studied (Lu & Cidlowski 2004; Lu & Cidlowski 2006). These two variants generate two

different GR isoforms, GR α and GR β , with a molecular weight of 97 and 94kDa, respectively. The human GR α isoform represents the classic glucocorticoid receptor and it has a high affinity for glucocorticoids compared to GR β , which acts as a negative regulator in the transcriptional activity of GR α . The human GR protein contains four main distinct domains: the N-terminal domain (amino-terminal A/B region), the DNA-binding domain (region C), the hinge region (D) and the ligand-binding domain (region E) (Figure 1.3-5) (Nicolaides et al. 2010).

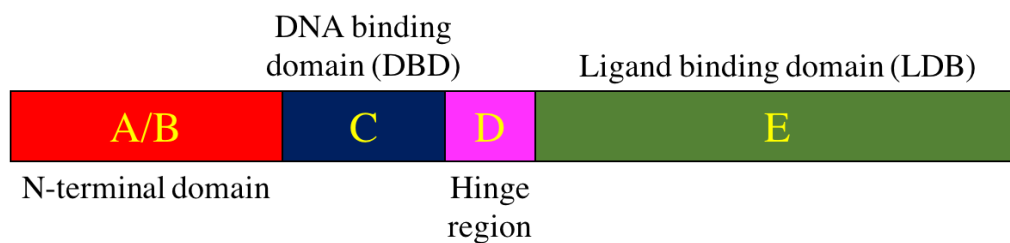


Figure 1.4-5 Structural organisation of the glucocorticoid receptor. All nuclear receptors share the same structure: an N-terminal domain (amino-terminal A/B region), a DNA-binding domain (region C), a hinge region (D) and a ligand-binding domain (region E).

During its inactive state, the GR sits in the cytoplasm as part of a multiprotein complex consisting of several heat shock proteins (HSPs). Glucocorticoid binding to the GR leads to a conformational change of the receptor and the GR homodimer dissociates from the HSP complex. Subsequently, the GR homodimer translocates to the nucleus, binds to glucocorticoid response elements (GREs) in the promoter region of glucocorticoid regulated genes and activates their transcription (Pratt 1993; Hollenberg & Evans 1988; Bamberger et al. 1996) (Figure 1.3-6). Additionally, the GR homodimer can be phosphorylated by tissue-specific kinases, leading to conformation changes in the receptor that either modulate

transcriptional activity or induce its degradation through ubiquitination (Gallagher-Beckley & Cidlowski 2009).

In addition to their classical genomic mechanism of action, glucocorticoids can also exert rapid non-genomic effects independently of gene expression and protein synthesis. These effects are predominantly mediated by G-protein coupled membrane-associated receptors on plasma and mitochondrial membranes (Spiga et al. 2014). Furthermore, non-genomic effects dependent on membrane-bound glucocorticoid receptors have also been reported (Nadal et al. 2001), as well as proteins that dissociate the glucocorticoids from the GR–multiprotein complex (Croxtall et al. 2000).

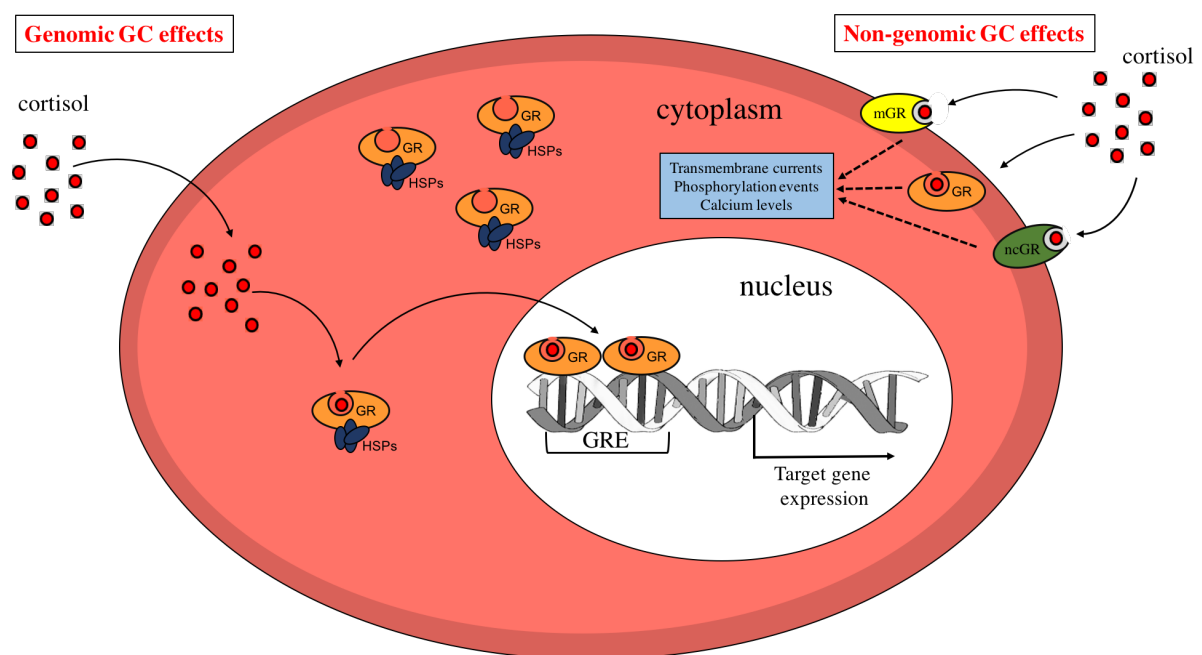


Figure 1.4-6 Schematic activation of the glucocorticoid receptor (GR) by cortisol. Cortisol enters the cells, binds to the glucocorticoid receptor in the cytoplasm and forms a cortisol-GR homodimer. Subsequently, the cortisol-GR homodimer translocates to the nucleus, binds to glucocorticoid response elements (GREs) in the promoter region of glucocorticoid regulated genes and activates their transcription (HSPs, heat-shock proteins). In addition, glucocorticoids (GC) can act in a non-genomic way through membrane bound GRs (mGR), non-classical GRs (ncGR) or through interaction between the cytoplasmic GR and other cytoplasmic signalling proteins.

The liver is the major site of cortisol metabolism where it can be reduced, oxidised or hydroxylated by a variety of enzymes, including the 11 β -HSD family enzymes and the A-ring reductases. The role of these enzymes will be further described in later sections. In the liver, cortisol predominantly exerts its effects is through binding the GR; however, non-genomic signalling by glucocorticoids has been recently reported in trout hepatocytes, involving modulation of intracellular Ca²⁺ (Vijayan et al. 2017).

1.4.1.1.3. Metabolic actions of glucocorticoids

Glucocorticoids are released in response to stress and play an important role in inflammation, cellular growth, development, body fluid homeostasis, carbohydrate, lipid and protein metabolism. However, glucocorticoid excess has been associated with metabolic abnormalities including central obesity, hypertension, hyperlipidemia and glucose intolerance. These effects are exemplified in patients with Cushing's disease, which is characterised by increased glucocorticoid secretion due to pituitary adenoma autonomously secreting ACTH (Wang 2005).

With regards to their role in carbohydrate metabolism, glucocorticoids mediate gluconeogenesis by increasing the expression of G6Pase and PEPCK (Lin et al. 1998; Petersen et al. 1988). However, chronic glucocorticoid excess promotes IR through visceral adipogenesis and leads to hyperglycaemia. Hyperglycaemia occurs as a result of impaired glucose uptake from the muscle and adipose tissue and increased gluconeogenesis and glycogenolysis in the liver (Geer et al. 2014). In addition, long-term exposure of pancreatic β -cells to glucocorticoids leads to IR and impaired glucose-stimulated insulin secretion (Beaudry & Riddell 2012).

Specifically with regards to lipid metabolism, glucocorticoids increase lipolysis from the visceral adipose tissue, which results in increased NEFA release into the portal circulation and direct delivery to the liver (Xu et al. 2009). In the liver, they promote VLDL generation and secretion and reduce apolipoprotein B degradation (Wang et al. 1995). They also induce the activity of enzymes involved in TAG synthesis, including FAS, ACC1 and ACC2, thus stimulating hepatic DNL and potentially promoting hepatic steatosis (Wang et al. 2012; Sørensen et al. 1992; Dolinsky et al. 2004). Although glucocorticoids do not seem to have any effect on CPT1 activity, the rate-limiting enzyme for fatty acid oxidation (Agius et al. 1986), they can induce ACC2 activity and limit oxidation through malonyl-CoA inhibition of CPT and there is evidence of impaired NEFA oxidation after glucocorticoid treatment in mice (Lettéron et al. 1997).

1.4.1.1.4. Pre-receptor glucocorticoid metabolism

The availability of glucocorticoids to bind to their receptors, and therefore regulate metabolism, is not only dependent on circulating levels but also on pre-receptor metabolism by a series of enzymes, including the 11 β -hydroxysteroid dehydrogenases (11 β -HSD, type 1 and 2) and the A-ring reductases (5 α -reductase type 1 and 2, AKR1D1). 11 β -HSD and 5 α -reductases have been well described in the past with regards to their important role in regulating aspects of the metabolic phenotype, therefore, their role will be covered very briefly. However, very little is known about AKR1D1 or its role in metabolism. 5 α -reductase type 1 and 2 and AKR1D1 activity will be discussed in detail in sections 1.3.2.1.4 and 1.5, respectively.

11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is 34kDa protein that is highly expressed in metabolic target tissues (liver, adipose tissue, muscle) and possesses both oxo-reductase and dehydrogenase activity. 11 β -HSD1 oxo-reductase activity converts the inactive

form of glucocorticoids cortisone to cortisol, thus amplifying local glucocorticoid action, whilst dehydrogenase activity drives the opposite reaction. *In vivo*, 11 β -HSD1 predominantly acts as an oxo-reductase (Tomlinson et al. 2004).

The emerging role of 11 β -HSD1 in the regulation of metabolic phenotype has been extensively described in animals and humans. 11 β -HSD1 activity in the liver is reduced in obese and NAFLD patients (Stewart et al. 1999; Ahmed et al. 2012), although this is not a consistent finding (Dube et al. 2014). However, in animal studies, pharmacological 11 β -HSD1 inhibition has been associated with improved glucose tolerance and insulin sensitivity (Alberts et al. 2002). Furthermore, global 11 β -HSD1 KO mice demonstrate increased hepatic insulin sensitivity and glucose tolerance (Morton et al. 2001). 11 β -HSD1 inhibition has also been associated with decreased hepatic glucose production rates in T2DM patients (Morgan & Tomlinson 2010).

11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) is a 44kDa protein and, like 11 β -HSD1, it is located in the ER membrane. As mentioned in section 1.3.1.1.2, 11 β -HSD2 is predominantly expressed in mineralocorticoid target tissues (e.g. kidney, colon), but not in hepatocytes, and is responsible for the inactivation of cortisol to cortisone to prevent glucocorticoid activation of the MR. Patients with mutations in 11 β -HSD2 demonstrate apparent mineralocorticoid excess (AME), a condition characterised by renal sodium retention and hypertension due to inappropriate activation of the MR by glucocorticoids (Stewart et al. 1996). In addition to its protective role in the activation of the MR, 11 β -HSD2 is also important in development, as it is expressed in the placenta and protects the fetus from increased maternal glucocorticoid levels (Burton & Waddell 1999).

1.4.1.2. Mineralocorticoids

1.4.1.2.1. Mineralocorticoid action

Aldosterone is the principal mineralocorticoid hormone in humans and plays a crucial role in sodium and potassium homeostasis, water retention and blood pressure control. The effects of aldosterone are mediated through its binding to the MR. The MR has a similar structure to the GR and consists of four major domains: a DNA-binding domain (DBD), an N-terminal domain (NTD), a ligand-binding domain (C terminal region, LBD) and a hinge region, which sits between the DBD and the LBD. Like cortisol, aldosterone binds to the receptor in the cytoplasm and, subsequently, the aldosterone-MR complex binds on the DNA to specific hormone response element, inducing target gene transcription (Tsai & O'Malley 1994). However, it is not known whether the MR has its own “mineralocorticoid response element” contrasting with the well-established glucocorticoid response elements. In addition, there is evidence of non-genomic aldosterone activity with rapid effects on intracellular pH and calcium dependent Na^+/H^+ exchange as well as via activation of G protein-coupled receptors (Christ et al. 1995; Gekle et al. 1996; Gros et al. 2011).

1.4.1.2.2. Metabolic actions of mineralocorticoids

Aldosterone plays a crucial role in water and electrolyte balance and blood pressure regulation. Increased aldosterone levels have been linked to arterial hypertension and this is exemplified in patients with primary aldosteronism (PA), with autonomous secretion of aldosterone from an adrenal adenoma (Stewart 1999). Hypertension is closely related with obesity (Garrison et al. 1987) and weight loss has been associated with decreased plasma aldosterone levels (Tuck et al. 1981). Furthermore, many patients with PA demonstrate a number of metabolic abnormalities including IR, impaired glucose metabolism as evidenced by increased fasting glucose levels and abnormal lipid profiles (Fallo et al. 2006; Ranade et

al. 2001). A more recent study of Arlt et al. (2017) has demonstrated increased glucocorticoid secretion in PA and this may be important in the prevalence of the metabolic phenotype in these patients. In addition, elevated serum aldosterone levels in obese patients have been linked to increased plasma TAG, waist circumference and HDL (Bochud et al. 2006; Kidambi et al. 2007). *In vitro*, aldosterone has also been shown to increase the mRNA expression of peroxisome-activated receptor- γ (PPAR γ), a key transcriptional regulator of adipogenesis (Caprio et al. 2007). In line with this, there is evidence of increased adipose tissue development following prolonged aldosterone treatment in adrenalectomised rats (Devenport et al. 1985).

1.4.1.2.3. Pre-receptor mineralocorticoid metabolism

Like glucocorticoids, mineralocorticoid concentrations are also controlled at pre-receptor level by AKR1D1 activity. Both aldosterone and corticosterone, the precursor of aldosterone, are inactivated by AKR1D1 to their 5 β -metabolites. However, it is not known how AKR1D1 activity on mineralocorticoids affect metabolism. AKR1D1 activity will be discussed in detail in section 1.5.

1.4.2. Gonadal steroidogenesis

Gonads (or sex glands) are mixed glands that are responsible for the synthesis of gametes (sex cells) and gonadal steroids. The gonadal steroids, also known as sex steroids, are divided into two main classes, androgens and oestrogens. Similar to adrenal androgens, gonadal androgen synthesis starts with the conversion of pregnenolone to DHEA in Leydig cells in males. However, Leydig cells express high levels of 3β -HSD and 17β -HSD resulting in a rapid conversion of DHEA to testosterone via the intermediates androstenediol and androstenedione. In females, gonadal androgen synthesis starts in theca cells of the ovaries, with the conversion of cholesterol to androstenedione. Subsequently, androstenedione is converted to either testosterone by 17β -HSD activity in the theca cells, or moves to the granulosa cells to be converted into estrone or testosterone and, finally, estradiol through aromatization (Burger 2002).

1.4.2.1. Androgens

1.4.2.1.1. The hypothalamic-pituitary-gonadal axis

The secretion of sex hormones by the gonads is under the control of the hypothalamus-pituitary-gonadal axis. Within the hypothalamus, Gonadotropin-releasing hormone (GnRH) is secreted by GnRH-expressing neurons and stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior portion of the pituitary gland. LH enters the circulation and is then transported to the Leydig cells of the testes in males and the theca cells of the ovaries in females to enhance the expression of StAR. Whilst the hormonal feedback in the males is simple, with testosterone feeding back negatively on LH and GnRH, feedback regulation in females is more complicated. Like in males, GnRH is released by the hypothalamus and results in the release of LH and FSH from the anterior pituitary. Subsequently, progesterone is released by the theca cells, whereas inhibin and estradiol are

released by the granulosa cells of the ovaries. Whilst inhibin always feeds back negatively the release of FSH from the pituitary, estradiol and progesterone act either as stimulators or inhibitors, depending on the stage of the menstrual cycle (Hall & Guyton 2011).

1.4.2.1.2. Androgen action

Testosterone is the major circulating androgen and its action is mediated by the androgen receptor (AR), a ligand-dependent transcription factor of the nuclear receptor family.

The AR gene is located on chromosome X and consists of eight exons. Two isoforms, AR-A and AR-B, have been identified for this gene with a molecular weight of 87kDa and 110kDa, respectively. Like other nuclear receptors, the AR consists of the following domains: a DNA-binding domain (DBD), an N-terminal domain (NTD), a ligand-binding domain (C terminal region, LBD) and a hinge region. Both isoforms possess similar functional activities (Gao & McPhaul 1998).

Androgen action is regulated via a multistep mechanism. Once testosterone enters the cell, it either directly binds to the AR or is converted by 5 α -reductase (5 α R) to the more potent form of testosterone, 5 α -dihydrotestosterone (5 α -DHT). Like glucocorticoids and mineralocorticoids, binding to the receptor leads to dissociation of HSPs, conformational change of the receptor and translocation of the homodimer to the nucleus. In the nucleus, the androgen-AR complex binds to the DNA and activates the transcription of androgen regulated genes.

Androgen action can also be mediated in a rapid, non-genomic way. The non-genomic effects are predominantly regulated by changes in Ca²⁺ (Foradori et al. 2008). Similar to glucocorticoids and mineralocorticoids, activation of G-coupled protein receptors, in particular the orphan GPRC6A receptor, also mediate the non-genomic effects of androgens

(Pi et al. 2010). In addition, changes in the structure of androgens can lead to activation of enzymes required for ATP hydrolysis, independently of receptor activation (Zylinska et al. 1999). A number of studies have also revealed the presence of a novel membrane-bound AR, but its role in androgens' actions still needs to be investigated (Foradori et al. 2008).

1.4.2.1.3. Metabolic actions of androgens

Androgens have extensive anabolic actions in many tissues and organs, and appear to play an important role in carbohydrate and fat metabolism. A number of studies have demonstrated a correlation between increased mortality due to CVD and testosterone deficiency (Khaw et al. 2007; Vikan et al. 2009; Araujo et al. 2011), and testosterone deficiency is present in 40% of T2DM male patients (Corona et al. 2011; Gibb & Strachan 2014). In addition, low testosterone levels have been linked to IR, increased fat deposition, obesity, dyslipidaemia and hypertension (Wang et al. 2011). Low levels of testosterone have been associated with an atherogenic lipoprotein profile, characterised by high LDL and TAG levels (von Eckardstein & Wu 2003), and a negative correlation between serum levels of testosterone and total and LDL-cholesterol has been observed in cross-sectional studies (Haffner 1993; Simon et al. 1997; Barud et al. 2002). In line with this, high serum testosterone levels have also been associated with insulin sensitivity (Pitteloud et al. 2005). Results from animal studies showed that mice treated with DHT have increased body weight and visceral fat mass, associated with increased TAG accumulation. Furthermore, DHT treatment increased expression of Sterol Regulatory Element-Binding Protein (SREBP2) and FAS, and decreased inactivating phosphorylation of ACC (McInnes et al. 2006). In contrast, Ding et al. (2006) showed that testosterone levels are associated with increased risk of T2DM in females and Lichtenstein et al. (1987) have also reported high testosterone levels with increased HDL cholesterol. A few

cross-sectional studies, however, have found no association between serum lipid measurements and endogenous testosterone levels (Kiel et al. 1989; Denti et al. 2000).

A number of studies have demonstrated a relationship between polycystic ovarian syndrome (PCOS) and NAFLD (Vassilatou 2014; Setji et al. 2006; Cerda et al. 2007). PCOS is one of the most common reproductive disorders and is often associated with clinical and biochemical androgen excess. Excess androgen activity also forms part of the diagnostic criteria of Rotterdam, along with oligoovulation (and/or anovulation) and polycystic ovaries, as detected by gynecologic ultrasound (Fauser 2004). PCOS shares many components with the metabolic syndrome, including lipid abnormalities, increased TAG, HDL and LDL and demonstrates a strong association with IR (Diamanti-Kandarakis et al. 2007).

1.4.2.1.4. Pre-receptor androgen metabolism

At pre-receptor level, androgens are metabolised by the A-ring reductases (5 α R type 1 and 2, AKR1D1). AKR1D1 activity will be discussed in detail in section 1.5.

The gene encoding 5 α R1 (SRD5A1) is located on chromosome 5, consists of 5 exons and is expressed in liver, skin, scalp and prostate and adipose tissue. SRD5A1 is also expressed in mouse liver. The enzyme encoded by this gene is hydrophobic protein with a pH optimum of 6-8.5 and a molecular weight of 29kDa (Russell & Wilson 1994).

The gene encoding 5 α R2 (SRD5A2) is located on chromosome 2 and consists of 5 exons. Like 5 α R1, 5 α R2 is also a hydrophobic protein with a pH optimum around 5 and a molecular weight of 28kDa. It shares less than 50% homology with 5 α R1 and is expressed in human, but not mouse, liver and in androgen-target organs, including epididymis and prostate (Andersson & Russell 1990; Russell & Wilson 1994).

Both isoforms are responsible for the conversion of testosterone to the more potent form of

testosterone, DHT. 5 α R also inactivate cortisol to 5 α -tetrahydrocortisol, but have a lower affinity towards this substrate compared to testosterone (Andersson & Russell 1990). Beyond testosterone and cortisol reduction, 5 α R convert progesterone, androstenedione, epitestosterone, aldosterone and deoxycorticosterone to their 5 α -reduced metabolites.

The role of 5 α R in the prevalence of the metabolic phenotype has been studied both *in vitro* and *in vivo*. In animal studies, 5 α R KO mice demonstrated increased hepatic TAG levels after American lifestyle-induced obesity syndrome diet (ALIOS), although they were protected against hepatocellular carcinoma (Joanna K. Dowman et al. 2013). In line with this, 5 α R deficiency was associated with IR, hepatic steatosis and increased fibrosis in mice (Livingstone et al. 2015). In contrast, results from observational clinical studies have revealed an association between enhanced 5 α R activity and IR as well as decreased 5 α R activity after weight loss (Tomlinson et al. 2008; Tomlinson et al. 2008). Increased 5 α R expression has also been linked to steatosis and NASH, as measured by increased 5 α -reduction of cortisol (Konopelska et al. 2009) and *in vitro* 5 α R inhibition, using either finasteride (5 α R2 inhibitor) or dutasteride (5 α R1 and 2 inhibitor), is able to decrease lipogenesis in human hepatocytes after cortisol treatment (Nasiri et al. 2015). However, a study from Dowman et al. (2013) demonstrated decreased 5 α R2 activity in NASH patients.

In addition to type 1 and 2, a type 3 5 α R has also been identified, but the role of the enzyme is not entirely clear (Nixon et al. 2012). A number of studies have demonstrated the ability of this enzyme to also metabolise testosterone and androstenedione to their 5 α -reduced metabolites (Uemura et al. 2008; Yamana et al. 2010). Furthermore, it assesses an important role in the reduction of polyprenol to dolichol and is mutated in a congenital glycosylation disorder (Cantagrel et al. 2010).

1.5. Bile acids

1.5.1. Bile acid synthesis

Bile acids (BAs) are produced via a series of enzymatic reactions in the cytosol, endoplasmic reticulum, mitochondria and peroxisomes within hepatocytes. In total, 17 enzymes are involved in BA synthesis and are responsible for the catalysis of the steroid ring as well the oxidative cleavage from the side chain of cholesterol (Chiang 2013). BAs are synthesised via two pathways: the classic (neutral) pathway and the alternative (acidic) pathway (Ferdinandusse & Houten 2006). Whilst the classic pathway occurs in the hepatocytes and is responsible for approximately 90% of total BA production, the alternative pathway is mainly extrahepatic and accounts for only 10% of total BA synthesis (Smith et al. 2009).

The first step of the classic BA synthesis involves the hydroxylation of cholesterol to 7 α -hydroxycholesterol by the enzyme 7 α -hydroxylase (CYP7A1), which is the only rate-limiting enzyme in BA synthesis (Chiang 1998). 7 α -hydroxycholesterol is then converted into 7 α -hydroxy-4-cholesten-3-one by HSD3B7 activity, followed by either 5 β -reduction by AKR1D1 to form 5 β -cholestan-3 α ,7 α -diol, or 12 α -hydroxylation by sterol 12 α -hydroxylase (CYP8B1) to form 7 α ,12 α ,dihydroxy-4-cholesten-3-one (Chiang 1998). 7 α ,12 α ,dihydroxy-4-cholesten-3-one is then reduced by AKR1D1 and generates 5 β -cholestan-3 α ,7 α ,12 α -triol. Subsequently, side chain oxidation of both 5 β -reduced intermediates by sterol 27-hydroxylase (CYP27A1, cytochrome p450) occurs in the mitochondria, followed by a three-carbon cleavage in the peroxisomes. The end products of BA synthesis are two C24 BAs: cholic acid (CA) and chenodeoxycholic acid (CDCA). CA and CDCA are the primary BAs and comprise approximately 80% of total BAs in bile in humans (Hofmann & Small 1967). (Figure 1.5-1).

The alternative pathway predominantly occurs in the macrophages and it begins with side-chain cleavage of cholesterol by CYP27A1 activity. In the alternative pathway, side-chain cleavage precedes steroid ring modifications, which are mediated by CYP7B1 and 3 β -HSD activity (Chiang 2013). The alternative pathway bypasses CYP7A1 activity and leads to production of CDCA, only (Figure 1.4-1). A few studies have also proposed that approximately 50% of total BA levels are synthesised via the alternative pathway (Princen et al. 1991; Vlahcevic et al. 1997).

CDCA can also be generated from oxidised metabolites in the liver through the action of a non-specific 7 α -hydroxylase (CYP7B1), which is widely expressed in all tissues (Chiang 2009). Furthermore, two minor pathways in the liver and in the brain are also present and produce BA intermediates by the activity of 25-hydroxylase and 24-hydroxylase, respectively (Figure 1.4-1).

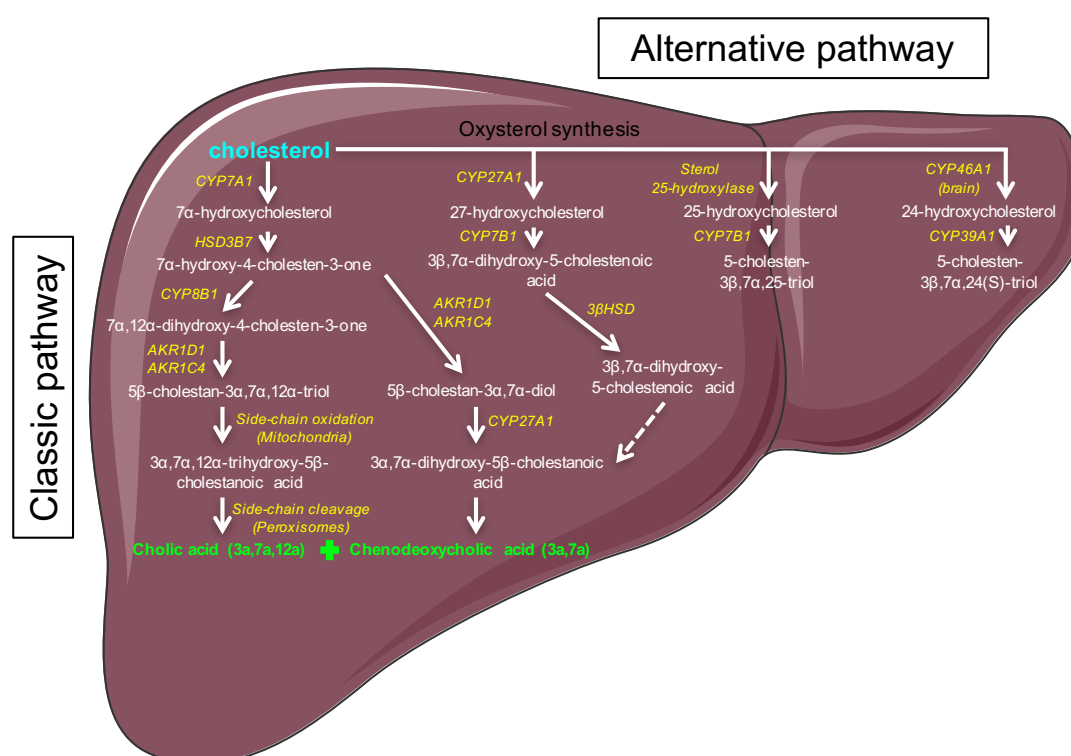


Figure 1.5-1 BA synthesis pathways in the liver. BAs are synthesised via two pathways: the classic (neutral) pathway and the alternative (acidic) pathway. The end products of BA synthesis are two C24 BAs: cholic acid (CA) and chenodeoxycholic acid (CDCA). Two minor pathways in the liver and in the brain are also present and produce BA intermediates by the activity of 25-hydroxylase and 24-hydroxylase, respectively.

BAs are amphipathic molecules with one hydrophobic and one hydrophilic side. The hydrophobic side is represented by the steroid side of the molecule where the alkyl chains of lipid molecules bind to, whilst the hydrophilic side is represented by the hydroxyl group, the amide carbonyl and the ionized acidic groups and faces the aqueous environment (Hofmann 1999) (Figure 1.5-2).

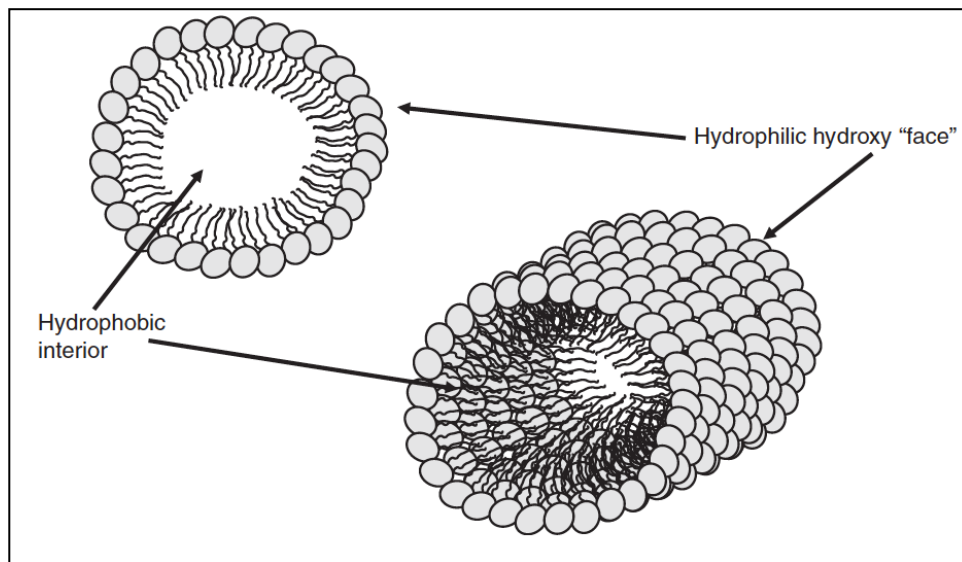


Figure 1.5-2 Structure of a mixed bile-acid/fatty-acid micelle, whereby the hydrophilic (OH groups of BAs) are radially arranged on the outside of the micelle and the hydrophobic moieties are arranged on the interior. As well as a classic micelle, a cylindrical mixed micelle structure is also shown. Picture taken from Stamp et al. (2008).

Before secretion in the bile, primary BAs are conjugated with either glycine or taurine at the carboxyl group to produce 8 possible conjugated BAs. The ratio of glycine to taurine conjugated BAs is approximately 3 to 1. Both CA and CDCA are hydrophobic BAs and conjugation increases their hydrophilicity, ionization and solubility at physiological pH (Lefebvre et al. 2009; Chiang 2013). Conjugated BAs also limit Ca^{2+} precipitation and reduce passive absorption. They are also resistant to cleavage by pancreatic carboxypeptidases (Chiang 2013). Conjugated CA and CDCA are then transported in the distal intestine and

colon, where they undergo de-conjugation and converted to the secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA) by bacterial 7α -hydroxylase, respectively.

1.5.2. Functions of bile acids

BAs are amphipathic, water-soluble steroids with detergent properties produced in the liver and act as important physiological agents to promote lipid digestion and absorption of dietary fat, cholesterol and fat-soluble vitamins in the small intestine. In addition, they are important for clearance of blood from waste products, mainly bilirubin, a toxic breakdown product of hemoglobin (Bauer et al. 2005; Mukhopadhyay & Maitra 2004).

BAs are the primary products of cholesterol catabolism, thus regulating cholesterol homeostasis and protecting the liver from cholesterol, TAG and toxic metabolites accumulation. BAs are stored in high concentrations in the gallbladder ($>300\text{mM}$) and together with bilirubin, heavy metals, lipophilic steroid and drug metabolites, they form the bile (Stamp et al. 2008).

Following storage in the gallbladder, BAs are secreted into the small intestine in the fed state, to enter the enterohepatic circulation. In the enterohepatic circulation, BAs help in lipid digestion and the release of lipid derivatives, including cholesterol, phospholipids, bilayers and fatty acids. Subsequently, these derivatives form mixed micelles with BAs, travel down to the jejunum and the lipids are transported to the portal veins through the epithelium. More than 95% of total BA content in the micelles is then reabsorbed by the distal ileum and returned to the liver for recirculation. Back in the liver, BAs inhibit their own synthesis by inhibiting CYP7A1 activity. During the enterohepatic circulation, the BA pool is circulated two to three times during digestion in humans (Hofmann & Small 1967) and each BA molecule can be recycled approximately 20 times (Garidel et al. 2007). The remaining 5% of BAs enter the colon, where they are converted into LCA and DCA (inactivated BAs) by

colonic bacteria and secreted in faeces. Small amounts of LCA are also circulated back to the liver and are sulfoconjugated by sulfotransferase (SULT2A1) to avoid detoxification.

Toxicity of BAs increases with hydrophobicity and is inversely related to the number of OH groups in the BA chain; LCA is the most hydrophobic and highly toxic BA with one OH group, followed by DCA and CDCA with two OH groups and CA with 3 OH groups (Stamp et al. 2008). Therefore, concentrations of BAs are tightly maintained by different mechanisms to avoid cellular damage. For example, in the fed state, conjugated BAs are first released into the duodenum in the form of BA anions to mix with food and are impermeable to cell membranes, thus keeping them with the gut lumen. CYP7A1 activity is predominantly repressed by hydrophobic BAs (i.e. DCA and CDCA) during the BA feedback whilst hydrophilic BAs do not have much effect (Heuman et al. 1989; Shefer et al. 1990; Xu et al. 1992; Bertolotti et al. 1991).

In addition to digestion and nutrient absorption, BAs also possess enteroprotective properties and they serve as antibacterial agents by limiting bacteria overgrowth (Inagaki et al. 2006). Furthermore, they accelerate hydrolysis of dietary proteins by inducing the action of pancreatic proteases (Gass et al. 2007).

1.5.3. Nuclear receptors involved in bile acid synthesis and metabolism

In addition to their role as digestive surfactants, BAs also act as signalling molecules and bind to specific nuclear receptors to perform their biological actions. Upon activation, these nuclear receptors mediate the expression of genes implicated in both BA synthesis and cholesterol metabolism. Furthermore, their activation allows BAs to regulate a variety of other physiological functions, including carbohydrate and lipid metabolism, energy homeostasis and immune response. Three nuclear receptors, Farnesoid X Receptor (FXR), Pregnane X Receptor (PXR), vitamin D receptor as well as a membrane receptor, the Gas

protein-coupled receptor TGR5, are directly activated by BAs. In addition, two other members of the nuclear receptor superfamily, peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR), also appear to be regulated by BAs or BA products. In this thesis, I have been mainly focused on the role of FXR and LXR in hepatic lipid and carbohydrate metabolism.

1.5.3.1. Farnesoid X Receptor (FXR)

FXR belongs to the metabolic receptor subclass of the nuclear receptor superfamily 1 and the gene encoding this receptor is located on chromosome 12. It is widely expressed in a variety of tissues, including liver, intestine, adipose tissue, pancreas and kidney (Huber et al. 2002). Four splice variants have been identified and they demonstrate different expression patterns and transcriptional activities (Boesjes et al. 2014). Both primary and secondary conjugated BAs are natural ligands of FXR and they bind with high affinity. CDCA is the most efficacious natural BA ligand, followed by LCA, DCA and finally CA. In addition, compounds not related to BAs have also been identified as FXR ligands, including androsterone, forskolin, epigallocatechin-3-gallate and cafestol (Wang et al. 2006; Howard et al. 2000; Li et al. 2012; Ricketts et al. 2007). Like other nuclear receptors, FXR is located in the cytoplasm and, upon activation, it translocates to the nucleus. In the nucleus, the FXR-ligand complex forms a heterodimer with Retinoid X Receptor (RXR) and binds to an FXR response element (FXRE) to regulate the expression of target genes (Zhang & Edwards 2008).

FXR regulates BA synthesis by inducing the expression of the small heterodimer partner (SHP), an inhibitory nuclear receptor (Lefebvre et al. 2009). Subsequently, SHP interacts with liver receptor homolog type 1 (LHR-1) leading to suppression of CYP7A1 activity (Lefebvre et al. 2009). Additionally, FXR activation in the intestine induces the expression of

the intestinal fibroblast growth factor 19 (FGF-19). FGF-19 is then transported to the liver, interacts with FGF-4 and activates the JNK pathway, resulting in inhibition of BA synthesis (Lefebvre et al. 2009). Other mechanisms related to BA absorption in the intestine and the liver may be, at least partially, regulated by FXR activation. For example, FXR seems to mediate the expression of intestinal bile-acid binding protein (IBAMP), a protein responsible for BA re-absorption in enterocytes, as well as the expression of Na⁺ taurocholate co-transporting polypeptide (NTCP), which uptakes BAs during enterohepatic circulation in the liver (Lefebvre et al. 2009).

In addition to BA synthesis, FXR plays a crucial role in lipid and carbohydrate metabolism. This will be discussed in detail in section 1.5.4.

FXR activation plays an important role in inflammation, driving an anti-inflammatory response. This role has been extensively described both *in vitro* and in animal studies. In primary human hepatocytes, FXR activation by the FXR agonist GW4064 and CDCA (potent FXR ligand) induced the expression of genes with antimicrobial properties and reduced pro-inflammatory cytokine expression (Inagaki et al. 2006). Furthermore, deletion of FXR in high-fat fed mice demonstrated bacterial overgrowth and higher levels of pro-inflammatory and pro-fibrogenic factors, including TNF α , inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), TGF β and type 1 collagen (Inagaki et al. 2006; Kong et al. 2009). In patients with NASH, hydrophobic and cytotoxic secondary BAs are higher compared to healthy individuals (Ferslew et al. 2015) and, in patients with T2DM and NASH, treatment with the FXR agonist WAY-362450 reduced hepatic inflammation, injury and fibrosis (Mudaliar et al. 2013).

1.5.3.2. Liver X Receptor (LXR)

The liver X receptor is a transcription factor and, like FXR, also a member of the nuclear receptor superfamily 1. Two isoforms, LXR α and LXR β , have been identified and are encoded by two different genes. The human LXR α gene is located on chromosome 11 and the LXR β gene is located on chromosome 19. Both isoforms are expressed in a variety of tissues with the highest expression in liver. However, whilst LXR β is widely expressed in almost all tissues and organs, LXR α expression is restricted to liver, kidney, intestine, adipose tissue, macrophages, lung and spleen (Chuu et al. 2007). Oxysterols, the oxidised derivatives of cholesterol that are generated when cholesterol levels are high, are the natural ligands of both LXR isoforms. The most known oxysterols include 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, 20(S)-hydroxycholesterol, and 27-hydroxycholesterol (Zhao & Dahlman-Wright 2010). In addition, there is also evidence that BAs activate LXR α , but not LXR β (Song et al. 2000). Similar to FXR, upon activation, LXR translocates to the nucleus and forms a heterodimer with RXR, with subsequent binding to LXR response elements (LXREs), inducing the expression of LXR regulated genes (Tate & Makishima 2006).

LXR plays a crucial role in the regulation of cholesterol metabolism and BA synthesis. Activation of the receptor by oxysterols leads to transcriptional up-regulation of CYP7A1, thus decreasing cholesterol levels by inducing BA synthesis in the liver (Lehmann et al. 1997). LXR activation also upregulates the expression of ATP-binding cassette transporter A1 (ABCA1) leading to enhancement of reverse cholesterol transport (Costet et al. 2000). In addition to ABCA1, several lipoproteins and lipid modulating enzymes that are implicated in reverse cholesterol transport are also regulated by LXR activity, including apolipoprotein E (ApoE) in macrophages and adipose tissue, ApoCI/ApoCIV/ApoCII in macrophages, ApoD

in adipose tissue, ApoAIV in liver, lipoprotein lipase (LPL) in liver and macrophages, phospholipid transfer protein (PLTP) and human cholesteryl ester transfer protein (CETP) (Laffitte et al. 2001; Mak et al. 2002; Hummasti 2004; Jakel et al. 2004; Zhang et al. 2001; Laffitte & Joseph et al. 2003; Cao et al. 2002; Luo & Tall 2000).

Further to its role in cholesterol homeostasis, LXR is a master regulator of fat metabolism. LXR activation induces the expression of SREBP-1c (Repa et al. 2000) and FAS (Joseph et al. 2002), leading to increased fatty acid and TAG synthesis. Furthermore, LXR up-regulates the expression of angiopoietin-like protein 3 (Angptl3), a liver-secreted protein that increases plasma TAG and fatty acids via LPL inhibition and lipolysis induction in the adipocytes, respectively (Koishi et al. 2002; Kaplan et al. 2003; Shimizugawa et al. 2002; Shimamura et al. 2003). Consistent with this, a study from Kalaany et al. (2005) revealed that LXR-KO mice were protected from obesity and demonstrated increased metabolic rates and impaired hepatic fatty acid synthesis, after feeding a high fat - high-cholesterol diet.

LXRs have also been implicated in carbohydrate metabolism. A number of studies have demonstrated the role of LXR in the up-regulation of genes involved in glucose storage and inhibition of gluconeogenesis in the liver, including PPAR γ , PEPCK, G6Pase and GCK (Kalaany & Mangelsdorf 2006). Moreover, LXR activation appears to up-regulate the expression of the insulin-sensitive glucose transporter (GLUT4) in white adipose tissue (Laffitte et al. 2003). Treatment with synthetic LXR agonists improved glucose tolerance and insulin sensitivity in obese and diabetic insulin-resistant rodents, respectively (Cao et al. 2003; Laffitte et al. 2003).

Importantly, there is evidence on the anti-inflammatory role of LXR, however, the exact molecular mechanisms of how LXR suppresses inflammation have not been fully elucidated. Data from SUMOylated LXR studies revealed that LXR can inhibit the gene expression of pro-inflammatory cytokines, including TNF α , IL-6 and IL-1 β (Venteclef et al. 2010).

Regulation of ABCA1 by LXR also appears to regulate inflammatory response; increased ABCA1 expression due to LXR activation results in reduced plasma cholesterol levels, thus activating Toll-like receptors which, in turn, inhibit the phosphorylation of proteins involved in the NF κ B pathway (Zhu et al. 2010; Cave et al. 2016). Finally, a recent study from Rahman et al. (2013) revealed that the steatotic effects of LXR activation are also regulated by C/EBP- β , a down-modulator of the IL-6 gene, indicating that LXR induces steatosis but protects against inflammation.

1.5.4. Metabolic actions of bile acids

Further to their role in facilitating nutrient absorption and cholesterol catabolism, BAs are master regulators of metabolic homeostasis and serum BA levels are now used as biomarkers for diagnosis of diabetes, obesity and NAFLD (Chiang 2013). Increased total BA content and altered BA composition have been associated with diabetes in rats, accompanied by increased CYP7A1 expression (Subbiah & Yunker 1984). In line with this, in diabetic patients, total BA and faecal BA levels were elevated and were decreased after insulin treatment (Bennion & Grundy 1977). In humans, CYP7A1, the rate-limiting enzyme in BA synthesis, appears to play a critical role in glucose metabolism and several studies have demonstrated glucose and insulin to increase CYP7A1 expression both *in vitro* and *in vivo* (Li et al. 2010; Li et al. 2012; Li et al. 2006).

Importantly, the pivotal role of BAs in regulating TAG metabolism occurs via their interaction with FXR. Activation of FXR by BAs inhibits fatty acid synthesis and TAG generation by inhibiting the expression of SREBP-1c and its target enzymes, including FAS, SCD and ACC (Watanabe et al. 2004; Cipriani et al. 2010; Xiong et al. 2014; Li et al. 2014). In line with this, a number of studies have demonstrated increased expression of FGF21 by FXR activation, resulting in SREBP-1c inhibition and reduced hepatic TAG levels in mice

(Cyphert et al. 2012; Xu et al. 2009). However, the mechanism of SREBP-1c repression by FXR has not been elucidated and may involve interference with LXR. A study from Sirvent et al. (2004) revealed that FXR activation, both *in vitro* and in animal models, increases the expression of VLDL receptor, which appears to be associated with increased uptake of VLDL in peripheral tissues (Tacke et al. 2000). Other FXR targets related to lipid metabolism include the microsomal TAG transfer protein (MTP) and ApoB, that are responsible for the formation and secretion of chylomicrons and VLDL particles in intestine and liver (Hirokane et al. 2004; Hussain 2002). FXR activation also induces TAG clearance; enhances the expression of human apolipoprotein C-II (94), which, in turn, activates lipoprotein lipase (LPL), resulting in lipolysis of TAG-rich lipoproteins. Moreover, it induces the expression of other apolipoproteins, including ApoA-V, ApoC-III and apoE (Houten et al. 2006; Claudel et al. 2005). Fatty acid oxidation due to up-regulation of pyruvate dehydrogenase kinase (PDK4) expression has also been associated with FXR activity, resulting in reduced plasma TAG (Savkur et al. 2005). Consistent with these findings, in animal studies, FXR-KO mice demonstrated elevated cholesterol, increased hepatic BA content, increased serum TAG levels, excessive hepatic lipid accumulation and pro-atherogenic serum lipoprotein profile (Sinal et al. 2000; Lambert et al. 2003).

In parallel with lipid metabolism, many studies have involved BAs and FXR in the regulation of glucose homeostasis. In animal studies, BAs activate FXR which, in turn, inhibits PEPCK expression via a SHP-dependent mechanism (Yamagata et al. 2004). In rat primary hepatocytes, both conjugated and unconjugated BAs activate protein kinase B (AKT), which phosphorylates FoxO1 and inhibits PEPCK and G6Pase in the gluconeogenesis pathway. AKT activation by BAs inhibits glycogen synthase kinase 3 β (GSK3 β) expression, thus resulting in impaired glycogen synthesis (Fang et al. 2007; Fang et al. 2004). However, in

other studies, FXR activation stimulated PEPCK expression in rat hepatocytes and diabetic mice (Stayrook et al. 2005; Zhang et al. 2006).

In addition to FXR, BAs also perform metabolic actions via their binding to other nuclear receptors. In enteroendocrine cells, BAs and TGR5 regulate glucagon-like peptide-1 (GLP-1) expression, an important regulator of glucose homeostasis, appetite, insulin and glucagon secretion (Katsuma et al. 2005; Thomas et al. 2009). Activation of the TGR5-signalling pathway by BAs increased mitochondrial activity and oxidative phosphorylation, which has been linked to insulin sensitisation, in rodent brown adipose tissue and human skeletal muscle cells (Watanabe et al. 2006). Sphingosin-1-phosphate receptor 2, another BA activated G-protein-coupled receptor that is expressed in hepatocytes, activates the insulin receptor/AKT1 pathway and regulates glucose and lipid metabolism, particularly via inhibiting GSK3 β expression (Dent et al. 2005). BAs have also been shown to regulate TAG metabolism via inducing expression of PPAR α and its target gene, CPT1, the rate-limiting enzyme in fatty acid oxidation (Pineda Torra et al. 2003).

1.5.5. Bile acid metabolism and NAFLD

The critical role of BAs and their target nuclear receptors (FXR, LXR, PPAR, TGR5) to regulate hepatic lipid and carbohydrate metabolism, has been described in section 1.4.4. FXR deletion results in hepatic steatosis, IR and hyperglycaemia with evidence suggesting decreased expression in NAFLD (Kunne et al. 2014; Cipriani et al. 2010; Yang et al. 2010). In addition, FXR agonism improved insulin sensitivity in NASH patients, showing early promise as a therapeutic strategy in NAFLD (Neuschwander-Tetri et al. 2015; Mudaliar et al. 2013). In humans, hepatic LXR expression is increased in NAFLD and activation drives lipid accumulation whilst suppressing the local inflammatory response (Ahn et al. 2014; Ito et al. 2015). In rodent models, LXR antagonism attenuates high fat diet induced hepatic steatosis

(Sim et al. 2014), but clinical translational data are lacking. In addition, PPAR α gene expression levels are low in NAFLD patients and decreased PPAR α expression levels are associated with visceral adiposity and IR (Francque et al. 2015).

1.6. 5 β -reductase (AKR1D1)

1.6.1. Gene structure and splice variants

5 β -reductase (AKR1D1) is a member of the aldo-keto-reductase (AKR) superfamily 1 of enzymes and is the first member of the 1D subfamily (AKR1D1), along with the rat (AKR1D2) and the rabbit (AKR1D3) 5 β -reductase isoform (Onishi et al. 1991; Faucher et al. 2008b). The gene is located on chromosome 7 in humans (Figure 1.6.1-1) (Chen & Penning 2014), whilst in mice and rats is located on chromosomes 6 and 4, respectively.

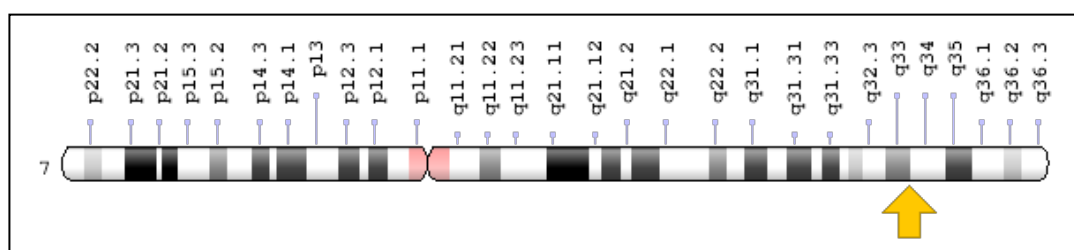


Figure 1.6-1 Chromosomal location of *AKR1D1* in humans. The cytogenetic location of *AKR1D1* is 7q33, which is the long term (q) arm of chromosome 7 at position 33. The molecular location is from 138,076,432 to 138,118,304 base pairs. Picture taken from <https://ghr.nlm.nih.gov>

The human gene consists of 9 exons and 8 introns and six transcript variants have been identified, four of which lead to functional protein isoforms (Figure 1.6.1-2). A long 3'-untranslated region rich in AT sequences in the mRNA of *AKR1D1* indicates that this gene undergoes quick degradation. Amongst all isoforms, a 326 amino acid AKR1D1 protein, encoded by an *AKR1D1-002* transcript, is the most well characterised. Transcript *AKR1D1-001* omits exon 5 and encodes a 285 amino acid AKR1D1 protein with identical N- and C-termini to the 326 amino acid isoform. However, the missing residues in the middle of the sequence, due to the lack of exon 5, may result in changes on the protein structure and subsequent problems with folding. In contrast, the *AKR1D1-006* transcript lacks exon 8, resulting in a 290 amino acid enzyme that misses the C-terminal flexible loop, which is

responsible for steroid binding. Therefore, although this isoform may fold correctly, it probably demonstrates decreased activity due to impaired affinity towards steroid substrates (Barski et al. 2013). Other isoforms may be present, but their full-length natures have not been determined yet.

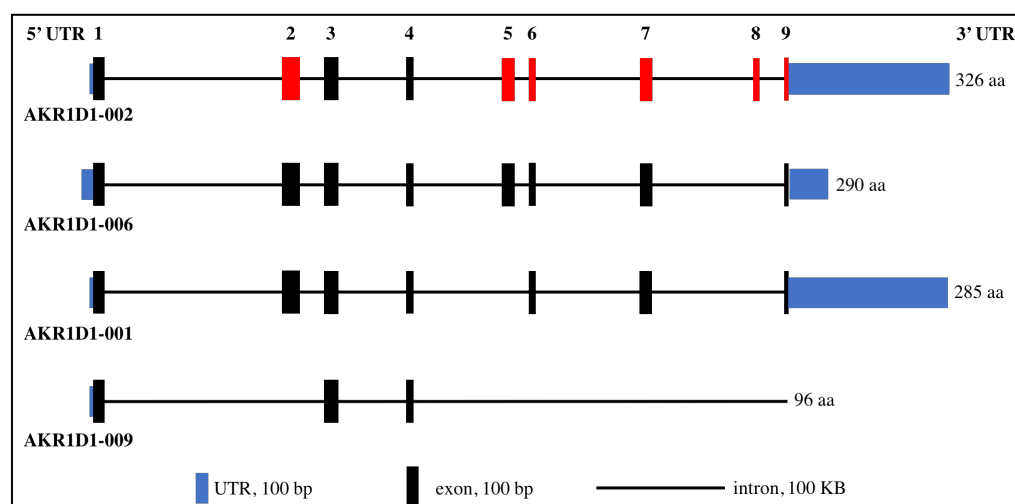


Figure 1.6-2 *AKR1D1* gene structures and splice variants. The nine exons are represented as filled boxes and numbered. The constitutive exons are shown in black and the alternatively spliced exons are in red.

The enzyme encoded by the full length gene has a molecular weight of 37kDa. It utilises NADPH as an electron donor and, although most aldo-keto reductases (AKRs) catalyse a reversible oxidoreduction, AKR1D1 catalyses a stereospecific irreversible double bond reduction between the 4th and the 5th carbon of the A-ring of steroids. It is also suggested to follow a bi-bi kinetic mechanism, with the cofactor NADPH binding first to the enzyme and NADP⁺ releasing last (Chen et al. 2014). 5 β -reduction is unique in steroid enzymology as it introduces a 90° bend and creates an A/B *cis*-ring junction, resulting in steroids with different properties from either the α,β -unsaturated or 5 α -reduced steroids, which possess a largely planar four-ring steroidal structure with an A/B *trans* ring-junction (Figure 1.5.1-3) (Jin et al. 2014).

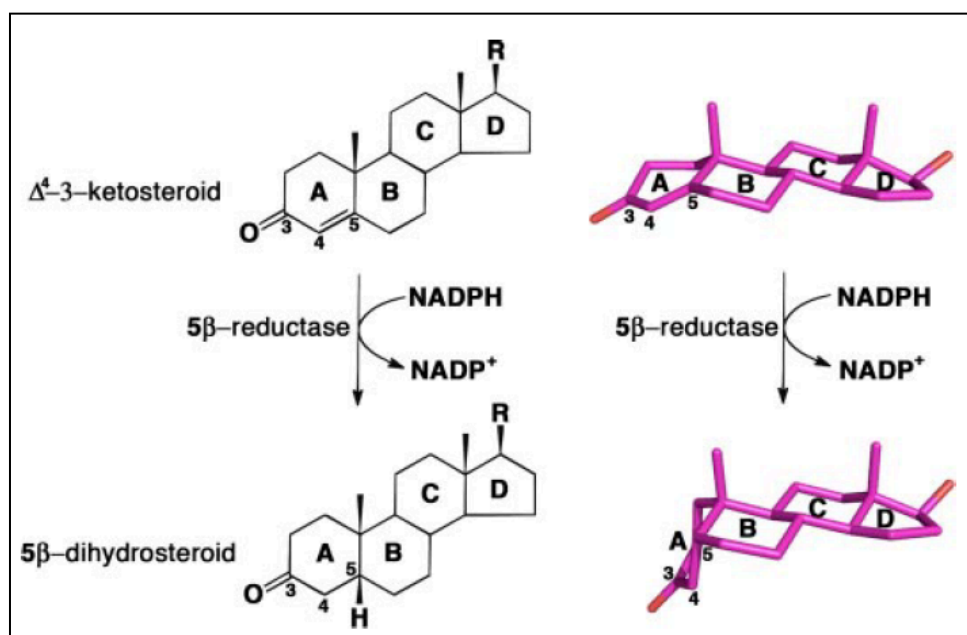


Figure 1.6-3 Reduction of the carbon–carbon double bond at C-5 of a Δ^4 -3-ketosteroid to form a 5β -dihydrosteroid as catalysed by AKR1D1. Steroid rings and selected carbon atoms are labelled according to standard steroid nomenclature. Picture and legend taken from Di Costanzo et al. (2008).

1.6.2. AKR1D1 substrate specificity

AKR1D1 has an important pre-receptor role in the regulation of steroid hormone action, as it generates all 5β -reduced dihydrosteroid metabolites for C19-C27 steroids (Chen & Penning 2014). Both cortisol and cortisone are metabolised to 5β -dihydrocortisol and 5β -dihydrocortisone respectively by AKR1D1, and are then subsequently converted, in a non-rate limiting step, to their tetrahydro-metabolites (tetrahydrocortisol, 5β -THF and tetrahydrocortisone 5β -THE) by 3α -HSD, respectively. AKR1D1 also inactivates progesterone and testosterone to 5β -pregnane-3,20-diol and 5β -DHT. Moreover, it inactivates the mineralocorticoid aldosterone and its precursor corticosterone (Figure 1.5-2). However, not all 5β -reduced metabolites are inactive; AKR1D1 activity generates a series of diverse active 5β -dihydrosteroids involved in neurotransmission, parturition, erythropoiesis and protection from xenobiotic insult (Chen et al. 2014; Chen et al. 2011).

In addition to its role in steroid hormone clearance, it has a crucial function in BA synthesis, as it uses the BA intermediates $7\alpha,12\alpha$ -dihydroxy-4-cholesten-3-one and 7α ,hydroxy-4-cholesten-3-one as substrates to catalyse the formation of CA and CDCA (Charbonneau & The 2001).

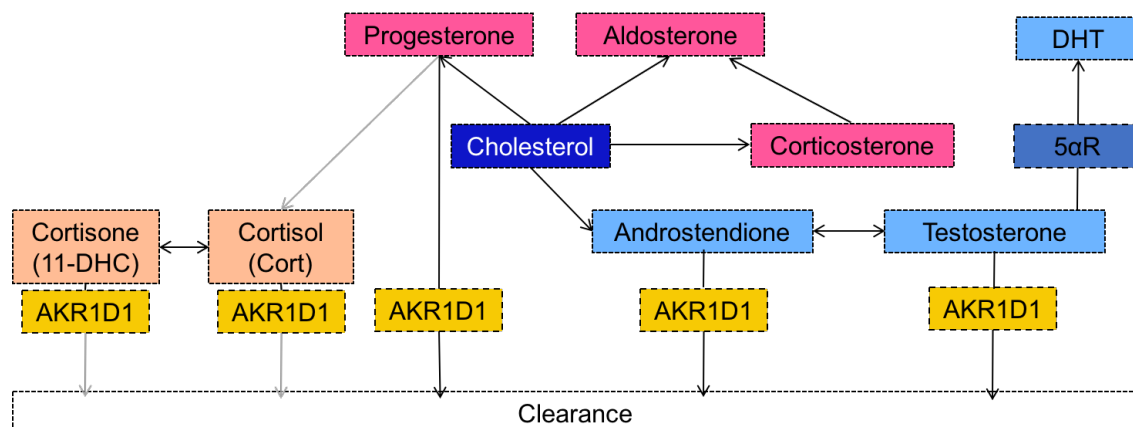


Figure 1.6-4 *AKR1D1* clears cortisone, cortisol, progesterone, aldosterone, corticosterone, androstenedione, and testosterone to their inactive 5 β -reduced metabolites.

Substrate specificity of AKR1D1 is debated and results from different studies have been conflicting. In 1990, Iyer et al. (1990) partially purified AKR1D1 protein from subcellular fractions of human liver and demonstrated that Δ^4 -3-ketosteroids, including androstenedione, progesterone, cortisone, and cortisol, were all active substrates, whilst testosterone was reported as an inactive AKR1D1 substrate. A study from Kondo et al. (1994) in COS cells demonstrated that the BA intermediates (cholestenones) were the preferred substrates of AKR1D1, whilst cortisol and testosterone 5 β -reduction was more limited by comparison. Furthermore, no reduction of androstenedione or progesterone was observed. In contrast, Charbonneau & The (2001) reported that, in HEK293 cells, AKR1D1 had the highest affinity towards androstenedione, testosterone, progesterone and 17-hydroxyprogesterone with

corticosteroids and mineralocorticoids acting as inferior substrates. It is likely that discrepancies in the data between different studies occurred due to different experimental conditions (e.g. cell homogenates, intact cells in culture) or due to low purification quality, which resulted in concomitant activity of other endogenous enzymes that also metabolise either the same substrates or their formed products. A number of studies have also tried to characterise the substrate specificity of AKR1D1 using purified recombinant AKR1D1 protein, but the data have been conflicting, probably due to differences in purification protocols (Chen et al. 2011; Okuda & Okuda 1984).

1.6.3. Tissue distribution

AKR1D1 is largely expressed in liver in humans (as in rats and mice), with a presence more than 10-fold higher than any other tissue (Chen & Penning 2014). A number of studies have also reported the presence of 5 β -reduced metabolites in foetal brain, uterus and plasma, indicative of AKR1D1 activity in these tissues (Sheehan et al. 2005; Lisboa et al. 1974). In particular, 5 β -pregnan-3,20-dione, the 5 β -reduced form of progesterone, has been reported as a potent tocolytic steroid with its concentrations to be negatively correlated to the active phase of the first stage of human labour (Sheehan et al. 2005; Kubli-Garfias et al. 1979). Low levels of AKR1D1 activity have also been found in testis, colon, skeletal muscle, adipose tissue and blood cells (Charbonneau & The 2001; Flicek et al. 2012; Kolker et al. 2012; Zhang et al. 2009). Although sex-related differences in AKR1D1 have been reported in rats, with increased enzyme activity and cytosolic content in males compared to females (Mode & Rafter 1985), no sexual dimorphism has been reported in humans (Baudrand et al. 2011). Sex-related differences in AKR1D1 activity have been reported in other animal species, including mouse, fish, bird and frog (Langlois et al. 2010; Barrett et al. 2011; Soma

et al. 2003). However, a study by Finken et al. (1999), revealed differences in glucocorticoid metabolism by AKR1D1 between men and women.

1.6.4. AKR1D1 structural biology

The human AKR1D1 protein is highly homologous with other members of the AKR family, including the AKR1C subfamily, aldose reductases and hydroxysteroid dehydrogenases (HSDs); in addition, it shares a 79% sequence homology with the rat isoform (AKR1D2) (Li et al. 2012; Jez et al. 1997). The crystal structure of ternary complexes of AKR1D1 have been extensively characterised by different groups using testosterone, cortisone, Δ^4 -androstene-3,17-dione, progesterone, and the 5 β -reduced metabolites 5 β -dihydroprogesterone and 5 β -dihydrotestosterone as substrates (Faucher et al. 2008a; Faucher et al. 2008b; Di Costanzo et al. 2008; Drury et al. 2009). In all studies, NADPH was used as a cofactor. AKR1D1 shares two important characteristics with the other members of the AKR family: a conserved protein fold that forms a characteristic (α/β)-barrel structure and a conserved mode in the centre of the barrel for substrate and cofactor binding. The members of the AKR family are composed of 323 amino acids of which four compose a tetrad most important in catalysis: Asp₅₀, Tyr₅₅, Lys₈₄, and His₁₁₇. The enzymes of the AKR1D subfamily, however, have three additional amino acids in their primary structures and, more importantly, a single amino acid substitution in their catalytic tetrad, from His₁₁₇ to Glu₁₂₀ (Faucher et al. 2008b; Mindnich et al. 2011). The Glu₁₂₀ residue does not play a direct role in catalysis; it is important for the proper positioning and binding of the substrate in the catalytic site (Faucher et al. 2008a). Furthermore, a study from Chen et al. (2012) has revealed the crucial role of the Glu₁₂₀ residue in the ability of AKR1D1 to perform its 5 β -reductase activity; through a single glutamate-to-histidine mutation in AKR1D1 (E120H), the enzyme eliminates its 5 β -reduction function and acts as a potent hydrogenase (3 β -HSD). In addition, site-directed mutagenesis

assays have identified the presence of an alternative binding site, which involves the Val₃₀₉ and Trp₂₃₀ residues, and is specific for testosterone and Δ^4 -androstene-3,17-dione. Whilst cortisone and progesterone bind perpendicularly to NADP⁺ in the catalytic cavity, testosterone and Δ^4 -androstene-3,17-dione bind parallel to NADP⁺ in the AKR1D1- NADP⁺ complex (Figure 1.5-5) (Faucher et al. 2008a; Di Costanzo et al. 2008). Efforts to characterise the binding of the BA precursors 7 α ,hydroxy-4-cholestenone and 7 α ,12 α ,dihydroxy-4-cholestenone, using the generated crystal structure of AKR1D1, have not been successful due to the insoluble nature of these compounds. However, through manual positioning, these substrates have been shown to also bind perpendicularly to NADP⁺, in the proximity of the Tyr₅₅ and Glu₁₂₀ residues in the binding cavity (Faucher et al. 2008b).

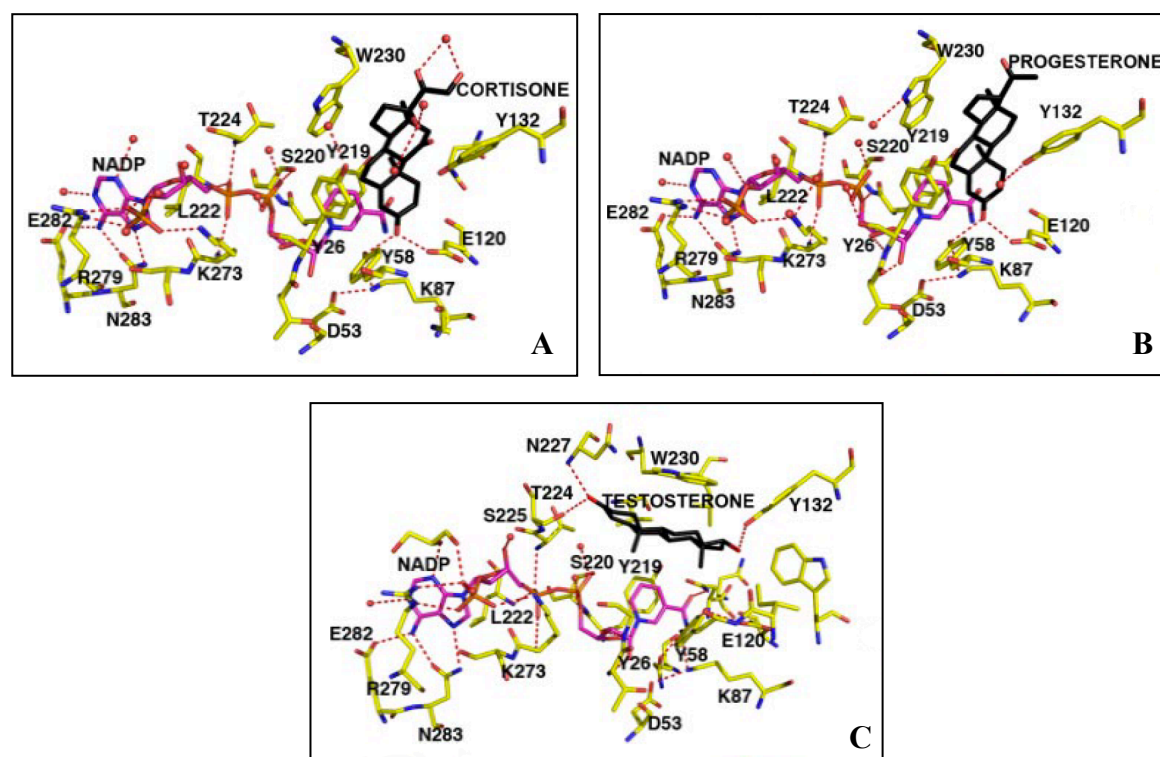


Figure 1.6-5 (A). AKR1D1-NADP⁺-cortisone, (B). AKR1D1-NADP⁺-progesterone and (C). AKR1D1-NADP⁺-testosterone complexes. Steroids are in black, protein carbon atoms in yellow and hydrogen bond interactions are indicated by red dashed lines. Pictures taken from (Di Costanzo et al. 2008)

1.6.5. AKR1D1 mutations

The first evidence for defects in AKR1D1 activity was reported in 1988 by Clayton et al. (1998) and Setchell et al. (1988) who demonstrated increased conjugated 7 α ,hydroxy-3-oxo-4-cholenoic acid and 7 α ,12 α ,dihydroxy-3-oxo-4-cholenoic acid levels in urine from infants with liver disease. In 1996, Clayton et al. (1996) reported an infant with chronic cholestatic liver disease and increased 3-oxo- Δ^4 BAs in urine; ursodeoxycholic acid treatment failed to improve liver disease but the patient responded to a combination of CDCA and CA indicating the importance of use of BAs that inhibit CYP7A1. In 1997, Sumazaki et al. were the first to sequence the AKR1D1 cDNA of a 21-days-old infant with 5 β -reductase deficiency and identified a single silent mutation at base number A753→G. However, there were no mutations that would have predicted defects in amino acid sequences of the AKR1D1 enzyme protein. Since then, a variety of single point mutations in patients with genetic 5 β -reductase deficiency have been reported. In 2003, Lemonde et al. reported three different patients with neonatal onset cholestasis and almost undetectable amounts of CDCA and CA. All three children demonstrated different mutations in the AKR1D1 gene; the first was homozygous for a missense mutation (662C>T) causing a Pro198Leu amino acid substitution in exon 6; the second patient was homozygous for a single base deletion (511 delT) causing an early stop codon in exon 5; the third patient was homozygous for a missense mutation (385C>T) causing a Leu106Phe amino acid substitution in exon 3 (Lemonde et al. 2003). However, other severe cases of 5 β -reductase deficiency have revealed significant differences in the biochemical findings and a number of studies have reported increased plasma CDCA concentrations, a high ratio of CDCA to CA or increased concentrations of total BAs (Kimura et al. 1991; Kimura et al. 2000; Kimura et al. 1998). A number of reasons may account for these findings. It is probable that these patients carry less severe AKR1D1 mutations resulting in residual AKR1D1 activity, which in turn leads to inhibition of the bile

salt export pump with reflux of BAs in the plasma. Reduced hepatic AKR1D1 mRNA levels due to increased mRNA degradation or secondary changes in the liver that lead to inactivation of AKR1D1, rather than mutations in AKR1D1, may also result in this phenotype. In 2004, another study from Gonzales et al. (2004) revealed two heterozygous mutations in the AKR1D1 gene in twin sisters with clinical and biochemical features of Δ^4 -3-oxosteroid 5 β -reductase deficiency. Sequencing of the 9 exons of the AKR1D1 gene revealed two non-conservative missense mutations in exons 4 (P133R, 467C>G) and 7 (R261C, 850C>T). CA treatment restored cholestasis, portal inflammation and fibrosis and five years later both girls had normal clinical features. Two more heterozygous mutations were reported a few years later, a single-nucleotide mutation in exon 6 of the AKR1D1 gene (737G>A) and another single mutation in exon 2 which caused an early stop codon at amino acid 50. Both patients were female infants who developed jaundice and hyper-3-oxo- Δ^4 bile aciduria; treatment with the secondary BA ursodeoxycholic acid (UDCA) and CDCA restored serum bilirubin and aminotransferase levels within the normal range, however, in one of the patients liver function continued to deteriorate and resulted in liver transplantation (Ueki et al. 2009). Finally, two heterozygous mutations were reported in 2012 in two male infants from China; the first patient was heterozygous for c.797G>A in exon 7 (p.R266Q), whilst the second patient had a nonsense mutation for c.396C>A as well as for c.722A>T (p.D241V) in exons 4 and 7, respectively. Both patients developed jaundice and UDCA and CDCA treatment improved liver transaminase concentrations, jaundice and total bilirubin levels (Zhao et al. 2012). The enzymatic properties of five of these mutations have been explored *in vitro* using HEK293 cells, confirming the adverse effects in BA formation (Drury et al. 2010). More recently, the P133R mutation was successfully purified in E.coli to further explore the effects of AKR1D1 mutations in bile acid deficiency (Chen et al. 2015).

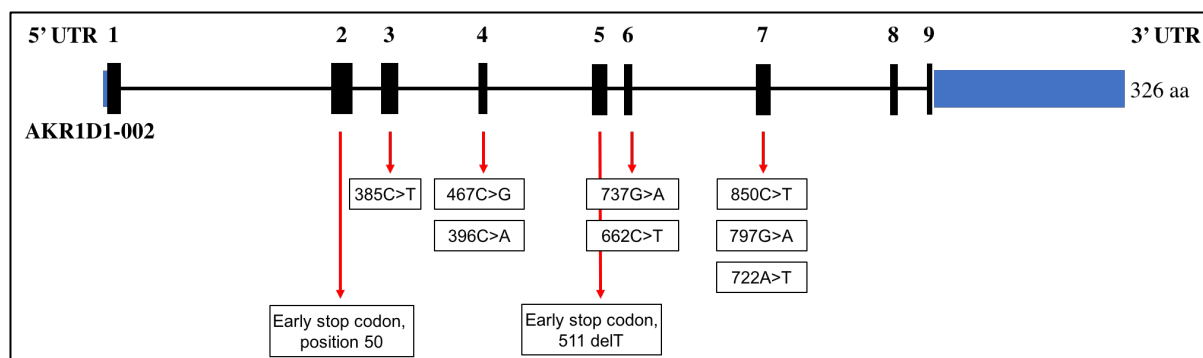


Figure 1.6-6 Diagram of AKR1D1 gene structure with found mutations on different exons.

1.6.6. Metabolic role of AKR1D1

Although the enzymology of AKR1D1 has been extensively described, to date, the putative role of this enzyme in the regulation of metabolic phenotype is almost entirely unexplored. A single study utilizing the measurement of urinary steroid hormone metabolites has demonstrated an association between increased AKR1D1 activity and hepatic steatosis (Westerbacka et al. 2003). In an animal study with obese Zucker rats, hepatic AKR1D1 mRNA expression was increased and insulin sensitisation partially reversed AKR1D1 activity (Livingstone et al. 2005). Consistent with this, increased AKR1D1 activity with enhanced cortisol clearance and a positive correlation between AKR1D1 activity and insulin levels has also been reported in women with PCOS (Gambineri et al. 2009). In contrast, decreased AKR1D1 activity has been associated with hypertension, as measured by increased plasma corticosterone levels in untreated hypertensive subjects compared to normotensive controls (Soro et al. 1995). It has also been suggested that in patients with mutations in 11 β -HSD2, who demonstrate apparent mineralocorticoid excess (AME), the subsequent elevated cortisol and cortisol metabolites levels due to decreased 11 β -HSD2 activity are responsible for inhibition of AKR1D1, resulting in impaired aldosterone clearance and increased mineralocorticoid levels (Faucher et al. 2008a). More recently, in an animal study, AKR1D1

deletion in mice resulted in decreased BA concentrations. AKR1D1-KO mice also demonstrated a sexually dimorphic metabolic phenotype, with female, but not male, KO mice being metabolically protected from high-fat diet and showing lower body weight gain, increased insulin sensitivity and improved glucose tolerance compared to wild type (Gathercole et al. 2015).

1.6.7. AKR1D1 inhibitors

Although there is evidence on the role of AKR1D1 in liver metabolism, to date, there are no specific inhibitors available for AKR1D1 that could help to investigate its exact functional role in metabolic phenotype and/or use these drugs as potential therapeutic interventions. However, a few studies have tried to explore the potential of known natural and pharmacological inhibitors to block AKR1D1 activity.

In a study from Okuda & Okuda (1984), 4-Chloromercuribenzoic acid (pCMB), a sulfhydryl organomercury compound that is used as a protease inhibitors, was used as a potential inhibitor of AKR1D1, using androstenedione as a substrate. pCMB was able to inhibit AKR1D1 activity in concentrations ranging from 0.01-0.1mM, with the highest inhibition of AKR1D1 (86.4%) in the presence of 0.1mM pCMB.

Finasteride is a selective 5 α R2 inhibitor and has been widely used a therapeutic agent for the treatment of benign prostatic hyperplasia and prostate cancer (Gormley 1996; Habib et al. 1997; Andriole et al. 1995; Bull et al. 1996). Although it was originally believed to act as a reversible competitive inhibitor, it was later found to be an irreversible mechanism-based inhibitor of 5 α R2 (Andersson & Russell 1990; Bull et al. 1996) . In a study of Drury et al. (2009), finasteride was tested as a potential AKR1D1 inhibitor, however, it failed to inhibit AKR1D1 activity and acted as a competitive inhibitor with low micromolar affinity ($K_i=2.1\mu\text{M}$).

More recently, non-steroidal anti-inflammatory drugs (NSAIDs) that have been proved to inhibit the human hydroxysteroid dehydrogenases AKR1C1-C4 were used as potential AKR1D1 inhibitors using testosterone as substrate, but failed to block the enzyme activity. In the same study, the natural inhibitors of AKR1C2 CDCA and ursodeoxycholic acid were tested against AKR1D1 activity; both BAs acted as non-competitive inhibitors for AKR1D1 with high low micromolar affinity ($K_i=3.2 \mu\text{M}$ and $K_i=9.8 \mu\text{M}$ for CDCA and ursodeoxycholic acid, respectively) (Chen et al. 2011).

1.7. Hypothesis and aims

AKR1D1 sits at the interface between two fundamentally important biological pathways (steroid hormone metabolism and BA synthesis) both of which have been shown to be potent regulators of metabolic phenotype. The putative role of AKR1D1 in this regard is almost entirely unexplored. We have therefore hypothesised that genetic manipulation of AKR1D1 has the potential to alter the availability of a variety of steroid hormones and BAs within the liver. Alterations in steroid hormone metabolism and BA formation, due to genetic manipulation of AKR1D1, can have an impact on the activation of specific nuclear receptors, including the GR, FXR and LXR, leading to alterations upon hepatocyte metabolic phenotype.

The aims of this thesis are:

- Develop a novel and improved *in vitro* human hepatocyte model that may more closely resemble primary human hepatocytes for the study of steroid hormone metabolism and metabolic disease (Chapter 3)
- Generate a human recombinant AKR1D1 protein and characterise the enzymology of AKR1D1 in cell free systems and to explore its potential as a tool for the development of selective inhibitors (Chapter 4)
- Define the pre-receptor role of AKR1D1 and its ability to regulate steroid hormone availability and formation of BAs, *in vitro* (Chapter 5)
- Explore the role of AKR1D1 to regulate the activation of nuclear receptors, including the GR, as a result of alterations in steroid hormone and BA availability and composition (Chapter 5)
- Define the role of AKR1D1 in carbohydrate and lipid metabolism in human hepatocytes (Chapter 6)
- Define the role of AKR1D1 in inflammation in human hepatocytes (Chapter 7)

Chapter 2 General Methods

The methods described in this chapter are commonly used within this thesis. Methods specific to individual chapters are found in the method sections of those chapters. Unless stated otherwise, methods were performed using Sigma Reagents (Gillingham, UK) and Cell Culture plastic ware from Corning (Artington, Surrey, UK).

2.1. Cell culture

2.1.1. HepG2, Hep3b and Huh7 cell culture

HepG2 cells are a hepatocyte-derived cellular carcinoma cell line that were derived from the liver tissue of a 15-year-old Caucasian American male from Argentina in 1975. It is an immortal cell line of epithelia-like tumourigenic cells, which secrete a variety of major plasma proteins and have been successfully grown in large-scale cultivation systems (Aden et al. 1979).

Hep3b cells are a well-differentiated hepatocyte-derived cellular carcinoma cell line that were derived from the liver tissue of an 8-year-old black male in the US in 1976 (Aden et al. 1979). It is an immortal cell line which contains an integrated hepatitis B virus genome and has been extensively used as an experimental model for the investigation of plasma protein biosynthesis and the relation of the hepatitis B virus genome to tumourigenicity (Knowles et al. 1980)

Huh7 cells are a hepatocyte-derived cellular carcinoma cell line that were originally derived from the hepatoma tissue of a 57-year-old Japanese male with well-differentiated hepatocellular carcinoma (Nakabayashi et al. 1982). Huh7.0 cells are an immortal cell line of epithelial-like tumourigenic cells and has been extensively used in hepatitis C and hepatic fat metabolism studies (Sainz et al. 2009; Mahdessian et al. 2014; Ohsaki 2006).

All three human hepatoma cell lines were maintained in Cell Bind 75cm² TC flasks in 15ml of Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 5% non-essential amino acids and were grown at 37°C in a 5% CO₂ setting. At 70-80% confluence, cells were trypsinised in 3ml Tryple Express (Invitrogen, Paisley, UK) and re-seeded into fresh flasks.

Cells that were frozen down for long-term storage were grown in 75cm² TC flasks. At 80% confluence, cells were washed, trypsinised and centrifuged at 240g for 5min. The cell pellet was resuspended in 1ml of freezing serum (FBS with 10% DMSO) and the cell mixture was transferred into 1.5ml cryovials and slowly frozen at a rate of 1°C/min in a cell freezing container (ThermoFisher Scientific, Hemel Hempstead, UK) at -80°C for 24h. Subsequently, cells were transferred to liquid nitrogen for long-term storage.

2.1.2. C3A cell culture

The C3A human cell line is a clonal derivative of HepG2/C3A that was isolated from a 15-year-old male with a liver tumour and they are well established as an *in vitro* model of the mature human liver (American Type Culture Collection).

Cells were maintained in Cell Bind 25cm² TC flasks in 5ml of Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 5% non-essential amino acids and were grown at 37°C in a 5% CO₂ setting. At 70-80% confluence, cells were washed and trypsinised in 1ml Tryple Express (Invitrogen, Paisley, UK) and re-seeded into fresh flasks.

Cells to be frozen were grown in 25cm² TC flasks. At 80% confluence, cells were washed, trypsinised and centrifuged at 500g for 5min. The cell pellet was resuspended in 1ml of freezing serum (FBS with 10% DMSO). Cell mixture was transferred into 1.5ml cryovials and slowly frozen at a rate of 1°C/min in a cell freezing container (ThermoFisher Scientific, Hemel Hempstead, UK) at -80°C for 24h. Subsequently, cells were transferred to liquid nitrogen for long-term storage.

2.1.3. Human Embryonic Kidney (HEK293) cell culture

The Human Embryonic Kidney cell line, also referred to as HEK293 cell line, is a specific cell line originally derived from human embryonic kidney cultures transformed with sheared adenovirus 5 DNA. The cell line was first generated in Alex van der Eb's laboratory in Leiden, The Netherlands and the original donor was an unknown, single and healthy foetus legally aborted under Dutch law (Gross 2001).

Cells were maintained in Cell Bind 75cm² TC flasks in 15ml of DMEM supplemented with 10% FBS and 5% non-essential amino acids and were grown at 37°C in a 5% CO₂ setting. At 70-80% confluence, cells were trypsinised in 3ml Tryple Express (Invitrogen, Paisley, UK) and re-seeded into fresh flasks.

Cells to be frozen were grown in 75cm² TC flasks. At 80% confluence, cells were washed, trypsinised and centrifuged at 500g for 5min. The cell pellet was resuspended in 1ml of freezing serum (FBS with 10% DMSO). Cell mixture was transferred into 1.5ml cryovials and slowly frozen at a rate of 1°C/min in a Mr. Frosty freezing container (ThermoFisher Scientific, Hemel Hempstead, UK) at -80°C for 24h. Following that, cells were transferred to liquid nitrogen for long-term storage.

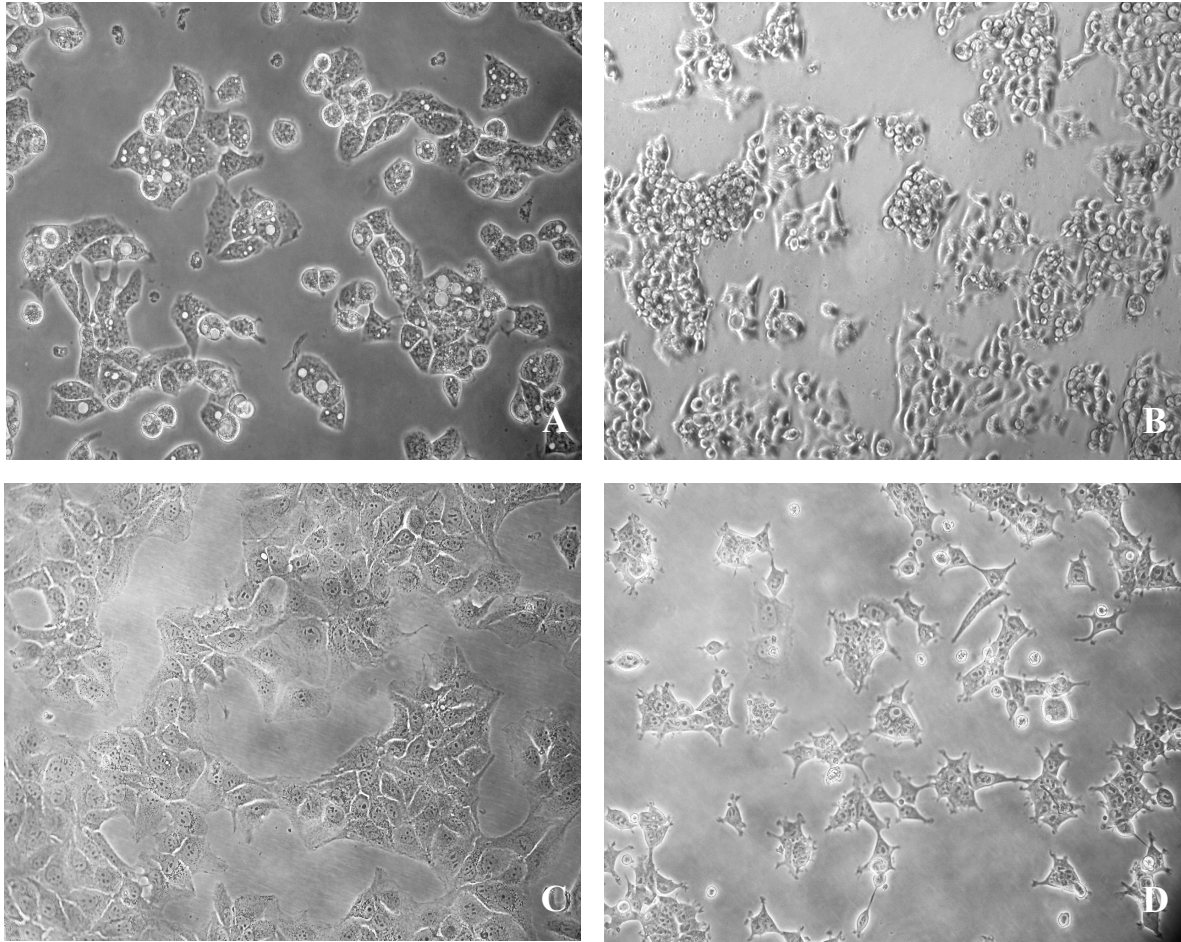


Figure 2.1.3-1 Human hepatoma (A). HepG2 cells, (B). C3A cells, (C). Huh7.0 cells and (D). Human Embryonic Kidney (HEK293) cells were cultured in DMEM or EMEM supplemented with 10% FBS and 5% non-essential amino acids and were grown at 37°C in a 5% CO₂ setting. (20x analysis).

2.2. Bacterial transformation

2.2.1. Principle

Artificial bacterial transformation is the process by which naked DNA molecules are inserted into not naturally transformable bacterial cells. This type of bacteria can receive exogenous genetic material through particular chemical or electrical treatments such as electroporation, heat shock, Ca^{2+} treatment or protoplast DNA uptake. However, as not all bacterial cells get transformed, a selection marker is often used (usually antibiotic resistance), to choose the successfully transformed cells. Cells are incubated in an antibiotic-containing media and DNA molecules are extracted from cells using purification methods.

2.2.2. Method

Alpha-Select Gold Efficiency cells were purchased by Bioline (Luckenwalde, Germany) and all plasmids were purchased by Origene Technologies (Rockville, USA). 2µl of plasmid DNA was added into 45µl of bacteria cells and put on ice for 45min. Cells were then heat-shocked at 42°C for 30sec and incubated at 37°C with 500µl of lysogeny broth (LB) growth medium for 1h. 50-100µl of the bacterial mix were then plated on an LB agar plate and the plate was incubated at 37°C overnight. One single bacterial colony was selected using a pipette tip and transferred into a 50ml falcon tube with 20ml of LB growth medium containing 100ng/ml penicillin. Tubes were incubated at 37°C overnight with constant agitation. Colonies were then centrifuged at 800g for 15min and plasmids were extracted using a commercially available Miniprep Kit (QIAGEN, Manchester, UK). The plasmid quality was measured using the Nanodrop ND-1000 system and the plasmid quantity was identified spectrophotometrically at OD₂₆₀. DNA has an absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the DNA purity of the

extraction. Only the DNA with the ratios in the range of 1.8 - 2.0 was used in the experiments.

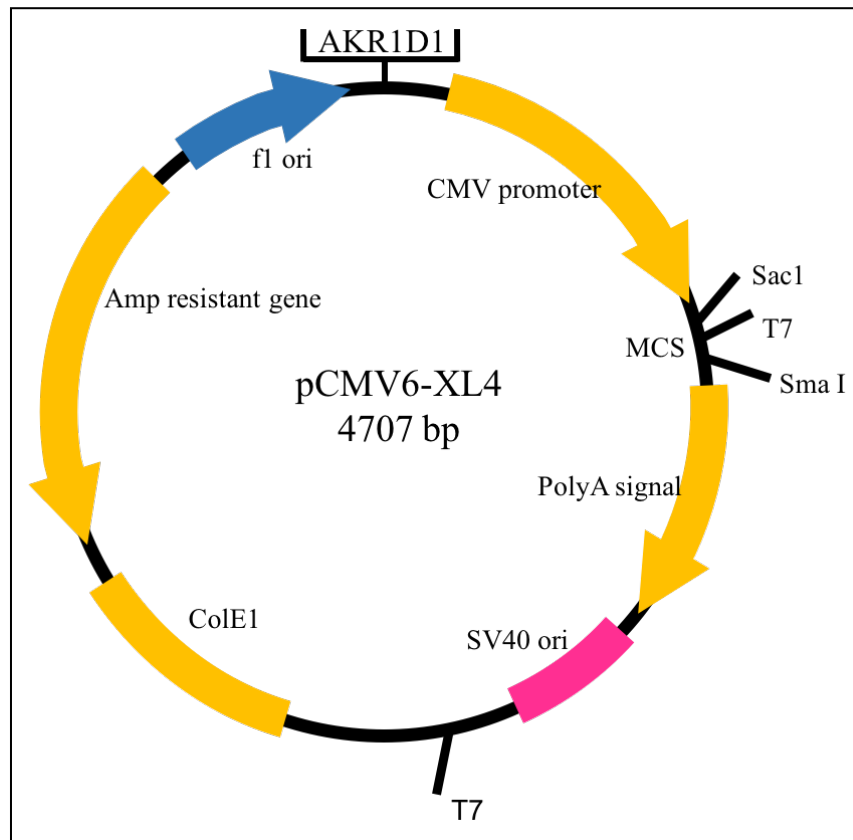


Figure 2.2.2-1 pCMV6-XL4 plasmid map with an AKR1D1 gene insert and Amp resistant gene (Origene Technologies).

2.3. Transfection

2.3.1. Principle

Transfection is the process whereby nucleic acids are introduced into eukaryotic cells. HepG2 hepatoma cells were tested to determine their suitability for transfection with the AKR1D1 expressing vector. In addition, they were also tested to determine whether they could be used as a suitable model for knockdown studies using small interference RNA (siRNA) transcripts.

Results showed that this cell line could be used as suitable transfection host (see chapters 5, 6 and 7).

2.3.2. Gene over-expression

24h prior to transfection, cells were plated in a 12-well Cell Bind plate (Corning, Flintshire, UK) to achieve cell confluence of ~60-70%. For the experiment 1µg of DNA and 2µL of X-tremeGENE DNA transfection Reagent (Roche, Hertfordshire, UK) was diluted in 100µL of OPTIMEM serum free media (Invitrogen, Paisley, UK). The mixture was vortexed and incubated at room temperature for 20min, and afterwards, was added drop-wise to each well (100µL/well). Finally, the plates were shaken gently and put into the incubator at 37°C for 48h.

2.3.3. Gene knockdown

12h prior to transfection, cells were plated in a 24-well Cell Bind plate (Corning, Flintshire, UK) to achieve cell confluence of ~60-70%. Three different AKR1D1 siRNA molecules (Table 2.3.3-1) and a Silencer® Select Negative siRNA Control were purchased from Invitrogen (Paisley, UK). To prepare siRNA/lipid solutions for each well of a 24-well plate, 20nmol of AKR1D1 siRNA were diluted in 25µL of OPTIMEM serum free media (Invitrogen, Paisley, UK). In a separate tube, 2.5µL of Lipofectamine RNAiMAX (Invitrogen, Paisley, UK) were diluted in 25µL of OPTIMEM. The contents of the two tubes were combined by gentle pipetting and incubated at room temperature for 5min. 50µL of the resulting transfection solution were added drop-wise to each well. Finally, the plates were shaken gently and put into the incubator at 37°C for 48h.

Increasing concentrations of all AKR1D1 siRNA variants (20, 40 and 100nM) were targeted against AKR1D1 expression in HepG2 cells and decreased mRNA expression was tested

using qPCR (Table 2.3-1, Figure 2.3.3-1). Based on the qPCR results, the AKR1D1 siRNA variant HSS1101183 was chosen for the knockdown studies.

Gene symbol	Assay ID	Interogated sequence (RefSeq)	Targeted exon (RefSeq)	siRNA location (RefSeq)
<i>AKR1D1</i>	HSS1101182	NM_001190906.1 NM_001190907.1	2	227
<i>AKR1D1</i>	HSS1101183	NM_001190906.1 NM_001190907.1	4	509
<i>AKR1D1</i>	HSS1101184	NM_001190907.1 NM_005989.3	5	566

Table 2.3.3-1 List of *AKR1D1* siRNA variants which are provided by Invitrogen.

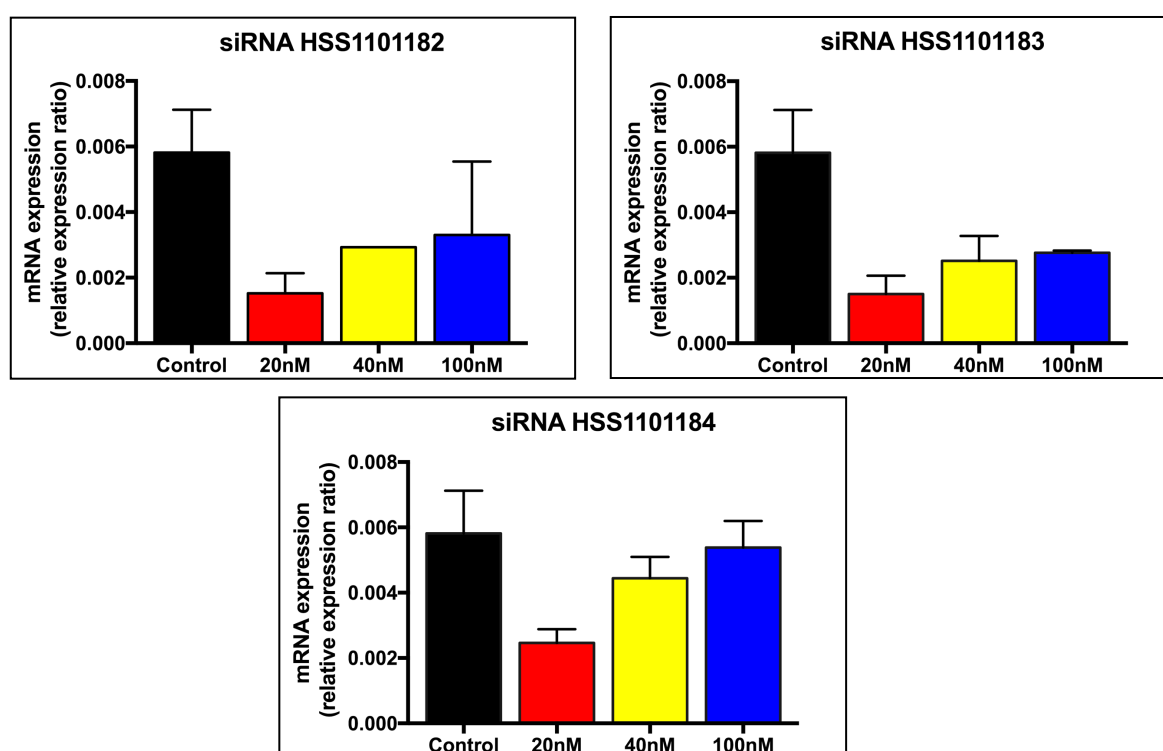


Figure 2.3.3-1 mRNA expression of *AKR1D1* following transfection with three *AKR1D1* siRNA variants in HepG2 cells, in a dose-dependent manner. Data are expressed as relative expression ratio values $\pm$ sd of n=2 experiments.

2.4. RNA extraction

2.4.1. Principle

RNA extraction is the purification of RNA from biological samples. This procedure is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA. Several methods are used in molecular biology to isolate RNA from samples; the most common of these is guanidinium thiocyanate-phenol-chloroform extraction (TRI-Reagent system).

2.4.2. Method

Total RNA was extracted from cells using the Tri-Reagent system (Sigma-Aldrich, Gillingham, UK). 24h after the transfection, the plates were put onto ice and 1ml of TRI-Reagent was added to each well. The wells were thoroughly scrubbed with a 1ml pipette tip in order to effectively detach the cells from the well surface. Afterwards, the cell lysates in Tri-Reagent were put into 1.5ml microtubes.

100µl of chloroform was added per 1ml of TRI-Reagent to each tube. The tubes were vigorously shaken for 15sec and then incubated at room temperature for 15min. The mixture was then centrifuged at 13200g for 15min at 4°C and, subsequently, the RNA was separated in the upper aqueous phase, with the DNA and proteins in the organic interface. 500µl of isopropanol, followed by 1ml of 75% ethanol, were then added in order to precipitate and wash the RNA. Finally, after the removal of ethanol, the RNA pellets were left at room temperature for 5min to dry and then dissolved in 20-50µl of nuclease-free water.

The RNA quality was measured using the Nanodrop ND-1000 system and the RNA quantity was identified spectrophotometrically at OD₂₆₀. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an

RNA preparation. Only the RNA with the ratios in the range of 1.8 - 2.0 was used in the experiments.

2.5. Reverse Transcription Polymerase Chain Reaction

2.5.1. Principle

Reverse transcription polymerase chain reaction (RT-PCR) is commonly used in molecular biology to convert single stranded RNA into complementary DNA (cDNA). The extracted RNA is heated to denature secondary structures and subsequently cooled to allow annealing of random hexamers to the RNA template. Once annealed, the enzyme reverse transcriptase is used to extend the RNA-bound primer, in the presence of an RNase inhibitor. The final cDNA product is then used as a template for exponential amplification using PCR.

2.5.2. Method

RT reactions were performed using an Applied Biosystems Reverse Transcription Kit (Applied Biosystems, Warrington, UK). 1µg of RNA was diluted in nuclease free water to a final volume of 10µl, and 10µl of 2x RT master mix were added to this RNA dilution. To generate a 2x RT master mix, components were added in the following order: 2µl of 10x RT buffer, 0.8µl of 25x dNTPs mix (100mM), 2µl of 10x RT Random primers, 1µl of MultiScribe reverse transcriptase, 1µl of RNase Inhibitor, and 3.2µl of nuclease free water. Samples were incubated at 25°C for 10min followed by 37°C for 120min, and then 85°C for 5min to denature the reverse transcriptase, using a thermal cycler (Applied Biosystems, Warrington, UK).

2.6. Quantitative Polymerase Chain Reaction

2.6.1. Principle

Quantitative PCR (qPCR) is a laboratory method of molecular biology for monitoring the amplification of a targeted DNA molecule as it accumulates during PCR in real time. A TaqMan oligonucleotide probe is designed for a region of interest and contains a fluorescent reporter dye on its 5' end and a quencher on its 3' end. The probe anneals downstream from one of the primer sites and as the primer extends, Taq polymerase removes and cleaves the probe that has annealed to the template. The cleavage of the probe releases the reporter from the quencher dye, thus removing the quenching effect and increasing the reporter dye signal. During each cycle, probe cleavage increases the amount of reporter dye signal, resulting in fluorescence intensity directly proportional to the amount of PCR product generated (Figure 2.6.1-1).

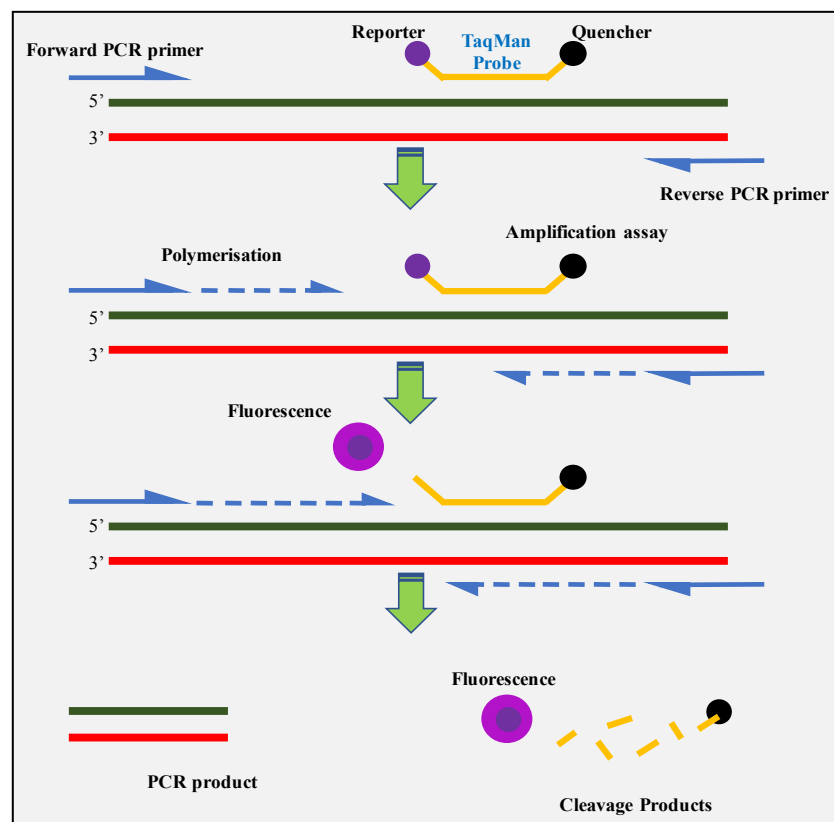


Figure 2.6.1-1 The mechanism of TaqMan qPCR. Separation of the quencher from the reporter dye allows detection of fluorescence.

2.6.2. Method

Prior to qPCR experiments, cDNA quality was examined using a Rotor Gene 6000 rotary real-time cycler (QIAGEN, Manchester, UK). 2µl of each cDNA sample was used to make a cDNA pool and a range of dilutions (1/10, 1/20, 1/40, 1/80, 1/160 and 1/320) in 10mM Tris-HCL was tested against the housekeeping gene 18s rRNA (Figure 2.6.2-1). Reactions were performed in 8µl volumes in 0.1ml tubes in triplicate. In all experiments, the following components were added per single reaction: 4µl of KlearKall 2x Master Mix with standard ROX (LGC Genomics, Middlesex, UK), 4µl of housekeeping gene and 3.6µl of cDNA. Life Technologies supplied all housekeeping genes. The reaction conditions were as follows: 95°C for 3min, then 40 cycles of 95°C for 3sec and 60°C for 20sec. Data were expressed as Ct values (Ct=cycle number at which logarithmic PCR plots cross a calculated threshold line).

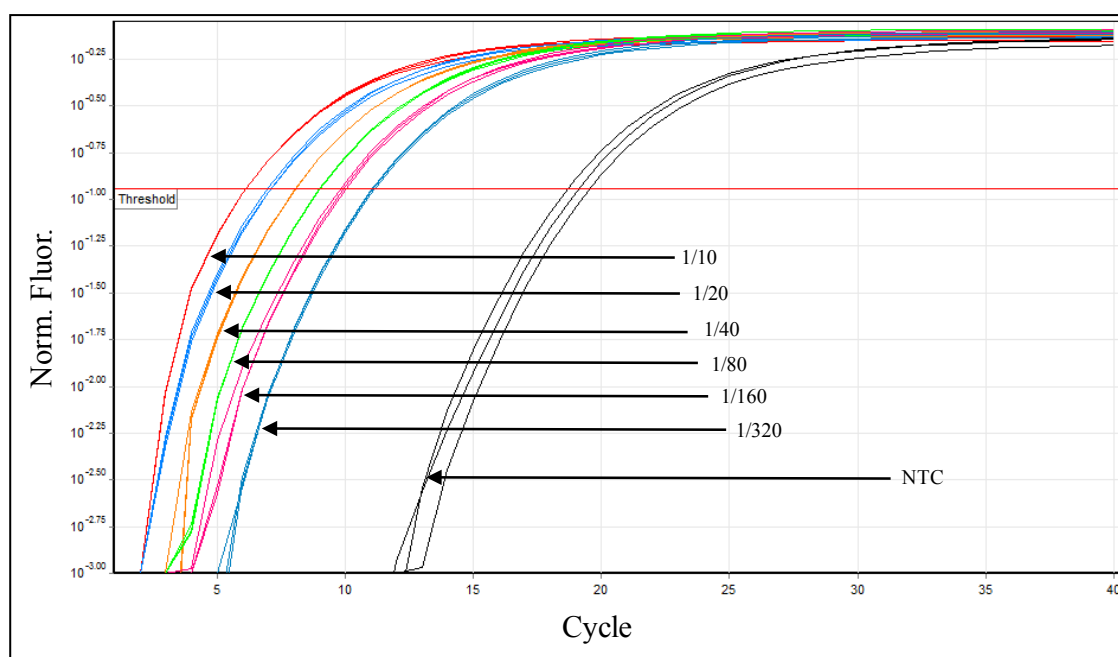


Figure 2.6.2-1 qPCR Amplification Curve. cDNA quality was examined using a Rotor Gene 6000 series in a range of dilutions (1/10, 1/20, 1/40, 1/80, 1/160, 1/320 and No Template Control [NTC]).

All qPCR experiments were conducted using an ABI 7900HT sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). Reactions were performed in 6ul volumes in 384-well plates in triplicate. In all experiments, the following components were added per single reaction: 3µl of KlearKall 2x Master Mix with standard ROX, 0.3µl of gene of interest and 2.7µl of cDNA. Life Technologies supplied all primers (Table 2.6-1). All target genes were labelled with FAM. The reaction conditions were as follows: 95°C for 3min, then 40 cycles of 95°C for 3sec and 60°C for 20sec.

Data were expressed as Ct values and used to determine the $\Delta/\Delta\text{Ct}$ calculation (Pfaffl 2001); it involves calculating the ΔCt (dCt) of each sample using the following calculation (where E is reaction efficiency – determined from a standard curve):

$$\Delta\text{Ct} = E^{[\min \text{ Ct-sampleCt}]}$$

Gene symbol	Exon boundary (RefSeq)	Assay location (RefSeq)	Amplicon length	Product order number	Gene symbol	Exon boundary (RefSeq)	Assay location (RefSeq)	Amplicon length	Product order number
<i>ABCA1</i>	6-7	947	80	Hs01059137	<i>IL1B</i>	3-4	190	91	Hs01555410
<i>ACCI</i>	31-32 31-32 29-30 30-31 35-36	4364 3931 4001 4112 5038	65	Hs01046047	<i>GAPDH</i>	7-8 8-8 7-7 8-8	870 928 822 836	157	Hs02786624
<i>ACC2</i>	1-2	667	88	Hs00153715	<i>CXCL8 (IL8)</i>	1-2	222	101	Hs00174103
<i>AFP</i>	13-14	1823	72	Hs01040598	<i>NOS2</i>	9-10	1265	67	Hs01075529
<i>AKR1D1</i>	1-2	170	103	Hs00818881	<i>PCK1</i>	8-9	1484	81	Hs00159918
<i>ALB</i>	6-7	784	104	Hs00609411	<i>PPARA</i>	4-5 5-6	556 640	62	Hs00947536
<i>CYP27A1</i>	8-9	1910	68	Hs01017992	<i>PYGL</i>	16-17 17-18	2205 2307	70	Hs00958087
<i>CYP7A1</i>	3-4	970	66	Hs00167982	<i>SCD</i>	2-3	798	129	Hs01682761
<i>DGAT1</i>	13-14	1373	64	Hs01020362	<i>SRD5A1</i>	2-3	654	120	Hs00602694
<i>DGAT2</i>	6-7 7-8	1146 1275	69	Hs01045913	<i>SLC2A6 (GLUT9)</i>	2-3 2-3	348	104	Hs00214042
<i>FAS</i>	3-4	399	62	Hs01005622	<i>SLC51A</i>	7-8	979	63	Hs00380895
<i>G6PC</i>	5-5 5-5	2249 2172	115	Hs02560787	<i>IL6</i>	3-4 2-3	450 259	95	Hs00174131
<i>NR1H4 (FXR)</i>	11-12 9-10 10-11 8-9 8-9 10-11	1828 1508 1661 1231 1243 1649	78	Hs01026590	<i>SREBF1</i>	13-14 12-13 12-13	2669 2441 2579	84	Hs01088679
<i>SLC2A1 (GLUT1)</i>	8-9		76	Hs00892681	<i>TTR</i>	3-4	470	56	Hs00174914
<i>SGK1</i>	10-11 8-9 8-9 7-8 8-9	1672 923 1102 746 878	81	Hs00178612	<i>TSC22D3 (GILZ)</i>	2-3 3-4 3-4 2-3 2-3	220 688 674 425 741	77	Hs00608272
<i>HNF4A</i>	3-4 3-4 3-4 4-5 4-5 4-5 4-5 3-4 3-4 3-4	502 323 323 593 545 545 545 323 502 502	49	Hs00230853	<i>SERPINA1</i>	2-3 2-3 4-5 2-3 4-5 3-4 4-5 4-5 4-5 3-4 3-4 3-4	908 887 1201 924 1220 1-28 1183 1180 1198 991 988 1088	93	Hs00165475
<i>18s</i>		41	69	Hs03003631_g1					

Table 2.6.2-1 List of primers which are provided by Applied Biosystems. The information in this table is gathered from: <http://www.thermofisher.com>.

2.7. Protein extraction

2.7.1. Principle

Monolayer adherent and semi adherent cells were lysed using detergent-containing buffers. Cell lysates were freeze thawed and sonicated to further break open the cell membranes and homogenise the sample. Cell debris and insoluble components are then removed by centrifugation.

2.7.2. Method

Monolayer adherent and semi adherent cells were washed twice with 1x PBS after cell medium removal. 150µl of lysis buffer containing RIPA Buffer (50mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0), protease inhibitor and phosphatase inhibitor cocktail (Fisher Scientific, Loughborough, UK) were added to each well, cells were scraped with a 1ml pipette tip and the lysate was transferred into a 1.5ml tube. Lysates were stored at -80°C overnight and then thawed on ice. Cell lysates were sonicated using a Jencons sonicator for ~30sec at 40% speed and homogenised samples were centrifuged at 10000g for 10min at 4°C to remove cell debris and insoluble components. The sample supernatant was then transferred to a new-labelled 1.5ml tube. Protein concentration was measured (see below) and samples were stored at -80°C.

2.8. Protein concentration measurements

2.8.1. Principle

Protein concentration was determined using the Bio-Rad DC protein (BioRad, Hertfordshire, UK) assay kit. This is a colourimetric assay for protein concentration following detergent solubilisation, which is based on the Lowry Method (Waterborg & Matthews 1984). The

assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. There are two steps which lead to colour development; the first reaction is between protein and copper in an alkaline medium followed by the reaction – a reduction of Folin reagent by the copper-treated protein. Folin reagent is reduced by 1, 2, or 3 oxygen atoms, primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Folin reagent reduction subsequently produces several possible reduced species which have a characteristic blue colour, with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

2.8.2. Method

Protein measurements were carried out in a 96-well plate according to the manufacturer protocol (BioRad, Herfordshire, UK). 5µl of each sample were analysed in duplicate in conjunction with a bovine serum albumin [BSA] standard curve. The range of standards were 0, 0.1, 0.2, 0.4, 0.8, 1, 1.5 and 2mg/ml. 25µl of a Reagent A+S (24.5µl alkaline copper tartrate solution (A) + 0.5µl surfactant solution (S)/ sample) were then added to each sample, followed by 200µl of Reagent B (Folin reagent). The plate was incubated at room temperature for 10min and absorbance was then measured at 750 nm on a SpectraMax Plus Microplate Reader (Molecular Devices, CA, USA).

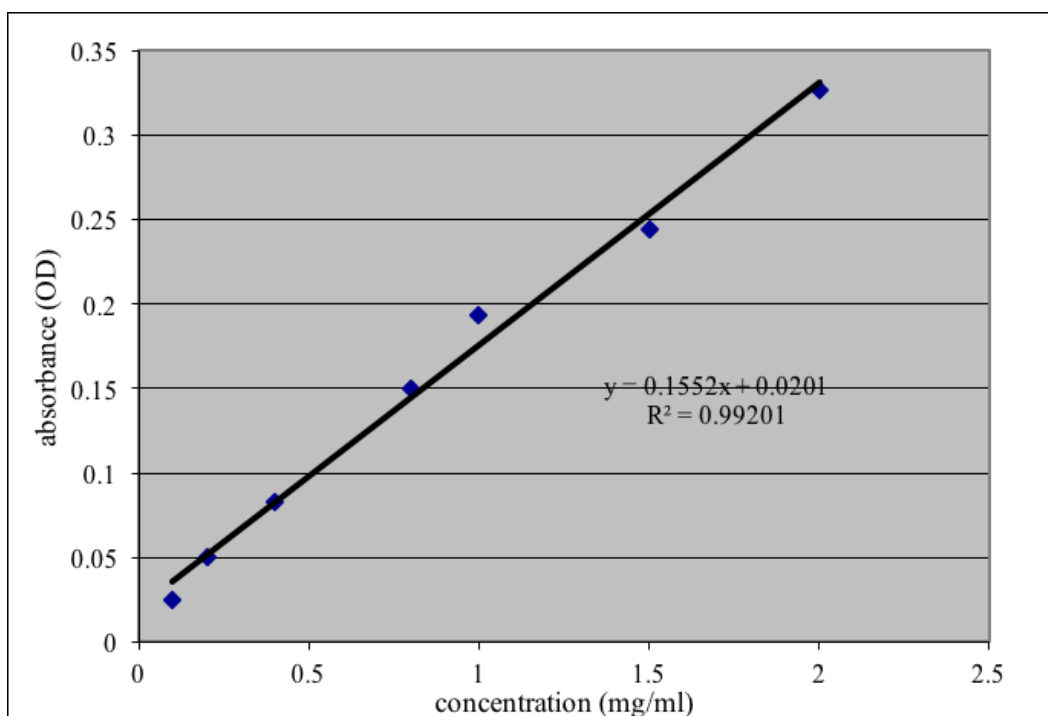


Figure 2.8.2-1 Representative protein standard curve.

2.9. Western blotting

2.9.1. Principle

Western blotting is used for the measurement of specific proteins in a mixture of protein samples. With this method, a mixed protein sample is separated based on molecular weight, and thus type, through gel electrophoresis (Mahmood & Yang 2012). The separated proteins are then transferred onto a membrane, usually nitrocellulose or polyvinylidene difluoride (PVDF). The membrane is blocked with a generic protein, usually milk proteins or BSA, to prevent antibodies from non-specific binding to the membrane. The membrane is then incubated with a primary antibody which binds specifically to the protein of interest, followed by a secondary antibody incubation, directed against the primary antibody. The secondary antibody is conjugated to an enzyme, usually horseradish peroxidase (HRP), which

is detected by the signal it produces by catalysing the oxidation of a chemiluminescent substrate. The signal is usually captured on a photographic film.

2.9.2. Method

TGX (Tris-Glycine eXtended) stain free Criterion gels and PVDF membranes were purchased by Bio-Rad using the V3 Western Workflow Blotting protocol. Approximately 20µg of each protein sample were mixed with an equal amount of 2x Laemmli sample buffer and boiled at 95°C for 5min. Samples were centrifuged at 16000g for 1min and, loaded to a TGX stain free gel and run at 240V for 30-45min. Proteins were transferred onto a PVDF membrane using the Trans-Blot Turbo system for 7min. To check for efficient transfer, membranes were incubated in ponceau stain for approximately 10min whilst on a rocking shaker, and then rinsed with distilled H₂O until the background was clean. Membranes were then blocked in 15ml of blocking buffer (Tris-Buffered Saline 0.1% Tween 5% Milk Powder) for 1h on a rocking shaker, rinsed three times with washing buffer (Tris-Buffered Saline 0.1% Tween) and incubated in primary antibody (Table 2.9-1) at 4°C overnight with constant agitation. Membranes were then washed three times for 15min in washing buffer and incubated in secondary antibody (dilution 1/2000) for 1h at room temperature. Membranes were then washed three times with washing buffer and 1ml of chemiluminescent substrate was applied on the membranes for antibody detection for 5min. Membranes were then transferred onto a cling film and signal was measured using the ChemiDocXS Touch Imaging System by Bio-Rad. Western blots were quantified by densitometry analysis using ImageJ (NIH, Bethesda, MD, <http://rsb.info.nih.gov/ij>).

Peptide/protein target	Antibody	Manufacturer	Species raised in; monoclonal or polyclonal	Dilution used
Glucose-6-phosphatase (G6PC)	Anti-glucose-6-phosphatase (HRP)	Abcam plc, Cambridge, UK	Rabbit, polyclonal	1/1000
PCK1 (PEPCK)	Anti-PCK1 (HRP)	Cell Signalling, Danvers, USA	Rabbit, monoclonal	1/1000
IRE1 α	Anti-IRE1 α (HRP)	Cell Signalling, Danvers, USA	Rabbit, monoclonal	1/1000
CHOP	Anti-CHOP (HRP)	Cell Signalling, Danvers, USA	Mouse, monoclonal	1/1000
β -tubulin	Anti- β -tubulin (HRP)	Cell Signalling, Danvers, USA	Rabbit, monoclonal	1/1000

Table 2.9.2-1 List of antibodies used for western blotting analysis.

2.10. Triglyceride concentration measurement

2.10.1. Principle

The triglyceride (TAG) assay kit by Instrumentation Laboratory (Milano, Italy) is used for the determination of TAG concentrations in plasma, serum, cell lysates, and tissue homogenate samples. The reaction starts from the enzymatic hydrolysis of TAG by lipase to glycerol and free fatty acids. The glycerol formed is then phosphorylated by glycerol kinase to glycerol-3-phosphate. Glycerol-3-phosphate is oxidised by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase catalyses the redox-coupled reaction of H₂O₂ with 4-aminoantipyrine (4-AAP) and N-Ethyl-N-(3-sulfopropyl)-m-anisidine (ESPA), leading to a purple colour production (Figure 2.10.1-1). The absorbance is measured at 540 nm.

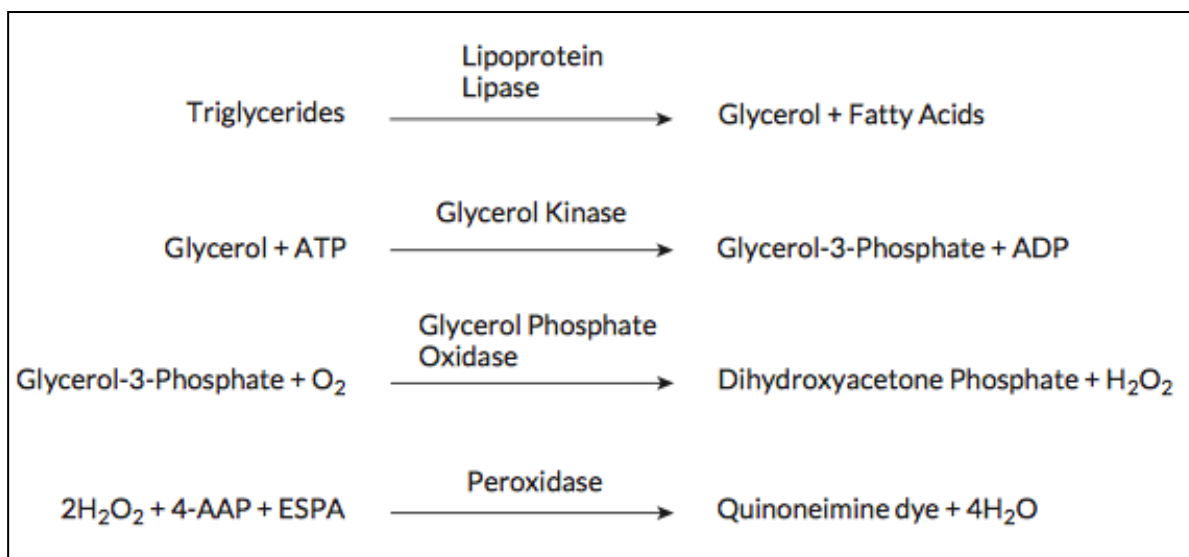


Figure 2.10.1-1 Series of reactions for the determination of triglyceride concentrations (Instrumentation Laboratory).

2.10.2. Method

Cells were cultured and treatments were carried out in 24 well plates. Cell medium was removed and the cells then washed with 1x PBS before 150µl 1% NP-40 lysis buffer (5M NaCl, 10% NP-40, 1M Tris-HCl, pH 8.0) were added to each well. Cells were incubated for 5min before scraping and samples were then transferred to -80°C in 1.5µl eppendorfs.

For the analysis, cell lysates were sonicated for 30sec, then heated at 95°C for 30min and spun at 12000g for 10min to remove cell debris. TAG concentration was then determined on a 650 iLAB Clinical Chemistry system (Instrumentation Laboratory, Milano, Italy) using the TAG assay kit (Instrumentation Laboratory, Milano, Italy). Prior to running samples, iLAB was calibrated using a range of 50, 100, 200 and 1000µM of glycerol standards. TAG concentration was determined as below:

$$\text{TAG concentration } (\mu\text{mol/l}) / \text{protein concentration (mg/ml)}$$

2.11. Glucose measurements

2.11.1. Principle

The glucose (Oxidase) IL Test kit is used for the quantitative *in vitro* determination of glucose in human serum, plasma, urine or cell medium samples, using the iLAB Chemistry System. It is a colourimetric method based on oxidation of D-Glucose to Gluconic acid and H₂O₂ by glucose oxidase and the subsequent use of H₂O₂ as a substrate for the production of red quinoneimine (Figure 2.11.1-1). The increase in absorbance generated by the red colour is proportional to the glucose concentration in the sample. The absorbance is measured at 510 nm.

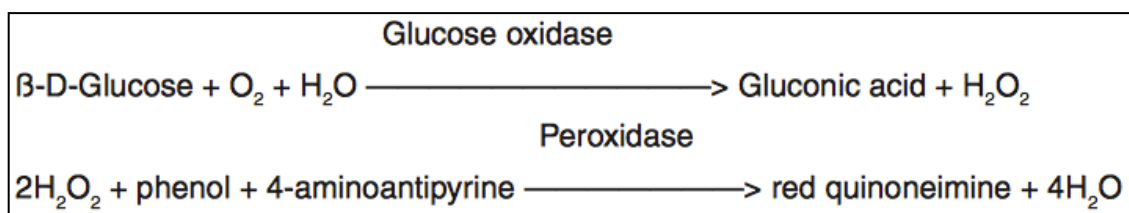


Figure 2.11.1-1 Series of reactions for the determination of glucose concentrations, using the iLAB Chemistry System.

2.11.2. Method

Cells were cultured and treatments were carried out in 24 well plates. Cell media samples were collected, transferred into 1.5ml tubes and stored in -80°C. Prior to running samples, iLAB 650 was calibrated using calibrated using 3 different concentrations (1, 2 and 5mmol/L) of glucose standard calibrator. 200µl of sample were then transferred into a separate 3ml iLAB standard sample cup. Results were finally normalised against protein concentration.

2.12. D-3-Hydroxybutyrate measurements

2.12.1. Principle

The D-3-Hydroxybutyrate (3OHB) kit by Randox (Crumlin, County Antrim, UK) is used for the quantitative *in vitro* determination of 3OHB in serum, plasma and cell medium samples. It is a kinetic enzymatic method based on the oxidation of 3OHB to acetoacetate by the enzyme 3-Hydroxybutyrate dehydrogenase (3-HBDH). Subsequent to this oxidation, the cofactor NAD⁺ is reduced to NADH and the associated change in absorbance can be directly correlated with the 3OHB concentration. 3OHB concentration was then determined on a 650 iLAB Clinical Chemistry system and the absorbance is measured at 505 nm.

2.12.2. Method

Cells were cultured and treatments were carried out in 24-well plates. Cell media samples were collected, transferred into 1.5ml tubes and stored in -80°C. Prior to running samples, iLAB 650 was calibrated using 3 different concentrations (62.5, 125 and 500µmol/L) of the CAL standard supplied in the Randox kit. 100µl of samples were then transferred into a separate 3ml iLAB standard sample cup. Results were finally normalised against protein concentration.

2.13. Lactate measurements

2.13.1. Principle

The lactate kit by Randox (Crumlin, County Antrim, UK) is used for the quantitative *in vitro* determination of lactate concentrations in serum, plasma and cell medium samples. It is a colourimetric method based on the oxidation of lactate to pyruvate and H₂O₂. Subsequent to this oxidation, H₂O₂ reacts with 4-aminoantipyrine and N-ethyl-N-(2,hydroxy-3-sulphopropyl) m-toluidine and generates purple product. The increase in absorbance generated by the purple colour is proportional to the lactate concentration in the sample. The absorbance is measured at 550 nm.

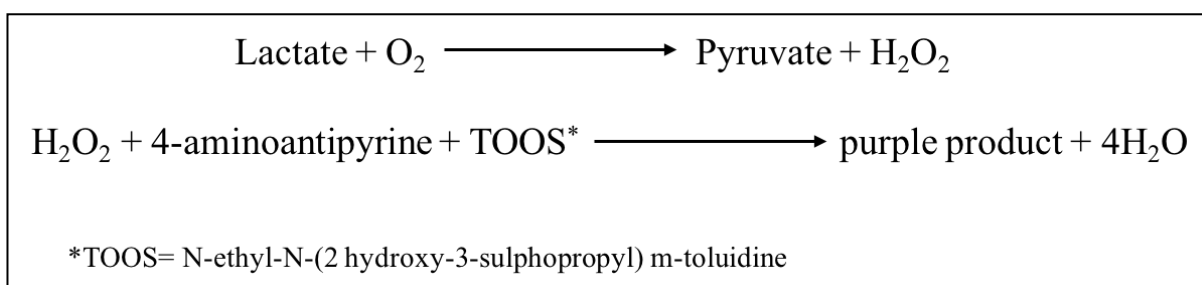


Figure 2.13.1-1 Series of reactions for the determination of lactate concentrations, using the iLAB Chemistry System.

2.13.2. Method

Cells were cultured and treatments were carried out in 24-well plates. Cell media samples were collected, transferred into 1.5ml tubes and stored in -80°C. Prior to running samples, iLAB 650 was calibrated using the CAL standard supplied in the Randox kit. 100µl of samples were then transferred into a separate 3ml iLAB standard sample cup. Results were finally normalised against protein concentration.

Chapter 3 Optimising human hepatocyte models for metabolic phenotype: effects with dimethyl sulfoxide (DMSO)

3.1. Introduction

Investigating the mechanisms that underpin the development and progression of NAFLD, as well as potential treatments, requires *in vitro* cellular models that mimic human hepatocyte metabolism and functionality. Primary human hepatocytes are the ideal *in vitro* cellular model to investigate aspects of liver function and metabolism as they most closely recapitulate the *in vivo* liver (Green et al. 2015). Although human primary hepatocytes are considered the gold standard, there are significant challenges in their use, such as availability of cells, donor variability, inability to proliferate and a short life span (Green et al. 2015). Therefore, a variety of human hepatoma cell lines have been generated to investigate human hepatocyte metabolism and function, including the HepG2, Hep3b, and Huh7 cell lines. The HepG2 cell line has been extensively used in cell-based metabolic studies (Javitt 1990); however, because of their malignant origin, they tend to present with an abnormal metabolic phenotype compared to human primary cells. As an example, HepG2 cells have increased rates of DNL and glycolysis (Daniels et al. 2014; Pereira da Silva et al. 2009). In addition, expression levels of various hormone-metabolising enzymes (e.g. AKR1D1, 5 α R1, 11 β -HSD1, 11 β -HSD2) seem to be abnormal, with increased AKR1D1 and 11 β -HSD2 expression whilst decreased SRD5A1 and 11 β -HSD1 expression in HepG2 cells compared to healthy liver tissue samples (Aviva RNAseq Tool 2017).

The amphipathic, molecule dimethyl sulfoxide [DMSO] has multiple effects on cellular function (Pal et al. 2012; Santos et al. 2003). A number of previous studies have examined whether DMSO treatment alters hepatoma cell line functionality. However, no study to date has examined in detail the impact of DMSO treatment on carbohydrate and lipid metabolism. DMSO treatment of HepG2 and Huh7 cells has been reported to enhance drug metabolism (Choi et al. 2009) and reduce lipid accumulation (Song et al. 2012), although the mechanisms

underpinning these observations remain to be elucidated. Moreover, it remains unclear whether DMSO treatment of hepatoma cell lines affects carbohydrate metabolism.

3.1.1. Hypothesis and aims

My hypothesis is that DMSO treatment will present a strategy to enhance human hepatoma cell metabolic functionality leading to the development of a novel and improved *in vitro* model that may more closely resemble primary human hepatocytes, including partial restoration of steroid metabolising enzymes. To elucidate this, human hepatoma cells will be exposed to DMSO treatment in a time-dependent manner and I aimed to:

- Explore the expression levels of AKR1D1 in four human hepatoma cell lines and compare them with the expression levels of AKR1D1 from other tissues, including human primary hepatocytes and immortalized adipocytes.
- Define the impact of DMSO treatment on gene expression markers of hepatocyte differentiation in human hepatoma cells
- Define the impact of DMSO treatment on cellular stress in human hepatoma cells
- Define the impact of DMSO on lipid and carbohydrate metabolism in human hepatoma cells.

To endorse my hypothesis, I will also measure gene expression levels of primary human hepatocyte cultures and compare them with the effect of DMSO treatment upon human hepatoma cells.

3.2. Methods

3.2.1. Human hepatoma cell culture

HepG2, Hep3b, Huh7 and C3A human hepatoma cells were plated in a 6-well Cell Bind plate (Corning, Flintshire, UK) at a density of 3.5×10^5 cells/ml and culturing was performed as described in section 2.1.1.

3.2.2. Primary human hepatocyte culture

Primary human hepatocyte cultures were performed by Dr Charlotte Green. Liver was obtained from patients undergoing surgery who had consented (NRES Committee South Central; Berkshire B 11/SC/0443) to the use of excess tissue (resection surplus) for research. Hepatocytes were isolated from whole liver tissue *via* a two stage collagenase digestion as previously described (Green et al. 2017). Cells were diluted to 1 million cells per ml in William's E buffer containing hepatocyte supplements before plating on Type I collagen coated plates. Cells were collected 24h post seeding for RNA isolation.

3.2.3. Immortalised adipocyte culture

The isolated stromo-vascular fraction of a paired subcutaneous abdominal and gluteal adipose tissue biopsy taken from a healthy 50 year old male (BMI 24.4 kg/m^2) was immortalised according to a modified version of a previously described protocol (Darimont et al. 2003; Todorčević et al. 2017). Preadipocytes were seeded onto 6-well plates (2×10^5 cells/well) and grown to confluence for 48h before 14-day stimulation with adipogenic medium comprising Dulbecco's modified Eagle's medium / Nutrient F-12 Ham supplemented with 2mM L-glutamine (Invitrogen, Paisley, UK); 17 μ M pantothenate; 100nM human insulin (Invitrogen, Paisley, UK); 1nM triiodo-L-thyronine; 33 μ M biotin; 10 μ g/ml transferrin; and 1 μ M

dexamethasone. For the first 4 days of differentiation, the adipogenic medium was supplemented with 2 μ M troglitazone (Sigma) and 250 μ M 3-isobutyl-1-methylxanthine (Sigma) (Todorčević et al. 2017). Adipogenic medium was changed on days 2, 4, 7, 10 and 12. RNA samples were courtesy of Mr. Nathan Denton.

3.2.4. Cell treatments

For all vehicle treatments, cells were cultured until 95% confluence, culture medium was then replaced with 1% ethanol complete with phenol-red free medium and samples were collected after 48h.

For all DMSO treatments, culture medium was replaced with 1% DMSO complete phenol-red free medium and cultures were incubated for 7, 14 and 21 days. During that time, 1% DMSO complete phenol-red free medium was replenished every 48h.

For fatty acid treatments (FA), cells were treated in phenol-red free media with 0.25% fatty acid-free BSA alone or conjugated to 45 μ M oleic, 30 μ M palmitic, and 25 μ M linoleic acid (OPL; physiological ratio 45:30:25%) for 24 or 48h.

3.2.5. Oil Red O staining

Oil Red O (Sudan Red 5B) is a lipid-soluble dye used for lipid staining on frozen sections or monolayer cells.

Oil Red O staining experiments were performed by Ms. Pippa Gunn. HepG2 cells were washed twice in PBS and fixed using 10% formalin for 1h. Cells were then washed in 60% isopropanol and left to dry. Staining was carried out by adding Oil Red O (6mM) (Sigma-Aldrich, Dorset, UK) for 15min at room temperature. Cells were then counter stained using haematoxylin and eosin and mounted onto slides using aqua mount.

3.2.6. RNA extraction and RT-PCR

Total RNA was extracted from cells using the Tri-Reagent system. Quantity and quality were determined spectrophotometrically at OD₂₆₀. One microgram of total RNA was used for reverse transcription performed as described in section 2.5.2.

3.2.7. Quantitative PCR

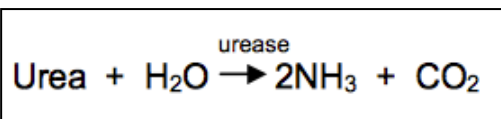
mRNA levels of all genes were determined using an ABI 7900HT sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). Reactions were performed as described in 2.6.2.

3.2.8. Protein extraction and immunoblotting

Total protein was extracted from cells and protein concentrations were measured as described in methods 2.7.2 and 2.8.2 respectively.

3.2.9. Urea, TAG, 3OHB and lactate measurements

Urea measurements were performed using the IL Urea Nitrogen Assay. The IL Urea Nitrogen assay is an enzymatic method suitable for the quantitative *in vitro* determination of urea in serum, plasma, urine and cell medium samples. The method is based on the following reaction:



Subsequently, the produced ammonium ions react with α -oxoglutarate and NADH to produce L-glutamate and NAD⁺. The decrease of absorbance at 340 nm due to NADH depletion is

proportional to the urea concentration.

Cell media samples were collected, transferred into 1.5ml tubes and stored in -80°C. Prior to running samples, the iLAB 650 was calibrated using the CAL standard supplied in the IL kit. 100µl of sample were then transferred into a separate 3ml iLAB standard sample cup. Results were normalised against protein concentration.

TAG (intracellular and media), 3OHB, glucose and lactate were analysed using Instrumentation Laboratory kits on an iLab 650 Clinical Chemistry analyser as described in methods 2.10.2, 2.11.2, 2.12.2 and 2.13.2 respectively. Phenol-red free media-containing FBS or lysis buffer were used as background controls.

3.2.10. Statistical analysis

For multiple comparisons, statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett corrections for comparison between control and different timepoints. Statistical analysis on qPCR data was performed on mean relative expression ratio values ($\text{Ratio} = \Delta C_{t[\text{target}]} / \Delta C_{t[\text{ref}]}$) (Pfaffl 2001). Data analysis was performed using Graphpad Prism software (Graphpad Software Inc, La Jolla, USA) and considered statistically significant at $P < 0.05$.

3.3. Results

3.3.1. Cell line characterisation

To define the best cellular model for exploring the *in vitro* role of AKR1D1 in human hepatocytes, the expression pattern of AKR1D1 was determined in four human hepatoma cell lines (HepG2, Hep3b, Huh7 and C3A cells), that have been previously used in metabolic research, using real-time PCR. With the exception of Hep3b, expression levels were similarly high in all hepatoma cell lines (HepG2, Huh and C3A cells) and primary human hepatocytes. AKR1D1 mRNA expression was also detected in differentiated human adipocytes from both gluteal and subcutaneous abdominal depots, albeit at much lower levels (Figure 3.3.1-1). Based on these findings, the HepG2 and the C3A cell lines were used for further optimisation.

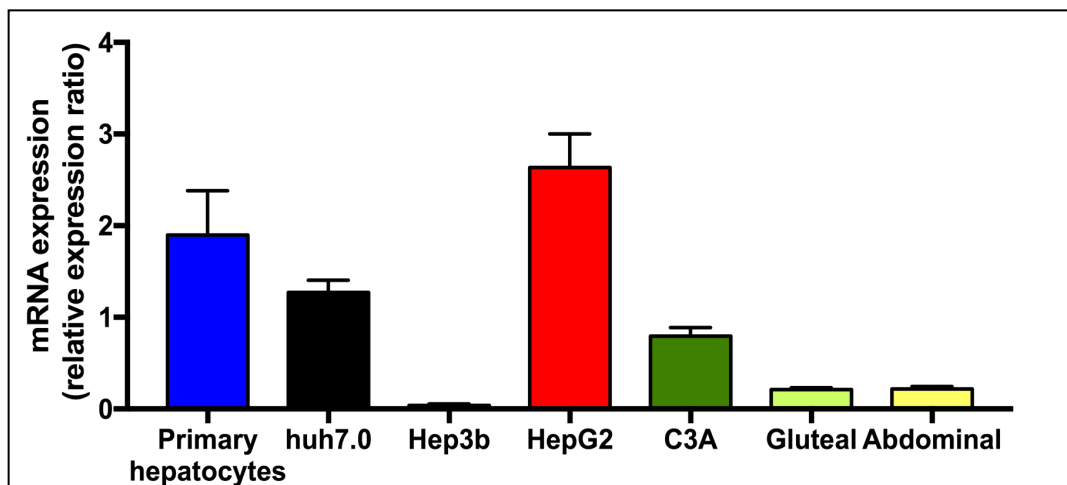


Figure 3.3.1-1 mRNA expression of AKR1D1 in primary hepatocytes, Huh7, Hep3b, HepG2, C3A, gluteal and abdominal immortalised cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments.

3.3.2. DMSO treatment of human hepatoma cell lines alters the expression of hepatic function markers

The mRNA expression of four markers of hepatic function (albumin, alpha-1-antitrypsin, HNF4A and transthyretin) were measured in confluent untreated and DMSO-treated HepG2 cells in a time-dependent manner. mRNA expression levels of many genes were higher in control untreated cells at day 0 compared to DMSO-treated HepG2 cells (Figure 3.3.2-1).

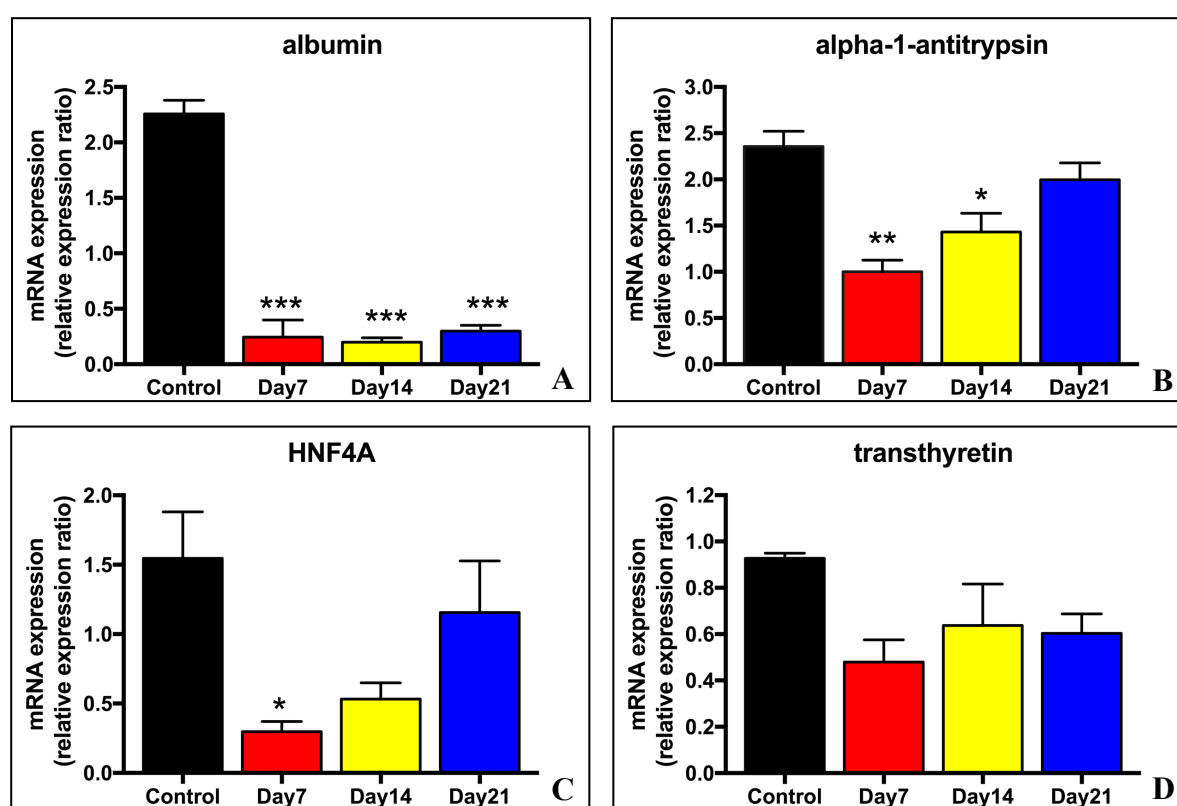


Figure 3.3.2-1 mRNA expression of (A). albumin, (B). alpha-1-antitrypsin, (C). HNF4A and (D). transthyretin in control and DMSO-treated HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control).

Alpha-fetoprotein (AFP) is a tumour marker associated with hepatocellular carcinoma. To explore whether DMSO had any beneficial effect on the tumourous phenotype of HepG2 cells, I measured the expression of *AFP* in untreated and DMSO treated HepG2 cells.

Notably, mRNA expression of AFP decreased after DMSO treatment, with a significant effect observed at day 7 which persisted up to day 21 of treatment (Figure 3.3.2-2).

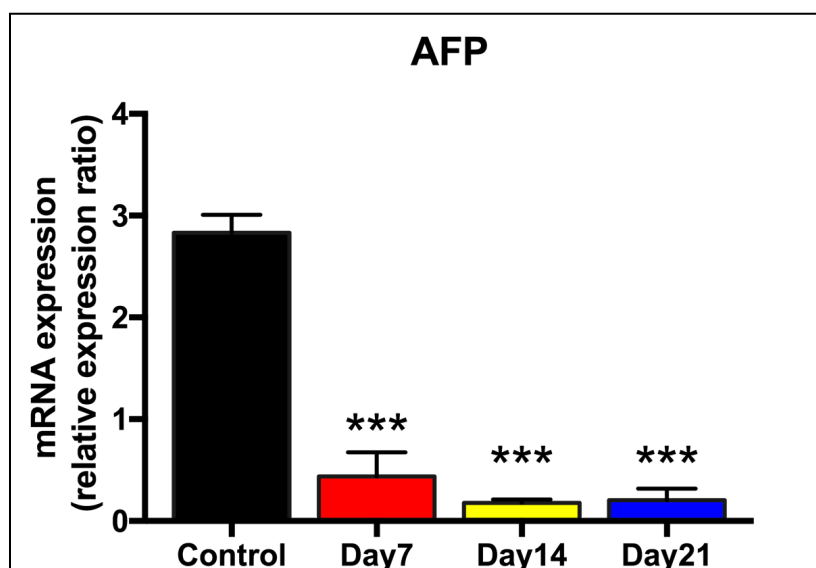


Figure 3.3.2-2 mRNA expression of the tumor marker alpha-fetoprotein (AFP) in control and DMSO-treated HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (*** $p<0.001$ vs. control).

To further confirm the effect of DMSO on human hepatoma cell lines, C3A cells were used as an additional cellular model. Similar observations, to HepG2 cells, were made in C3A cells treated with DMSO and absolute changes in mRNA expression levels are presented in Table 3.3-1.

Gene	Control	Day 7	Day 14	Day 21
albumin	3.59 $\pm$ 0.38	1.34 $\pm$ 0.38***	0.42 $\pm$ 0.06***	0.75 $\pm$ 0.09***
alpha-1-antitrypsin	2.18 $\pm$ 0.14	1.29 $\pm$ 0.12***	1.49 $\pm$ 0.17**	2.40 $\pm$ 0.11
HNF4A	0.98 $\pm$ 0.15	0.45 $\pm$ 0.008*	0.44 $\pm$ 0.08*	0.83 $\pm$ 0.14
transthyretin	2.86 $\pm$ 0.28	1.39 $\pm$ 0.20***	0.93 $\pm$ 0.05***	1.37 $\pm$ 0.11***
AFP	4.078 $\pm$ 0.1	1.58 $\pm$ 0.26***	0.33 $\pm$ 0.08***	0.51 $\pm$ 0.18***

Table 3.3-1 mRNA expression of the hepatic function markers in control and DMSO-treated C3A cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control).

In order to ascertain if DMSO treatment of HepG2 cells induced a phenotype reflective of primary human hepatocytes, the mRNA expression profile of hepatic function markers at day 7 of DMSO treatment was compared with that of primary cultures of human hepatocytes. In untreated HepG2 cells expression of these markers far exceeded primary cultures, with significant decrease after 7 days. In cell culture, when cells reach high density (HD), they stop proliferating due to contact inhibition. After 7 days of DMSO treatment cells had reached 100% confluence and, although not measured, based on their appearance, it could be speculated that cells had decreased proliferation. DMSO treatment (7 days) reduced mRNA levels of liver function makers to levels, although still higher, closer to those seen in primary human hepatocytes (Figure 3.3.2-3).

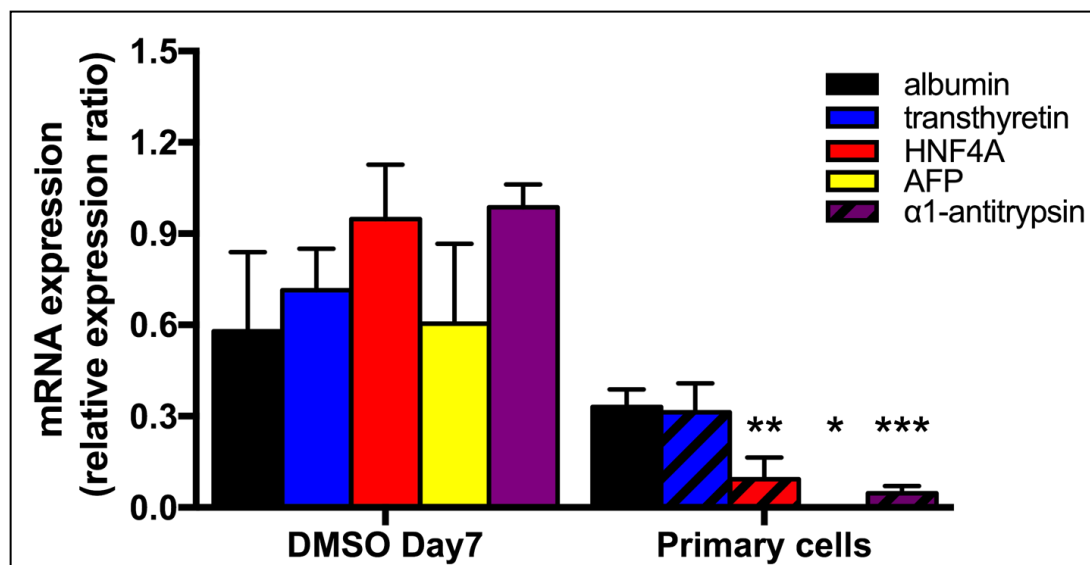


Figure 3.3.2-3 mRNA expression of hepatic function markers in DMSO-treated HepG2 cells for 7 days and in primary human hepatocytes, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments.

Urea secretion, a measure of hepatic cell function, was not compromised by DMSO treatment in HepG2 cells (Figure 3.3-4).

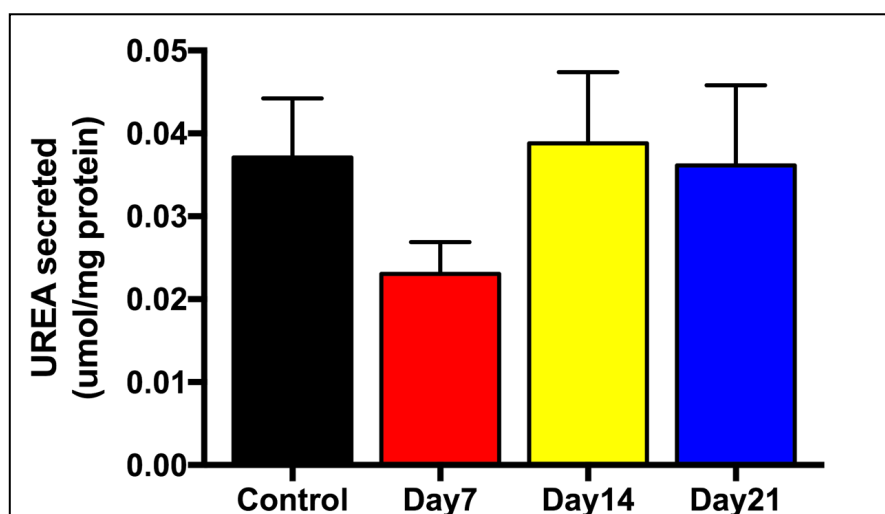


Figure 3.3.2-4 Urea levels in control and DMSO-treated HepG2 cell media. Data are expressed as mean $\pm$ se of $n=3$ experiments.

3.3.3. DMSO treatment of HepG2 cells decreases the expression of ER stress markers

ER stress has the potential to influence hepatocyte functionality and metabolism and therefore markers of convergent ER stress pathways were measured across the time course of the experimental protocol. Inositol-requiring enzyme 1 (IRE1 α) is a well-established ER stress marker and protein expression decreased after 7 days of DMSO treatment, whilst there was no significant change in CHOP protein levels (Figure 3.3.3-1).

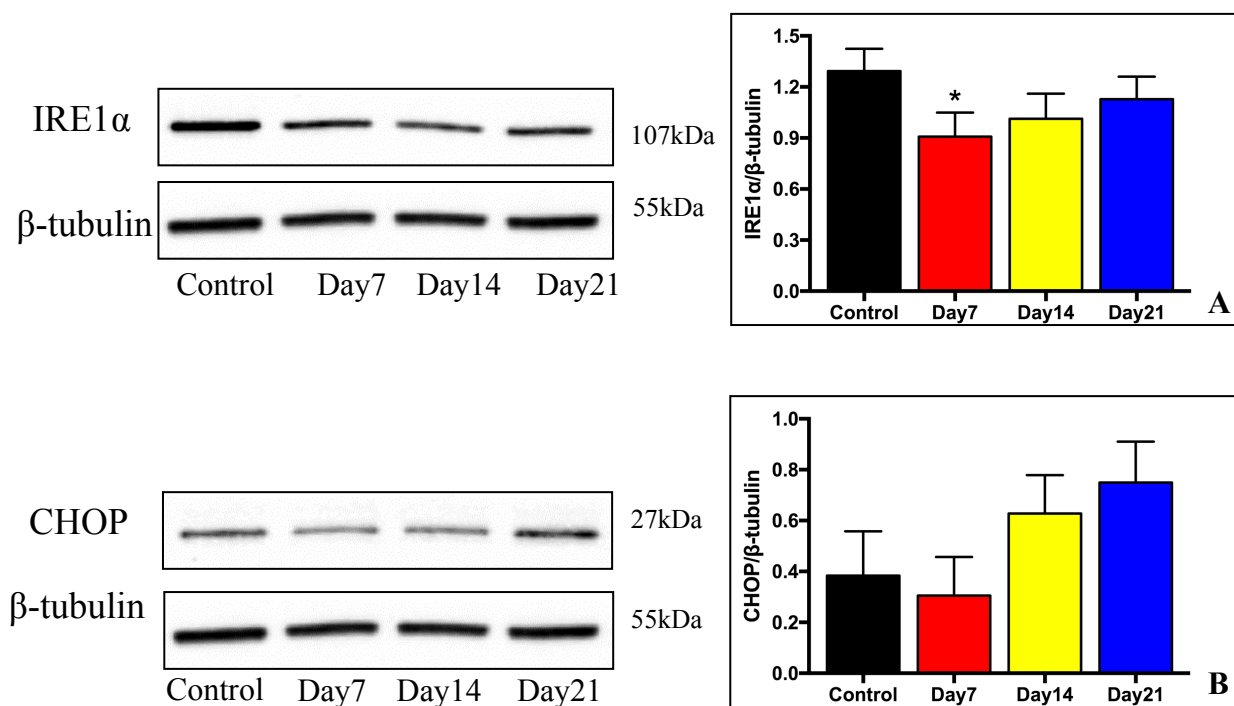


Figure 3.3.3-1 Protein expression of the ER stress markers (A). IRE and (B). CHOP in control and DMSO-treated HepG2 cells. Data are presented as mean $\pm$ se of $n=3$ experiments and quantified relative to β -tubulin. Representative western blots are shown (* $p<0.05$ vs. control)

3.3.4. DMSO treatment changes the lipid metabolic profile of HepG2 cells

The mRNA expression of fatty acid synthetic and oxidation genes *ACC1*, *ACC2*, *DGAT1*, *DGAT2*, *FAS* and *SCD* were significantly decreased in HepG2 cells after 7 days of DMSO treatment, with no further reduction after 14 and 21 days. (Figure 3.3.4-1).

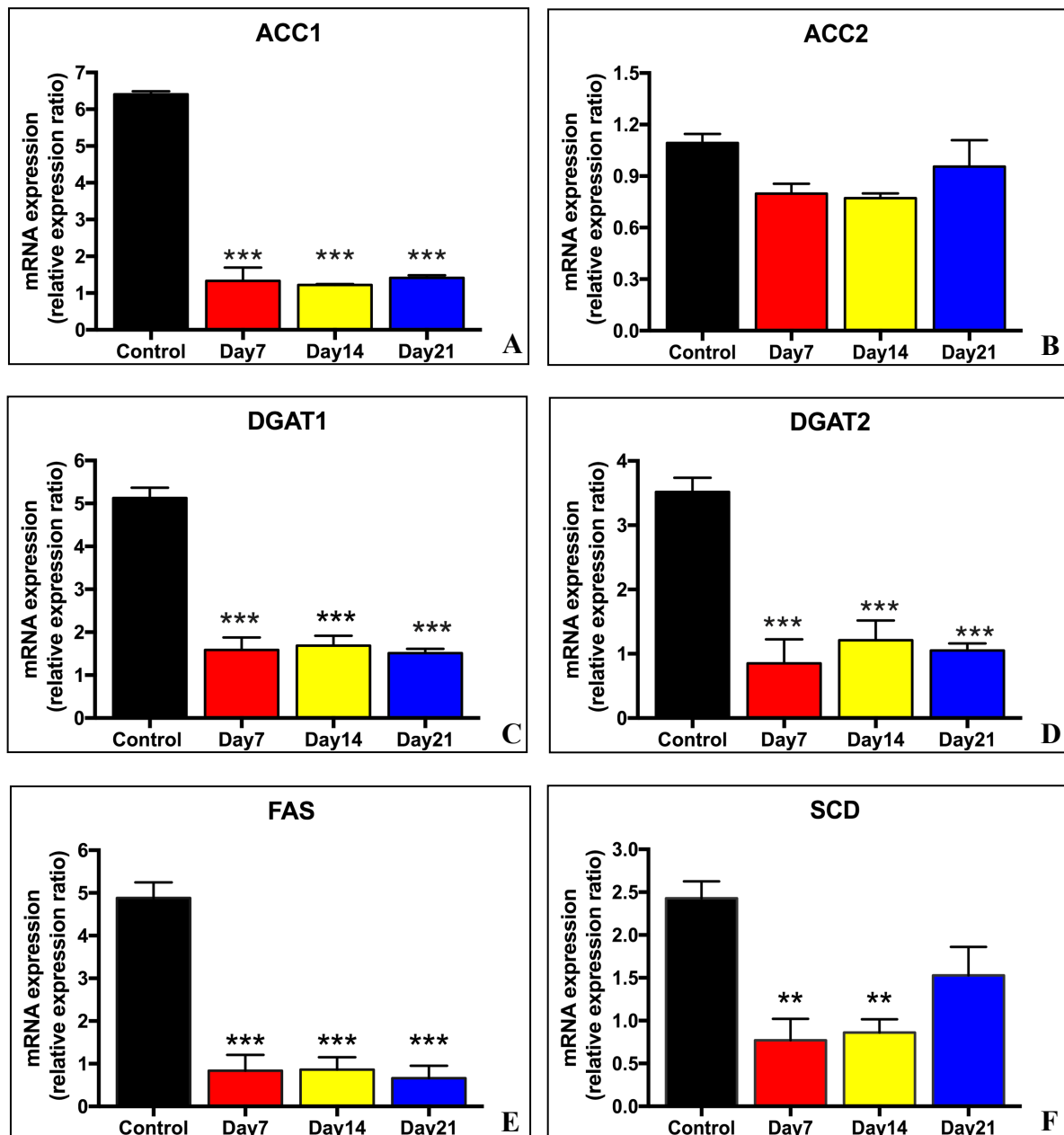


Figure 3.3.4-1 mRNA expression of (A). *ACC1*, (B). *ACC2*, (C). *DGAT1*, (D). *DGAT2*, (E). *FAS* and (F). *SCD* in control and DMSO-treated HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control).

In line with my findings in HepG2 cells, similar suppression in the mRNA expression of all fatty acid synthetic and oxidation genes mentioned above was observed in C3A human hepatoma cells, after 7, 14 and 21 days of treatment with DMSO. (Table 3.3-2).

Gene	Control	Day 7	Day 14	Day 21
<i>ACC1</i>	5.44±0.79	2.06±0.15**	1.16±0.03***	2.09±0.03**
<i>ACC2</i>	2.60±0.25	1.82±0.23*	1.52±0.10**	2.21±0.1
<i>DGAT1</i>	4.77±0.29	1.79±0.27***	0.97±0.40***	2.14±0.03***
<i>DGAT2</i>	3.05±0.33	0.82±0.11***	0.45±0.05***	0.88±0.04***
<i>FAS</i>	3.98±0.42	1.69±0.32**	1.82±0.23**	2.70±0.18
<i>SCD</i>	2.04±0.20	1.75±0.28	1.09±0.20*	2.04±0.29

Table 3.3-2 mRNA expression of fatty acid synthetic and oxidation genes in control and DMSO-treated C3A cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of n=3 experiments (* p <0.05, ** p <0.01, *** p <0.001 vs. control).

Fatty acids within the hepatocyte can be partitioned into esterification (to synthesise TAG) or oxidation (resulting in the generation of 3OHB) pathways. Compared with untreated control cells, DMSO-treated HepG2 cells had significantly lower concentrations of intracellular TAG at all time points (209.7 ± 9.83 (control) vs. 95.7 ± 12.3 (day 7), 93.0 ± 9.78 (day 14), and 93.7 ± 7.07 nmol/mg protein (day 21), $p < 0.05$) (Figure 3.3.4-2). These observations were mirrored by a decrease in intracellular neutral lipid (TAG) staining with Oil Red O, after 7 days of DMSO treatment (Figure 3.3.4-3).

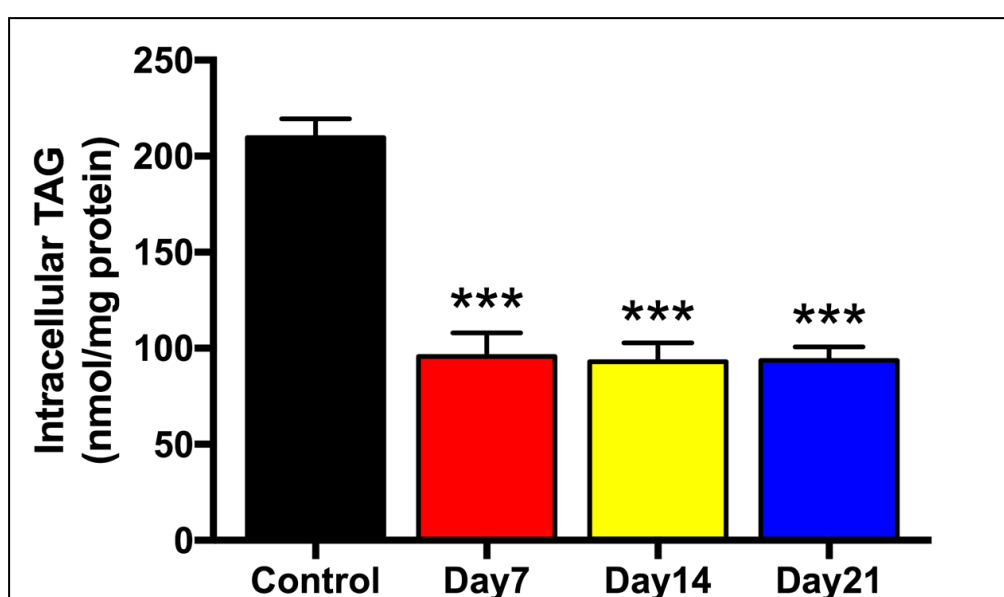


Figure 3.3.4-2 Intracellular TAG levels in control and DMSO-treated HepG2 cells. Data are expressed as mean $\pm$ se of $n=3$ experiments (*** $p < 0.001$ vs. control).

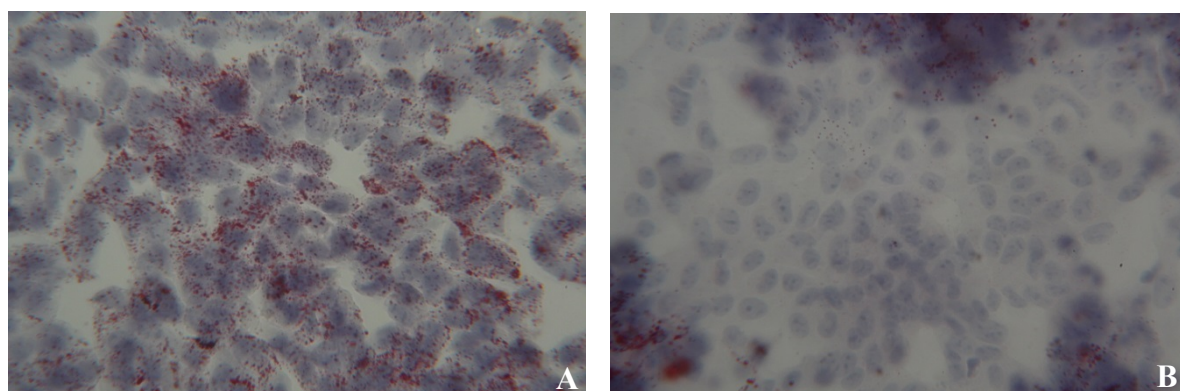


Figure 3.3.4-3 Intracellular neutral lipid content in control (A) and DMSO-treated (B) HepG2 cells for 7 days, oil red O staining, 20x analysis. Images courtesy of Ms. Pippa Gunn.

Removal of fatty acids from the hepatocyte can occur either by secretion of TAG from the cell or through oxidation pathways (namely ketogenesis and the TCA cycle). 3OHB, a product of ketogenesis is often used as a marker of hepatic fatty acid oxidation; 3OHB concentrations were significantly increased in HepG2 cell medium after treatment with 1% DMSO for 7 days and 14 days (8.36 ± 1.36 (control) vs. 13.44 ± 0.86 (day 7), $12.12 \pm 1.06 \mu\text{mol/mg protein}$ (day 14), $p < 0.05$) (Figure 3.3.4-4) in comparison with untreated control cells. Unlike primary hepatocytes, HepG2 cells secrete very low levels of TAG (Wang et al. 1988). Whilst DMSO treatment appeared to increase the TAG concentration in the media from HepG2 cells in a time dependent manner, this did not achieve statistical significance (Figure 3.3.4-5).

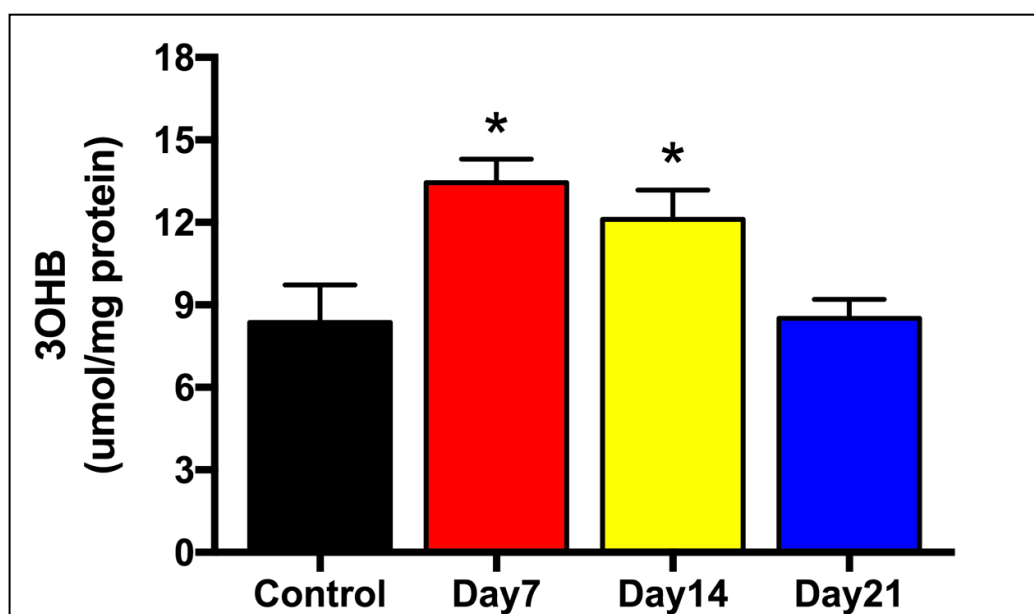


Figure 3.3.4-4 3OHB concentrations in control and DMSO-treated HepG2 cell media. Data are expressed as mean $\pm$ se of $n=3$ experiments (* $p < 0.05$ vs. control).

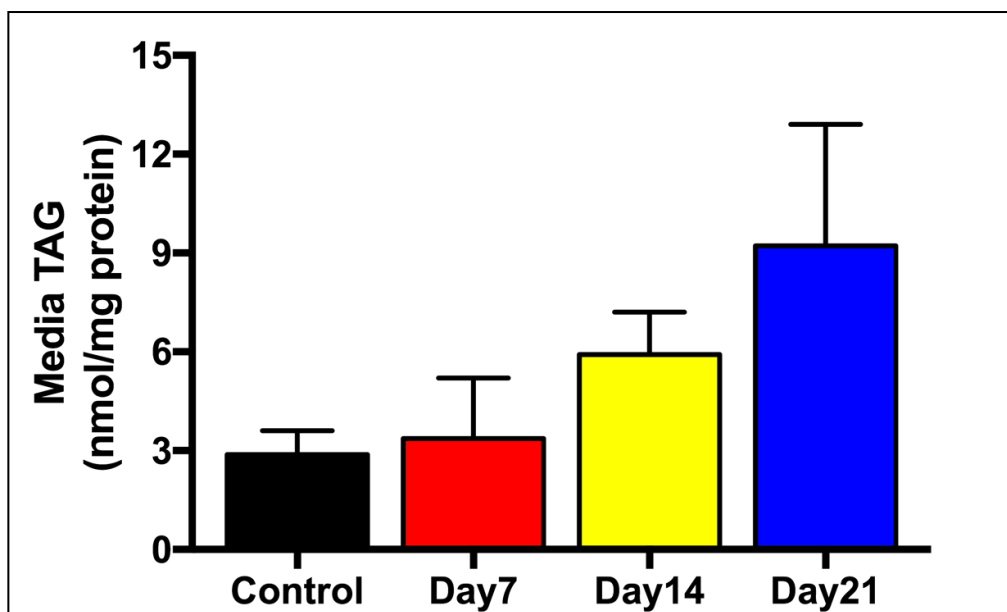


Figure 3.3.4-5 TAG concentrations in control and DMSO-treated HepG2 cell medium in a time-dependent manner. Data are expressed as mean $\pm$ se of $n=3$ experiments.

3.3.5. Fatty acid treatment does not have any impact upon lipid metabolic phenotype after DMSO treatment in HepG2 cells.

In order to determine whether fatty acid supplementation in addition to DMSO treatment had any further impact upon lipid metabolic phenotype, control and DMSO-treated cells (7 days) were treated with 45 μ M oleic, 30 μ M palmitic, and 25 μ M linoleic acid (OPL; physiological ratio 45:30:25%) for 24 or 48h. Although I observed increased intracellular TAG and cell media 3OHB concentrations as well as decreased mRNA expression of FAS and ACC1 after 48h of FA treatment in control HepG2 cells, no further effect was observed in the presence of DMSO after 7 days of treatment (Figure 3.3.5-1 and 3.3.5-2).

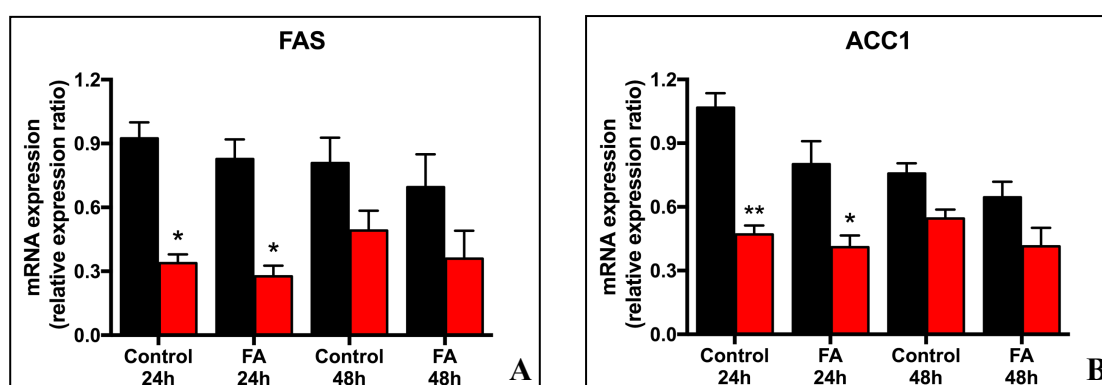


Figure 3.3.5-1 mRNA expression of (A). FAS and (B). ACC1 in control (black bars) and DMSO-treated HepG2 cells (red bars) for 7 days, in the presence or absence of 45 μ M oleic, 30 μ M palmitic, and 25 μ M linoleic acid, for 24 or 48h, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of n=3 experiments (*p<0.05, **p<0.01 vs. control).

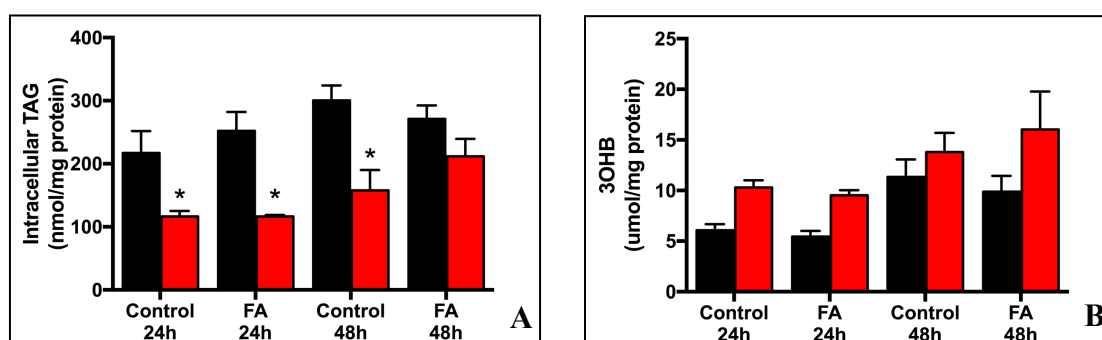


Figure 3.3.5-2 (A). Intracellular TAG and (B). cell media 3OHB levels in control and DMSO-treated HepG2 cells for 7 days, in the presence or absence of 45 μ M oleic, 30 μ M palmitic, and 25 μ M linoleic acid, for 24 or 48h. Data are expressed as mean $\pm$ se of n=3 experiments (*p<0.05 vs. control).

3.3.6. DMSO treatment alters glucose metabolism in HepG2 cells.

In untreated control HepG2 cells, the mRNA expression of PEPCK and G6Pase, which are related to glucose production, along with glyceraldehyde 3-phosphate dehydrogenase, (GAPDH) which is a key component of glycolysis, were low. DMSO treatment significantly increased the mRNA expression of these genes in a time-dependent manner (Figure 3.3.6-1).

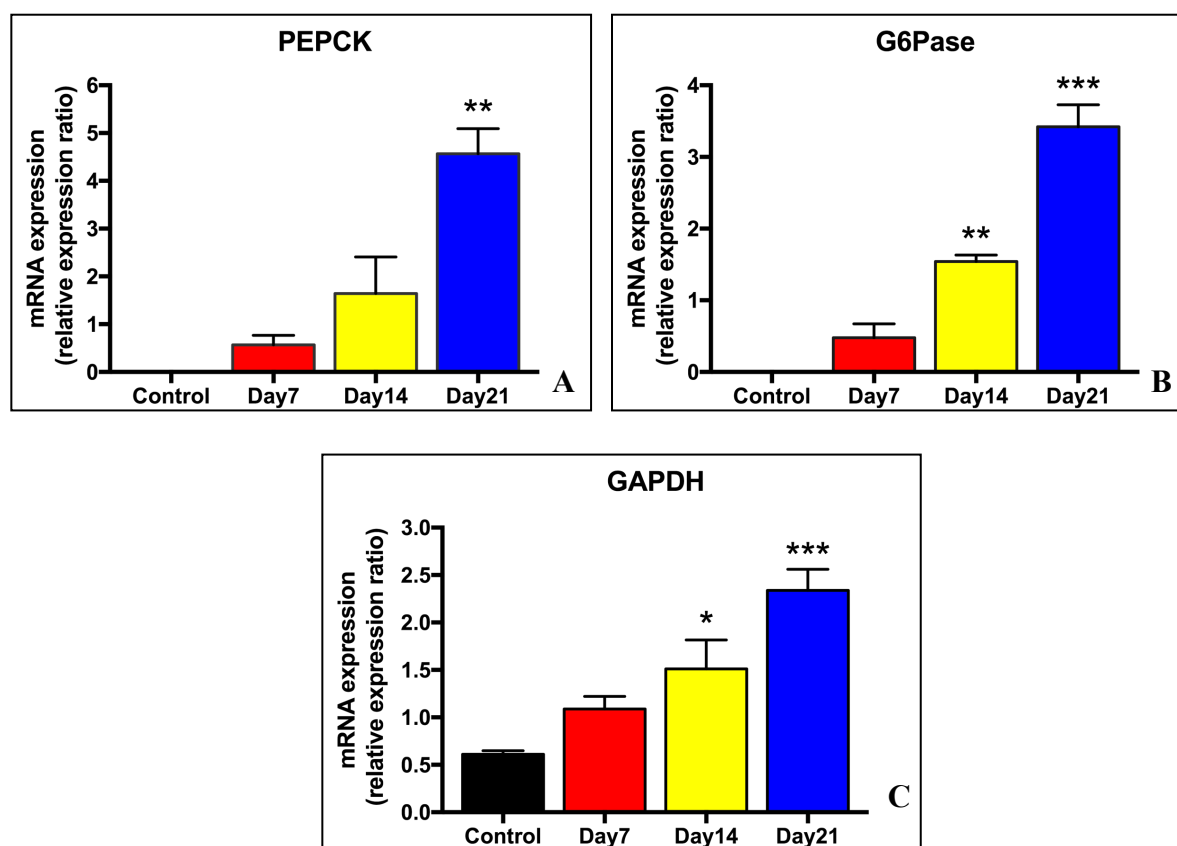


Figure 3.3.6-1 mRNA expression of (A). PEPCK, (B). G6Pase and (C). GAPDH in control and DMSO-treated HepG2 cells in a time-dependent manner, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control).

Similar changes, with increased mRNA expression in PEPCK, G6Pase and GAPDH were observed in C3A human hepatoma cells, after treatment with DMSO for 7, 14 and 21 days (Table 3.3-3).

Gene	Control	Day 7	Day 14	Day 21
<i>PEPCK</i>	0.01±0.002	0.19±0.013*	0.30±0.12**	0.88±0.13***
<i>G6PC</i>	0.03±0.006	0.03±0.007	0.08±0.02**	0.12±0.02***
<i>GAPDH</i>	0.26±0.05	1.86±0.20***	1.80±0.19***	2.77±0.23***

Table 3.3-3 mRNA expression of glucose metabolic genes in control and DMSO-treated C3A cells in a time-dependent manner, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control).

Changes in mRNA expression of PEPCK and G6Pase were mirrored by changes at protein level (Figure 3.3.6-2).

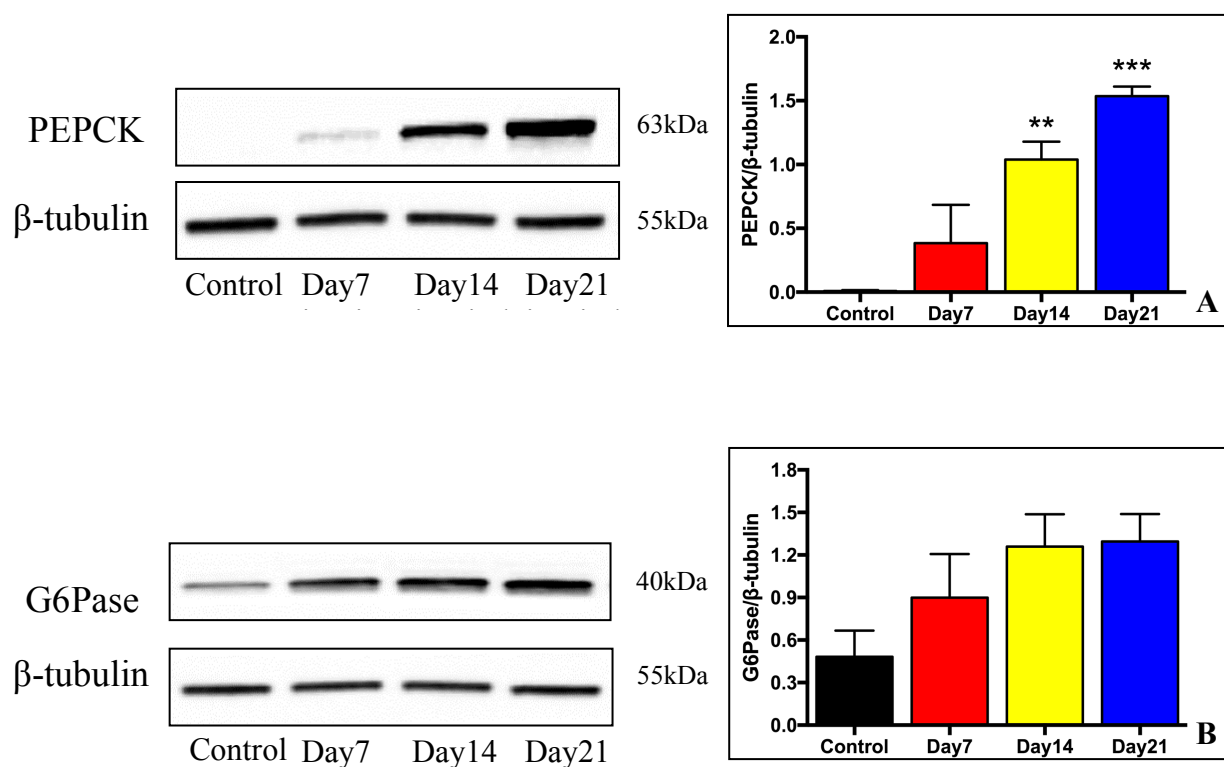


Figure 3.3.6-2 Protein expression of (A). PEPCK and (B). G6Pase in control and DMSO-treated HepG2 cells in a time-dependent manner. Data are presented as mean $\pm$ se of $n=3$ experiments and quantified relative to β -tubulin. Representative western blots are shown.

Whilst glucose concentration in the media did not change significantly (Figure 3.3-15A), 21 days of DMSO treatment decreased media lactate concentrations (3.93 ± 0.4 (control) vs. $3.18 \pm 0.36 \mu\text{mol}/\text{mg}$ protein (day 21), $p < 0.05$) (Figure 3.3.6-3).

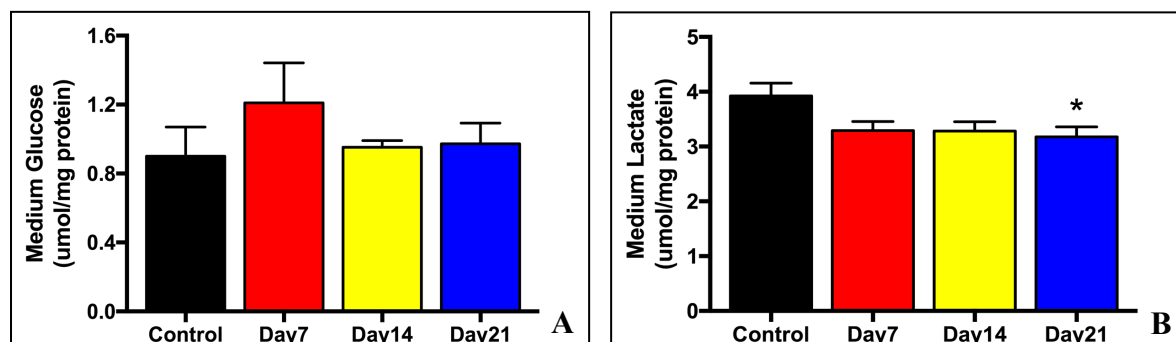


Figure 3.3.6-3 The media concentrations of (A). glucose and (B). lactate in control and DMSO-treated HepG2 cells in a time-dependent manner. Data are expressed as mean $\pm$ se of $n=3$ experiments (* $p < 0.05$ vs. control).

3.3.7. DMSO treatment effects on the expression of genes associated with steroid clearance and BA synthesis in HepG2 cells.

Based on published RNAseq data from Aviva Systems Biology (<http://www.avivasysbio.com>), the expression levels of SRD5A1, which is responsible for cortisol clearance and dihydrotestosterone production, are lower in HepG2 cells compared to healthy human liver tissue. In my study, DMSO treatment increased the mRNA expression of SRD5A1 in HepG2 cells. In contrast, AKR1D1 expression was significantly decreased after DMSO treatment in HepG2 cells (Figure 3.3.7-1).

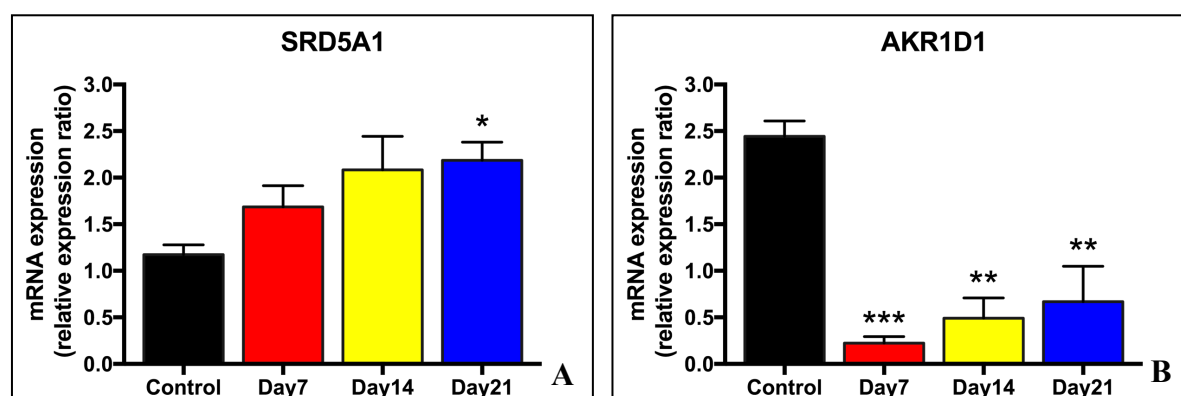


Figure 3.3.7-1 mRNA expression of (A). SRD5A1 and (B). AKR1D1 in control and DMSO-treated HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of n=3 experiments (* p <0.05, ** p <0.01, *** p <0.001 vs. control).

Although AKR1D1 expression was significantly decreased, DMSO treatment had no effect in the mRNA expression of the other key components of BA synthesis, CYP7A1 and CYP27A1 (Figure 3.3.7-2).

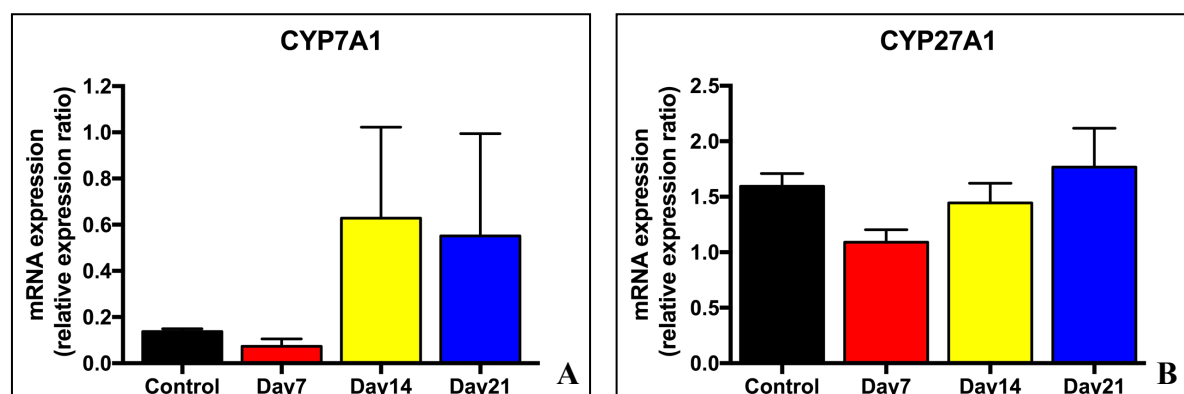


Figure 3.3.7-2 mRNA expression of (A). CYP7A1 and (B). CYP27A1 in control and DMSO-treated HepG2 cells after 7, 14 and 21 days, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments.

3.4. Discussion

Liver diseases are major causes of mortality and morbidity in humans (Younossi et al. 2013), therefore in order for effective treatment strategies to be developed, the cellular mechanisms involved in their aetiology need to be defined. As a consequence, there is a necessity for *in vitro* cellular models that recapitulate human hepatic metabolism. Primary cultures of human hepatocytes still represent the gold-standard, but their limitations including availability, inability to proliferate and donor variability, hamper their utility. In addition, in many circumstances the primary culture tissues that are available are from individuals with underlying liver disease and therefore their physiology and function may already be altered. Alternate models of human hepatocytes, including cell-lines that have been derived from hepatomas (e.g. HepG2, Huh7, Hep3b), offer the potential to be useful for metabolic studies, but have limitations, principally in understanding how closely they reflect human metabolism. Previous reports have suggested that DMSO treatment induces differentiation and drug metabolism in human hepatoma cell lines (Isom et al. 1985; Choi et al. 2009), although the molecular mechanisms are not fully understood.

By supplementing 1% DMSO to cell culture medium of HepG2 cells I observed changes in metabolic functionality over a time course of 21 days, with significant differences occurring at 7 days. Earlier time points were also tested (2 and 4 days of treatment) but little effect was observed (data not shown). In particular, I have shown that DMSO treatment reduces intracellular TAG accumulation which appears to be mediated through the suppression of lipogenic gene expression. In contrast, I observed a significant increase in the expression of key genes involved in intracellular glucose metabolism.

Hepatocyte functionality has previously been assessed by the measurement of markers of cell differentiation and viability. The most frequently measured markers include albumin, alpha-

1-antitrypsin, transthyretin and HNF1A (Nikoozad et al. 2014; Sainz & Chisari 2006). I measured markers of liver functionality that have been shown to be key markers in human hepatocyte physiology and found robust changes in the expression of these genes such that, they more closely resembled primary human hepatocytes. Although I did not directly assess cell viability, previous reports have demonstrated DMSO treatment does not cause cytotoxicity in concentrations up to 2% (Wang et al. 2012), double the concentration used in my experiments. One of the advantages of using hepatoma cell lines is their ability to proliferate reflecting their origin from hepatocellular carcinoma. Amongst the reported effects of DMSO is its ability to decrease proliferation and induce or maintain differentiation (Sainz & Chisari 2006). Following DMSO treatment, I observed a reduction in the malignant phenotype as evidenced by a significant decrease in AFP mRNA expression. DMSO is known to upregulate tumour suppressor PTEN expression in leukemic HL60 cells (Lee et al. 2005) and tumour suppressor protein, HLJ1 in lung cancer cell lines (Wang et al. 2012). Taken together, treatment with 1% DMSO in cell culture media has the ability to enhance hepatocyte functionality of hepatoma-derived cell lines to be more closely resemble human primary hepatocytes.

Lipid synthesis in the context of hepatocellular carcinoma (HCC) has been reported to be associated with increased HCC survival allowing it to survive at times of metabolic stress (Wang et al. 2016). The decrease in lipogenic gene expression and TAG accumulation I observed provides further evidence that I have reduced the malignant phenotype of the HepG2 cells. In addition, primary rat hepatocytes and cells from healthy human liver tissue have been reported to accumulate lower levels of intracellular lipid content compared to HepG2 cells (Gibbons et al. 1994; Green et al. 2014). In my study, I have clearly demonstrated that DMSO treatment affects lipid metabolism. The mRNA expression of genes involved in DNL (FAS, ACC1, ACC2) and TAG synthesis (DGAT1, DGAT2), were

decreased with a consequent reduction in intracellular TAG accumulation. Song et al. (2012) demonstrated that low dose DMSO (0.01%) treatment of HepG2 cells decreased palmitate-induced TAG accumulation; however, in the absence of palmitate supplementation and in contrast to my findings, intracellular TAG content did not differ between untreated and DMSO treated cells. It is possible that the difference between these data and my observations are a reflection of the different doses of DMSO used in the respective protocols.

Fatty acid oxidation is impaired in HepG2 cells compared to parenchymal hepatocytes, as measured by CPT1 activity (Agius 1988). Consistent with this, Gibbons et al. (1994) failed to detect any ketone body formation in oleate-treated HepG2 cells. In my study, fatty acid oxidation in DMSO-treated HepG2 cells was increased compared to untreated cells, even in the absence of exogenous fatty acids and this may have contributed to the observed decrease in intracellular TAG accumulation.

Previous studies have reported TAG secretion from HepG2 cells to be impaired when compared to primary rat hepatocytes (Wang et al. 1988), although culture conditions may also have an effect (e.g. glucose concentrations, variability in fetal serum) (Clavey et al. 1999). In my study, I demonstrated that DMSO treated cells had increased TAG levels in the cell medium compared to untreated controls, in a time-dependent manner, which again suggests the adoption of a more primary hepatocyte-like phenotype.

The liver plays an important role in glucose metabolism regulating the balance between exogenous glucose utilisation and endogenous production through gluconeogenesis. Indicative of their HCC origin, HepG2 cells have impaired glucose production, as demonstrated by downregulated gluconeogenic gene expression (Wang et al. 2013). In my study, DMSO treatment induced gluconeogenesis in a time-dependent manner, as indicated by increased PEPCK and G6Pase expression. Carbohydrate metabolism differs in hepatoma

cells compared to primary hepatocytes as the former maintain energy production through anaerobic glycolysis (Hugo-Wissemann et al. 1991). I observed a significant decrease in lactate production after 7 days of DMSO treatment, again suggestive of a more hepatocyte-like phenotype.

AKR1D1 is predominantly expressed in the liver and is an important regulator of both steroid hormone clearance and BA synthesis. In my study, I observed increased levels of AKR1D1 expression in HepG2 cells, compared to human primary hepatocytes, and that expression was significantly reduced after DMSO treatment in a time-dependent manner. These findings demonstrate that DMSO-treated HepG2 cells could be used as an improved model for the study of the role of AKR1D1 in the human liver, as the mRNA expression levels more closely resemble those in human primary hepatocytes.

Whilst I have demonstrated robust findings with regards to the effective role of DMSO in optimising the metabolic profile of HepG2 cells, there are some limitations to my experiments. Studies were undertaken using standard culture medium containing 25mM glucose (4.5g/L) which is non-physiological and may induce cellular stress and inflammation (Green et al. 2015). This needs to be considered when interpreting my data although all relevant control cells were cultured in the same glucose concentrations (11mM glucose for primary hepatocytes). DMSO treated cells did not require passaging and I did not directly assess cell proliferation, although others have reported that DMSO at concentrations up to 0.1% enhances proliferation in HepG2 cells (Song et al. 2012); however, at concentrations exceeding 0.5%, proliferation rates decrease and at concentrations below 2% cell viability is not impaired (Wang et al. 2012). Whilst I have demonstrated the impact of DMSO treatment on the metabolic profile of HepG2 cells, untreated (control) HepG2 cells continue to proliferate rapidly, therefore, it was not possible to match experimental treatment times with

control cells. The observed differences in the mRNA expression levels between untreated and DMSO-treated cells are not likely to be influenced by cell confluence, as the untreated cells were also confluent at the time of mRNA collection; however, a combination of both cell confluence and DMSO treatment cannot be excluded. In addition, the exact molecular mechanism that underpins the effects of DMSO on gene expression still remains unclear. A general -omics approach, including RNA sequencing and metabolites profiling, would help to elucidate the exact role of DMSO on lipid, carbohydrate and cancer metabolism.

In conclusion, I have shown that 1% DMSO treatment attenuates lipid accumulation, induces fatty acid oxidation and TAG secretion, augments gluconeogenesis and decreases AKR1D1 expression in HepG2 cells such that they more closely resemble primary human hepatocytes. This represents a significant development and enhancement of the currently available *in vitro* model systems to study hepatocyte biology and has important implications, not only for the study of the pathological processes that contribute to disease, but also their response to specific therapeutic interventions.

Chapter 4 Enzymology of AKR1D1

4.1. Introduction

AKR1D1 (5 β -reductase) is a cytosolic monomeric protein of 37kDa and belongs to the Aldo-keto-reductase family 1 of enzymes, which is an expanding family of NAD(P)(H)-dependent oxidoreductases (Chen et al. 2014). The gene is located on chromosome 7 in humans and it consists of 9 exons and 8 introns; six transcript variants have been identified, four of which lead to functional protein isoforms (Barski et al. 2013). The enzyme encoded by this gene utilises NADPH as an electron donor and catalyses a stereospecific irreversible double bond reduction between the 4th and the 5th carbon of the A ring of steroids. Detailed descriptions of the published studies that relate to its enzymology are described in section 1.5.

AKR1D1 is largely expressed in liver and plays an important role in steroid hormone metabolism and bile acid synthesis, leading to formation of the primary bile acids, CA and CDCA (Charbonneau & The 2001; Chen & Penning 2014). In addition, AKR1D1 activity has been reported in other tissues, including the brain and the placenta, and leads to the formation of active 5 β -dihydrosteroids, that are involved in neurotransmission, parturition and erythropoiesis (Chen et al. 2014; Chen et al. 2011).

Historically and more recently, a number of research groups have tried to characterise the substrate specificity and the kinetic parameters of AKR1D1, including the Michaelis-Menten constant, K_m , the maximal rate, V_{max} , the turnover number, k_{cat} , and the catalytic efficiency of the reaction, which is determined by the ratio of k_{cat}/K_m . V_{max} defines the maximum rate of reaction when the enzyme is saturated with substrate and K_m represents the concentration of substrate which permits the enzyme to achieve half V_{max} ; k_{cat} is the number of times each enzyme site converts substrate to product per unit time and the ratio k_{cat}/K_m determines how fast the enzyme reacts with the substrate once it encounters the substrate. However, as discussed in section 1.5.2, due to difficulties in purification of the enzyme and the generation

of a highly homogenous protein, results have been conflicting (Okuda & Okuda 1984; Iyer et al. 1990; Kondo et al. 1994; Charbonneau & The 2001; Chen et al. 2011) and have raised the possibility that multiple forms of this enzyme might be important to perform 5 β -reduction activity.

Considering the critical role of AKR1D1 in steroid hormone metabolism and formation of BAs, the generation of potent pharmacological inhibitors or allosteric activators for this enzyme would be of benefit and, through *in vitro* and *in vivo* studies; I could explore its functional role and use these compounds as potential therapeutics. However, difficulties in determining the substrate specificity of AKR1D1 and describing its enzyme kinetics has created problems in identifying specific inhibitors/activators and, to date, no highly potent inhibitors/activators for AKR1D1 are available.

4.1.1. Hypothesis and aims

As AKR1D1 plays a critical role in both steroid hormone metabolism and bile acid synthesis, I believe this enzyme would be a putative metabolic target. Therefore, the aim of this chapter was to characterise AKR1D1 enzymology by the following:

- Generate a human recombinant AKR1D1 protein
- Develop a high throughput NADPH-dependent fluorimetric screening assay with a potential view to large scale screening for potent selective inhibitors (in collaboration with European Lead Factory [ELF], see section 8.3.2).
- Determine the substrate specificity of human AKR1D1
- Define the kinetic properties of human AKR1D1
- Explore and identify specific AKR1D1 inhibitors

4.2. Methods

4.2.1. AKR1D1 construct

The construct encoding the *AKR1D1* gene was provided by the Structural Genomic Consortium (SGC) of the University of Oxford, in collaboration with Prof Udo Oppermann and was translated into the 326 aminoacid AKR1D1 isoform. The gene was inserted into a pNIC-CTHF vector with C-terminal hexa-HIS tag, preceded by a TEV protease cleavage site to facilitate subsequent protein purification.

Coding DNA sequence:

```
TTAAGAAGGAGATATACTATGATGGATCTCAGTGCTGCAAGTCACCGCATAC
CTTAAGTGATGGAAACAGCATTCCCATCATCGGACTTGGTACCTACTCAGAA
CCTAAATCGACCCCTAAGGGAGCCTGTGCAACATCGGTGAAGGTTGCTATTG
ACACAGGGTACCGACATATTGATGGGGCCTACATCTACCAAATGAACACGA
AGTTGGGGAGGCCATCAGGGAGAAGATAGCAGAAGGAAAGGTGCGGAGGGA
AGATATCTTCTACTGTGGAAAGCTATGGGCTACAAATCATGTCCCAGAGATG
GTCCGCCCAACCCTGGAGAGGACACTCAGGGTCCTCCAGCTAGATTATGTGG
ATCTTTACATCATTGAAGTACCCATGGCCTTTAAGCCAGGAGATGAAATATAC
CCTAGAGATGAGAATGGCAAATGGTTATATCACAAGTCAAATCTGTGTGCCA
CTTGGGAGGCGATGGAAGCTTGCAAAGACGCTGGCTTGGTGAAATCCCTGGG
AGTGTCCAATTTTAACCGCAGGCAGCTGGAGCTCATCCTGAACAAGCCAGGA
CTCAAACACAAGCCAGTCAGCAACCAGGTTGAGTGCCATCCGTATTTACCCC
AGCCAAAACCTCTTGAAATTTTGCCAACAACATGACATTGTCATTACTGCATAT
AGCCCTTTGGGGACCAGTAGGAATCCAATCTGGGTGAATGTTTCTTCTCCACC
TTTGTAAAGGATGCACTTCTAAACTCATTGGGGAAAAGGTACAATAAGACA
GCAGCTCAAATTGTTTTGCGTTTCAACATCCAGCGAGGGGTGGTTGTCATTCC
TAAAAGCTTTAATCTTGAAAGGATCAAAGAAAATTTTCAGATCTTTGACTTTT
CTCTCACTGAAGAAGAAATGAAGGACATTGAAGCCTTGAATAAAAATGTCCG
CTTTGTAGAATTGCTCATGTGGCGCGATCATCCTGAATACCCATTTTCATGATG
AATACGCAGAGAACCTCTACTTCCAATC
```


Final protein sequence:

```
MMDLSAASHRIPLSDGNSIPIIGLGTSEPKSTPKGACATSVKVAIDTGYRHIDGA  
YIYQNEHEVGEAIREKIAEGKVRREDIFYCGKLWATNHVPEMVRPTLERTLRVL  
QLDYVDLYIIEVPMFAFKPGDEIYPRDENGKWL YHKS NLCATWEAMEACKDAG  
LVKSLGVSNFNRRQLELILNKPGLKHKPVSNQVECHPYFTQPKLLKFCQQHDI  
TAYSP LGTSRNPIWVNVSSP LLDALLNSLGKRYNKTA AQIVLRFNIQRGVVVI  
PKSFNLERIKENFQIFDFSLTEEEMKDIEALNKNVRFVELLMWRDHPEYPFHDEY  
AENLYFQSHHHHHHDYKDDDDK
```

4.2.2. Transformation

AKR1D1 construct was inserted into BL-21 Rosetta bacteria cells by a standard heat-shock procedure. A number of colonies from the transformation were then used to inoculate 1ml of LB media containing 50µg/ml kanamycin, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture.

4.2.3. Expression

A fresh transformation was used to inoculate 20ml of LB media containing 50µg/ml kanamycin, which was placed in a 37°C shaker overnight. The next day this starter culture was used to inoculate 3x 1L of TB media (5 ml starter culture into each) containing 50µg/ml kanamycin. When the OD₆₀₀ reached 0.8, usually after 6 to 7h, the temperature was reduced to 18°C. After a further hour, the cells were induced by the addition of 0.5mM IPTG. The expression was continued overnight.

4.2.4. Cell harvest

Cells were centrifuged at 1000g for 10min at 4°C. The cells were resuspended in 30ml of Lysis Buffer (50mM KPO₄ pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole; 0.5mM TCEP). The resuspended cell pellet was stored in a -80°C freezer.

4.2.5. Purification

Protease inhibitor cocktail (cOmplete™, Roche, Hertfordshire, UK), 1u/ml nuclease inhibitor (Benzonase® Nuclease, Sigma) and lysozyme (100mg/50ml pellet) were added to the thawed cell pellet and mixed by inversion. The cell pellet was lysed by sonication (on ice), 10sec on, 10sec off, for a total of 10min sonication.

The lysate was then centrifuged at 17000g for 45min (Sorvall SS-34). The supernatant was loaded onto a Ni Sepharose column (4ml bed volume) and was washed with 10 Column Volumes (CV) of lysis buffer, 10 CV of wash buffer (as lysis buffer plus 30mM Imidazole). The protein was eluted into 3CV of the same buffer plus 400mM Imidazole.

Protein was further purified using the AKTA purifier and an S200 Superdex Hi-load column. The sample was loaded on the pre-equilibrated S200 gel filtration column in the same buffer but without imidazole (50mM KPO4 pH 7.5; 500mM NaCl; 5% Glycerol; 0.5mM TCEP) at 1.0ml/min, 1.5CV. Eluted proteins were collected in 2ml fractions and analysed on SDS-PAGE. The purified protein was concentrated in a VivaSpin15 (10 K MWCO) and stored at 4°C. Protein concentration was determined spectrophotometrically at 280 nm using Nanodrop (MW = 40279.1, Extinction Coefficient= 51340).

4.2.6. Cell-free high throughput activity assay

The high throughput activity assay, was based on previous described protocols by Faucher et al. (2008) and Chen et al. (2011). Incremental amounts of substrate were added to 5µM AKR1D1 in 100mM potassium phosphate (pH 6.0) in the presence of 20µM NADPH and 4% acetonitrile to give a final volume of 100µl in 37°C. NADPH reduction was then measured using a BMG Labtech Flurostar fluorescence spectrophotometer ($\lambda_{excitation}$ =340 nm, $\lambda_{emission}$ =460 nm) for 35min.

4.3. Results

4.3.1. Expression and purification of AKR1D1

The expression of AKR1D1 by E.coli BL-21 was induced with 0.50mM IPTG. The recombinant AKR1D1 enzyme showed a high degree of purity following purification by Ni-NTA affinity chromatography. Samples from the different stages of purification were collected to check purity and the results are shown in figure 4.3.1-1. Subsequently, the flow through from the Ni-NTA column was collected and loaded onto a HiLoad 16/60 superdex-200 column (GE Healthcare, UK) for final purification. The eluted recombinant AKR1D1 protein was detected, as expected, at approximately 37kDa and was determined through a calibration curve using the molecular weight standards dextran (200kDa), albumin (66kDa), carbonic anhydrase (29kDa), cytochrome C (12.4kDa) and aprotinin (6.5kDa) (Figure 4.3.1-2 & 3).

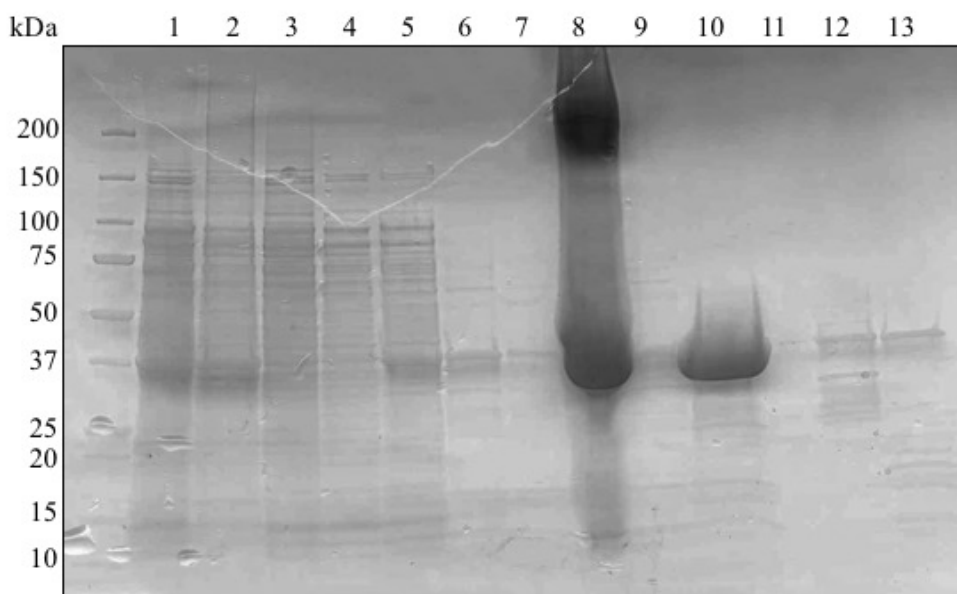


Figure 4.3.1-1 SDS-PAGE of human AKR1D1 protein purification steps, expressed in BL-21 bacteria cells. Lanes: **Lane 1.** total cell lysate, **Lane 2.** cell pellet, **Lane 3.** cell supernatant, **Lane 4.** flow through, **Lane 5.** lysis buffer 6-7. 30mM imidazole, **Lane 8.** 400mM imidazole, **Lane 10.** Purified protein, **Lane 11-13:** elution fractions.

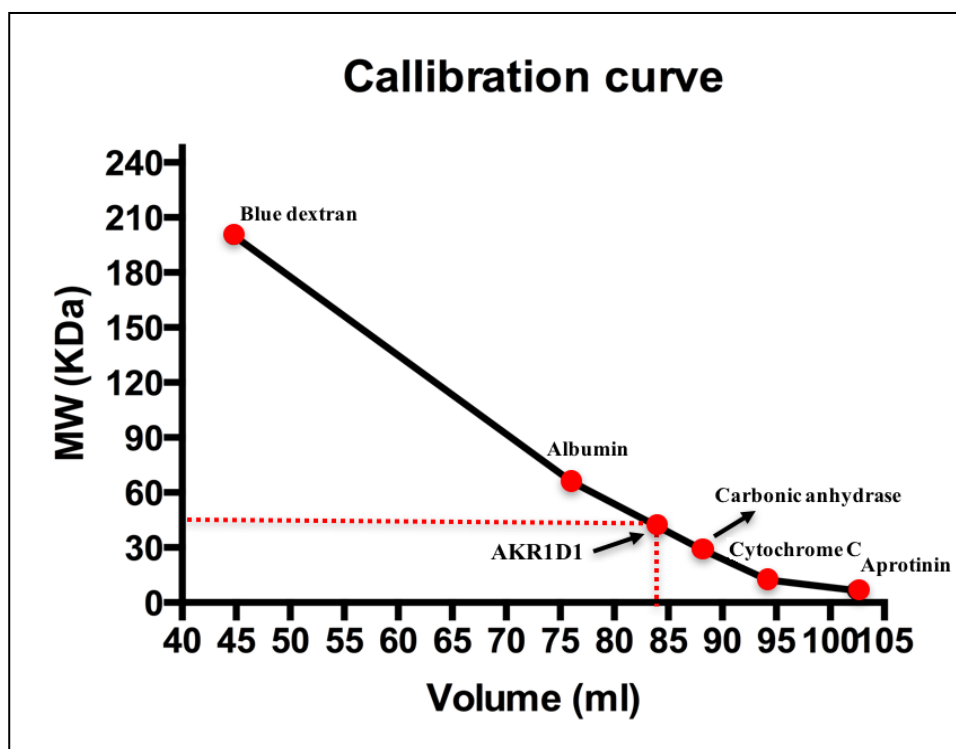


Figure 4.3.1-2 Determination of the molecular weight of purified AKR1D1 by gel filtration through HiLoad 16/60 superdex-200 column. Marker proteins used for calibration: dextran (200kDa), albumin (66kDa), carbonic anhydrase (29kDa), cytochrome C (12.4kDa) and aprotinin (6.5kDa).

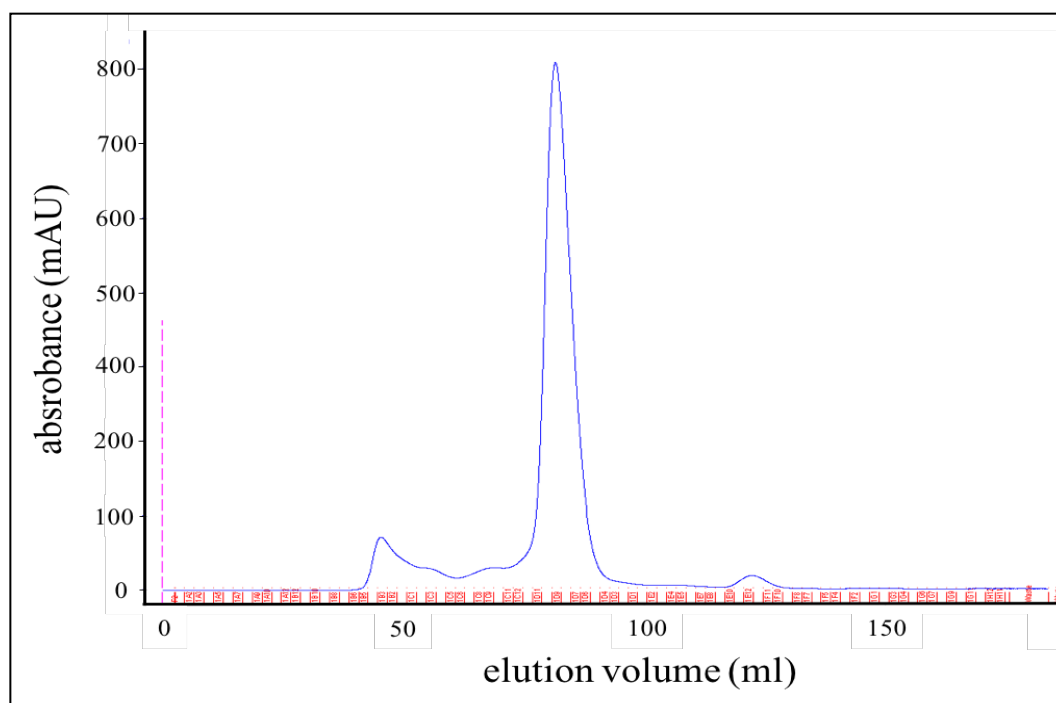


Figure 4.3.1-3 Gel filtration chromatogram documenting the final purification step of AKR1D1. Protein sample was loaded onto a preparative HiLoad 16/60 Superdex 200 column, eluted with a flow rate of 1 ml/min and the absorbance at 280 nm was recorded.

4.3.2. Substrate specificity of AKR1D1

The first step to determine the substrate specificity of AKR1D1 included the development and optimisation of a high throughput assay which would be able to catalyse the 5 β -reduction of a variety of C21-C27 steroids. Using cortisol as a substrate, I first explored enzyme activity and NADPH decrease reached the linear phase of substrate after 35min of incubation. Similar findings were observed when testosterone and cortisone were used as substrates (Figure 4.3.2-1). In addition, increasing amounts of substrate were tested and a concentration of 40 μ M was chosen to ensure that substrate concentration is not rate-limiting for this assay.

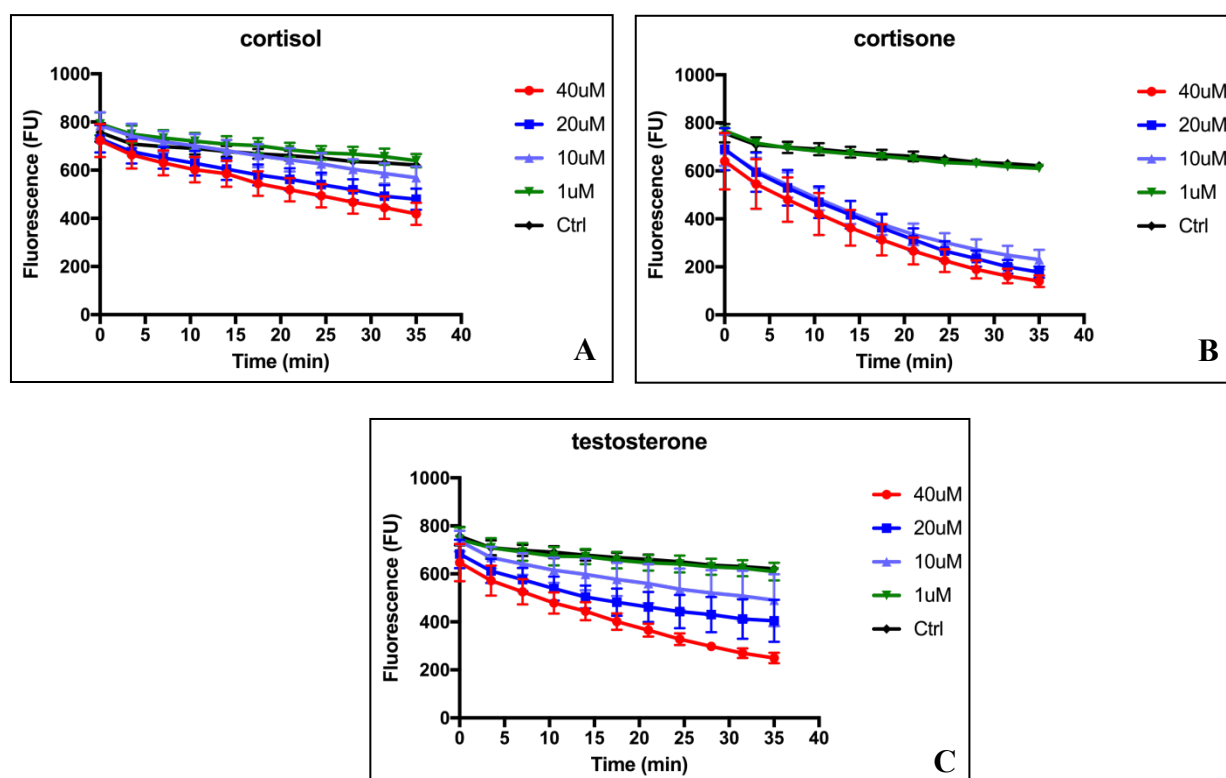


Figure 4.3.2-1 AKR1D1 activity as measured by NADPH reduction using incremental concentrations of (A). cortisol, (B). cortisone and (C). testosterone as substrates. Data are expressed as mean $\pm$ se of n=8-10 experiments.

Seven steroid hormones (cortisol, cortisone, testosterone, androstenedione, progesterone, aldosterone and corticosterone) and two BA precursors (7 α ,hydroxy-cholestenone and

7 α ,12 α ,dihydroxy-cholestenone) were examined as substrates for AKR1D1 by measuring NADPH reduction in a time-dependent manner. For the assay, a modified version of a previously published procedure (Chen et al. 2011) was developed: 40 μ M of substrate were incubated with 5 μ M of recombinant AKR1D1 protein, in the presence of 20 μ M NADPH at 37°C. AKR1D1 has a pH optimum of 6.0; to achieve this, 100mM potassium phosphate were added to the final mix. The progress curves for steroid reduction were generated by monitoring the disappearance of NADPH fluorescence.

From the steroid hormones investigated, AKR1D1 exhibited the highest activities towards cortisone and aldosterone (Figure 4.3.2-2A and 2E), followed by testosterone, androstenedione and corticosterone (Figure 4.3.2-2G, 2B and 2C). Lower activities were exhibited towards cortisol and the bile acid precursor 7 α ,hydroxy-cholestenone and that was demonstrated by the modest NADPH reductions compared to controls (Figure 4.3.2-2D and 2H). The BA precursor 7 α ,12 α ,dihydroxy-cholestenone was also tested against AKR1D1, but, similar to 7 α ,hydroxy-cholestenone, low activity was observed (data not shown). For all the steroids tested, I was not able to detect the formed 5 β -reduced products.

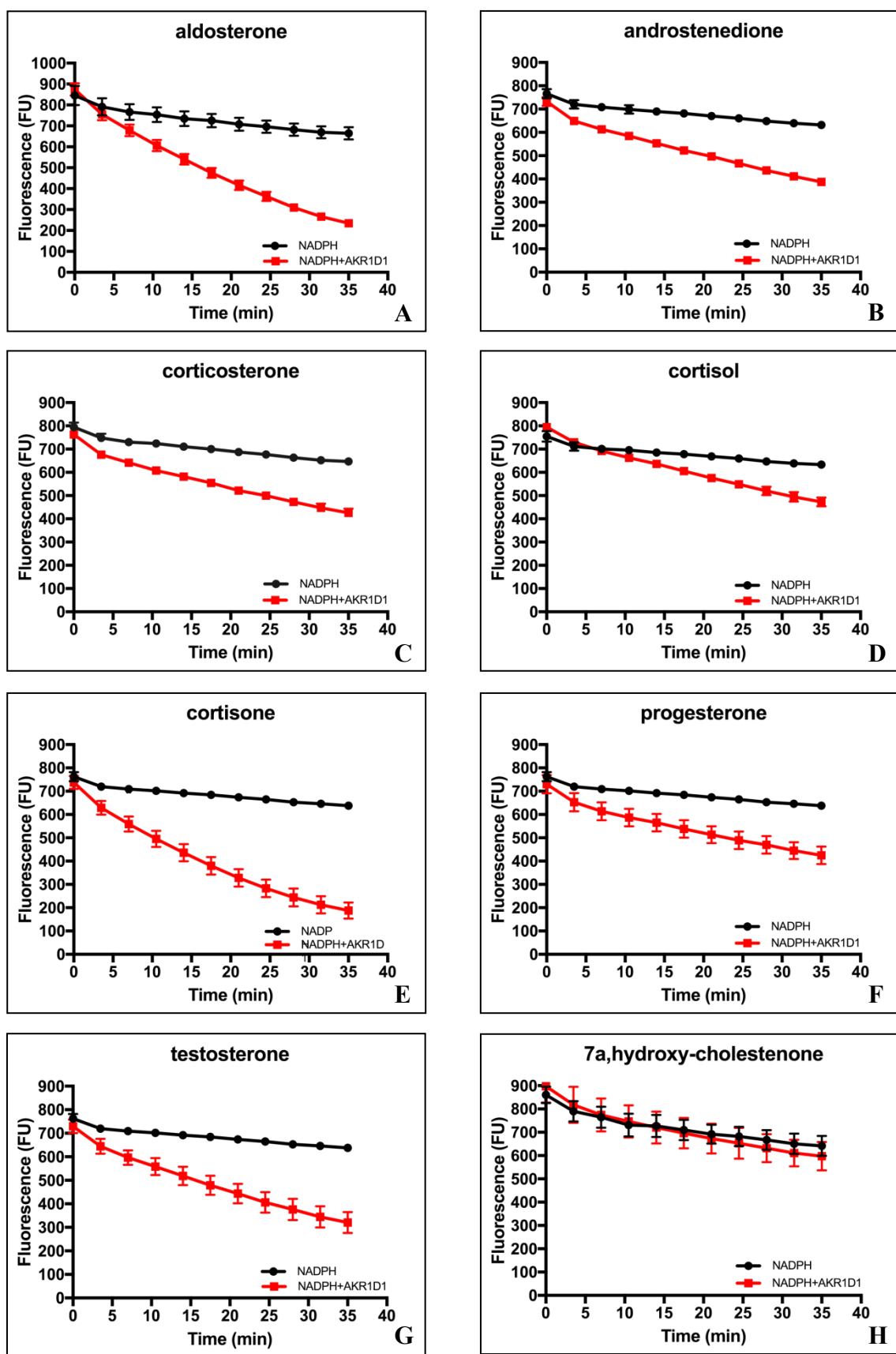


Figure 4.3.2-2 AKR1D1 activity as measured by NADPH reduction using (A). aldosterone, (B). androstenedione, (C). corticosterone, (D). cortisol, (E). cortisone, (F). progesterone, (G). testosterone and (H). 7 α , hydroxy-cholestenone as substrates. Data are expressed as mean $\pm$ se of $n=8-10$ experiments.

4.3.3. Enzyme kinetic analysis of AKR1D1

Further to substrate specificity, enzyme kinetic experiments were performed to generate the main Michaelis-Menten kinetic values of the maximum velocity ($V_{\max}$) and the substrate concentration needed to achieve half-maximum velocity (K_m). 5 μ M of recombinant AKR1D1 were tested against different concentrations of substrate (0.6-40 μ M), in the presence of 20 μ M NADPH at 37°C. In addition to the $V_{\max}$ and K_m values, I calculated additional enzyme kinetic parameters, including the turnover number (k_{cat}) as well as the catalytic efficiency of the reaction (k_{cat}/K_m).

From all substrates tested, aldosterone demonstrated the highest $V_{\max}$ value followed by cortisone, reflecting fast catalysis of the reaction by AKR1D1. AKR1D1 showed the highest k_{cat}/K_m values for aldosterone, cortisone, androstenedione and testosterone, whereas the lowest values were observed for cortisol and the two BA precursors, 7 α ,hydroxy-cholestenone and 7 α ,12 α ,dihydroxy-cholestenone. The results are summarized in Table 4.3-1. Michaelis-Menten plots using the Henri - Michaelis-Menten equation $V=V_{\max}/(K_m+[S]+([S]^2/K_i))$ as well as Lineweaver-burk plots were generated (Figures 4.3.3-1 and 4.3.3-2).

Substrate	$V_{\max}$ (FU/min)	K_m (uM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{ uM}^{-1}$)
cortisone	13.577	5.44	0.73	0.134
aldosterone	15.429	6.65	0.83	0.124
androstenedione	6.546	3.1	0.35	0.113
testosterone	10.5	7.2	0.73	0.101
progesterone	4.743	3.29	0.25	0.077
corticosterone	5.634	4.36	0.30	0.069
cortisol	8.571	25.42	0.46	0.018
7 α ,12 α ,dihydroxy-cholestenone	2.789	15.82	0.15	0.009
7 α ,hydroxy-cholestenone	4.737	25.77	0.24	0.009

Table 4.3-1 AKR1D1 kinetic values of AKR1D1 using C21-C27 steroid substrates. K_m (μ M), k_{cat} (min^{-1}) and k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$) were determined with 20 μ M NADPH as co-enzyme.

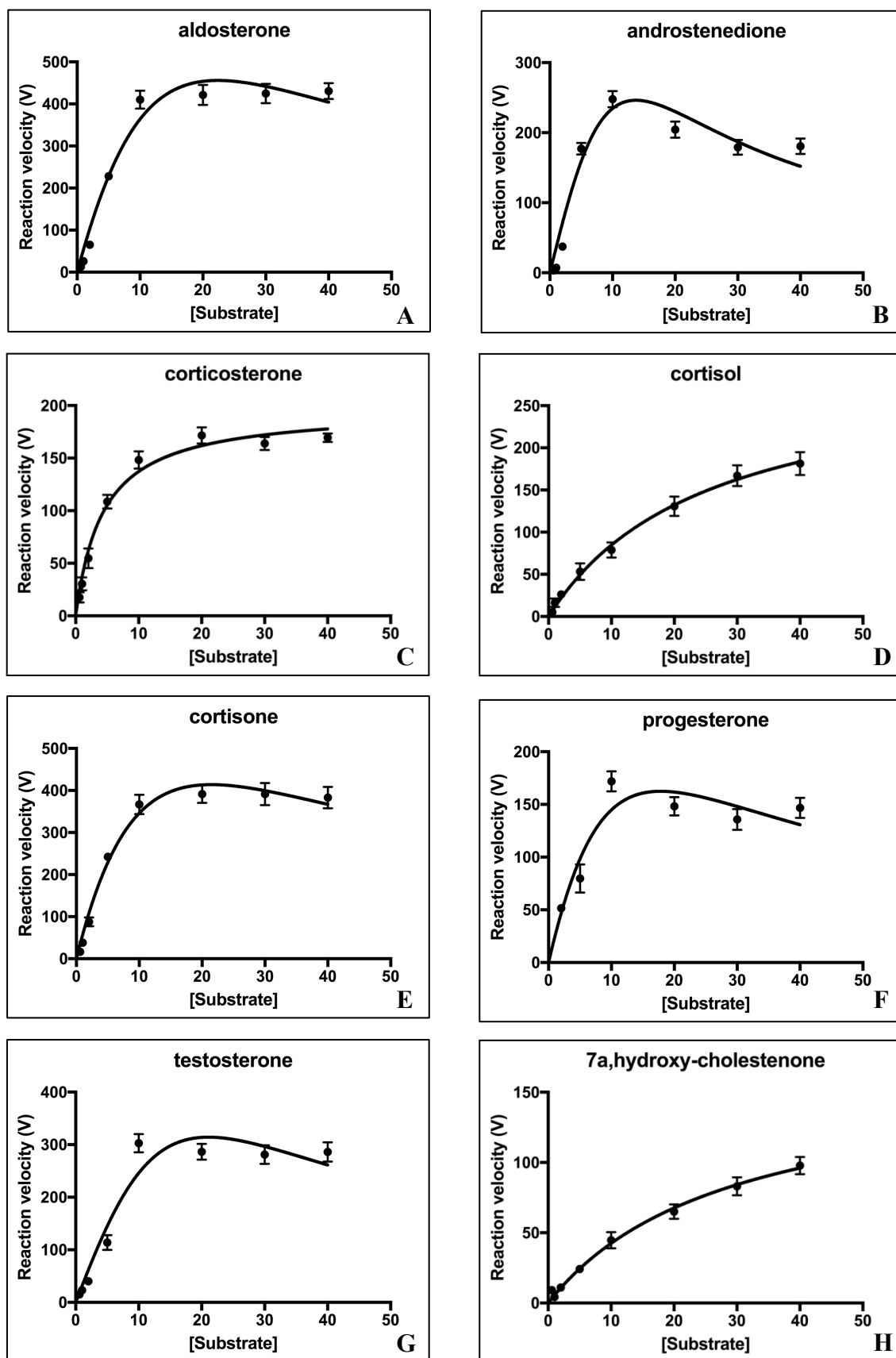


Figure 4.3.3-1 Michaelis-Menten plot of the AKR1D1 kinetics using (A). aldosterone, (B). androstenedione, (C). corticosterone, (D). cortisol, (E). cortisone, (F). progesterone, (G). testosterone and (H). 7 α ,hydroxy-cholestenone as substrates. Data were fitted in the Henri-MM equation: $V = V_{max} / (K_m + [S] + ([S]^2 / K_i))$.

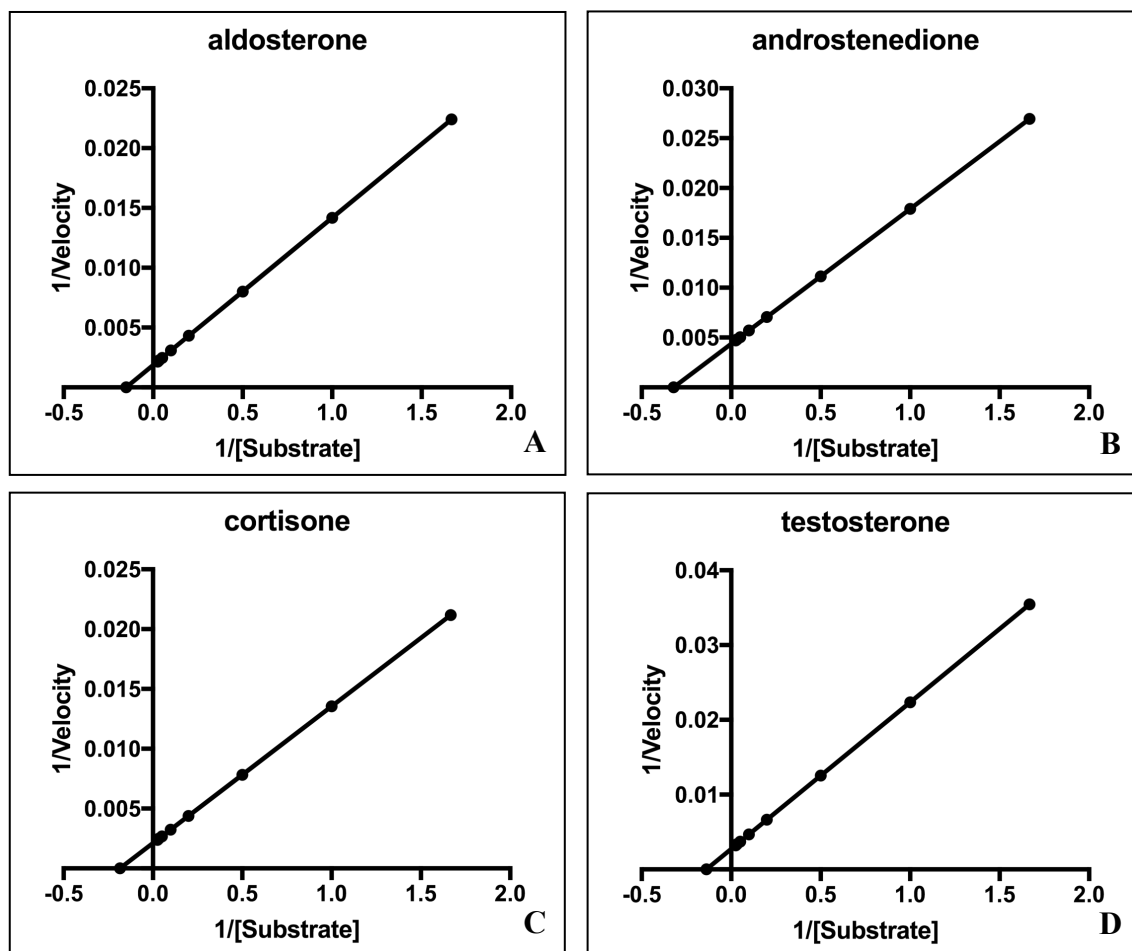


Figure 4.3.3-2 Lineweaver-burk plots for the 4 AKR1D1 substrates with the highest catalytic efficiency: (A). aldosterone, (B). androstenedione, (C). cortisone and (D). testosterone.

4.3.4. Inhibition of AKR1D1

Using the high throughput assay described above, we explored the potential of two 5 α R inhibitors, finasteride and dutasteride, to inhibit AKR1D1 activity. Finasteride (selective 5 α R2 inhibitor) partially inhibited AKR1D1 activity, in a dose-dependent manner, when using aldosterone and cortisone as substrates and, to a lesser extent, when androstenedione and testosterone were used as substrates (finasteride 0-500 μ M, substrate 40 μ M, AKR1D1 5 μ M, NADPH 20 μ M, 37°C) (Figure 4.3.4-1A, 1B, 1D, 1G). Dutasteride (non-selective 5 α R1 and 5 α R2 inhibitor), under the same conditions as described above, was even less potent than

finasteride and significantly decrease AKR1D1 activity only in the highest inhibitor concentrations. Neither finasteride nor dutasteride had any effect on AKR1D1 activity when cortisol, 7 α ,hydroxy-cholestenone or 7 α ,12 α ,dihydroxy-cholestenone were used as substrates (Figure 4.3.4-1C, 1H). Surprisingly, finasteride increased the enzyme activity in the presence of progesterone (Figure 4.3.4-1F).

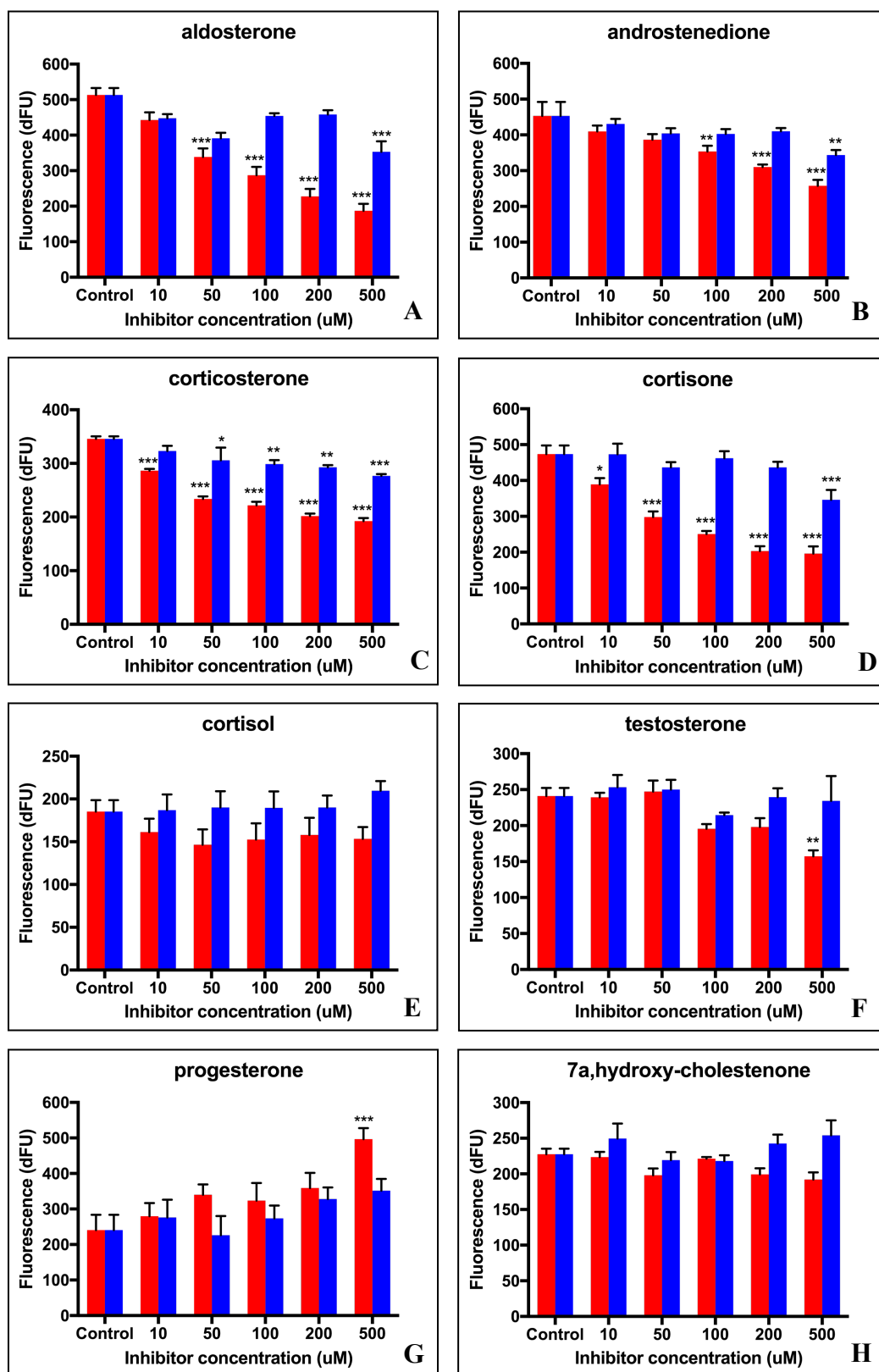


Figure 4.3.4-1 AKR1D1 inhibition by finasteride (red bars) and dutasteride (blue bars) using (A). aldosterone (B). androstenedione, (C). corticosterone, (D). cortisone, (E). cortisol, (F). testosterone, (G). progesterone and (H). 7 α ,hydroxy-cholestenone as substrates. Data are expressed as mean $\pm$ se of $n=8-10$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to control).

4.4. Discussion

In the current study, I generated a homogenous human recombinant AKR1D1 protein with a signal/background (S/B) ratio value of 100 and Z' factor value of 0.9, indicative of high enzyme stability. These results agree with Chen et al. (2011) who first generated a highly homogenous and stable protein using a similar protocol. With the parallel development of a high throughput activity assay, I have demonstrated broad substrate specificity for AKR1D1, but with a wide variability in enzymatic constants, reflecting differing substrate affinities and catalytic efficiencies. Efforts to explore whether the two 5 α R inhibitors, finasteride and dutasteride, are able to act as AKR1D1 inhibitors revealed partial inhibition of AKR1D1 activity by finasteride but not dutasteride.

Previous work exploring AKR1D1 substrate specificity have reported conflicting data. Iyer et al. (1990), who purified AKR1D1 from human liver specimens, reported that the Δ^4 -3-ketosteroids, including androstenedione, progesterone, cortisone and cortisol, were all active substrates, whilst testosterone had no specificity towards AKR1D1. In contrast, Kondo et al. (1994) demonstrated that, in COS cells, the C27 BA intermediates (cholestenones) were the preferred substrates of AKR1D1, whilst 5 β -reduction of cortisol and testosterone was limited. Furthermore, no 5 β -reduction of androstenedione or progesterone was observed. In another study using HEK293 cells, AKR1D1 had the highest affinity towards androstenedione, testosterone, progesterone and 17-hydroxyprogesterone, whilst corticosteroids and mineralocorticoids acted as inferior substrates (Charbonneau & The 2001). It is likely the discrepancy in findings can be, at least partially, explained by low purification enzyme quality or variation among the different *in vitro* models.

Results from studies where human recombinant AKR1D1 protein was generated have also been confusing. Chen et al. (2011) revealed that human recombinant AKR1D1 protein could

metabolise cholestenones, aldosterone, androstenedione, testosterone and corticosterone with high affinity, whilst cortisone and cortisol reduction was limited. However, a more recent study from Chen et al. (2014), demonstrated data that agreed with my findings; using human recombinant AKR1D1 protein and a high-throughput screening protocol similar to mine, they showed that the C19-C21 steroids, including aldosterone, cortisone, testosterone and corticosterone were highly metabolised by AKR1D1. In contrast, and in agreement with my results, 5 β -reduction of the C27 BA intermediates 7 α ,hydroxy-cholestenone and cholestenone was slow and with low turnover numbers.

These data reveal that AKR1D1, regardless the level of affinity for each steroid, accepts substrates with large size differences, indicating that the active site of the enzyme is not strongly dependent on the size of the C17 side chain of the steroid. In addition, results from crystal structure studies for AKR1D1 demonstrated that the steroid binding pocket of this enzyme resembles a cylindrical cavity and, upon binding, the steroid A ring buries deep at the bottom of the cavity, where the active site sits; the C17 side chain of the steroid remains unaffected and protrudes towards the enzyme surface (Di Costanzo et al. 2008).

Differences in substrate specificity and kinetic values for each steroid suggest there may be differences in the rate-determining step of each reaction. In my study, the small C19-C21 steroid substrates were highly metabolised by AKR1D1, suggesting perhaps that steroids of smaller size fully fit in the active site of the enzyme. In contrast, the C17 tail of the long C27 steroids may interact with the outer surface of the enzyme, resulting in additional substrate-enzyme interactions and affecting binding affinity and the velocity of the reaction. Furthermore, as mentioned in section 1.5.4, there is evidence of differences in the orientation of each substrate within the binding pocket of AKR1D1; Di Costanzo et al. (2008) and Faucher et al. (2008a) reported that testosterone and Δ^4 -androstene-3,17-dione do not bind

the same way as cortisone and progesterone and that may account for differences in substrate specificity and kinetic values amongst different steroids.

Besides substrate specificity and enzyme kinetics, I also explored potential inhibitors of AKR1D1. A study from McNeilly et al. (2010) showed that BAs, especially CDCA, act as competitive inhibitors of rat AKR1D1 and repress the activity of the human AKR1D1 promoter, with confounding effects on glucocorticoid metabolism and total BA levels. In contrast, Chen et al. (2011) reported that CDCA and the secondary BA ursodeoxycholic acid are non-competitive inhibitors of AKR1D1. Previous studies attempted to explore the inhibitory effect of the selective 5 α R2 inhibitor finasteride on AKR1D1 using testosterone as a substrate, but demonstrated low micromolar affinity and high K_i values (Drury et al. 2009). My data endorsed these findings, and only partial inhibition of AKR1D1 activity by high doses of finasteride was observed. The AKR1D1-NADP⁺ complex is responsible for the reduction of the double bond between the 4th and the 5th carbon of the A ring of steroids (Δ^4 -ene reduction). However, this complex is not able to reduce the double bond between the first and second carbon in the same ring (Δ -1-2-ene) (Drury et al. 2009). Both finasteride and dutasteride carry a Δ -1-2-ene (Figure 4.3.4-2), thus making it impossible for AKR1D1 to reduce this bond and potentially explaining the inability of these inhibitors to block AKR1D1 activity.

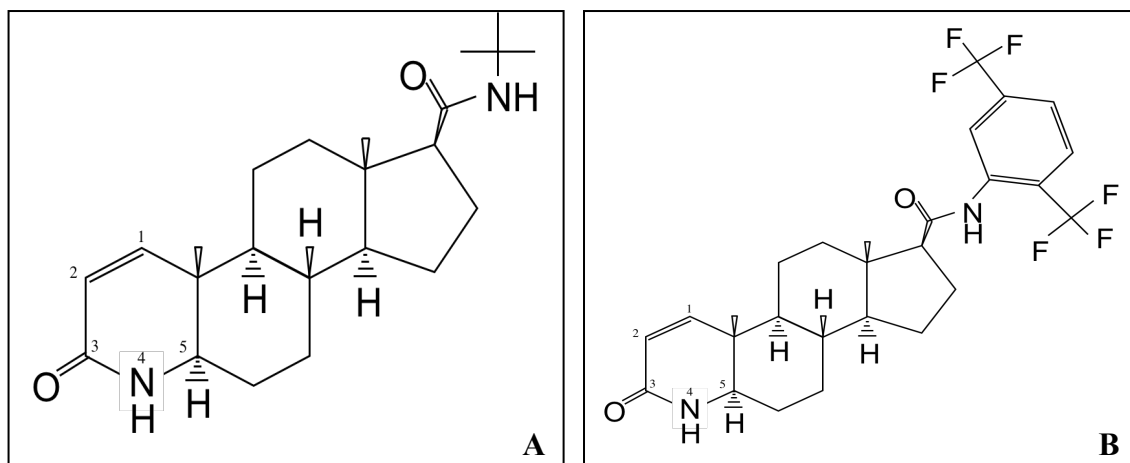


Figure 4.4-1 (A). *finasteride* structure and (B). *dutasteride* structure, with a double bond between the first and the second carbon of the A ring.

My study does come with some limitations. The AKR1D1 recombinant protein was generated using a prokaryotic cell-free system; although these systems lead to high-yield protein production, post-translational modifications for functional proteins are restricted in these cells and only limited modifications are possible. Therefore, differences between *in vitro* and recombinant AKR1D1 proteins may occur and the *in vitro* protein may possess slightly different or extra properties compared to the recombinant one. In addition, in my cell-free system, I have not taken into account multiple substrate availabilities with subsequent substrate competition that normally occurs in *in vitro* and *in vivo* systems; when the concentration of a substrate is below the enzyme's K_m , competition between substrates is low, whilst at substrate concentrations above K_m , competition can be significant. Moreover, AKR1D1 performs irreversible reactions, therefore substrate competition can be compared to enzyme inhibition; depending on their concentration, different substrates can act as inhibitors of each other's reactions and that can influence the kinetics of the enzyme. It is also possible that substrate inhibition could be responsible for discrepancies in the results from previous studies who tried to characterise AKR1D1 substrate specificity; crystal structure studies from Faucher et al. (2008) revealed that the activity of AKR1D1 can be strongly reduced even in

the presence of relatively low substrate concentrations. It should also be mentioned that, in the cell-free system assay I used, AKR1D1 was the only enzyme tested for the different steroid substrates; things are more complex *in vitro* where other enzymes can compete for the same substrate. For instance, cortisol can also be converted to 5 α -dihydrocortisol by 5 α -reductase and this enzyme demonstrates higher affinity towards cortisol compared to AKR1D1 (Nixon et al. 2012). The K_m for 5 α -reduction of testosterone is also low ($<0.1\mu\text{M}$); it is, then, possible that the different routes of clearance regulate intracellular hormone levels by first pass metabolism. It is possible that the actual effect of AKR1D1 activity on these substrates to be even more limited *in vitro* and be participating in steroid clearance only when steroid concentrations are above physiological levels. As mentioned in section 1.5.1, 4 different isoforms of AKR1D1 have been identified; although, in this study, the longest isoform was generated, alternative isoforms were not able to be detected and potential differences in the properties amongst the different isozymes cannot be excluded.

Further analysis of enzyme kinetic parameters could not be determined due to absence of suitable methodology and equipment. In addition, detailed inhibition studies and determination of specific K_i values for both finasteride and dutasteride should be assessed. Additional substrate inhibition studies should also be performed, to further enlighten the role of steroid substrates upon AKR1D1 activity.

In conclusion, I have demonstrated the successful generation of a human recombinant protein through which I was able to examine the substrate specificity and enzyme kinetic properties of AKR1D1. These experiments pave the way for the development of a potent selective inhibitor screening (see section 8.2.3), with the potential to also identify allosteric activators of AKR1D1. This will help to define the role of AKR1D1, both *in vitro* and *in vivo*, to regulate cellular and whole-body phenotype.

Chapter 5 AKR1D1 regulates glucocorticoid availability, GR receptor activation and BA synthesis

5.1. Introduction

AKR1D1 is highly expressed in the liver where it uses the C19-C27 steroids (including cortisol, cortisone, testosterone and progesterone) as substrates to generate all 5 β -reduced dihydrosteroid metabolites. Both cortisol and cortisone are metabolised to 5 β -dihydrocortisol and 5 β -dihydrocortisone respectively by AKR1D1, and are then subsequently converted, in a non-rate limiting step, to their tetrahydro-metabolites (tetrahydrocortisol, 5 β -THF and tetrahydrocortisone, 5 β -THE) by 3 α -HSD. AKR1D1 also plays a crucial role in BA synthesis, as it is present in both arms of the classic BA synthesis pathway.

Steroid hormones, including glucocorticoids and androgens, are potent regulators of metabolic phenotype and have been implicated in driving hepatic lipid accumulation. Glucocorticoid excess, androgen deficiency in men, and excess in women are all associated with NAFLD (Hazlehurst & Tomlinson 2013). Steroid hormone action is regulated at a pre-receptor level within key target tissues, including the liver, by a series of enzymes that can either enhance their clearance or augment their action. In this regard, the 11 β -HSD isozymes and the 5 α Rs have been extensively described. 11 β -HSD1 catalyses the conversion of inactive cortisone to active cortisol and global deletion of 11 β -HSD1 has been associated with increased insulin sensitivity, protection from hepatic steatosis, reduced expression of lipolytic enzymes in adipose tissue and a beneficial metabolic phenotype in mice (Morton et al. 2001; Morgan et al. 2014; Wang et al. 2015). In line with this, hepatic over-expression of 11 β -HSD1 has been linked to increased hepatic TAG content in mice (Paterson et al. 2004). In humans, there is evidence of decreased hepatic 11 β -HSD1 activity in obese patients (Stewart et al. 1999). Similarly, deletion of 5 α R1 has been associated with increased hepatic lipid accumulation in mice (Dowman et al. 2013), whilst, in urinary steroid profiles from obese patients, 5 α R activity increased with indexes of IR (Tomlinson et al. 2008).

Glucocorticoids predominantly perform their actions through activation of the glucocorticoid receptor (GR) and there is an emerging role for the GR in the control of carbohydrate and lipid metabolism. Upon glucocorticoid binding in the cytosol, the GR translocates to the nucleus and upregulates the expression of key enzymes involved in glucose metabolism (e.g. PEPCK, G6Pase) (Patel et al. 2014; Moraitis et al. 2017), alongside increases in the expression of genes implicated in fatty acid synthesis (e.g. ACC1, ACC2) (Wang et al. 2012). In addition to steroid hormones, BAs are now established as potent signalling molecules that regulate metabolic phenotype and BA synthesis is also regulated by at least 14 enzymes. Abnormal activity of enzymes implicated in BA synthesis have been associated with decreased in normal BA synthesis and unusual BA composition (Sundaram et al. 2008).

5.1.1. Hypothesis and aims

A limited number of studies have tried to characterise the ability of AKR1D1 to metabolise steroid hormones and BA intermediates (Chen et al. 2011, Chen & Penning 2014, Rižner & Penning 2014), by using purified recombinant protein. However, little is known about the ability of this enzyme to regulate steroid hormone availability and BA synthesis *in vitro*. To investigate this, I genetically manipulated AKR1D1 gene expression through over-expression and transient knockdown assays and aimed to:

- Define the ability of AKR1D1 to regulate glucocorticoid availability in human hepatocytes
- Define the ability of AKR1DI to regulate glucocorticoid receptor activation in human hepatocytes
- Define the ability of AKR1D1 to regulate BA synthesis and secretion in human hepatocytes.

5.2. Methods

5.2.1. HepG2, Huh7 and HEK293 cell culture

HepG2 human hepatoma, Huh7 human hepatoma and Human Embryonic Kidney (HEK293) cells were cultured as described in sections 2.1.1 and 2.1.3, and plated in 12-well and 24-well Cell Bind plates (Corning, Flintshire, UK) at a density of 1.6×10^5 and 0.8×10^5 cells/ml, respectively.

5.2.2. Transfection studies

For the over-expression studies, AKR1D1 cDNA was cloned into the pCMV6-XL4 vector (Origene Technologies, Rockville, USA) and transiently transfected into HepG2 and HEK293 cells as described in section 2.3.2.

For knockdown studies, AKR1D1 siRNA molecules were purchased from Invitrogen (Paisley, UK) and transiently transfected into HepG2 cells as described in section 2.3.3.

In all experiments investigating activation of the GR, cells were plated in 12-well Cell Bind plates (Corning, Flintshire, UK) and co-transfected with 1 μ g of AKR1D1 vector, 0.5 μ g of GRE reporter (QIAGEN, Manchester, UK), a mixture of inducible GRE-responsive firefly luciferase construct and a constitutively expressing Renilla luciferase construct. The amount of transfection reagent was increased to 3 μ l/well and the mixture was diluted in 100 μ l of OPTIMEM serum free media (Invitrogen, Paisley, UK). The final mixture was vortexed and incubated at room temperature for 20min, and afterwards, was added drop-wise to each well (100 μ L/well). Finally, the plates were shaken gently and put into the incubator at 37°C for 48h.

5.2.3. Cell treatments

For all cortisone treatments, HepG2 and HEK293 cells were treated with serum free and phenol-red free media containing 200nM cortisone, after 48h of transfection, in a time-dependent manner.

For all cortisol treatments, HepG2 and HEK293 cells were also treated with serum free and phenol-red free media containing 100, 200 or 500nM cortisol, after 48h of transfection, in a time-dependent manner.

For all BA measurement assays, HepG2 and Huh7 cells were treated with serum free and phenol-red free media, after 48h of transfection.

5.2.4. RNA extraction and RT-PCR

Total RNA was extracted from cells using the Tri-Reagent system. Quantity and quality were determined spectrophotometrically at OD₂₆₀. One microgram of total RNA was used for reverse transcription performed as described in section 2.5.2.

5.2.5. Quantitative PCR

mRNA levels of *AKR1D1* and GR receptor regulated genes (*DUSP*, *SGKI*, *GILZ*) were determined using an ABI 7900HT sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). Reactions were performed as described in section 2.6.2.

5.2.6. Protein measurements

Total protein was extracted from cells and protein concentrations were measured as described in sections 2.7.2 and 2.8.2, respectively.

5.2.7. Dual luciferase assay

Dual luciferase assays were performed with the dual luciferase reporter assay system (Promega Corp., Madison, USA). HepG2 and HEK293 cells were transfected and treated with cortisol (100, 200 and 500nM, 24h). Cell media was then removed, cells were washed twice with 1x PBS and 250µL of 1x passive lysis buffer were added in the wells. After allowing lysis for approximately 30min on a rocker at room temperature, a 10µl aliquot was used for luminescence measurements with an EnSpire Multimode plater reader (PerkinElmer, Massachusetts, USA). The following steps were used for luminescence measurements: 50µl of the firefly luciferase reagent (LARII) were added to the test sample, followed by addition of 50µl of the Renilla luciferase reagent and firefly quenching (Stop & Glo). The data were represented as the ratio of firefly to Renilla luciferase activity (Fluc/Rluc).

5.2.8. Gas Chromatography-Mass Spectrometry (GC-MS)

Cortisol and cortisone as well as their 5β-reduced metabolites 5β-tetrahydrocortisol (5β-THF) and 5β-tetrahydrocortisone (5β-THE) were measured by gas chromatography-mass spectrometry (GC-MS). The method was adapted from a historic method used for more than 20 years (Shackleton 1986). 1mL of culture media was collected, and 2ul of 10ug/ml of the internal standard cortisol-d4 were added to the samples. Samples were heated for 20min to denature proteins and steroids were then extracted by solid-phase extraction using 4ml of methanol (SPE). Samples were then evaporated under N₂ at 55-60°C and were derivitised overnight to form methyloxime trimethylsilylethers after the addition of ~50ul of MO-pyridine and 75ul of TMSi. 2 aliquots of 10ul of external standard solution containing known concentrations of cortisone, cortisol, 5β-THE, 5β-THF, 5α-THF and cortisol-d4 were also derivitised. Incubated media samples containing 200nM of cortisone and 500nM of cortisol in the absence of cells were also extracted and used as negative controls. The final derivative

was dissolved in 55 μ L cyclohexane, which was transferred to an autosampler vial for GC-MS analysis. An Agilent 5973 instrument (www.agilent.com) was used in a selected ion monitoring mode and the following steroids were identified: cortisol cortisone, 5 β -THE, 5 β -THF, 5 α -THF and cortisol-d4. Cortisol was positively identified by comparison to an authentic reference standard that gave a double peak at approximately 24.17min, monitored ion was 605. Cortisone was positively identified by comparison to an authentic reference standard that gave a double peak at approximately 23.2min, monitored ion was ion 531. The monitored ions for 5 β -THE and 5 β -THF were 578 and 562, respectively and were positively identified at approximately 18.87min and 19.95min, respectively.

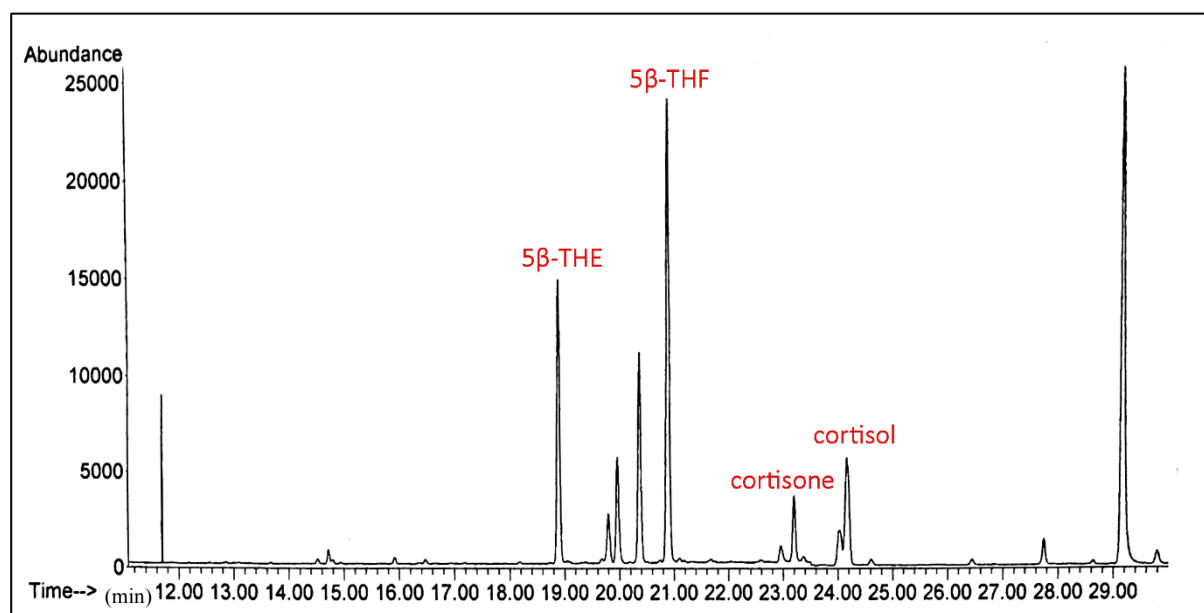


Figure 5.2.8-1 Representative total GC-MS ion chromatogram of derivatives of cortisone, cortisol and their 5 β -reduced metabolites 5 β -tetrahydrocortisone (5 β -THE) and 5 β -tetrahydrocortisol (5 β -THF).

5.2.9. Total BA, CA and CDCA ELISA assay

Total BA content was measured using a fluorimetric assay according to the manufacturer's protocol (Cell Biolabs Inc., San Diego, USA). Phenol-red free cell media samples were collected after 24h of serum free media treatment and 50 μ L of sample were added into a 96-well microtiter black plate. 50 μ L of Assay reagent were then added to each well, followed by

100µl of 1x Assay buffer. The plate was incubated for 45-60min at room temperature and read at an excitation wavelength of 560 nm and an emission wavelength of 590 nm with an EnSpire Multimode plater reader (PerkinElmer, Massachusetts, USA).

Total CA levels were measured using a colorimetric competitive ELISA according to the manufacturer's protocol (Cell Biolabs Inc., San Diego, USA). Phenol-red free cell media samples were collected after 24h of treatment and 50µl of sample were added into a CA conjugate coated plate for 10min. 50µl of anti-CA antibody were then added to each well and the plate was incubated at room temperature for 1h. Wells were washed 3 times with 1x wash buffer and 100µl of secondary antibody-HRP enzyme conjugate were added to each well. The plate was then incubated for another 1h at room temperature, washed and 100µl substrate solution were added to each well. Reaction was stopped after 2-30min by adding 100µl of stop solution per well. Absorbance was measured on a SpectraMax Plus Microplate Reader (Molecular Devices, CA, USA) at 450 nm. According to the manufacturer's protocol, the CA antibody demonstrated 11.9% cross reactivity with CDCA.

Total CDCA levels were measured using a colorimetric competitive ELISA according to the manufacturer's protocol (Cell Biolabs Inc., San Diego, USA). Phenol-red free cell media samples were collected after 24h of treatment and 50µl of sample were added into a CDCA conjugate coated plate for 10min. 50µl of anti-CDCA antibody were then added to each well and the plate was incubated at room temperature for 1h. Wells were washed 3 times with 1x wash buffer and 100µl of secondary antibody-HRP enzyme conjugate were added to each well. The plate was then incubated for another 1h at room temperature, washed and 100µl substrate solution were added to each well. Reaction was stopped after 2-30min by adding 100µl of stop solution per well. Absorbance was measured on a SpectraMax Plus Microplate Reader (Molecular Devices, CA, USA) at 450 nm. According to the manufacturer's protocol, the CA antibody demonstrated 4% cross reactivity with CDCA.

5.2.10. Statistical analysis

Data are presented as mean $\pm$ standard error. Two-tailed, paired t-tests were used to compare single treatments to control. For comparisons between control and different treatments, statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett corrections. To compare mean differences between groups that had been split on multiple treatments, doses or times, two-way analysis of variance (ANOVA) with Sidak corrections was used. Statistical analysis on qPCR data was performed on mean relative expression ratio values (Ratio= $\Delta C_{t[\text{target}]}/\Delta C_{t[\text{ref}]}$) (Pfaffl 2001). Data analysis was performed using Graphpad Prism software (Graphpad Software Inc, La Jolla, USA) and considered statistically significant at $P < 0.05$.

5.3. Results

5.3.1. AKR1D1 over-expression enhances glucocorticoid clearance in HepG2 cells

Based on the mRNA expression profile of AKR1D1 in hepatoma cell lines (see section 3.3.1), the HepG2 human hepatoma cell line was chosen as the cellular model for the genetic manipulation of AKR1D1.

HepG2 cells were transfected for 48h with either empty pCMV6-XL4 vector (EV) or AKR1D1 vector and increased mRNA expression of AKR1D1 was confirmed using qPCR (Figure 5.3.1-1). Further western blotting experiments were also performed, however, the commercially available AKR1D1 antibodies did not provide reliable results.

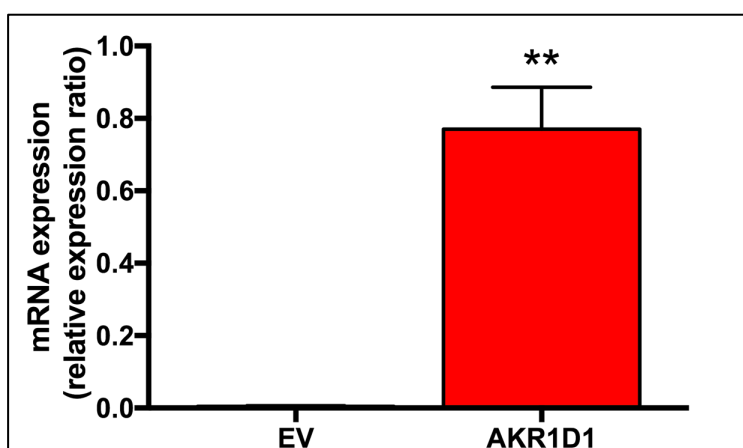


Figure 5.3.1-1 mRNA expression levels of AKR1D1 following over-expression in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments (** $p<0.01$, AKR1D1 compared to EV).

Cells were treated with either 200nM cortisone for 2, 4, 6 and 8h or 500nM cortisol for 4, 8 and 24h as AKR1D1 substrates. Changes in AKR1D1 gene expression were paralleled by functional activity, as measured by cortisol and cortisone clearance as well as 5 β -tetrahydrocortisol [5 β -THF] and 5 β -tetrahydrocortisone [5 β -THE] generation (5 β -reduced metabolites) (Figure 5.3.1-2).

AKR1D1 over-expression increased cortisone and cortisol clearance and generation of the 5 β -reduced metabolites, in a time-dependent manner, confirming the previous gene expression data (Figure 5.3.1-3).

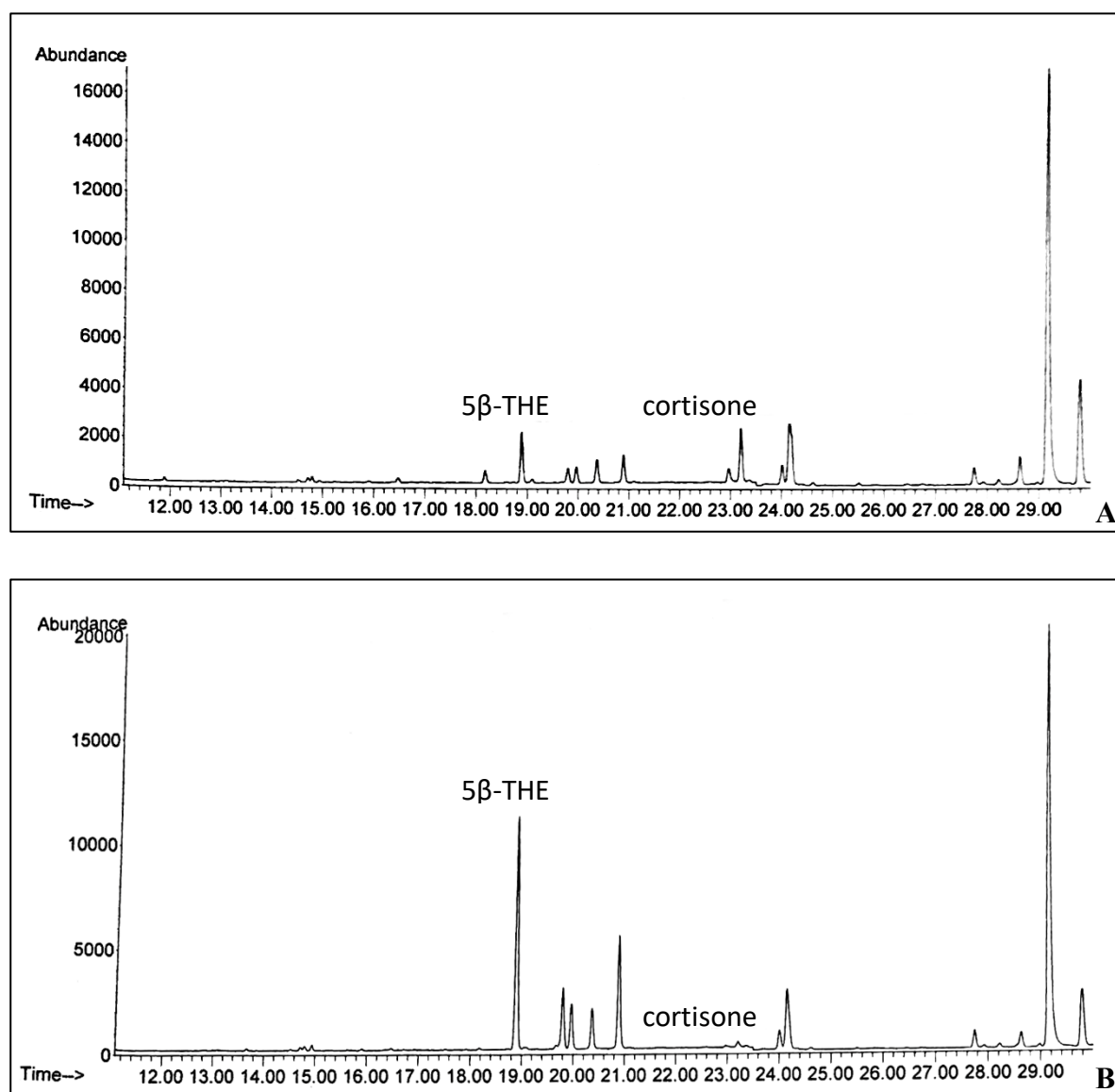


Figure 5.3.1-2 Total GC-MS ion chromatogram showing the peaks of derivatives of cortisone and its 5 β -reduced metabolite 5 β -tetrahydrocortisone (5 β -THE) in (A). empty vector-transfected HepG2 cells and (B). AKR1D1-transfected HepG2 cells, after treatment with 200nM cortisone for 4h.

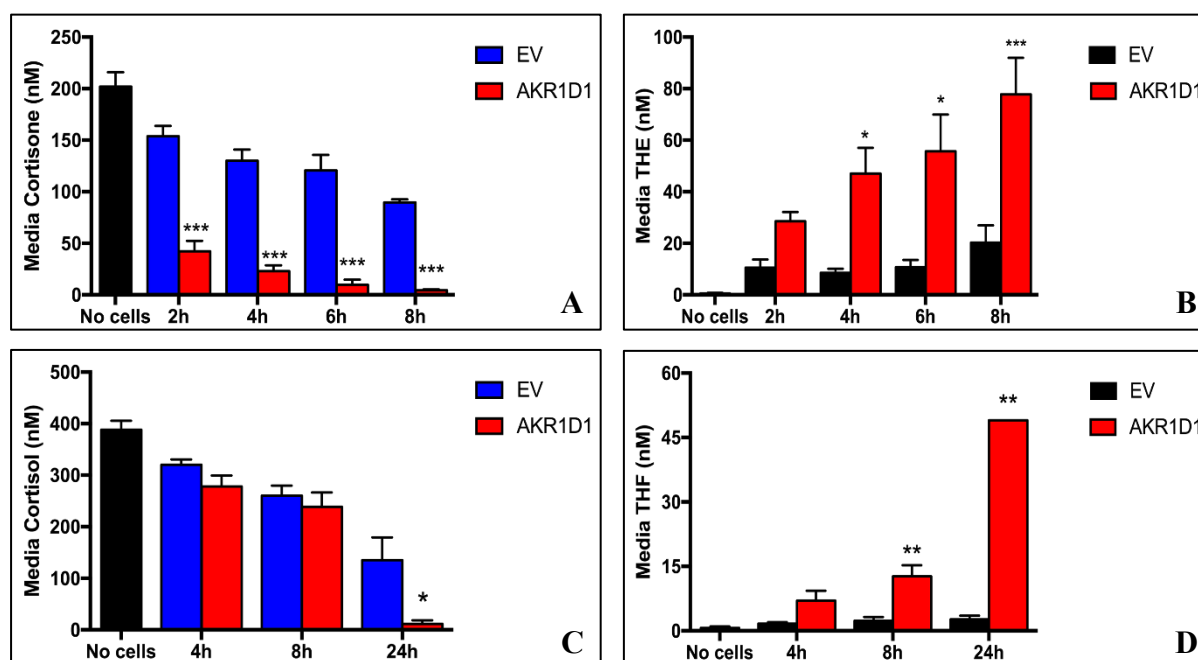


Figure 5.3.1-3 Glucocorticoid clearance in HepG2 cells, as measured by (A). cortisone clearance, (B). 5 β -tetrahydrocortisone (5 β -THE) generation, (C). cortisol clearance and (D). 5 β -tetrahydrocortisol (5 β -THF) generation, in a time-dependent manner. Results are expressed as mean $\pm$ se of $n=4$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, AKR1D1 compared to EV).

In addition to cortisol and 5 β -THF, we detected cortisone and 5 β -THE levels in the cortisol-treated media samples, indicative of dehydrogenase activity (Figure 5.3.1-4). Given the mRNA expression data in chapter 3, it is likely that 11 β -HSD2 activity catalysed the conversion of cortisol to cortisone and, followed AKR1D1 over-expression, the generated cortisone was converted to 5 β -THE. Due to the protocol used, we were not able to quantify the total steroid recovery proportion or detect any other metabolites downstream the 5 β -reduction pathway. Moreover, no 5 α -THF was detected in either EV or AKR1D1 transfected HepG2 cells, indicative of impaired 5 α -reductase activity in these cells.

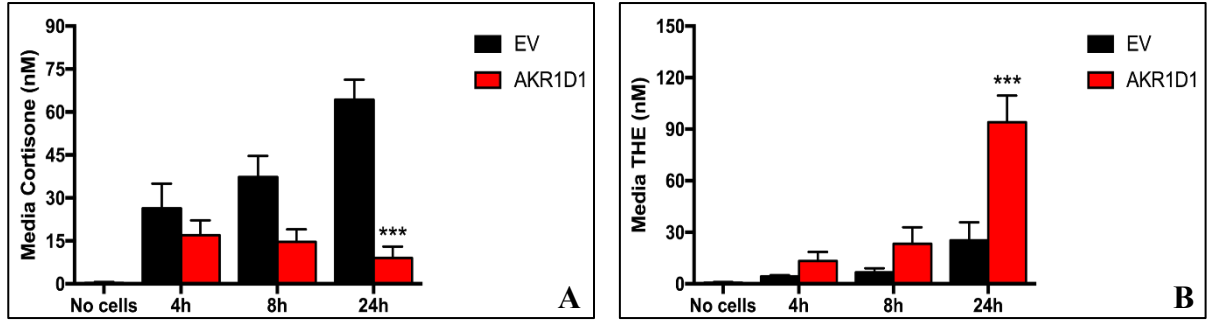


Figure 5.3.1-4 (A). Cortisone generation and (B). 5 β -tetrahydrocortisone (5 β -THE) generation, after 500nM cortisol treatment, in HepG2 cells, in a time-dependent manner. Results are expressed as mean $\pm$ se of n=4 experiments (***) p <0.001, AKR1D1 compared to EV).

5.3.2. AKR1D1 knockdown decreases glucocorticoid clearance in HepG2 cells

To complement the over-expression studies, AKR1D1 knockdown experiments were performed using a specific AKR1D1 siRNA transcript and compared with scrambled siRNA, as negative controls. Cells were transfected for 48h and AKR1D1 knockdown was confirmed by qPCR.

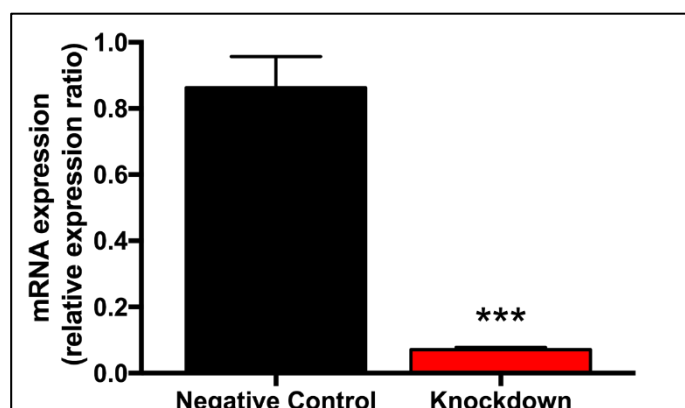


Figure 5.3.2-1 mRNA expression levels of AKR1D1 following transient knockdown in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=7$ experiments (** $p<0.001$, compared to negative control).

Cells were then treated with 200nM cortisone for 2, 4 and 8h and decreases in AKR1D1 activity were confirmed by decreased cortisone clearance and decreased 5 β -THE generation (Figure 5.3.2-2 & 3).

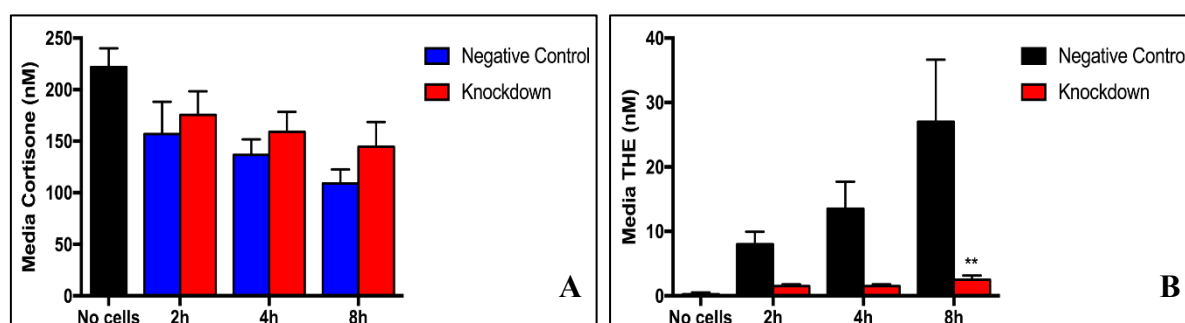


Figure 5.3.2-2 Glucocorticoid clearance in HepG2 cells, as measured by (A). cortisone clearance and (B). 5 β -tetrahydrocortisone (5 β -THE) generation, in a time-dependent manner. Results are expressed as mean $\pm$ se of $n=4$ experiments (** $p<0.01$, compared to negative control).

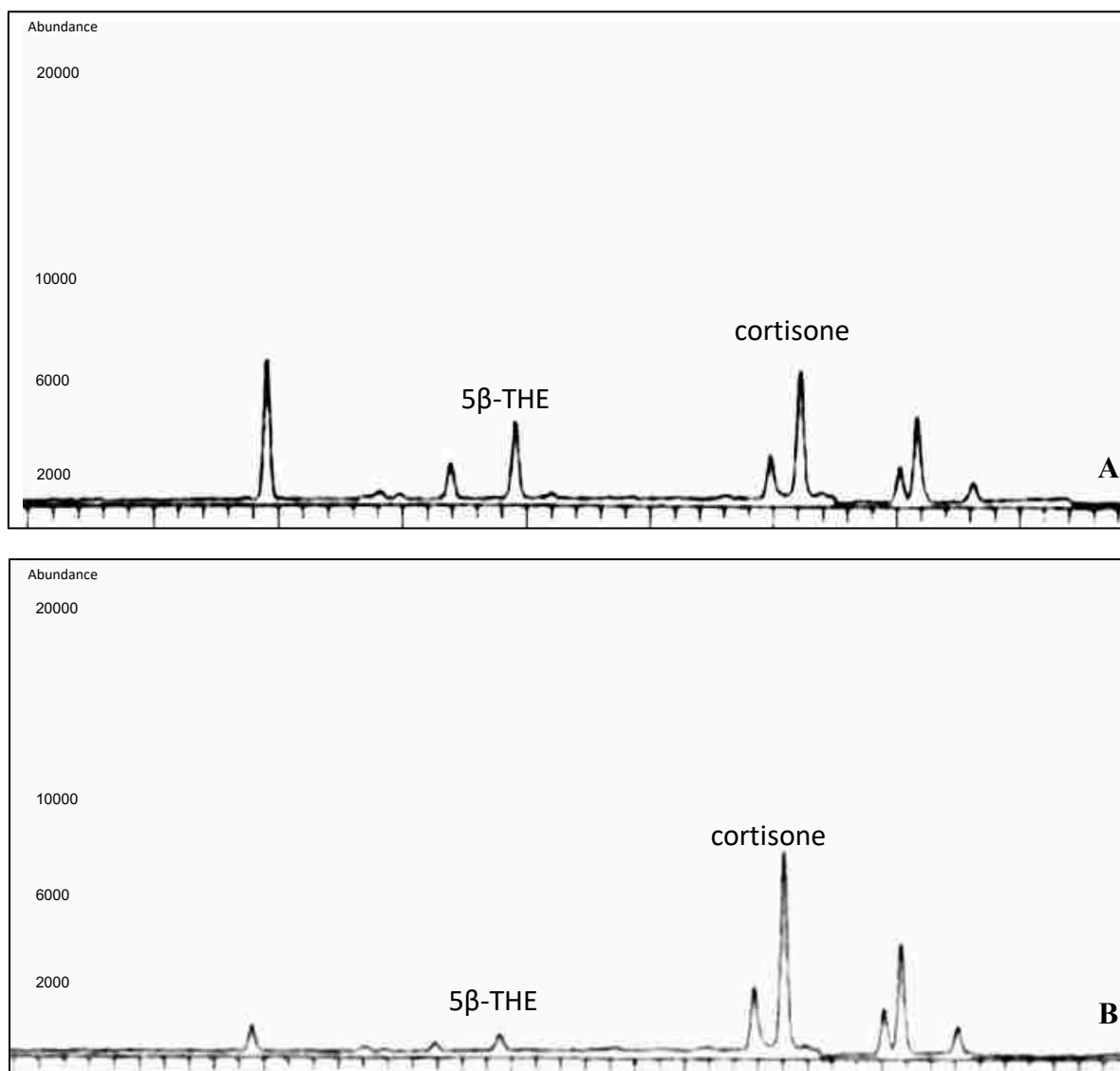


Figure 5.3.2-3 Total GC-MS ion chromatogram showing the peaks of derivatives of cortisone and its 5 β -reduced metabolite 5 β -tetrahydrocortisone (5 β -THE) in (A). negative control HepG2 cells and (B). AKR1D1 knockdown HepG2 cells, after treatment with 200nM cortisone for 4h.

5.3.3. AKR1D1 over-expression enhances glucocorticoid clearance in HEK293 cells

HepG2 cells also express several other steroid metabolizing enzymes (including 11 β -HSD2 and 5 α R1) (Figure 5.3.3-1), therefore, in order to understand the precise contribution of AKR1D1 in glucocorticoid metabolism and ensure that the observed changes in response to AKR1D1 over-expression were not due to action of other enzymes, additional experiments were performed in HEK293 cells. HEK293 cells do not express any of the A-ring reductases, including 5 α R1 and 2 and AKR1D1 (undetectable mRNA levels).

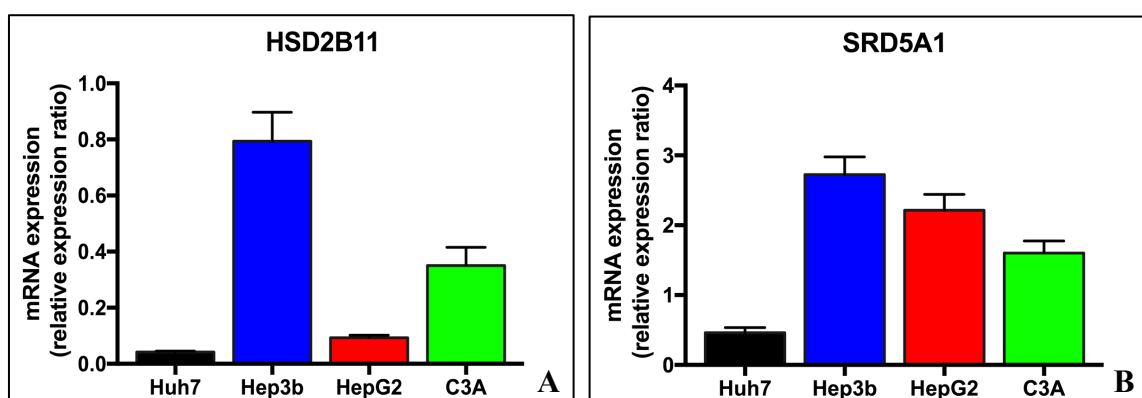


Figure 5.3.3-1 mRNA expression of (A). *HSD2B11* and (B). *SRD5A1*, in Huh7, Hep3b, HepG2 and C3A human hepatoma cell lines. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments.

HEK293 cells were transfected for 48h with either empty pCMV6-XL4 vector (EV) or AKR1D1 vector and increased mRNA expression of AKR1D1 was confirmed using qPCR (Figure 5.3.3-2).

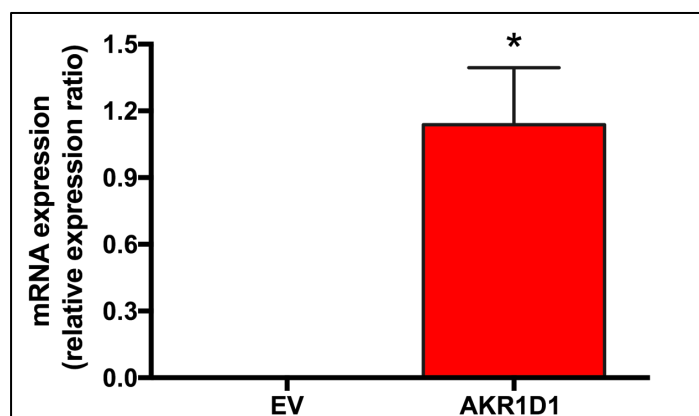


Figure 5.3.3-2 mRNA expression levels of *AKR1D1* following over-expression in HEK293 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (* $p<0.05$, *AKR1D1* compared to EV).

Cells were treated with either 200nM cortisone for 2, 4, 6 and 8h or 500nM cortisol for 4, 8 and 24h. Cortisol and cortisone clearance were increased as was 5 β -tetrahydrocortisol [5 β -THF] and 5 β -tetrahydrocortisone [5 β -THE] generation (Figure 5.3.3-3). In contrast to the findings in HepG2 cells, HEK293 cells were not able to generate cortisone and/or 5 β -THE after cortisol treatment due to lack of 11 β -HSD2 expression in these cells.

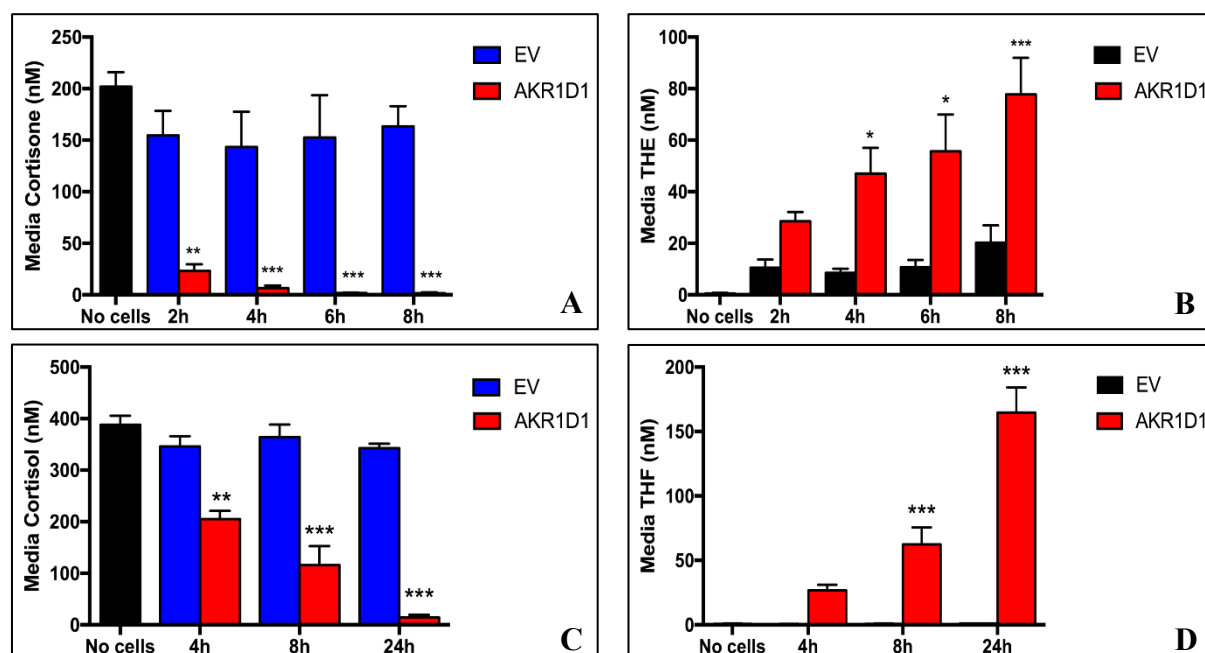


Figure 5.3.3-3 Glucocorticoid clearance as measured by (A). cortisone clearance, (B). 5 β -tetrahydrocortisone (5 β -THE) generation, (C). cortisol clearance and (D). 5 β -tetrahydrocortisol (5 β -THF) generation, in HEK293 cells. Results are expressed as mean $\pm$ se of $n=3$ experiments (** $p<0.01$, *** $p<0.001$, *AKR1D1* compared to EV).

5.3.4. AKR1D1 over-expression regulates GR receptor activation in HepG2 cells

In order to determine whether the change in glucocorticoid availability mediated by alteration in AKR1D1 activity was affecting activation of the GR, over-expression studies were repeated in HepG2 cells, after co-transfection with the glucocorticoid receptor element (GRE) luciferase reporter.

Cortisol treatment (in the absence of any manipulation of AKR1D1 expression) for 24h induced a significant activation of the GR in a dose-dependent manner (Figure 5.3.4-1).

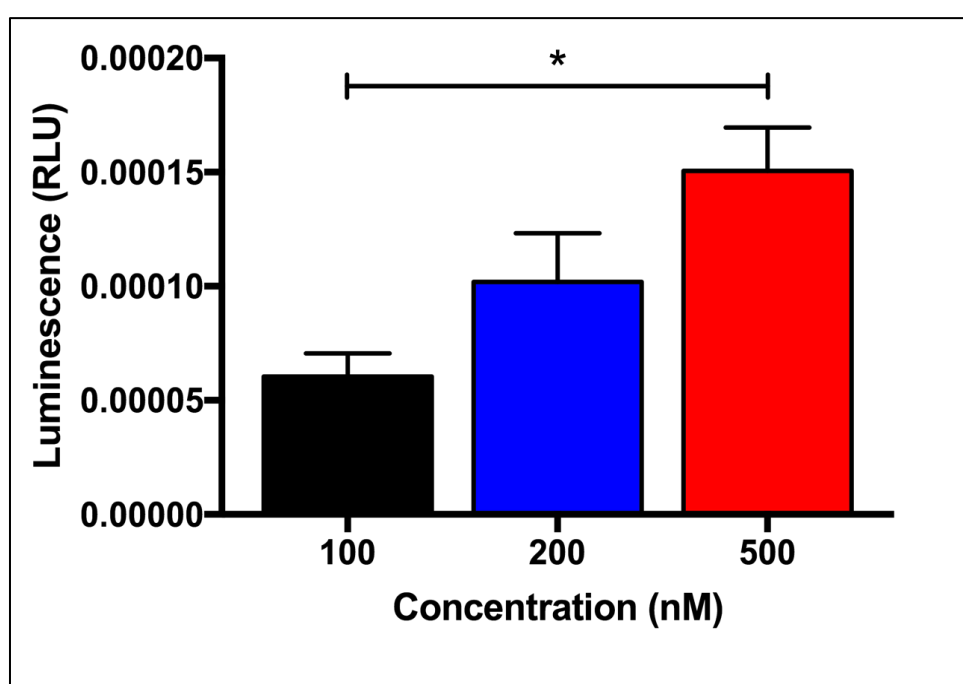


Figure 5.3.4-1 Activation of the glucocorticoid receptor in HepG2 cells after treatment with cortisol in a dose-dependent manner, as measure by luminescence. Results are expressed as mean $\pm$ se of $n=5$ experiments (* $p<0.05$).

Over-expression of AKR1D1 in HepG2 cells decreased GR activation, following treatment with 500nM, compared to empty vector controls (Figure 5.3.4-2).

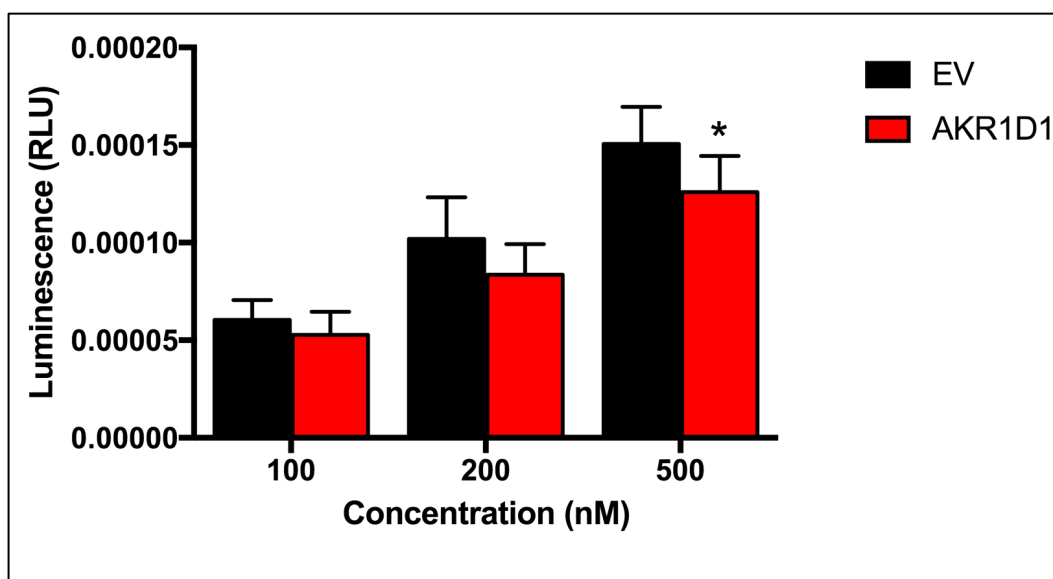


Figure 5.3.4-2 Activation of the glucocorticoid receptor in control (EV) and over-expressed (AKR1D1) HepG2 cells after treatment with cortisol in a dose-dependent manner, as measure by luminescence. Results are expressed as mean $\pm$ se of $n=5$ experiments (* $p<0.05$, AKR1D1 compared to EV).

To endorse my findings, I examined the mRNA expression of the known glucocorticoid regulated genes SGK1, DUSP and GILZ (Ayroldi & Riccardi 2009; Webster et al. 1993; Isikbay et al. 2014) after AKR1D1 over-expression in HepG2 cells treated with cortisol (500nM) for 24h. Consistent with the previous data, AKR1D1 over-expression significantly decreased the mRNA expression of SGK1 and DUSP, after 500nM cortisol treatment (Figure 5.3.4-3). GILZ mRNA expression remained unchanged after AKR1D1 over-expression (data not shown).

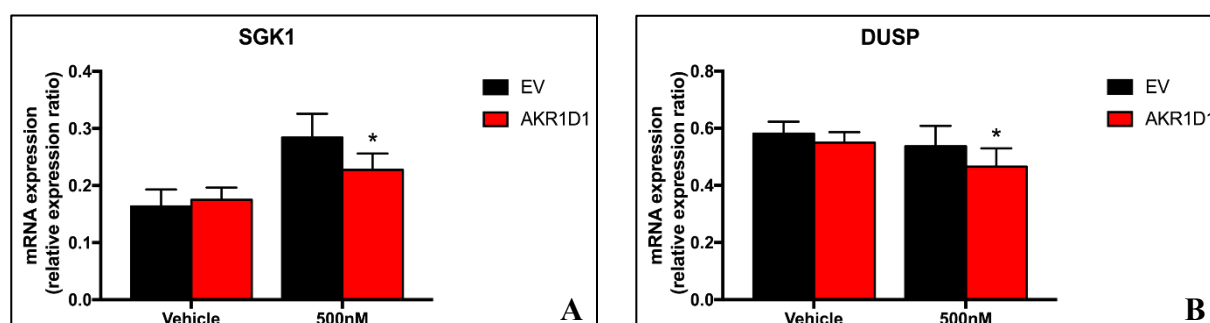


Figure 5.3.4-3 mRNA expression levels of (A). SGK1 and (B). DUSP between empty vector control (EV) and AKR1D1 over-expressed HepG2 cells, treated with either vehicle (0.1% DMSO) or 500nM cortisol. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments (* $p<0.05$, AKR1D1 compared to EV).

5.3.5. AKR1D1 over-expression regulates GR receptor activation in HEK293 cells

As cortisol acts a substrate for many metabolizing enzymes expressed in the liver (11 β -HSD1, 5 α R1, 5 α R2), I hypothesised that, although significant, the small differences in GRE activation between control and over-expressed HepG2 cells could be the result of reduced cortisol levels due to its clearance by other enzymes. For this reason, I repeated the GRE activation assay in HEK293 cells. Although this cell line is not of hepatocyte origin, they represent a “cleaner” cellular system to examine GRE activation, as they lack expression of any of the cortisol-metabolizing enzymes.

HEK293 cells were co-transfected with either empty or AKR1D1 vector and GRE reporter for 48h and subsequently treated with 500nM cortisol for 24h.

Cortisol treatment (without alteration of AKR1D1 expression) for 24h caused activation of the glucocorticoid receptor in a dose-dependent manner (Figure 5.3.5-1).

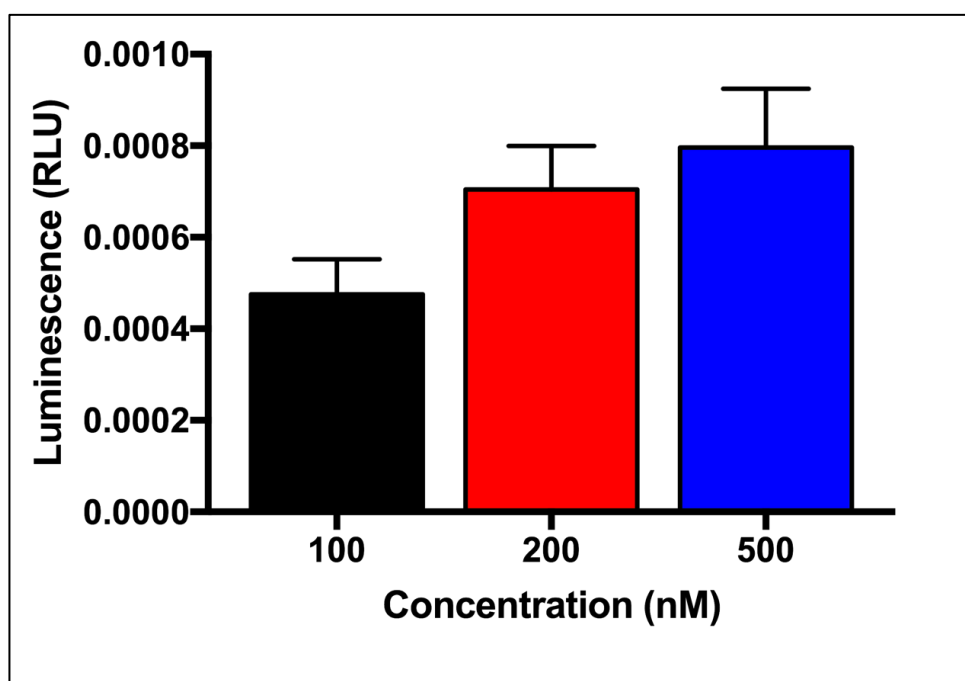


Figure 5.3.5-1 Activation of the glucocorticoid receptor in HEK293 cells after treatment with cortisol in a dose-dependent manner, as measure by luminescence. Results are expressed as mean $\pm$ se of n=4 experiments.

AKR1D1 over-expression significantly decreased GRE activation after 100, 200 and 500nM of cortisol treatment, as measured by luminescence (Figure 5.3.5-2).

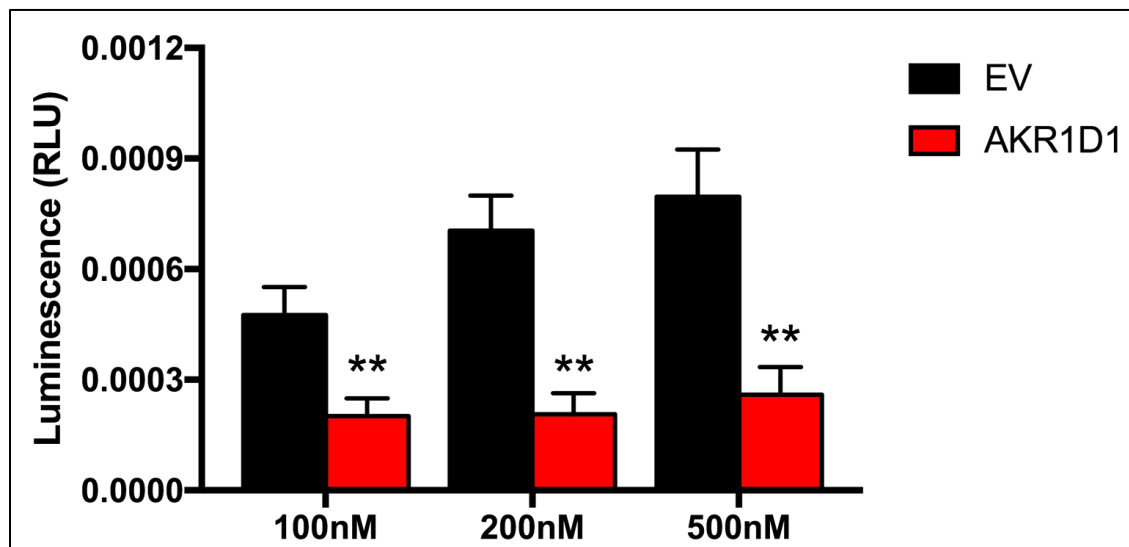


Figure 5.3.5-2 Activation of the glucocorticoid receptor in control (EV) and AKR1D1 over-expressed HEK293 cells after treatment with cortisol in a dose-dependent manner, as measure by luminescence. Results are expressed as mean $\pm$ se of n=4 experiments (** p <0.01, AKR1D1 compared to EV).

I repeated mRNA expression analysis of the glucocorticoid receptor regulated genes DUSP, SGK1 and GILZ, using the conditions described in section 5.3.4. AKR1D1 over-expression significantly decreased the mRNA expression of SGK1 and GILZ, after 100nM and 500nM cortisol treatment in HEK293 cells (Figure 5.3.5-3). DUSP mRNA expression remained unchanged after AKR1D1 over-expression (data not shown).

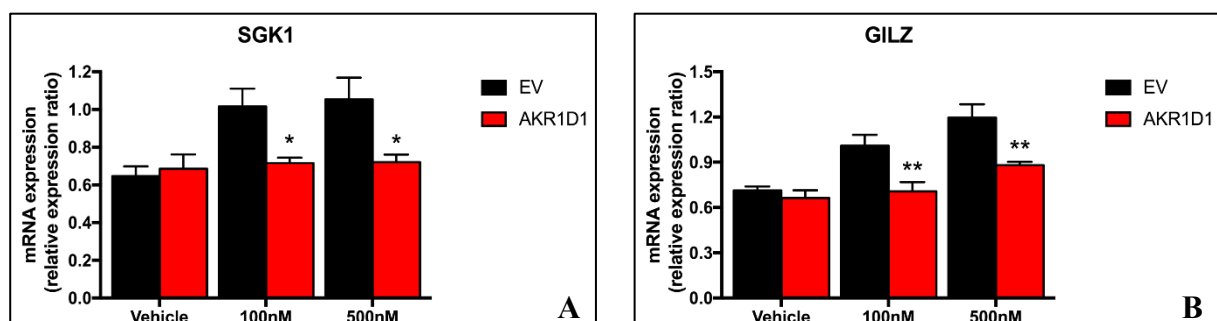


Figure 5.3.5-3 mRNA expression levels of (A). SGK1 and (B). GILZ between empty vector control (EV) and AKR1D1 over-expressed HEK293 cells, treated with vehicle (0.1% DMSO), 100nM or 500nM cortisol. Data are expressed as relative expression ratio values $\pm$ se of n=3 experiments (* p <0.05, ** p <0.01, AKR1D1 compared to EV).

5.3.6. AKR1D1 knockdown regulates BA synthesis and secretion

In addition to glucocorticoid metabolism, I explored the effect of genetic manipulation of AKR1D1 on BA synthesis and secretion in human hepatocytes. AKR1D1 knockdown significantly increased the mRNA expression of CYP7A1, the rate-limiting step of BA synthesis, as well as increasing the expression of the BA transporter SLC51A (Figure 5.3.6-1).

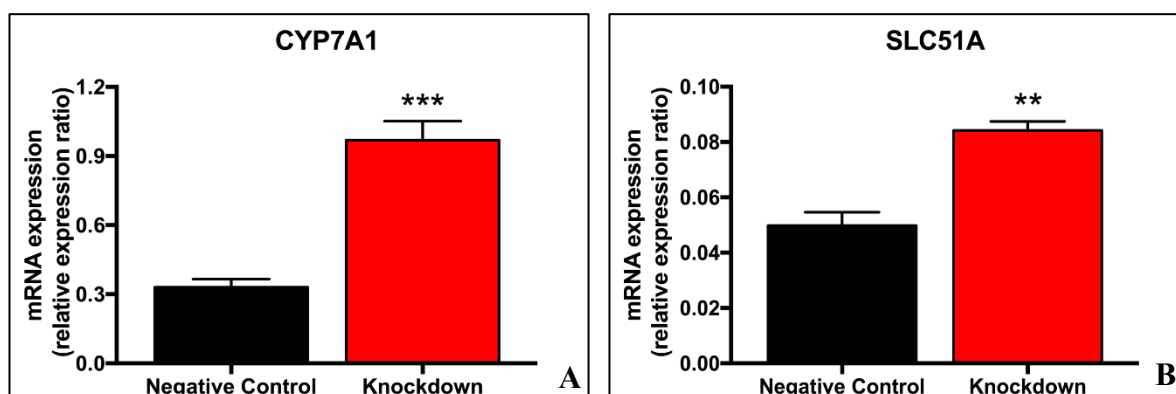


Figure 5.3.6-1 mRNA expression levels of (A). CYP7A1 and (B). SLC51A following transient AKR1D1 knockdown in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=4-7$ experiments (** $p<0.01$, *** $p<0.001$, compared to negative control).

AKR1D1 knockdown reduced total BA levels in the media of HepG2 cells after 48h of transfection (Figure 5.3.6-2).

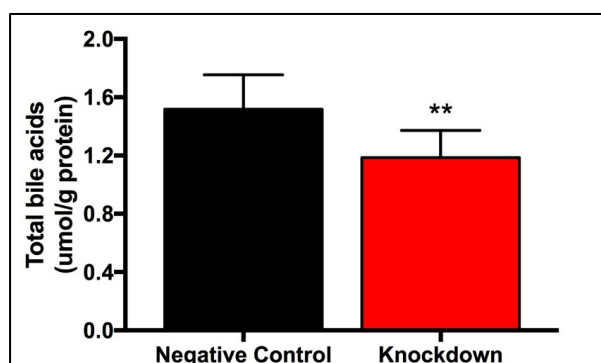


Figure 5.3.6-2 Total BA levels in the media of HepG2 cells, following transient AKR1D1 knockdown for 48h, as measured by ELISA. Results are shown as mean $\pm$ se of $n=9$ experiments (** $p<0.01$, compared to negative control).

AKR1D1 is a crucial enzymatic step in both arms of the classic BA synthesis pathway, which leads to the generation of CA and CDCA. AKR1D1 knockdown in HepG2 cells significantly increased CDCA concentrations in the media, consistent with activation of the alternative pathway of BA synthesis (Figure 5.3.6-3).

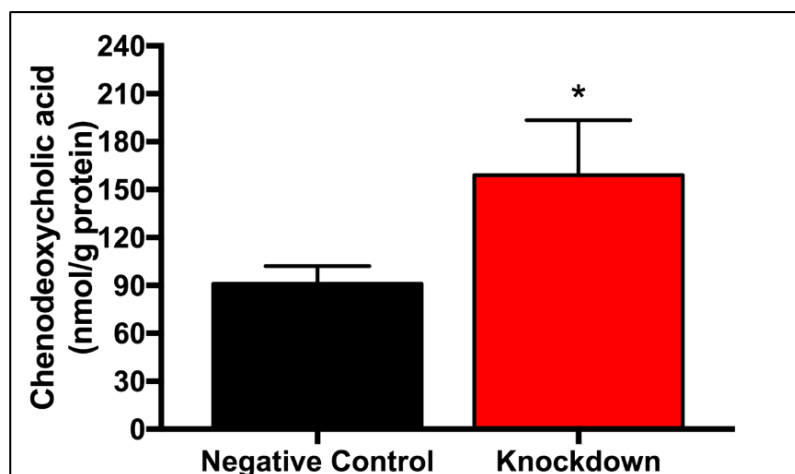


Figure 5.3.6-3 CDCA levels in the media of HepG2 cells, following AKR1D1 knockdown, as measured by ELISA. Results are shown as mean $\pm$ se of $n=7$ experiments (* $p<0.05$, compared to negative control).

Additional experiments were performed to determine the levels of CA in the media of HepG2 cells. HepG2 cells generated low levels of CA, compared to CDCA (Figure 5.3.6-4A) but surprisingly, AKR1D1 knockdown significantly increased CA concentration (Figure 5.3.6-4B).

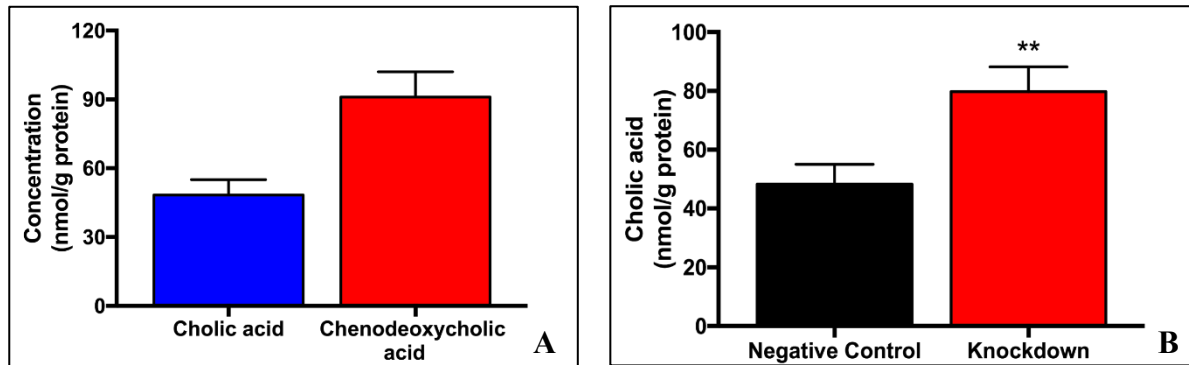


Figure 5.3.6-4 (A). CA and CDCA levels in HepG2 cells, and (B). CA levels in HepG2 cells, following *AKR1D1* knockdown, as measured by ELISA. Results are expressed as mean $\pm$ se of $n=7-8$ experiments (** $p<0.01$, compared to negative control).

To determine if the changes in HepG2 cells were reproducible, Huh7 human hepatoma cells were used as an alternative hepatocyte model for the study of BA synthesis. Similar to HepG2 cells, Huh7 cells were transiently knocked down *AKR1D1* for 48h and mRNA expression was measured by qPCR (Figure 5.3.6-5).

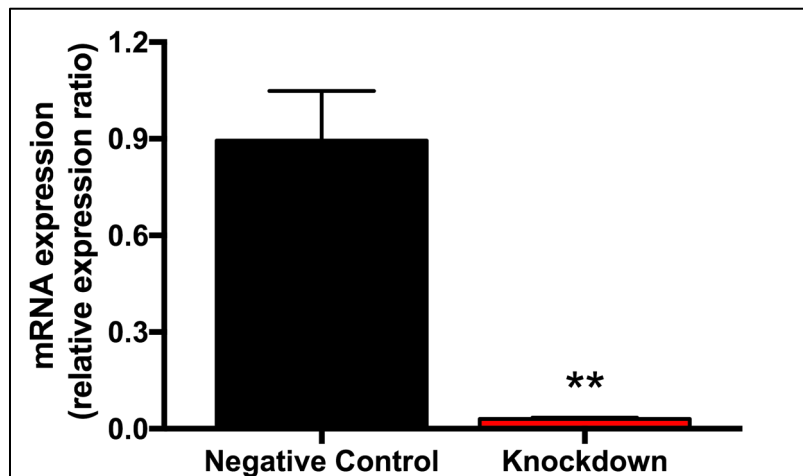


Figure 5.3.6-5 mRNA expression levels of *AKR1D1* following transient knockdown in Huh7 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments (** $p<0.01$, compared to negative control).

In contrast to the results from the HepG2 cells, in Huh7 cells, both CA and CDCA levels were reduced in the media of *AKR1D1* knocked down cells compared to negative controls (Figure 5.3.6-6).

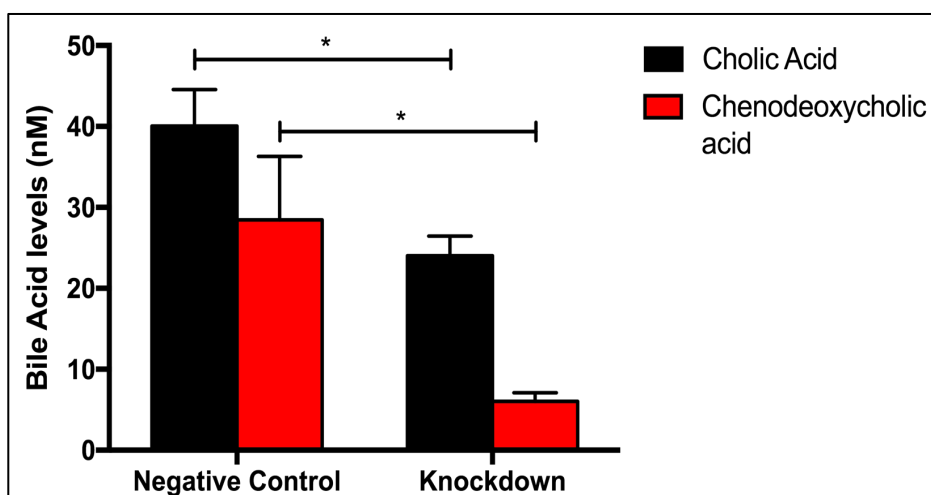


Figure 5.3.6-6 CA and CDCA levels in the media of Huh7 cells, following *AKR1D1* knockdown, as measured by ELISA. Results are expressed as mean $\pm$ se of $n=5$ experiments (* $p<0.05$, compared to negative control).

5.4. Discussion

I have demonstrated, for the first time, that manipulation of AKR1D1 expression and activity is able to regulate steroid hormone availability and BA synthesis in human hepatocytes. AKR1D1 over-expression leads to increased glucocorticoid clearance and decreased GR activation, whilst AKR1D1 knockdown leads to decreased glucocorticoid clearance as well as impaired BA secretion and altered BA availability.

In chapter 3 I characterised the enzyme kinetics and substrate specificity of AKR1D1 using a purified recombinant protein and we showed that this enzyme is able to inactivate a variety of glucocorticoid hormones to their 5 β -reduced metabolites. Although the ability of AKR1D1 to reduce cortisol levels *in vitro* has been described in COS and HEK293 cells (Kondo et al. 1994; Charbonneau & The 2001) in this chapter I have shown that the activity and expression of AKR1D1 acts as a critical regulator of glucocorticoid availability within human hepatocytes. AKR1D1 over-expression in HepG2 hepatoma cells, followed by cortisone and cortisol treatment, revealed that both steroids can be inactivated by this enzyme within hepatocytes, but to a different extent. The rate of cortisone depletion as well as 5 β -tetrahydrocortisone generation was faster than the rate observed with cortisol, confirming the previous substrate specificity data and demonstrating cortisone as a better *in vitro* substrate. Importantly, when cortisol was used as a substrate, cortisone generation and clearance with subsequent 5 β -THE generation was also observed, due to existing 11 β -HSD2 activity in HepG2 cells. As HepG2 cells lack 11 β -HSD1 expression, we did not detect any cell media cortisol levels, after cortisone treatment.

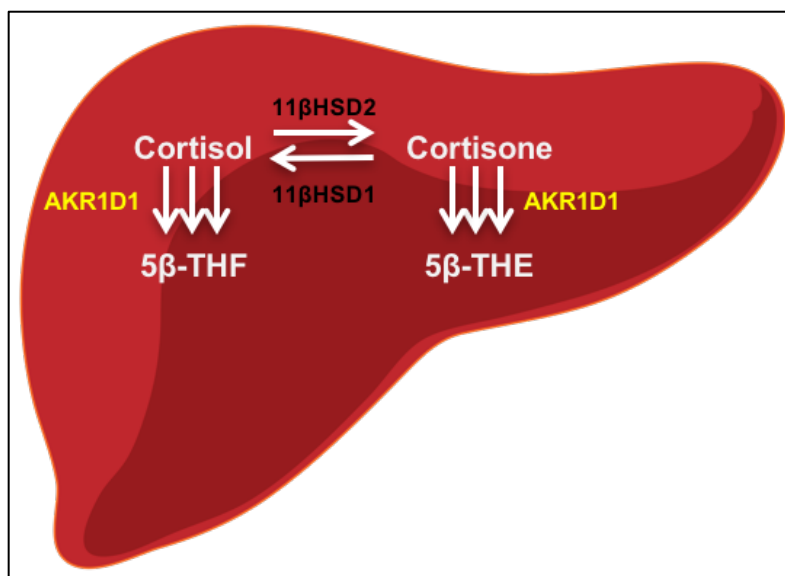


Figure 5.3.6 Pre-receptor metabolism of cortisone and cortisol by AKR1D1 within human liver.

AKR1D1 knockdown modified glucocorticoid availability and reduced cortisone clearance as well as 5β-THE generation. Although the differences in cell media cortisone levels between negative controls and knocked down cells did not reach statistical significance, effective knockdown was confirmed by the significantly decreased 5β-THE levels generated, compared to negative controls.

In addition to AKR1D1, human hepatocytes express a variety of enzymes that inactivate glucocorticoids; therefore, the use of an alternative “cleaner” cellular model was important to define the exact level of AKR1D1 activity *in vitro*. HEK293 cells do not express any of the enzymes that could potentially reduce cortisol or cortisone cell media concentrations (AKR1D1, 11β-HSD1, 11β-HSD2, 5αR1 or 5αR2). Thus, it was of interest that cortisol clearance was increased, after AKR1D1 over-expression, to even higher levels than to those observed in HepG2 cells.

BAs are the end products of cholesterol catabolism and, in addition to their crucial role in intestinal digestion and nutrient absorption, they also act as important signalling molecules and key regulators of systemic metabolism (Li & Chiang 2014). CA and CDCA are the

primary BAs in humans and are synthesised from cholesterol through two dominant pathways: the classic (or neutral) pathway and the alternative pathway (Ma & Patti 2014). CYP7A1 is the rate-limiting enzyme in the synthesis of BAs from cholesterol via the classic pathway and is regulated by BA availability via feedback inhibition (Chiang 2009). In HepG2 cells, AKR1D1 knockdown decreased total BA secretion in the cell media and compensatory increased CYP7A1 expression. In addition, compensatory increase of the BA transporter SLC51A was observed.

Both CA and CDCA are generated in approximately equal levels through the classical pathway, whereas the alternative pathway generates mainly CDCA (Chiang 1998). In my study, I observed an unusual BA pattern in the cell media of HepG2 cells, with CDCA being the main product of BA synthesis, while CA was produced in low amounts. This finding agrees with previous studies using HepG2 cells, where CDCA was found to be the predominant BA in the cell media (Everson & Polokoff 1986, Javitt et al. 1989). Other primary BAs may also be present in the cell media (di- and trihydroxycholestanoic acid), but we were not able to measure their levels, due to lack of available assays in our lab. We also demonstrated that AKR1D1 knockdown increased the levels of CDCA in the cell media, indicative of increased activation of the alternative pathway. Interestingly, when we measured CA levels, we observed increased CA appearance in the cell media after AKR1D1 knockdown, which is a paradox, considering the expected impaired classical pathway activity. Regarding this, Everson & Polokoff (1986) had observed small amounts of CA in the HepG2 cell media that were not generated through the classical pathway, therefore suggesting the presence of an alternate pathway of CA synthesis in these cells. Considering that CA is also generated from the metabolism of 25-hydroxycholesterol (Vlahcevic et al. 1980, Salen et al. 1975), it has been proposed this pathway may be the main source of CA in

HepG2 cells. Therefore, increased CA levels in the AKR1D1 knocked down HepG2 cells may reflect concurrent 25-hydroxylation of cholesterol towards CA generation.

A study from Amuro et al. (1994) demonstrated higher cell media CDCA concentrations than CA concentrations using Huh7 cells, supporting the hypothesis that CDCA is the predominant primary BA synthesised in patients with hepatocellular carcinoma (Nakayama et al. 1987; Amuro et al. 1982). However, this finding contradicts our results, where CA levels were higher to CDCA levels in the cell media, indicative of variation across different cell sublines. As expected, AKR1D1 knockdown significantly decreased cell media CA levels, confirming decreased AKR1D1 activity. Importantly, AKR1D1 knockdown significantly decreased CDCA generation, indicative of inability of these cells to activate the alternative pathway.

It is likely that differences between my data and results from other studies may appear due to the methodology used, as ELISA assays are not the gold standard method for measuring BA levels. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are considered the gold standard methods for BA profiling and could help to separate 5 β - and 5 α -reduced BAs.

Taken together, I have demonstrated that manipulation of AKR1D1 is able to regulate BA synthesis. Although AKR1D1 over-expression had no effect in BA synthesis (data not shown), AKR1D1 knockdown altered both total BA levels and BA composition, concordant with observations in patients with mutations in AKR1D1 (Seki et al. 2013; Lemonde et al. 2003; Ueki et al. 2009). As previously mentioned, these patients predominantly demonstrate unusual BA synthesis, including 5 α -reduced and 3-oxo Δ^4 BAs and develop cholestasis and jaundice. Exogenous CDCA treatment has been shown to improve liver function and reduced unusual BA levels; it is, then, possible, that the switch from the classic to the alternative BA synthesis pathway we observed to be a compensatory mechanism to increase hepatic CDCA

levels and prevent liver damage. Also, given the phenotypic spectrum of patients with AKR1D1 mutations, it is possible that impaired steroid clearance and subsequent abnormal steroid hormone levels due to decreased AKR1D1 activity in these patients may have an additive effect on the observed phenotypes.

Chapter 6 AKR1D1 regulates carbohydrate and lipid metabolism in human hepatocytes

6.1. Introduction

In chapter 5, I demonstrated that 5 β -reductase (AKR1D1) has an important *in vitro* pre-receptor role in the regulation of steroid hormone action as it metabolises both cortisol and cortisone to their tetrahydro-metabolites (tetrahydrocortisol, 5 β -THF and tetrahydrocortisone, 5 β -THE). Steroid hormones, including glucocorticoids and androgens are potent regulators of metabolic phenotype and have been implicated in driving hepatic lipid accumulation. Glucocorticoid excess, androgen deficiency in men, and excess in women are all associated with NAFLD (Hazlehurst & Tomlinson 2013). Previous studies have already demonstrated the important role of pre-receptor metabolism of hormone action by other enzymes. As examples, the isozymes of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) interconvert cortisol (the active major circulating glucocorticoid) with inactive cortisone; 11 β -HSD2 inactivates cortisol to cortisone (largely in mineralocorticoid sensitive tissues such as the kidney), whilst 11 β -HSD1 regenerates cortisol from cortisone and amplifies local glucocorticoid action. In parallel, the 5 α -reductases (5 α R1 and 5 α R2) both inactivate glucocorticoids, and simultaneously generate the most potent androgen, dihydrotestosterone (5 α -DHT) from less active testosterone. Genetic and pharmacological manipulation of both 11 β -HSD1 and 5 α R activity has been demonstrated to have a profound impact upon steroid hormone action and metabolic phenotype both *in vivo* and *in vitro*, including the development of hepatic steatosis (Dowman et al. 2013; Nasiri et al. 2015; Hermanowski-Vosatka et al. 2005; Masuzaki et al. 2001; Morton et al. 2001).

However, in addition to its role in steroid hormone clearance, AKR1D1 has a crucial function in BA synthesis and BA composition. Patients with mutations in AKR1D1 develop a rare and severe form of neonatal cholestasis, although spontaneous recovery in adulthood is reported (Palermo et al. 2008; Gonzales et al. 2009). BAs are now established as potent signalling

molecules that regulate metabolic phenotype acting through receptors, including FXR. Oxysterols, including BA intermediary substrates are also able to activate LXR α (Janowski et al. 1996). FXR and LXR α are both nuclear hormone receptors and their activation has been implicated in the pathogenesis of NAFLD. FXR deletion results in hepatic steatosis, IR and hyperglycaemia with evidence suggesting decreased expression in NAFLD (Yang et al. 2010; Kunne et al. 2014; Cipriani et al. 2010). FXR agonism has already shown early promise as a therapeutic strategy in NAFLD (Neuschwander-Tetri et al. 2015; Janowski et al. 1996). In contrast, LXR α expression is increased in NAFLD (Ahn et al. 2014) and activation drives lipid accumulation. In rodent models, LXR antagonism attenuates high fat diet induced hepatic steatosis (Sim et al. 2014), but clinical translational data are lacking.

6.1.1. Hypothesis and aims

Although it is now evident that AKR1D1 plays a crucial role in both steroid hormone availability and BA synthesis, there is no data on whether this enzyme plays any role in carbohydrate and/or lipid metabolism within human liver.

Utilising genetic manipulation of AKR1D1 gene expression in HepG2 human hepatoma cells, through over-expression and transient knockdown assays I aimed to:

- Define the ability of AKR1D1 to regulate carbohydrate metabolism in human hepatocytes
- Define the ability of AKR1D1 to regulate lipid metabolism in human hepatocytes
- Explore the mechanisms underpinning the effects of AKR1D1 to regulate hepatic carbohydrate and lipid metabolism, using FXR agonism and LXR antagonism.

6.2. Methods

6.2.1. HepG2 cell culture

HepG2 human hepatoma cells were cultured as described in section 2.1.1, and plated in 12-well and 24-well Cell Bind plates (Corning, Flintshire, UK) at a density of 1.6×10^5 and 0.8×10^5 cells/ml, respectively.

6.2.2. Transfection studies

For the over-expression studies, the AKR1D1 cDNA was cloned into the pCMV6-XL4 vector (Origene Technologies, Rockville, USA) and transiently transfected into HepG2 and HEK293 cells as described in section 2.3.2.

For the knockdown studies, AKR1D1 siRNA molecules were purchased from Invitrogen (Paisley, UK) and transiently transfected into HepG2 cells as described in section 2.3.3.

6.2.3. Cell treatments

For all vehicle treatments, HepG2 cells were treated with serum free and phenol-red free media containing 0.1%DMSO for 24h, after 48h of transfection.

For all cortisol treatments, HepG2 cells were treated with serum free and phenol-red free media containing 500nM cortisol for 24h, after 48h of transfection.

For GW4064 treatments, HepG2 cells were treated with serum free and phenol-red free media containing 5 μ M of GW4064 for 24h, after 48h of transfection.

For 22-S hydroxycholesterol (22-S HC) treatments, HepG2 cells were treated with serum free and phenol-red free media containing 10 μ M of 22-S HC for 24h, after 48h of transfection.

For GSK2033 treatments, HepG2 cells were treated with serum free and phenol-red free media containing 100nM of GSK2033 for 24h, after 48h of transfection.

6.2.4. RNA extraction and RT-PCR

Total RNA was extracted from cells using the Tri-Reagent system. Quantity and quality were determined spectrophotometrically at OD₂₆₀. One microgram of total RNA was used for reverse transcription performed as described in section 2.5.2.

6.2.5. Quantitative PCR

mRNA levels of all genes were determined using an ABI 7900HT sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). Reactions were performed as described in 2.6.2.

6.2.6. Protein measurements

Total protein was extracted from cells and protein concentrations were measured as described in methods 2.7.2 and 2.8.2, respectively.

6.2.7. Glycogen measurements

Intracellular glycogen levels were measured using a colorimetric assay kit according to the manufacturer's protocol (Biovision, Inc., California, USA). Cells were rapidly homogenized with 200ul ddH₂O for 10min on ice and the homogenates were then boiled for 10min to inactivate enzymes. 50ul of each sample were transferred to a 96-well plate, 2ul of hydrolysis enzyme mix were added to each well and samples were incubated at room temperature for 30min. 48ul of reaction mix containing glycogen development buffer, development enzyme mix and probe were then added to each well and incubated at room temperature for another 30min. Absorbance was measured on a SpectraMax Plus Microplate Reader (Molecular Devices, CA, USA) at 450 nm.

6.2.8. TAG, 3OHB, glucose and lactate measurements

TAG (intracellular and media), 3OHB, glucose and lactate were analysed using Instrumentation Laboratory kits on an ILab 650 Clinical Chemistry analyser as described in methods 2.10.2, 2.11.2, 2.12.2 and 2.13.2 respectively.

6.2.9. Lipid extraction and gas chromatography-mass spectrometry (GC-MS)

Total intracellular lipid was extracted and measured as previously described. (Folch et al. 1957; Burdge et al. 2000).

Intracellular DNL was measured by the uptake of deuterium from $^2\text{H}_2\text{O}$ (Finnigan GasBench-II, ThermoFisher Scientific, UK) into intracellular TAG and phospholipid [^2H]-palmitate. [^2H]-palmitate enrichments were determined by GC-mass spectrometry (GC-MS) using a 5890 GC coupled to a 5973N MSD (Agilent Technologies; CA, USA). Ions with mass-to-charge ratios (m/z) of 270 ($M+0$) and 271 ($M+1$) were determined by selected ion monitoring (Semple et al. 2009).

6.2.10. RNA sequencing

Total RNA was extracted using the Tri-Reagent system (Sigma-Aldrich, Dorset, UK) and enriched for polyA-tailed mRNA using oligo (dT) beads. The Illumina TruSeq Stranded mRNA HT Sample Prep Kit was used to prepare cDNA libraries for sequencing. In-house 8bp indexes (Lamble et al. 2013) were used to multiplex samples (10-plex), which were then sequenced over 1 lane of an Illumina HiSeq4000 machine using HiSeq 3000/4000 PE Cluster Kit and SBS Kit. Paired-end sequencing (75bp) was performed at a depth of ~25 million read pairs per sample.

Reads were mapped with STAR 2.5.1b (Dobin et al. 2013) on default settings with GENCODE version 19 (Harrow et al. 2012) as transcriptome and GRCh37 as genome reference. Gene level reads counts for all protein-coding and long intergenic non-coding RNA (lincRNA) transcripts present in GENCODE version 19 were quantified in a strand-specific manner with feature Counts (Liao et al. 2014) from the Subread package v1.5.0-p2. For plotting purposes, I also normalized the gene counts to transcripts per million (TPM).

Differential expression analysis was performed using limma (Law et al. 2014) in R 3.2.2 on voom-normalised gene counts for all autosomal protein-coding and lincRNA genes that were expressed at > 1 count per million (CPM) in all knockdown and/or all control samples. A paired model was fitted to the data, and significance was determined by empirical Bayes moderated t-statistics implemented in limma. Differentially regulated genes were defined by a false discovery rate (Benjamini-Hochberg method) adjusted p-value < 1%. Differential expression analysis and figure generation were performed by Dr. Martijn Van de Bunt and Ms. Sara Althari.

6.2.11. Statistical analysis

Data are presented as mean $\pm$ standard error. Two-tailed, paired t-tests were used to compare single treatments to control. For comparisons between control and different treatments, statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett corrections. To compare mean differences between groups that had been split on multiple treatments, doses or times, two-way analysis of variance (ANOVA) with Sidak corrections was used. Statistical analysis on qPCR data was performed on mean relative expression ratio values (Ratio= $\Delta\text{Ct}_{[\text{target}]}/\Delta\text{Ct}_{[\text{ref}]}$) (Pfaffl 2001). Data analysis was performed using Graphpad Prism software (Graphpad Software Inc, La Jolla, USA) and considered statistically significant at $P < 0.05$.

6.3. Results

6.3.1. AKR1D1 over-expression does not regulate glucose uptake or triglyceride accumulation in HepG2 cells

Over-expression assays were performed using HepG2 cells. HepG2 cells were transfected for 48h with either empty pCMV6-XL4 vector (EV) or AKR1D1 vector and increased mRNA expression of AKR1D1 was confirmed using qPCR (Figure 6.3.1-1).

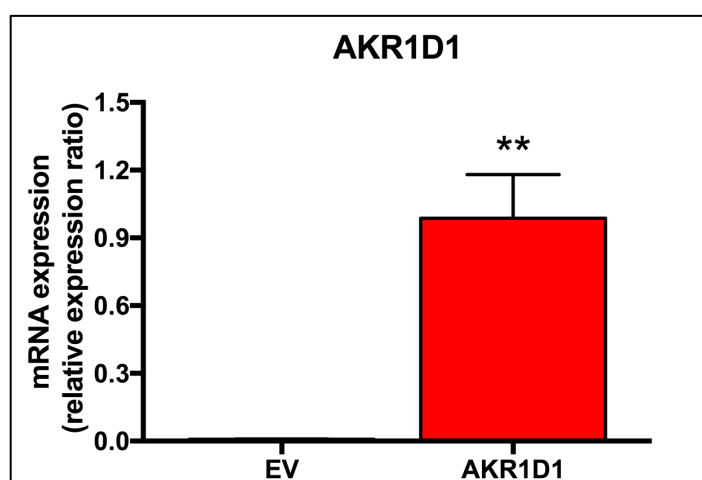


Figure 6.3.1-1 mRNA expression levels of AKR1D1 following over-expression in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments (** $p<0.01$, AKR1D1 compared to EV).

Cells were then treated with serum free and phenol-red free media for 24h and changes in mRNA expression of genes implicated in carbohydrate metabolism were tested. AKR1D1 over-expression had no effect on the expression of the HepG2 glucose transporters GLUT1 or GLUT9. In addition, no difference between EV and AKR1D1 over-expressed cells in the expression of GAPDH and glycogen phosphorylase was observed (Figure 6.3.1-2).

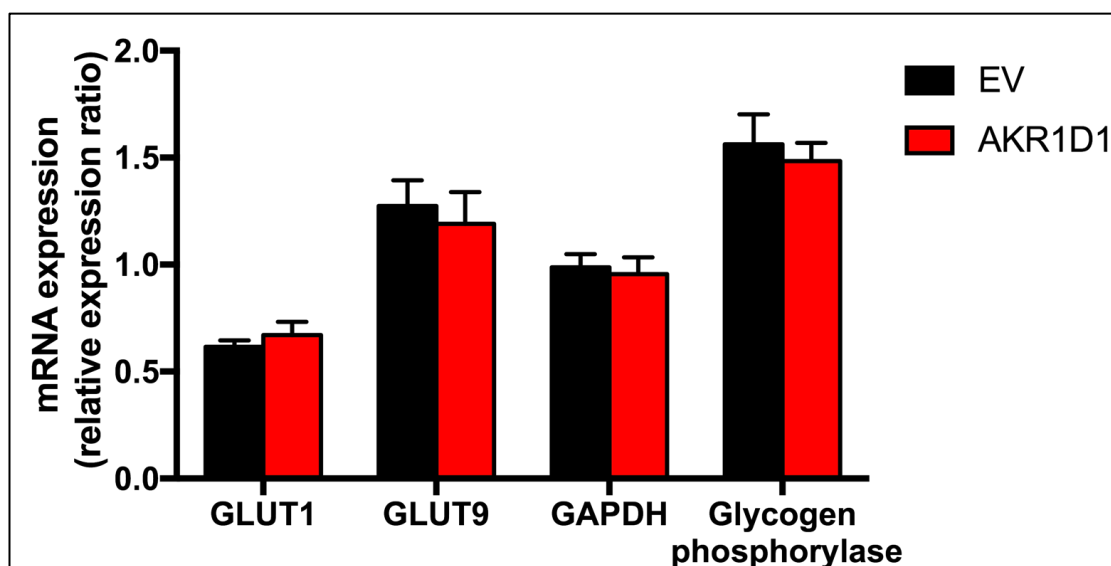


Figure 6.3.1-2 mRNA expression levels of GLUT1, GLUT9, GAPDH and glycogen phosphorylase following AKR1D1 over-expression in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments.

Similarly, I investigated whether AKR1D1 over-expression had any effect on lipogenic gene expression in HepG2 cells. FAS, ACC1, SCD, PPAR α and sterol regulatory element-binding protein 1c (SREBF1) mRNA expression analysis, using qPCR, revealed no differences between EV and AKR1D1 over-expression in HepG2 cells (Figure 6.3.1-3).

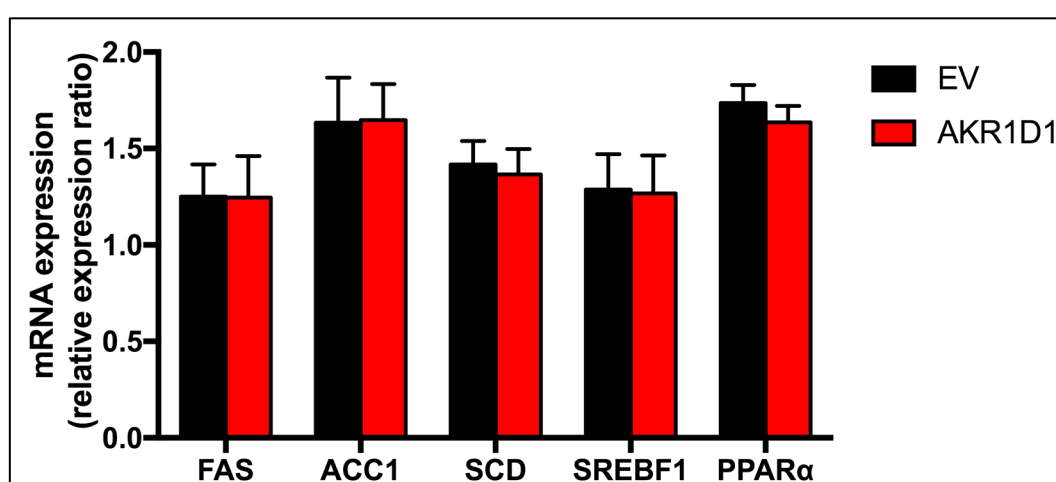


Figure 6.3.1-3 mRNA expression levels of FAS, ACC1, SCD, SREBF1 and PPAR α following AKR1D1 over-expression in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments.

Consistent with these findings, total intracellular TAG accumulation showed no difference between EV and AKR1D1 over-expression in HepG2 cells (Figure 6.3.1-4).

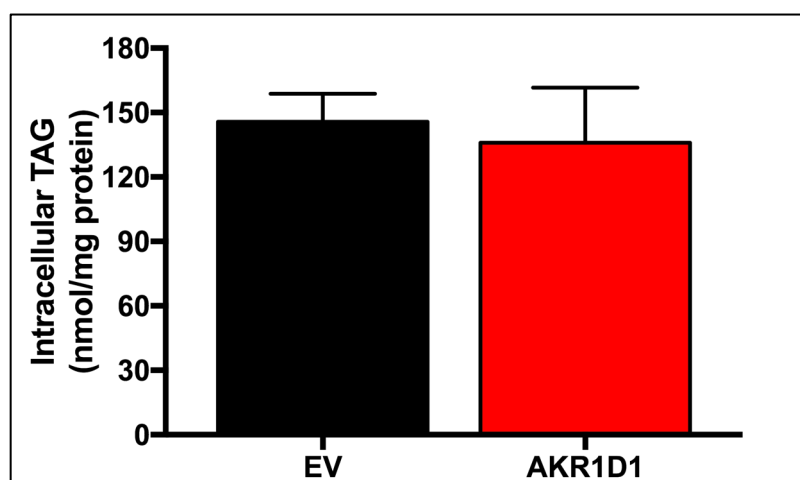


Figure 6.3.1-4 Intracellular TAG content following AKR1D1 over-expression, in HepG2 cells. Data were normalised to protein levels. Data are expressed as mean $\pm$ se of $n=3$ experiments.

6.3.2. AKR1D1 knockdown defines discrete dysregulated metabolic pathways

To gain broader insight into the role of AKR1D1 in human hepatocytes, transcriptome analysis of AKR1D1 knockdown in HepG2 cells was undertaken. RNA sequencing was used to determine expression with 75 nucleotide paired-end reads on the Illumina HiSeq4000 platform, which were aligned to the GENCODE version 19 transcriptome reference. Normalized gene counts were converted to log₂ transformed counts per million (cpm) and ranked on the basis of differential expression as determined by empirical Bayes moderated t-statistics. Differentially regulated genes were defined by a false discovery rate adjusted p-value (Benjamini-Hochberg corrected) threshold of 1% (Figure 6.3.2-1A). A total of 465 transcripts were differentially expressed as a function of AKR1D1 knockdown ($p = 4.06 \times 10^{-3}$; logFC = -4.29) compared to HepG2 cells treated with scrambled control. The top 10% of differentially regulated genes are presented in the heatmap in figure 6.3.2-1B. Amongst these genes, *AKR1D1* expression was significantly decreased whilst *CYP7A1* expression was significantly increased, confirming the previous qPCR data. To facilitate wider exploration of molecular processes affected by AKR1D1 knockdown, a differential expression gene set obtained with a more relaxed significance threshold (FDR 5%) was used to search for enrichment of biological pathways annotated in the Kyoto Encyclopaedia of Genes and Genomes (KEGG). This analysis identified discrete dysregulated pathways impacting upon a variety of cellular phenotype, notably, those impacting upon metabolic pathways, insulin action and fatty acid storage and utilization (Figure 6.3.2-1C).

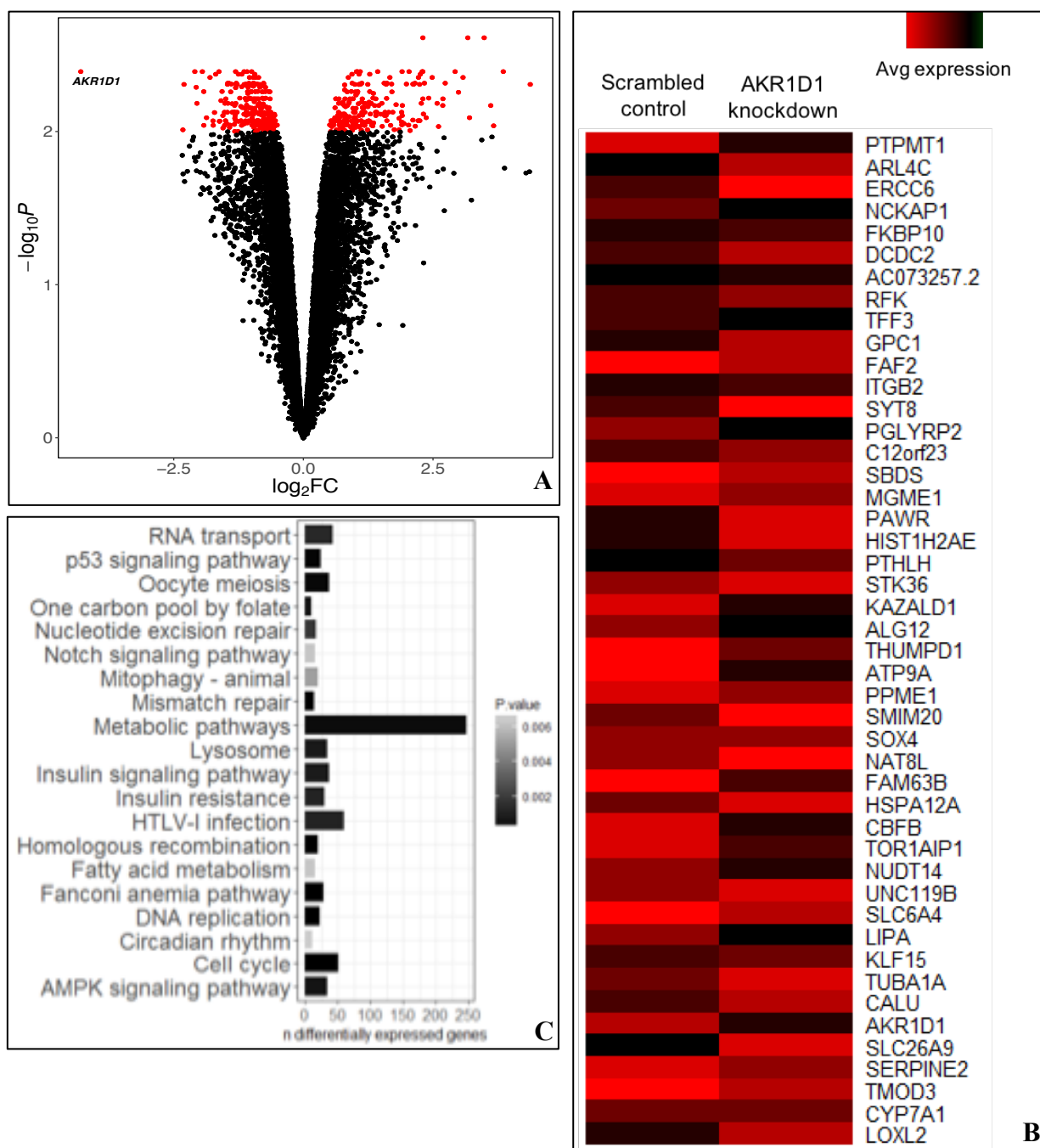


Figure 6.3.2-1 (A). Transcriptomic profiling of *AKR1D1* knockdown in HepG2 cells. Volcano plot illustrating fold-change in expression ($\log_2FC$) against statistical significance ($-\log_{10}$ adjusted P-values) for all genes. Red dots represent differentially expressed genes as a function of *AKR1D1* knockdown (FDR corrected p-values < 0.01). (B). Heat map of the expression changes (normalised counts per million) of the top 10% differentially expressed genes (FDR 1%) in *AKR1D1* knockdown HepG2 cells (red and black bars represent up- and down-regulation, respectively). (C). Top KEGG pathways illustrated as a function of the number of differentially expressed genes contributing to each annotated pathway and coloured with a gradient defined by the statistical significance of pathway enrichment.

6.3.3. AKR1D1 knockdown regulates carbohydrate and lipid metabolism in human hepatocytes

In light of the pathway analysis from the RNA sequencing data that highlighted the pronounced dysregulation of metabolic genes, assessments of carbohydrate and lipid metabolism were performed in HepG2 cells with AKR1D1 knockdown experiments, using a specific AKR1D1 siRNA transcript (see section 2.3.3) and compared with scrambled siRNA, as negative controls. Cells were transfected for 48h and AKR1D1 knockdown was confirmed by qPCR (Figure 6.3.3-1).

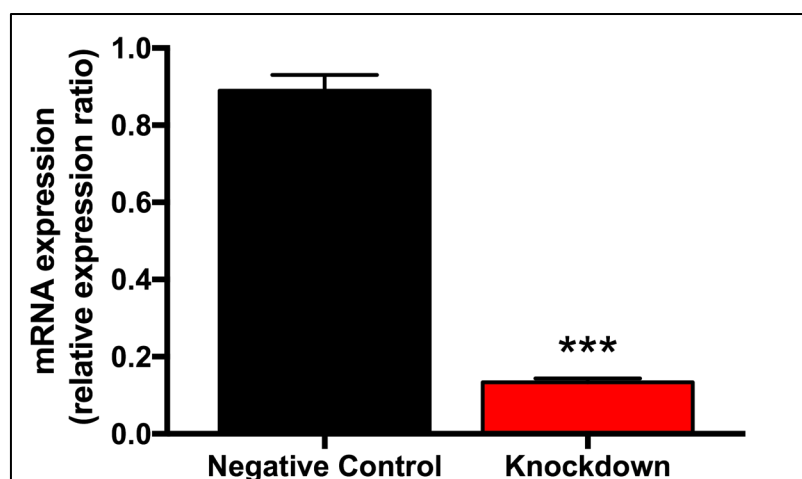


Figure 6.3.3-1 mRNA expression levels of AKR1D1 following transient knockdown in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=6$ experiments ($***p<0.001$, compared to negative control).

HepG2 cells were treated with serum free and phenol-red free media for 24h and the mRNA expression of selected genes involved in carbohydrate metabolism determined. In contrast to AKR1D1 over-expression, AKR1D1 knockdown increased the mRNA expression of the glucose transporters *GLUT1* and *GLUT9*. In addition, it significantly decreased the expression of *GAPDH* and glycogen phosphorylase, suggesting decreased glycolysis and glycogenolysis, respectively (Figure 6.3.3-2).

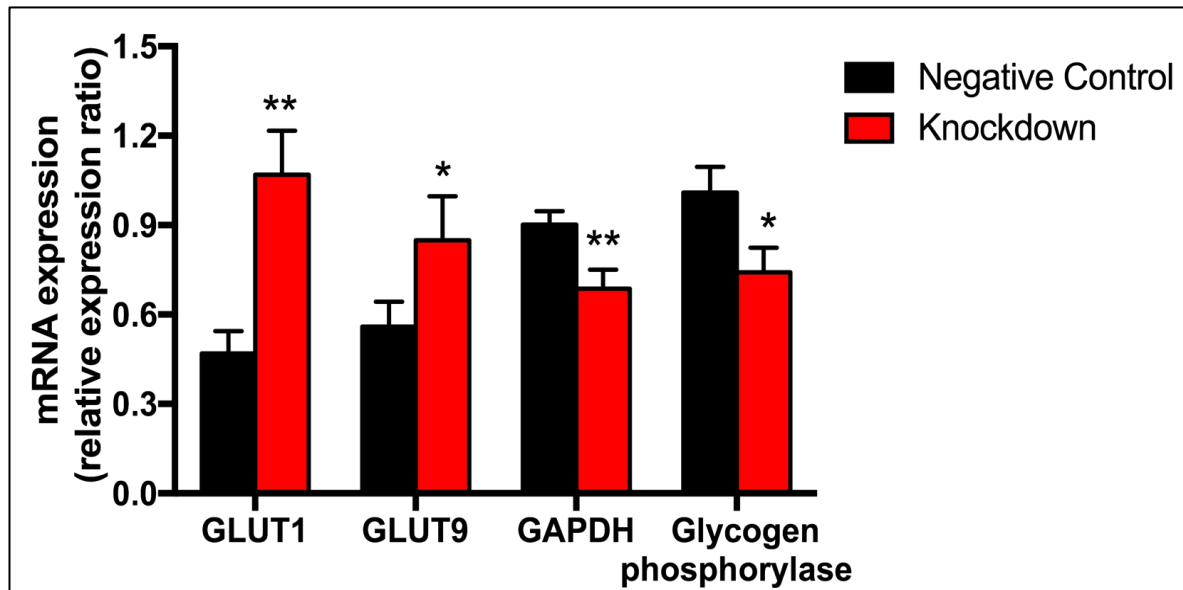


Figure 6.3.3-2 mRNA expression levels of GLUT1, GLUT9, GAPDH and glycogen phosphorylase, following AKR1D1 knockdown in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5-7$ experiments (* $p<0.05$, ** $p<0.01$, compared to negative control).

Consistent with these findings, media glucose concentrations were decreased (negative control: 15.34 ± 1.51 vs. AKR1D1 knockdown: 12.11 ± 0.9 nmol/mg, $p<0.05$) (Figure 6.3.3-3A) whilst intracellular glycogen content increased (negative control: 0.019 ± 0.003 vs. AKR1D1 knockdown: 0.023 ± 0.003 mg/ml, $p<0.05$) (Figure 6.3.3-3B). Additional experiments using 5mM glucose media (1g/L) demonstrated that there was no residual glucose remaining in the cell media 24h following transfection (data not shown).

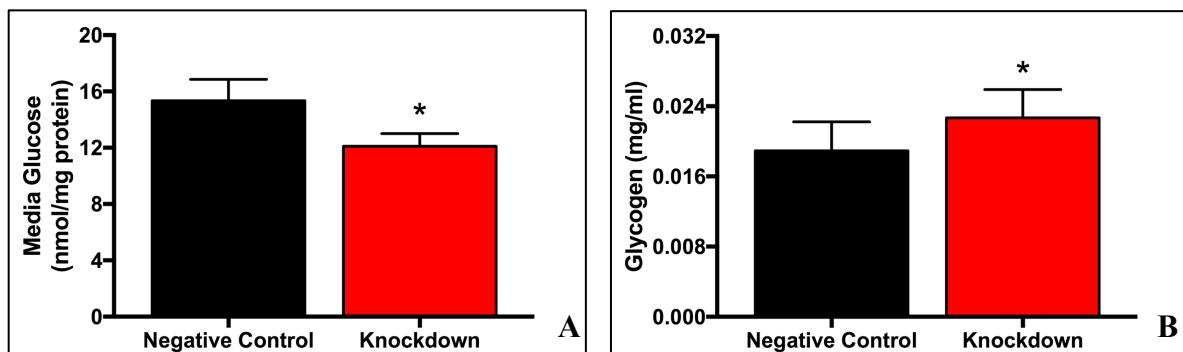


Figure 6.3.3-3 (A). Media glucose levels and (B). intracellular glycogen content following AKR1D1 knockdown in HepG2 cells. Data were normalised to protein levels. Data are expressed as mean $\pm$ se of $n=5-6$ experiments (* $p<0.05$, compared to negative control).

We observed significant increases in lipogenic gene expression (*FAS*, *ACCI*, *SCD*, *SREBF1*) and reductions in the expression of enzymes regulating lipid export (ATP-binding cassette subfamily A1 – *ABCA1*) and oxidation (*PPARα*) (Figure 6.3.3-4). The qPCR data from these genes agreed with the previous RNA sequencing data, thus, validating the RNA sequencing method.

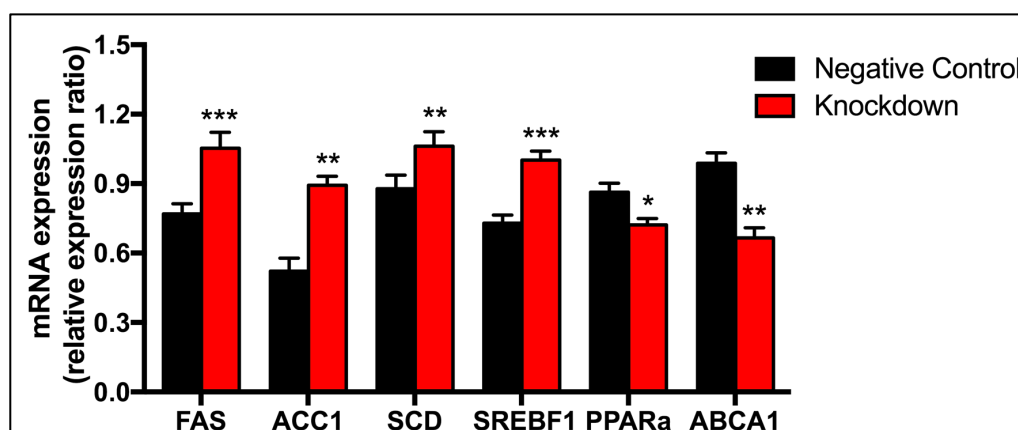


Figure 6.3.3-4 mRNA expression levels of *FAS*, *ACCI*, *SCD*, *SREBF1*, *PPARα* and *ABCA1*, following *AKR1D1* knockdown in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5-7$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to negative control).

Endorsing the changes in lipid gene expression, total intracellular TAG accumulation increased (negative control: 56.9 ± 8.8 vs. *AKR1D1* knockdown: 74.1 ± 9.0 nmol/mg, $p<0.001$) whilst fatty acid oxidation as measured by cell media 3OHB concentrations decreased (negative control: 18.7 ± 2.3 vs. *AKR1D1* knockdown: 11.4 ± 2.6 μ mol/mg, $p<0.01$) (Figure 6.3.3-5).

Additional experiments using low glucose media (1g/L) treatments demonstrated the same consistent effect on both cellular TAG content (negative control: 34.6 ± 4.86 vs. *AKR1D1* knockdown: 48.3 ± 3.76 nmol/mg, $p<0.05$) and media 3OHB concentrations (negative control: 8.32 ± 1.23 vs. *AKR1D1* knockdown: 3.48 ± 0.52 μ mol/mg, $p<0.05$). However, the magnitude of the effect was smaller, probably reflecting lower substrate concentrations (Figure 6.3.3-6).

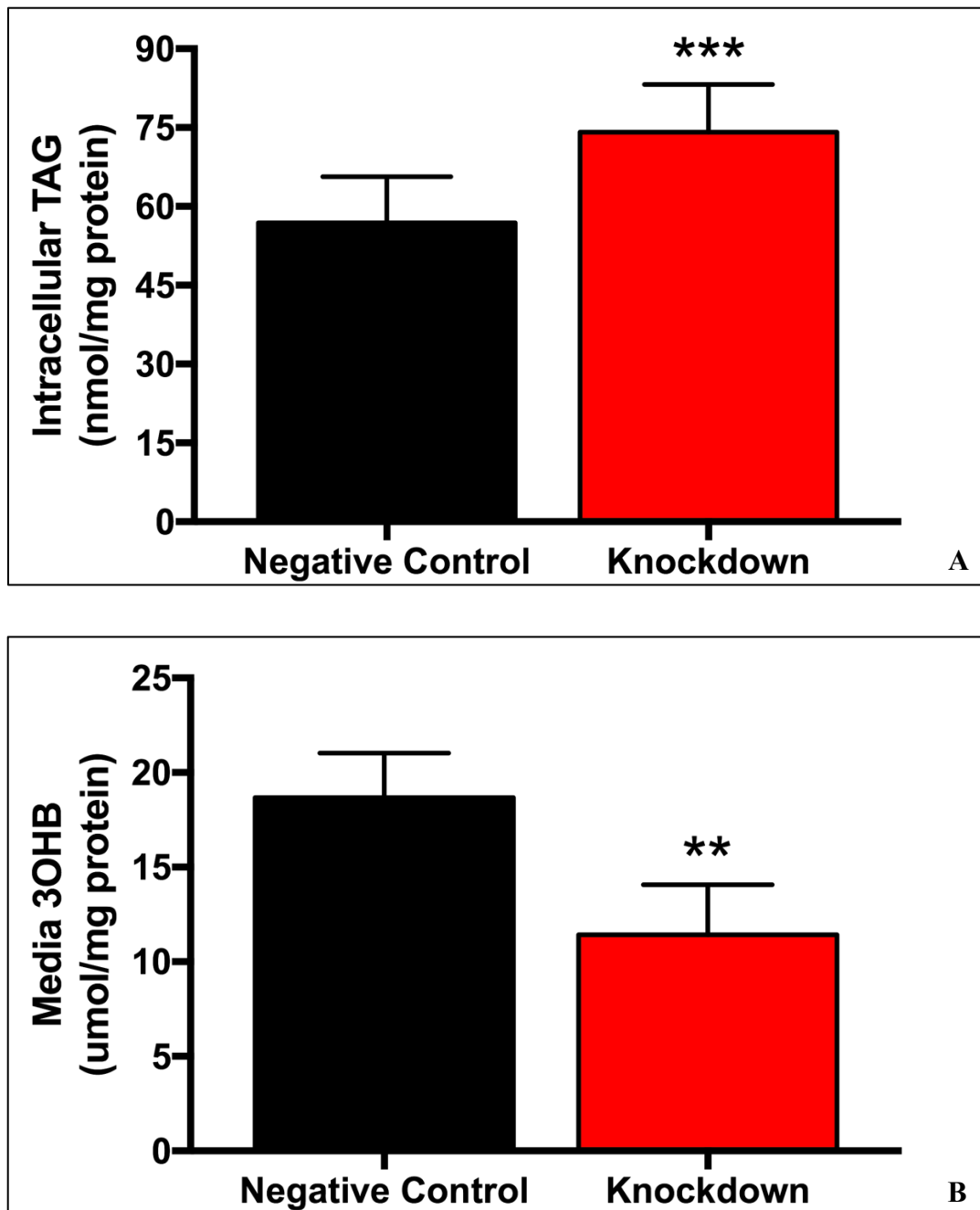


Figure 6.3.3-5 (A). Intracellular TAG content and (B). media 3OHB levels, following AKR1D1 knockdown, in HepG2 cells. Data were normalised to protein levels. Data are expressed as mean $\pm$ se of $n=5-8$ experiments (** $p<0.01$, *** $p<0.001$, compared to negative control).

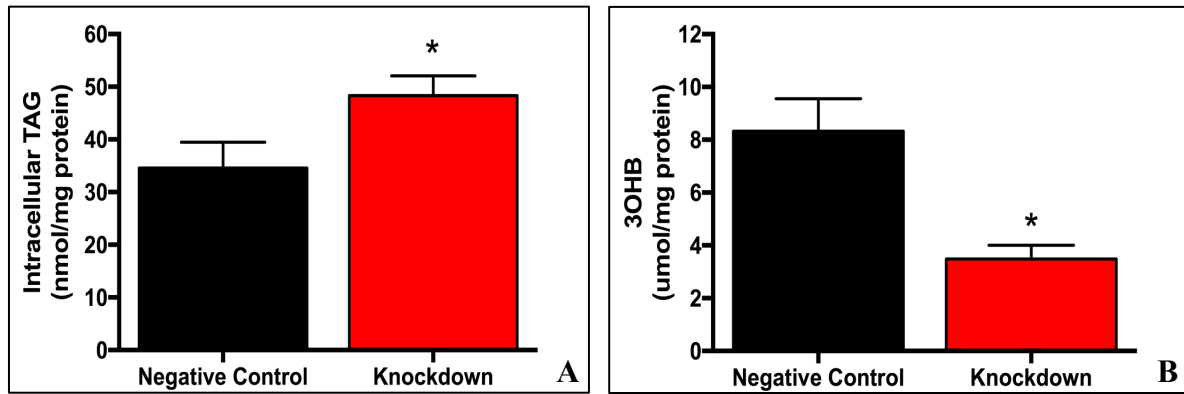


Figure 6.3.3-6 (A). Intracellular TAG content and (B). media 3OHB levels, following *AKR1D1* knockdown, in low glucose treated (1g/L) HepG2 cells. Data were normalised to protein levels. Data are expressed as mean $\pm$ se of $n=3$ experiments (* $p<0.05$, compared to negative control).

In addition, we explored TAG secretion levels, by measuring TAG concentration in the cell media of HepG2 cells pre-and-post *AKR1D1* knockdown; however, TAG was not detected (data not shown). Lactate levels, in the media also remained unchanged (data not shown).

6.3.4. AKR1D1 changes fatty acid composition and drives DNL in human hepatocytes

The fatty acid composition of intracellular TAG and phospholipid fractions, in HepG2 cells were explored using gas chromatography analysis. In agreement with the SCD gene expression data, AKR1D1 knockdown increased fatty acid desaturation, as measured by increased palmitoleic acid abundance (16:1/16:0 ratio) both in intracellular TAG and phospholipid fraction (Figure 6.3.4-1).

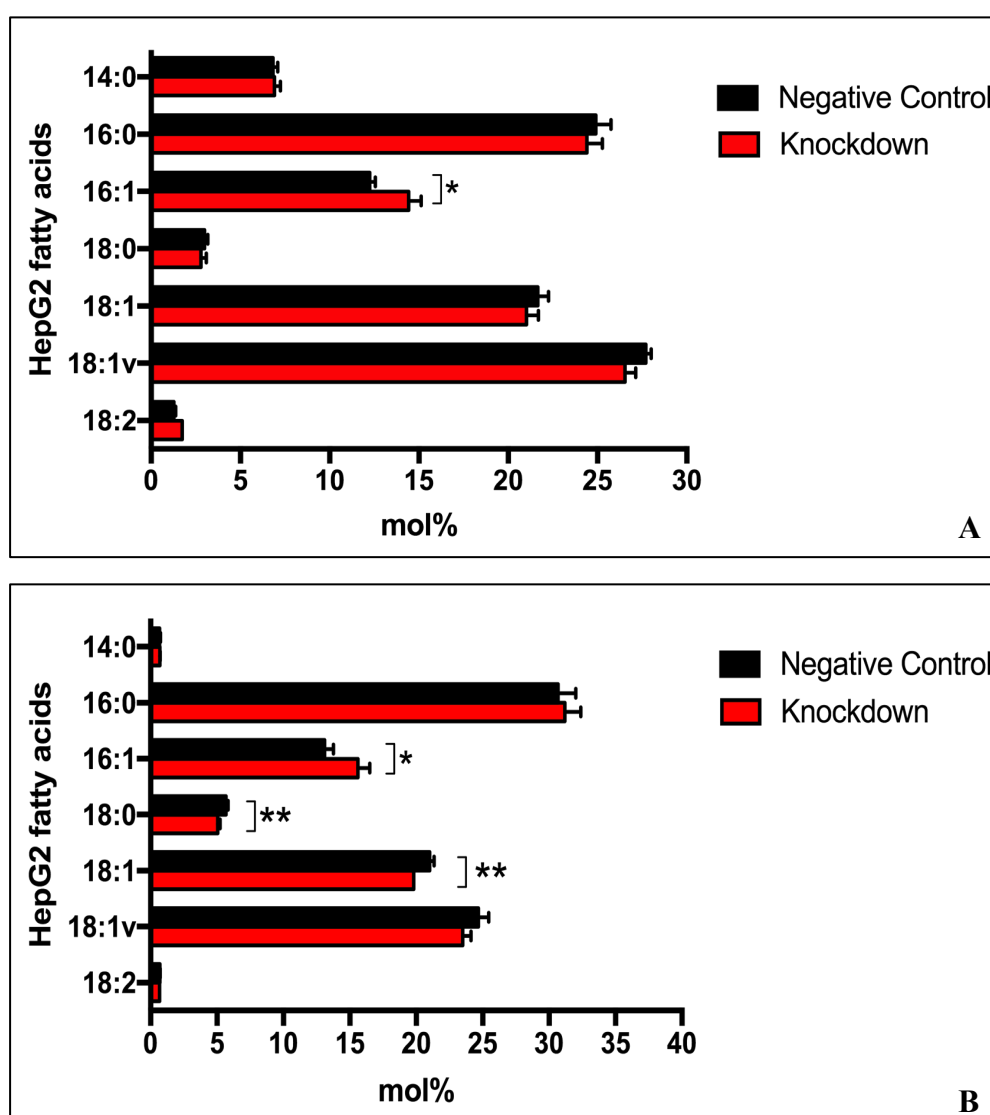


Figure 6.3.4-1 Fatty acid composition in (A). TAG and (B). phospholipid fraction, following AKR1D1 knockdown in HepG2 cells. Data are presented as mol% $\pm$ se of $n=6$ experiments (* $p<0.05$, ** $p<0.01$, compared to negative control).

TAG accumulation in hepatocytes can occur via a number of different pathways. DNL is the synthesis of palmitate from non-lipid precursors and this was measured by incorporation of deuterium from $^2\text{H}_2\text{O}$ into cellular TAG. AKR1D1 knock down increased newly synthesised palmitate, indicative of increased DNL, both in intracellular TAG and phospholipids, as measured by palmitate synthesis (Figure 6.3.4-2).

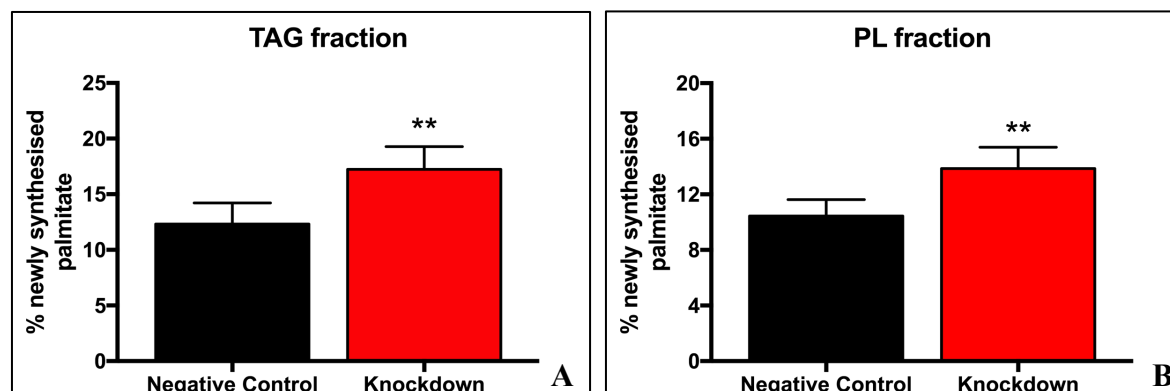


Figure 6.3.4-2 Palmitate synthesis in (A). intracellular TAG and (B). phospholipid (PL) fraction, as measured by incorporation of ^2H from $^2\text{H}_2\text{O}$ into TAG and phospholipid palmitate respectively following AKR1D1 knockdown, in HepG2 cells. Data are presented as % $\pm$ se of $n=6$ experiments (** $p<0.01$, compared to negative control).

6.3.5. Impact of AKR1D1 knockdown on lipid metabolism is not driven by glucocorticoids

In order to determine whether the observed changes in carbohydrate and lipid metabolism following AKR1D1 knockdown were driven by changes in glucocorticoid availability, HepG2 cells were treated with 500nM of cortisol for 24h and gene expression analysis was performed. Cortisol treatment failed to regulate AKR1D1, CYP7A1, FAS, ACC1, SREBF1 or SCD mRNA expression, suggesting that the phenotype that we observed in our AKR1D1 knockdown experiments was unlikely to be due to changes in glucocorticoid availability (Figure 6.3.5-1).

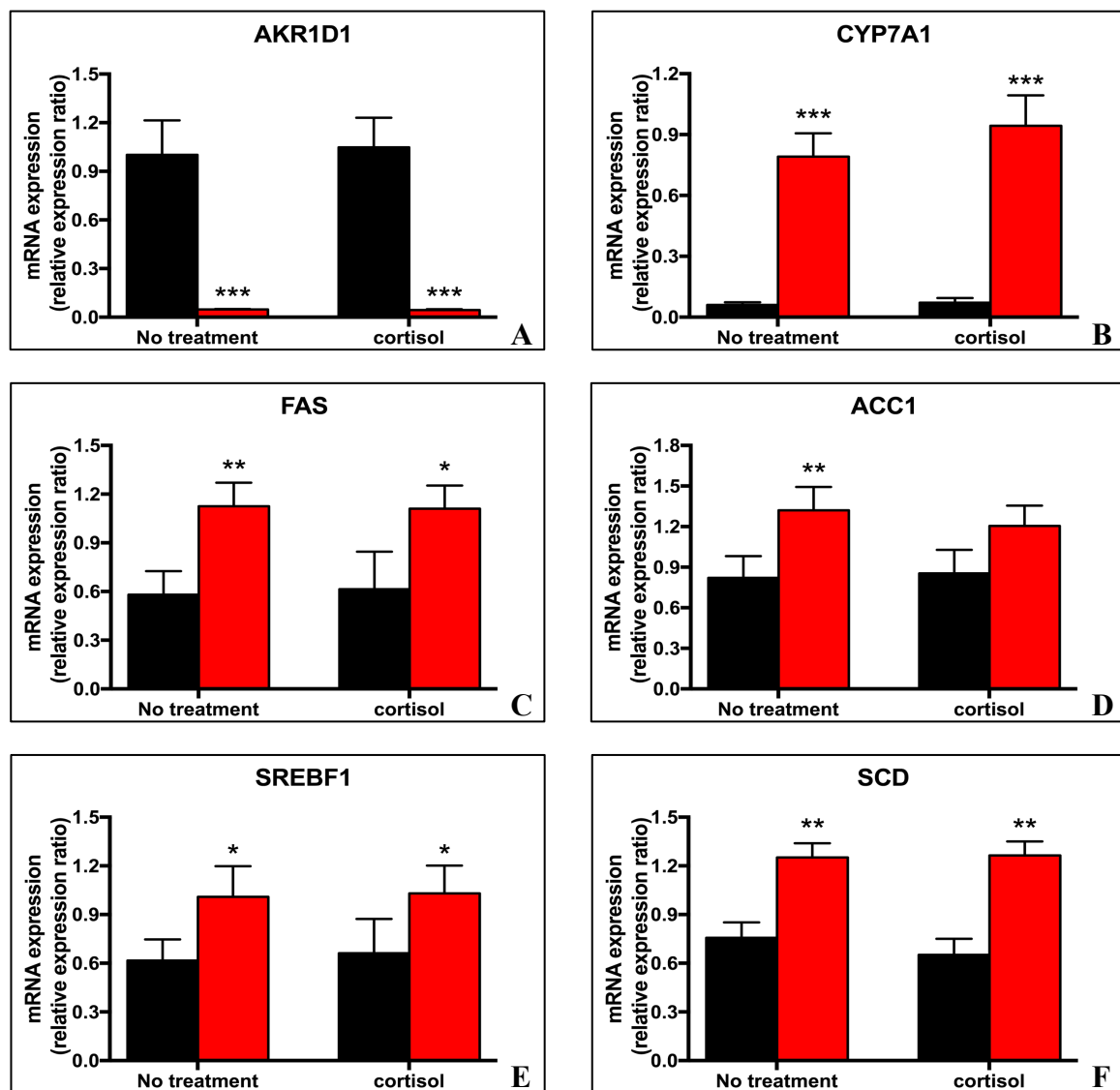


Figure 6.3.5-1 mRNA expression of (A). *AKR1D1*, (B). *CYP7A1*, (C). *FAS*, (D). *ACC1*, (E). *SREBF1* and (F). *SCD*, in negative control (black bars) and *AKR1D1* knockdown (red bars) HepG2 cells, pre- and post- cortisol treatment, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=4$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to negative control).

Consistent with these findings, intracellular TAG accumulation was not regulated by cortisol treatment (Figure 6.3.5-2).

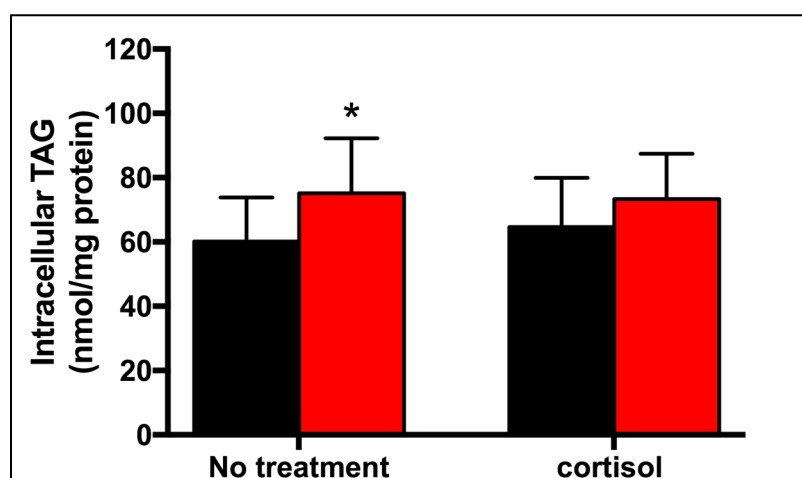


Figure 6.3.5-2 Intracellular TAG content, in negative control (black bars) and AKR1D1 knockdown (red bars) HepG2 cells, pre- and post- cortisol treatment (500nM, 24h). Data were normalised to protein levels. Data are expressed as mean $\pm$ se of $n=4$ experiments (* $p<0.05$, compared to negative control).

6.3.6. Impact of AKR1D1 knockdown on lipid metabolism is driven largely, but not exclusively, by alteration in FXR activation

As cortisol treatment failed to regulate the lipogenic gene expression observed after AKR1D1 knockdown, I tested the hypothesis that the phenotype observed could arise from decreased FXR activation (due to decreased BA synthesis and availability) or increased LXR activation secondary to increased accumulation of oxysterols and BA precursors. I proposed that FXR activation or LXR antagonism would have the potential to rescue the phenotype in our cells.

HepG2 cells were treated with the FXR agonist, GW4064 (5 μ M, 24h), the LXR α antagonist 22-S hydroxycholesterol (22-S HC) (10 μ M, 24h) and the LXR β antagonist GSK2033 (100nM, 24h). GW4064 decreased *CYP7A1*, *FAS* and *SCD* expression, whilst the LXR antagonist 22-S hydroxycholesterol (22-s HC) (10 μ M, 24h) decreased *CYP7A1*, *SCD* and *SREBF1*; the LXR β antagonist GSK2033 had no effect on the lipogenic gene expression (Figure 6.3.6-1).

AKR1D1 knockdown significantly increased the expression of *CYP7A1*, *FAS*, *ACCI*, *SCD* and *SREBF1*. Treatment with the FXR agonist, GW4064, normalized the expression of all target genes to levels seen in the negative scrambled controls. However, co-treatment with the LXR α antagonist, 22-S HC, partially restored the expression levels of *CYP7A1* and *FAS* only and had no impact on the induction of expression of the other genes caused by AKR1D1 knockdown (Figure 6.3.6-2). Taken together these data provide strong evidence that the observed metabolic effects of genetic manipulation of AKR1D1 knockdown are largely mediated by altered FXR activation.

Additional functional activity assays where total intracellular TAG content and media 3OHB concentrations were measured, failed to reflect the differences observed in lipogenic mRNA expression analysis (data not shown).

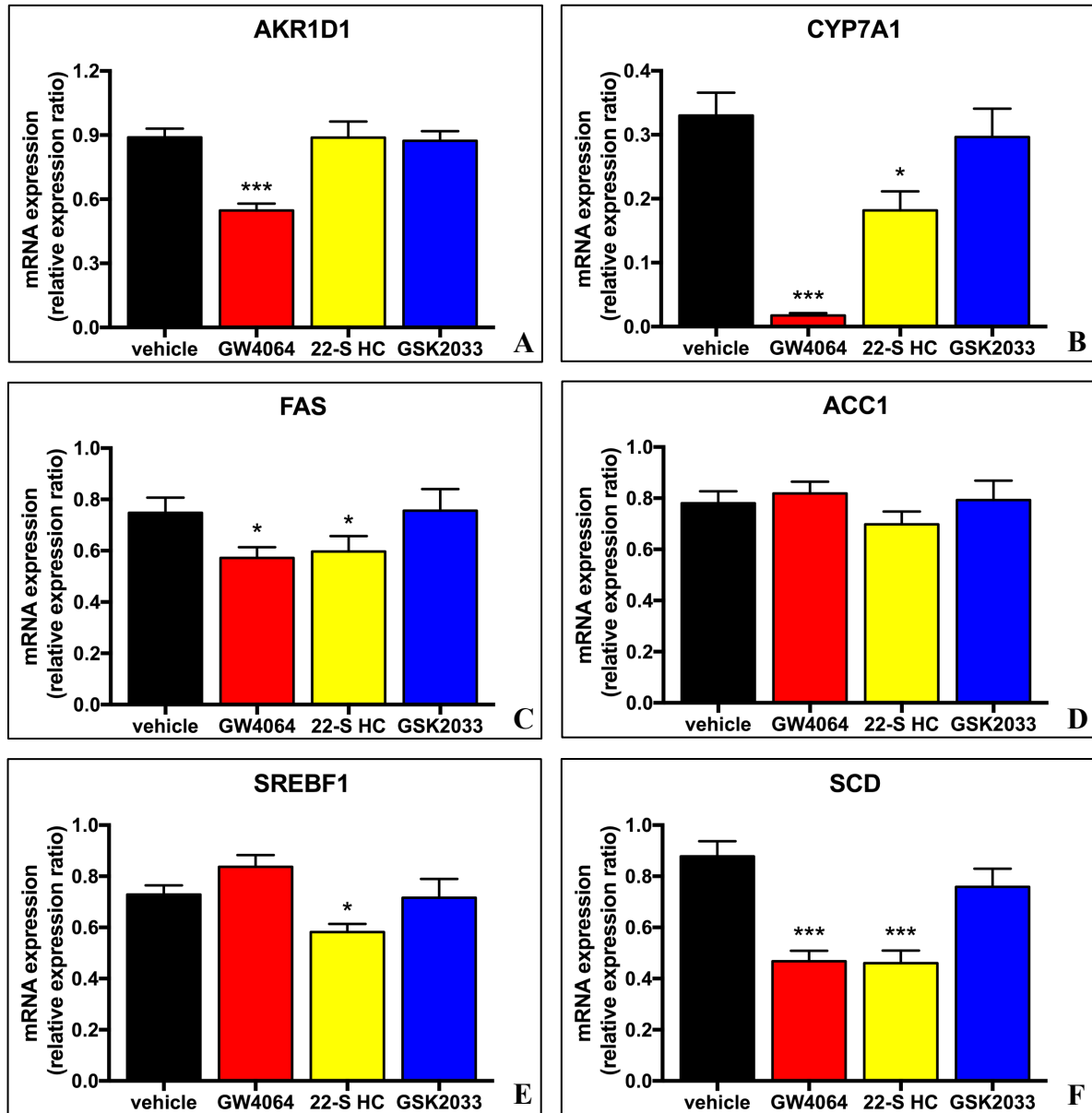


Figure 6.3.6-1 mRNA expression of (A). *AKR1D1*, (B) *CYP7A1*, (C). *FAS*, (D). *ACC1*, (E). *SREBF1* and (F). *SCD*, following GW4064 (5 μ M), 22-S HC (10 μ M) and GSK2033 (100nM) treatment for 24h in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=6$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to vehicle).

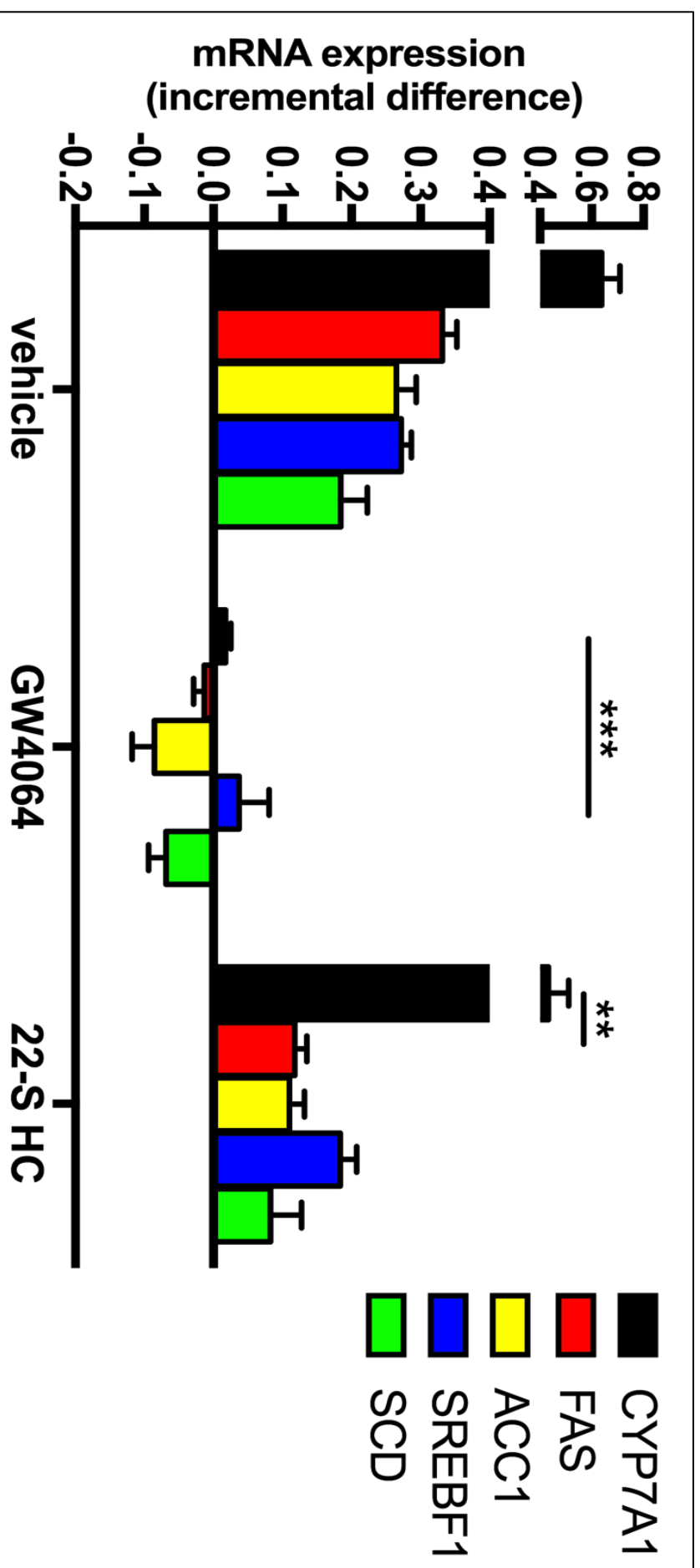


Figure 6.3.6-2 Incremental differences in mRNA expression of CYP7A1, FAS, ACC1, SREBF1 and SCD following GW4064 (5 μ M), 22-S HC (10 μ M) and GSK2033 (100nM) treatment for 24h in AKR1D1 knocked down HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values *se of n=6* experiments (* $p < 0.01$, ** $p < 0.001$, compared to vehicle).

6.4. Discussion

AKR1D1 plays an important role in BA synthesis, in addition to its ability to regulate steroid hormone availability, within the liver. Both BAs and steroid hormones are potent regulators of the metabolic phenotype and have been implicated in hepatic lipid accumulation; however, in this regard, the role of AKR1D1 is almost entirely unexplored. In this chapter, I have demonstrated, for the first time, that manipulation of AKR1D1 expression and activity is able to regulate fundamental metabolic processes within human hepatocytes that govern lipid accumulation and carbohydrate metabolism.

Over-expression of AKR1D1 did not show any effect on carbohydrate or lipid metabolism in human hepatoma HepG2 cells, either at mRNA or functional activity level. There are a number of reasons that may account for this lack of effect. AKR1D1 is already highly expressed in HepG2 cells, compared to primary human hepatocytes, as demonstrated by previous qPCR data. Therefore, additive over-expression assessments would not be able to reveal any specific metabolic effects. An alternative hepatocyte model where AKR1D1 levels are lower to those seen in primary human hepatocytes would help to enlighten the issue. In addition, as the metabolic effects observed seem to be the result of reduced FXR activation and/or increased LXR α action, due to alterations in BA levels and composition, AKR1D1 over-expression does not modulate hepatic metabolism, as it is not the rate-limiting step in BA synthesis. However, silencing of AKR1D1 could potentially become rate-limiting for BA synthesis and, therefore, have an impact on carbohydrate and lipid metabolism.

Glucose levels are tightly regulated in the human body to provide the sufficient energy needed to preserve carbohydrate homeostasis and abnormalities in glucose transport have been linked to NAFLD, T2DM and the development of hepatocellular carcinoma (Keembiyehetty et al. 2006; Amann et al. 2009; Kasai et al. 2009). Although GLUT2 is the

major glucose transporter in primary human hepatocytes (Gould & Holman 1993), GLUT1 and GLUT9 are the major contributors to glucose influx in HepG2 cells (Takanaga et al. 2008). Genetic deletion of AKR1D1 lead to increased glucose flux, as observed by changes in GLUT1 and GLUT9 mRNA expression, alongside reduced cell media glucose levels, suggesting increased glucose transport into the cells. However, in the absence of insulin stimulation, the mechanisms that underpin the increased glucose influx as a consequence of AKR1D1 knockdown are not clear. NAFLD is associated with increased gluconeogenesis in human liver (Sunny et al. 2011). HepG2 cells are routinely cultured in high glucose media, and it is therefore possible that cells prefer to induce glucose influx rather than producing glucose *via* gluconeogenesis, which could potentially lead to higher energy consumption. Subsequently, once glucose enters the cell, it is then used as substrate to increase intracellular glycogen levels. Consistent with this, both Müller et al. (1997) and Clore et al. (1992) demonstrated increased intracellular hepatic glycogen content in both obese non diabetic and T2DM patients.

Lipid accumulation within hepatocytes is the initial, pre-requisite step in the development of NAFLD. Whilst fatty acid delivery from unchecked lipolysis in adipose tissue arising as a consequence of adipose tissue IR remains the predominant source of TAG within hepatocytes, the synthesis of TAG via DNL has become increasingly important with the development of NAFLD (Lambert et al. 2014; Diraison et al. 2003). In addition, there is clear evidence that limitation of β -oxidation of lipid within hepatocyte mitochondria fuels further lipid accumulation (Koliaki et al. 2015; Rector et al. 2010). AKR1D1 knockdown had a dual effect on DNL and β -oxidation. However, as there is an inverse correlation between hepatic DNL and β -oxidation, where increased malonyl-CoA production leads to decreased oxidation via CPT1 inhibition (Abu-Elheiga et al. 2001), it remains unclear whether the observed decrease in β -oxidation is directly attributable to AKR1D1 knockdown.

The mechanisms underpinning my observations would not appear to be related to alterations in glucocorticoid availability, which would have predicted to reduce lipid accumulation as a consequence of decreased cortisol availability, but more likely are due to changes in BA synthesis. In line with this, none of the target genes we tested were regulated by cortisol treatment, supporting the hypothesis that the phenotype was driven by changes in FXR activation.

In chapter 5, I demonstrated that AKR1D1 knockdown resulted in decreased BA synthesis and altered BA composition, and a compensatory increase in the rate-limiting enzyme of BA synthesis, CYP7A1, was observed. BAs are natural ligands of the nuclear receptor FXR and are involved in the regulation of hepatic glucose metabolism (Prawitt et al. 2011), but the data have been conflicting. A number of studies have demonstrated that FXR activation by CA resulted in decreased PEPCCK, G6Pase and fructose-1,6-bisphosphatase (FBS1) expression (Yamagata et al. 2004; Ma et al. 2006) while, others, have shown repressed PEPCCK and G6Pase expression in FXR-deficient mice (Duran-Sandoval et al. 2005). AKR1D1 knockdown in HepG2 cells decreased G6Pase mRNA expression; however, the RNAseq data revealed increased PEPCCK and FBS1 mRNA expression.

FXR also regulates lipid homeostasis; activation of the receptor represses the activity of genes involved in TAG synthesis, including inhibition of the expression of SREBP-1c and its lipogenic target gene FAS (Fiorucci et al. 2009; Cariou 2008). Consistent with these findings, AKR1D1 knockdown increased lipogenic gene expression, including ACC1, SREBF1, FAS and SCD, and that increase was abolished after FXR activation with the FXR agonist GW4064. These results demonstrate that the effects of AKR1D1 knockdown on lipid metabolism are largely driven by FXR activation, but further experiments need to be performed to elucidate the exact mechanism. Furthermore, we demonstrated that FXR regulates AKR1D1 expression by decreasing its expression upon activation by GW4064. In

contrast, Valanejad et al. (2017) recently showed that FXR activation by the same agonist had no effect on the regulation of AKR1D1 expression, using the same cell line. This discrepancy between data may reflect variations across different sublines of HepG2 cells, which highlights some of the limitations in the use of hepatoma cells and raises the importance of endorsing some of the findings using primary cultures of healthy human hepatocytes.

LXR receptors have been involved in BA and fatty acid metabolism and oxysterols are the endogenous ligands for both LXR isoforms (LXR α , LXR β). AKR1D1 knockdown increased the expression of *CYP7A1* due to decreased FXR activation by BAs. However, it has also been shown that LXR α stimulates *CYP7A1* expression in rodents (Peet et al. 1998); consistent with this, in my study, treatment with the LXR α antagonist 22-S hydroxycholesterol was able to partially reduce *CYP7A1* expression after AKR1D1 knockdown in HepG2 cells. There are many published studies that have demonstrated the regulation of lipogenic gene expression, including *SREBP-1c*, *FAS*, *ACCI* and *SCD*, by LXR activation (Joseph et al. 2002; Yoshikawa et al. 2001; Schultz et al. 2000; Tabor et al. 1998). In agreement with these findings, 22-S hydroxycholesterol reduced the effect of AKR1D1 knockdown on lipogenic gene expression, although to a lesser extent when compared to FXR agonism.

The role of LXR β in hepatic lipogenesis has been conflicting. A study from Repa et al. (2000) demonstrated that LXR α , but not LXR β , deficient mice had reduced lipogenic gene expression compared to wild type mice. In contrast, a recent finding from Ducheix et al. showed that LXR β deficiency reduced the effects of the synthetic LXR ligand T0901317 on lipogenic gene expression in mice. In our study, treatment with the LXR β antagonist GSK2033 had no effect on lipogenic gene expression in HepG2 cells. In total, these data

highlight the additive role of LXR in the phenotype observed after AKR1D1 knockdown in HepG2 cells.

In conclusion, I have demonstrated a novel role of AKR1D1 in human hepatocyte function and have shown that manipulation of AKR1D1 expression and activity regulate carbohydrate and lipid metabolism. In addition, I have demonstrated that the observed effects are largely driven by FXR and LXR activation. Further studies using both human hepatoma cells and primary human hepatocytes should be performed to elucidate the exact role of AKR1D1. Multiple knockdown studies, including both AKR1D1 and FXR should have an even more detrimental effect on carbohydrate and lipid metabolism. In contrast, a double AKR1D1/LXR α knockdown model could potentially demonstrate a less dramatic phenotype compared to AKR1D1 knockdown, only. A more detailed analysis on the RNA sequencing data could potentially provide extra mechanistic insight with respect to FXR and/or LXR signalling. In addition, additional experiments using chromatin immunoprecipitation (ChIP) protocols could help to explore whether FXR agonism/antagonism is able to regulate AKR1D1 expression. The use of a pharmacological inhibitor of AKR1D1 in this context would significantly add to the literature, but to date, there are currently no specific AKR1D1 inhibitors available.

Chapter 7 AKR1D1 regulates inflammation in human hepatocytes

7.1. Introduction

In chapter 5, I demonstrated that AKR1D1 regulates glucocorticoid availability as well as GR activation in human hepatocytes. Although synthetic glucocorticoids have been extensively used as anti-inflammatory and immunosuppressive drugs, the metabolic effects of prolonged glucocorticoid treatment have been associated with IR, T2DM, steatohepatitis (NASH) (Coutinho & Chapman 2011; Patel et al. 2017) and these factors can mediate secondary “hits” that lead to hepatocellular injury and liver inflammation (Dandona et al. 2004; Neuschwander-Tetri 2010; Shoelson et al. 2007). In addition, recent studies have suggested the involvement of GR in the pathogenesis of NASH/NAFLD and hepatic inflammation, as it antagonizes NF- κ B signalling through direct physical protein-protein interactions; binding of glucocorticoids to the receptor regulates targeted gene expression in the cell nucleus and, subsequently, these alterations lead to up-regulation of anti-inflammatory protein synthesis and reduced pro-inflammatory gene expression (Starick et al. 2015; Lim et al. 2015; Woods et al. 2015). Regulation of glucocorticoid metabolism at pre-receptor level has also been associated with hepatic inflammation; patients with NASH have demonstrated induced 11 β -HSD1 and decreased 5 α R activity, resulting in increased glucocorticoid generation and decreased glucocorticoid clearance, respectively. The net effect is to maximize glucocorticoid availability and this may serve to limit the inflammatory response. (Ahmed et al. 2012).

In addition, I have shown that AKR1D1 regulates BA synthesis and secretion in human hepatocytes, by decreasing total BA content and altering BA composition. There is extensive literature on the inflammatory role of BAs and their ability to trigger cytokine and chemokine secretion (Zhu et al. 2016). In addition, abnormalities in hepatic BA transport and composition lead to toxic BA accumulation with subsequent cell death pathway stimulation and induced oxidative stress (Trauner et al. 2008; Smit et al. 1993; Schaap et al. 2013).

In chapter 6, I demonstrated that AKR1D1 regulates both carbohydrate and lipid metabolism in human hepatocytes, leading to increased lipid accumulation, increased palmitic acid synthesis (via DNL) and impaired fatty acid oxidation. *In vitro* fatty acid treatments, including palmitic acid, have been associated with toxicity and apoptosis, autophagy through mitochondrial dysfunction and ER stress in liver cells (Liu et al. 2010; Park et al. 2014). Although Kupffer cells and other immune cells are responsible in regulating the immune and inflammatory response within the liver, with stellate cells co-ordinating the fibrotic response (Weiskirchen & Tacke 2014), hepatocytes are also involved in stimulating fibrosis, mainly through reactive oxygen species (ROS) production and apoptosis regulation (Nieto et al. 2002; Basu et al. 2006). Furthermore, hepatocytes are able to produce cytokines and chemokines in response to pathological conditions (Dong et al. 1998), and recent studies have demonstrated that fatty acid exposure, including linoleic acid, induces inflammation in human hepatoma cells (Vinciguerra et al. 2009; Maruyama et al. 2014).

In addition to their crucial role in hepatic lipid and glucose metabolism, activation of BA nuclear receptors, including FXR and TGR5, can exert anti-inflammatory and immunomodulatory effects by inhibiting the expression of inflammatory regulators in response to NF- κ B signalling (Wang et al. 2008). Furthermore, activation of the liver X receptors (LXRs) is associated with repression of inflammatory gene expression, including the toll-like receptor (TLR), interleukin 1 β (IL-1 β), IL-6, inducible nitric oxide synthase (iNOS), interleukin 8 (IL-8) and tumor necrosis alpha (TNF α) (Ogawa et al. 2005; Joseph et al. 2003; Myhre et al. 2008; Bi et al. 2016).

7.1.1. Hypothesis and aims

Based on these findings as well as the findings in chapter 6, I hypothesised that AKR1D1 knockdown has the potential to drive inflammation within human hepatocytes, either through

increased intracellular lipid accumulation/impaired fatty acid oxidation or through elevated concentrations of toxic BA intermediates. Using genetic manipulation of AKR1D1 gene expression in HepG2 and Huh7 human hepatoma cells, through over-expression and transient knockdown assays my aims were to:

- Define the ability of AKR1D1 knockdown to regulate inflammation in HepG2 cells.
- Define the ability of AKR1D1 over-expression and knockdown to regulate inflammation and/or ER stress in Huh7 cells.
- Explore the mechanisms underpinning the regulation of inflammatory phenotype by AKR1D1 using glucocorticoid treatments, FXR agonism and LXR antagonism.

7.2. Methods

7.2.1. HepG2 and Huh7 cell culture

HepG2 human hepatoma cells were cultured as described in section 2.1.1, and plated in 24-well Cell Bind plates (Corning, Flintshire, UK) at a density of 0.8×10^5 cells/ml, respectively. Huh7 human hepatoma cells were cultured as described in section 2.1.1, and plated in 12-well and 24-well Cell Bind plates (Corning, Flintshire, UK) at a density of 1.3×10^5 and 0.6×10^5 cells/ml, respectively.

7.2.2. Transfection studies

For the over-expression studies, the AKR1D1 cDNA was cloned into the pCMV6-XL4 vector (Origene Technologies, Rockville, USA) and transiently transfected into Huh7 cells as described in section 2.3.2.

For the knockdown studies, AKR1D1 siRNA molecules were purchased from Invitrogen (Paisley, UK) and transiently transfected into HepG2 and Huh7 cells as described in section 2.3.3.

7.2.3. Cell treatments

For all vehicle treatments, HepG2 cells were treated with serum free and phenol-red free media containing 0.1%DMSO for 24h, after 48h of transfection.

For all cortisol treatments, HepG2 cells were treated with serum free and phenol-red free media containing 500nM cortisol for 24h, after 48h of transfection.

For GW4064 treatments, HepG2 cells were treated with serum free and phenol-red free media containing 5 μ M of GW4064 for 24h, after 48h of transfection.

7.2.4. RNA extraction and RT-PCR

Total RNA was extracted from cells using the Tri-Reagent system. Quantity and quality were determined spectrophotometrically at OD₂₆₀. One microgram of total RNA was used for reverse transcription performed as described in section 2.5.2.

7.2.5. Quantitative PCR

mRNA levels of all genes were determined using an ABI 7900HT sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). Reactions were performed as described in 2.6.2.

7.2.6. Protein extraction and immunoblotting

Total protein was extracted from cells and protein concentrations were measured as described in methods 2.7.2 and 2.8.2 respectively.

7.2.7. IL-1 β , IL-6 and IL-8 ELISA assays

IL-6 levels were measured using colorimetric ELISA assay according to the manufacturer's protocol (Abcam, Cambridge, UK). Phenol-red free cell media samples were collected after 24h of treatment and centrifuged at 1000g for 10min to remove debris. 100 μ l of sample were added into an anti-IL-6 96-well coated plate followed by 50 μ L of 1X biotinylated anti-IL-6 and plate was covered and incubated for 3h at room temperature. Wells were washed 3 times with 1x wash buffer, 100 μ L of 1X streptavidin-HRP solution were added into all wells and plate was incubated for 30min at room temperature. Wells were washed 3 times with 1x wash buffer and 100 μ L of chromogen TMB substrate solution were added into each well and incubated in the dark for 10-20min at room temperature. 100 μ L of stop reagent were then

added into each well to stop the reaction and absorbance was measured on a SpectraMax Plus Microplate Reader (Molecular Devices, CA, USA) at 450 nm.

IL-8 and IL-1 β media levels were also measured using the same principle, according to the manufacturer's protocol (Abcam, Cambridge, UK).

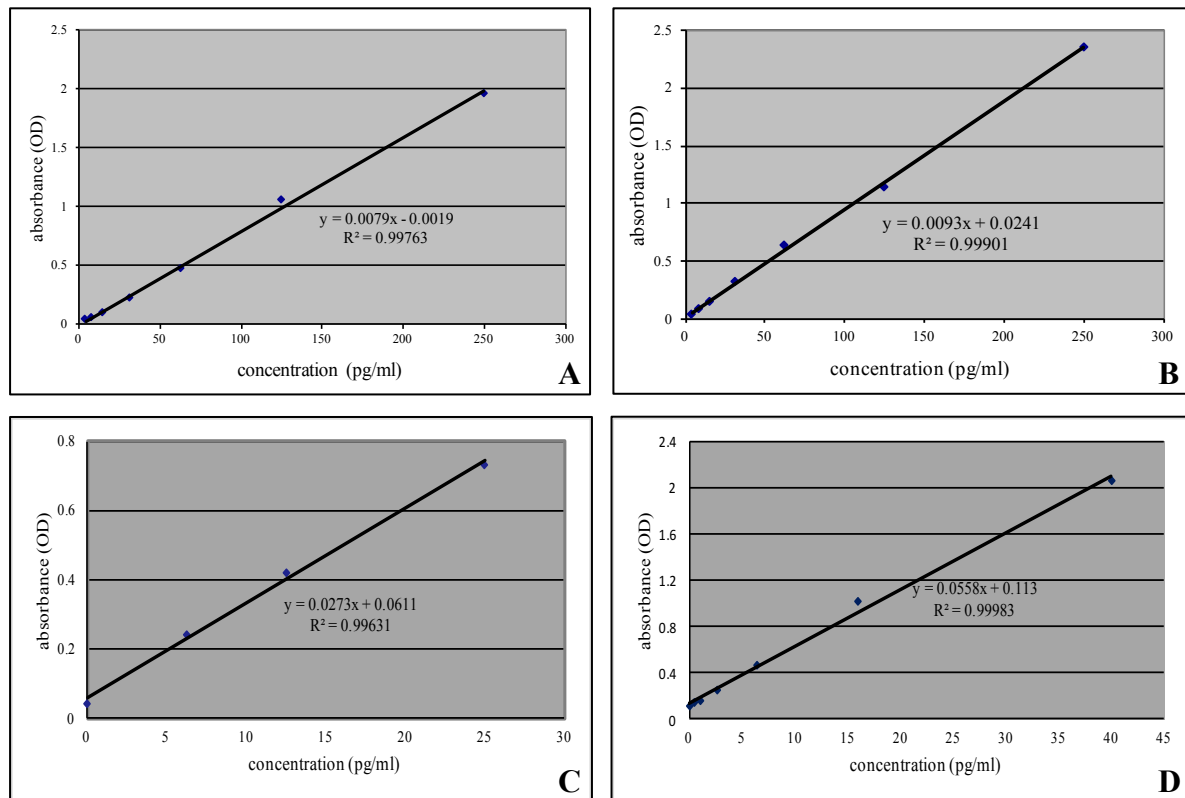


Figure 7.2.7-1 Standard curve of (A). HepG2 IL-8, (B). Huh7 IL-8, (C). Huh7 IL-6 and (D). Huh7 IL-1 β ELISA assay.

7.2.8. Statistical analysis

Data are presented as mean $\pm$ standard error. Two-tailed, paired t-tests were used to compare single treatments to control. For comparisons between control and different treatments, statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett corrections. To compare mean differences between groups that had been split on multiple treatments, doses or times, two-way analysis of variance (ANOVA) with Sidak corrections was used. Statistical analysis on qPCR data was performed on mean relative

expression ratio values ($\text{Ratio} = \Delta C_{t[\text{target}]} / \Delta C_{t[\text{ref}]}$) (Pfaffl 2001). Data analysis was performed using Graphpad Prism software (Graphpad Software Inc, La Jolla, USA) and considered statistically significant at $P < 0.05$.

7.3. Results

7.3.1. AKR1D1 knockdown regulates inflammation in HepG2 cells

As previously described, AKR1D1 was knocked down in HepG2 cells using using a specific AKR1D1 siRNA transcript and compared with scrambled siRNA as a negative control. AKR1D1 knockdown was confirmed by qPCR. mRNA expression of pro-inflammatory cytokine IL-8 increased as did the expression of the inducible nitric oxide synthase (*iNOS*) (Figure 7.3.1-1).

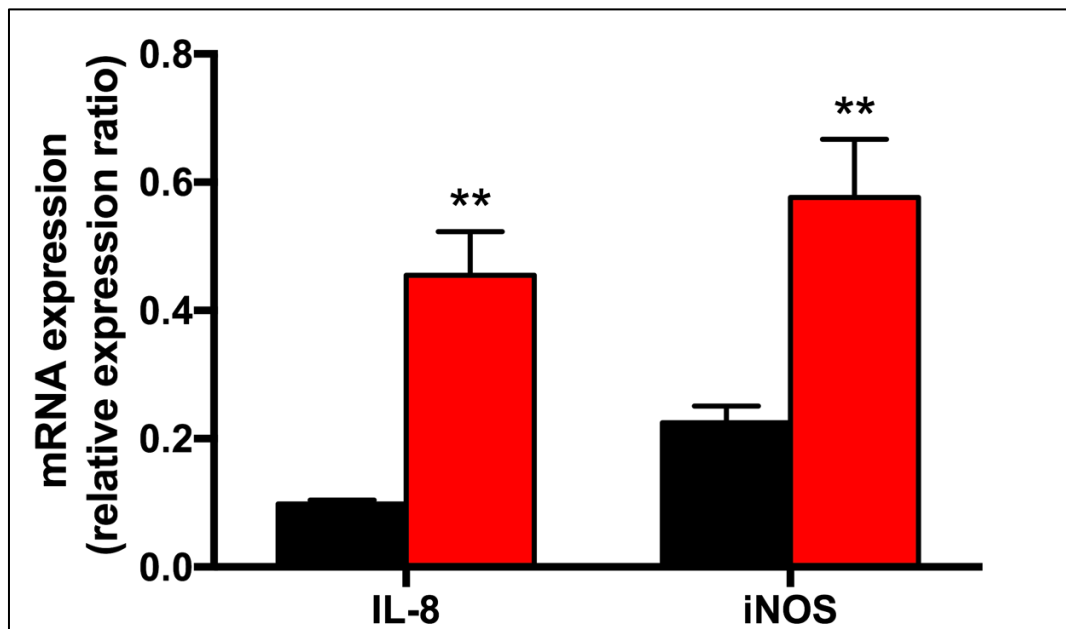


Figure 7.3.1-1 mRNA expression levels of IL-8 and iNOS in negative control (black bars) and AKR1D1 knockdown HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=6$ experiments (** $p<0.01$, compared to negative control).

In addition to mRNA expression, IL-8 secretion in the cell media was measured using a commercially available ELISA kit. AKR1D1 knockdown significantly increased IL-8 secretion after 24h of treatment with serum free media (Figure 7.3.1-2).

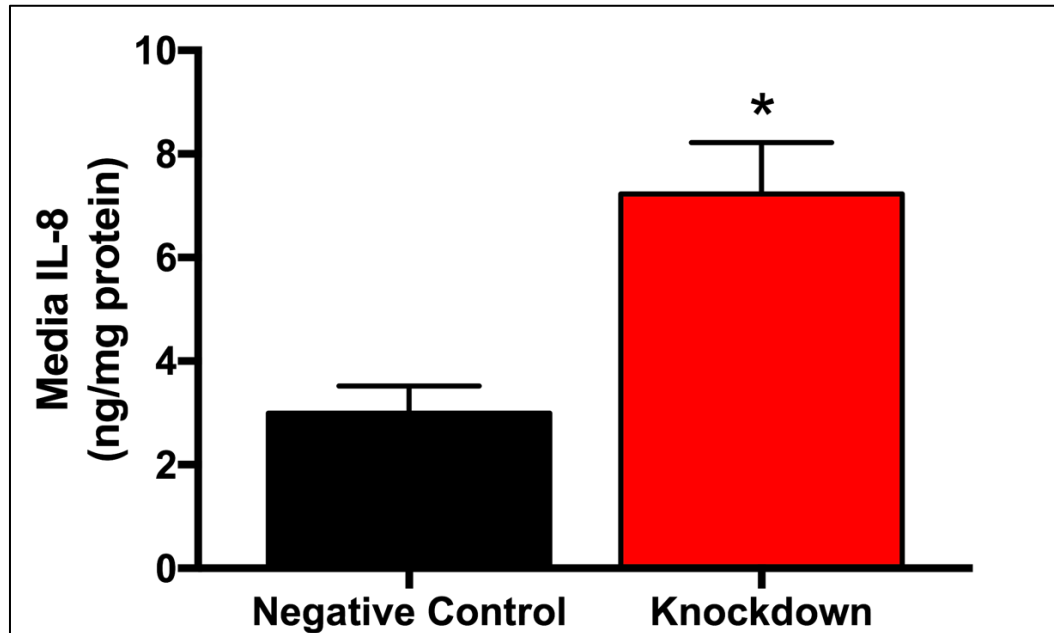


Figure 7.3.1-2 Media levels of IL-8 following AKR1D1 knockdown in HepG2 cells, as measured by ELISA. Data were normalised to protein values. Data are expressed as mean $\pm$ se of $n=6$ experiments (* $p<0.05$, compared to negative control).

The expression of target proteins implicated in the NF- κ B pathway as the key regulatory step in the inflammatory response were investigated. Protein levels of I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- α), the endogenous inhibitor of NF- κ B, were not affected by AKR1D1 knockdown. Moreover, protein expression of JNK, which plays a critical mechanistic role in NAFLD development (Czaja 2010), was explored but no significant expression was detected (data not shown). Additional experiments exploring pro-inflammatory cytokines *IL-1 β* , *IL-6* and *TNF α* did not reveal any mRNA expression in HepG2 cells. As a consequence, the inflammatory response was examined in an alternative hepatoma cell line.

7.3.2. AKR1D1 knockdown regulates inflammation and ER stress in Huh7 cells

Based on the previous mRNA expression analysis, HepG2 cells seemed to be a poor model of inflammation, expressing and secreting a very limited number of pro-inflammatory cytokines. As Huh7 human hepatoma cells have also been validated in this context, therefore, a series of experiments to further determine the impact of AKR1D1 knockdown on inflammatory phenotype were undertaken.

Huh7 cells were knocked down using the same AKR1D1 siRNA transcript, and compared with scrambled siRNA, as negative controls. mRNA expression was confirmed by qPCR (Figure 7.3.2-1).

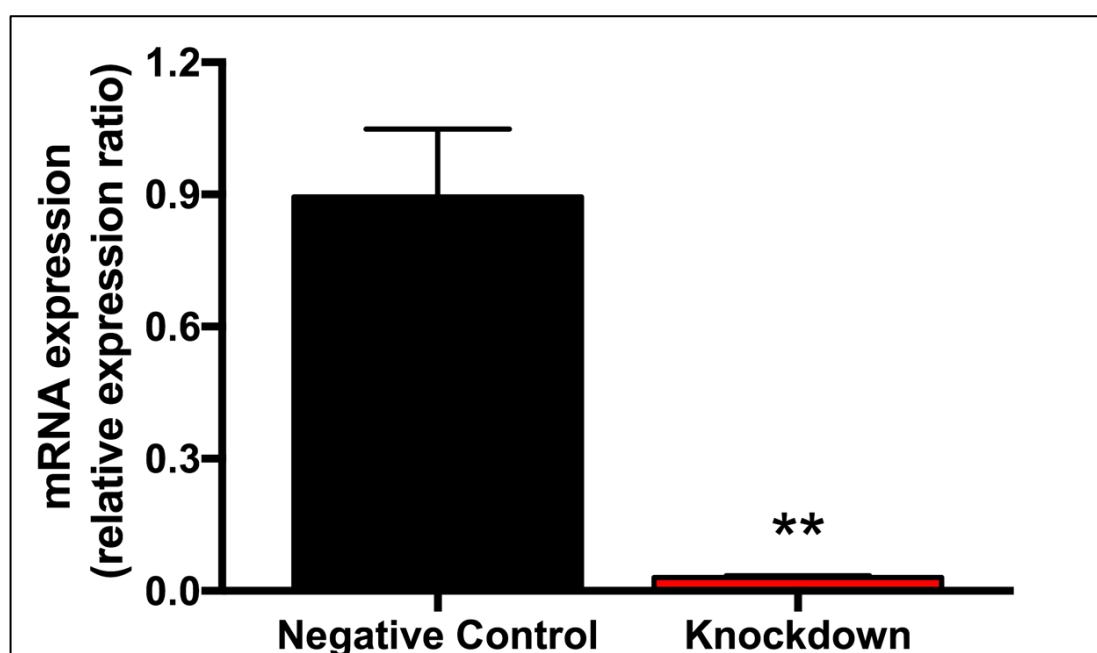


Figure 7.3.2-1 mRNA expression levels of AKR1D1 following transient knockdown in Huh7 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments ($***p<0.01$, compared to negative control).

Subsequently, Huh7 cells were treated with serum free media for 24h and mRNA expression of pro-inflammatory cytokines IL-1 β , IL-6, IL-8 and TNF α as well as *iNOS* were explored. AKR1D1 knockdown significantly increased the expression of *IL-1 β* , *IL-6*, *IL-8* and *iNOS*, as measured by qPCR (Figure 7.3.2-2). TNF α mRNA expression levels were low in Huh7 cells and were not regulated by AKR1D1 knockdown (data not shown).

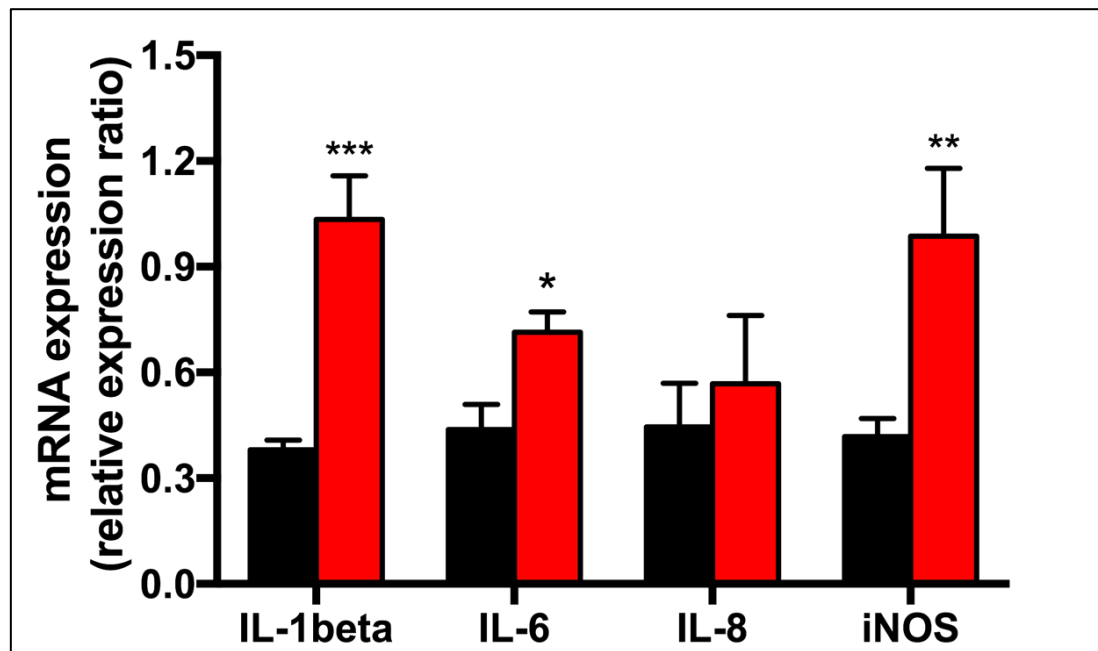


Figure 7.3.2-2 mRNA expression levels of *IL-1 β* , *IL-6*, *IL-8* and *iNOS*, following *AKR1D1* knockdown in Huh7 cells (red bars), as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=5$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to negative control (black bars)).

In addition, IL-1 β , IL-6 and IL-8 secretion in the cell media were measured, using commercially available ELISAs. AKR1D1 knockdown significantly increased IL-6 and IL-8 secretion after 24h of treatment with serum free media (Figure 7.3.2-3). No IL-1 β secretion was detected in the media from either negative control or AKR1D1 knocked down Huh7 cells.

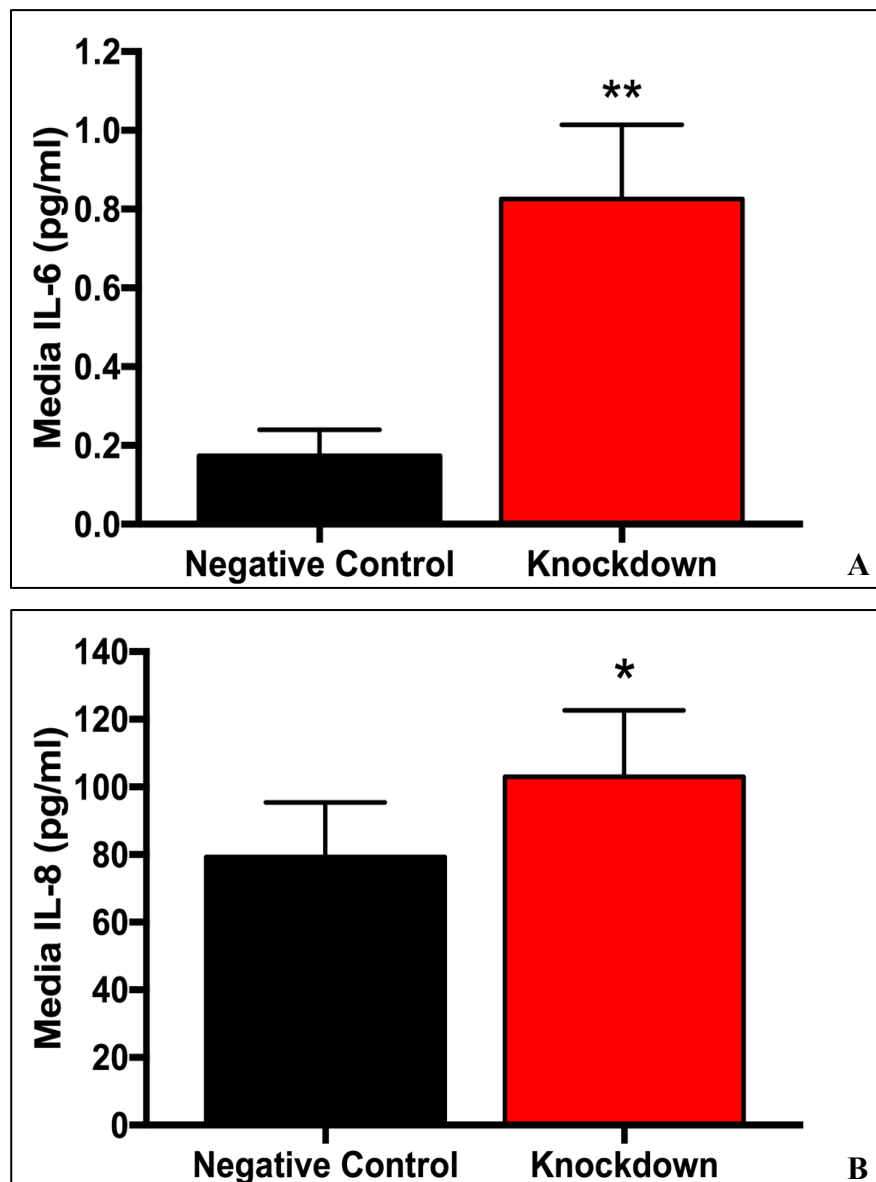


Figure 7.3.2-3 Media levels of (A). IL-6 and (B). IL-8, following AKR1D1 knockdown in Huh7 cells, as measured by ELISA. Data are expressed as mean $\pm$ se of n=5 experiments (*p<0.05, **p<0.01, compared to negative control).

Next, the expression of target proteins implicated in the NF- κ B pathway were investigated. Protein levels of I κ B α were reduced by AKR1D1 knockdown (Figure 7.3.2-4).

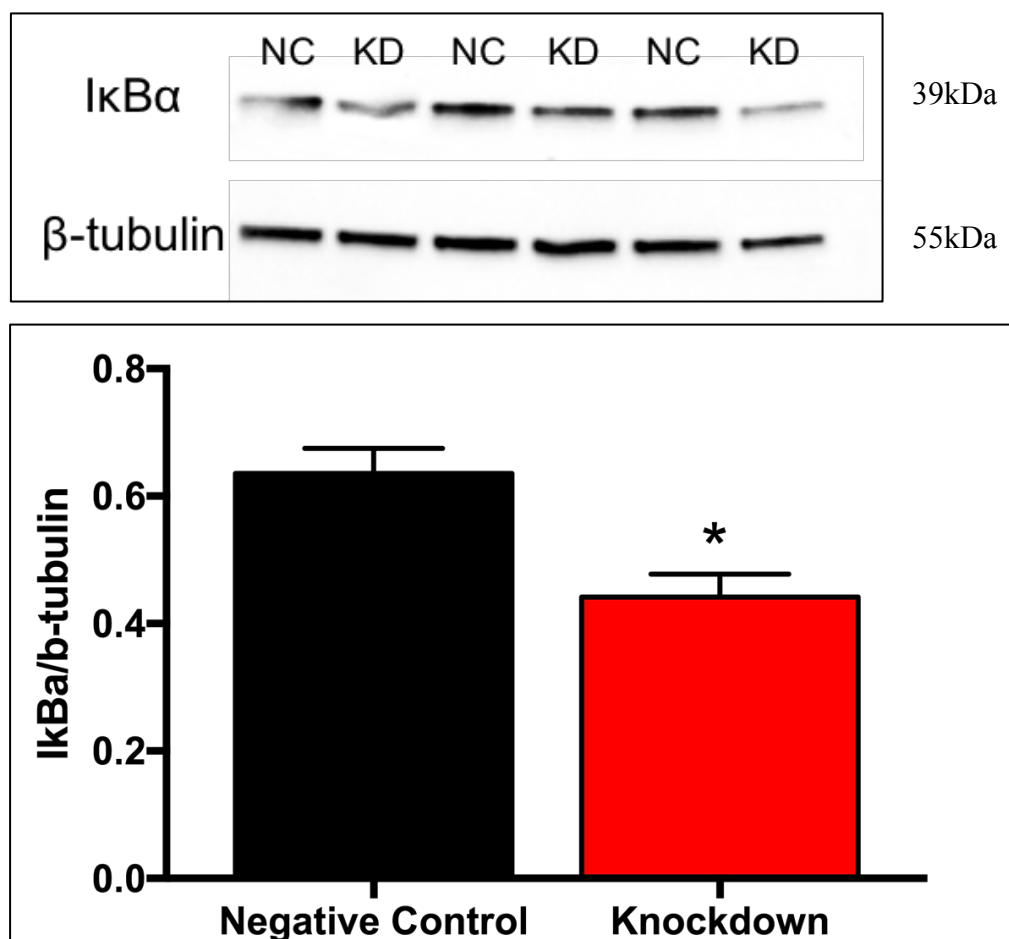


Figure 7.3.2-4 Protein expression levels of the endogenous inhibitor of NF- κ B, I κ B α , following AKR1D1 knockdown (KD) in Huh7 cells, compared to negative controls (NC). Data are expressed as mean $\pm$ se of n=3 experiments and quantified relative to β -tubulin. Representative western blots are shown. (* p <0.05, compared to negative control).

As in HepG2 cells, protein expression of JNK was not detected (data not shown).

Recent studies have proposed ER stress response to play a crucial role in both the development and progression of NASH (Puri et al. 2008; Rinella et al. 2011; Zhang et al. 2012). In addition to inflammatory response, AKR1D1 knockdown increased protein expression of IRE1 α in Huh7 cells, indicative of increased ER stress (Figure 7.3.2-5).

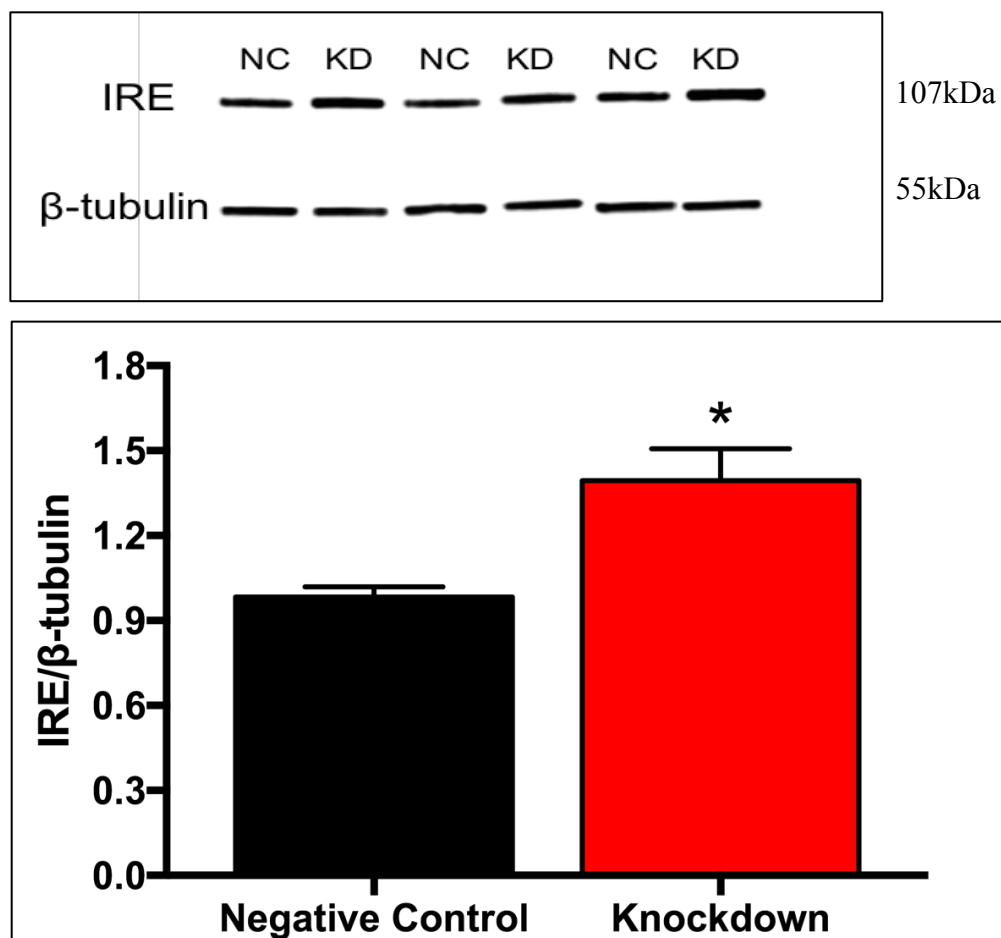


Figure 7.3.2-5 Protein expression levels of the ER stress marker IRE1 α , following AKR1D1 knockdown (KD) in Huh7 cells, compared to negative controls (NC). Data are expressed as mean $\pm$ se of n=3 experiments and quantified relative to β -tubulin. Representative western blots are shown. (* p <0.05, compared to negative control).

Additional western blotting experiments exploring the levels of another ER stress CHOP were performed, but no protein expression was observed (data not shown).

7.3.3. AKR1D1 over-expression does not regulate inflammation in Huh7 cells

To complement the knockdown studies, AKR1D1 was over-expressed in Huh7 cells using a pCMV6-XL4+AKR1D1 vector and compared with an empty vector (EV), as negative controls. Increased mRNA expression of AKR1D1 was confirmed using qPCR (Figure 7.3.3-1).

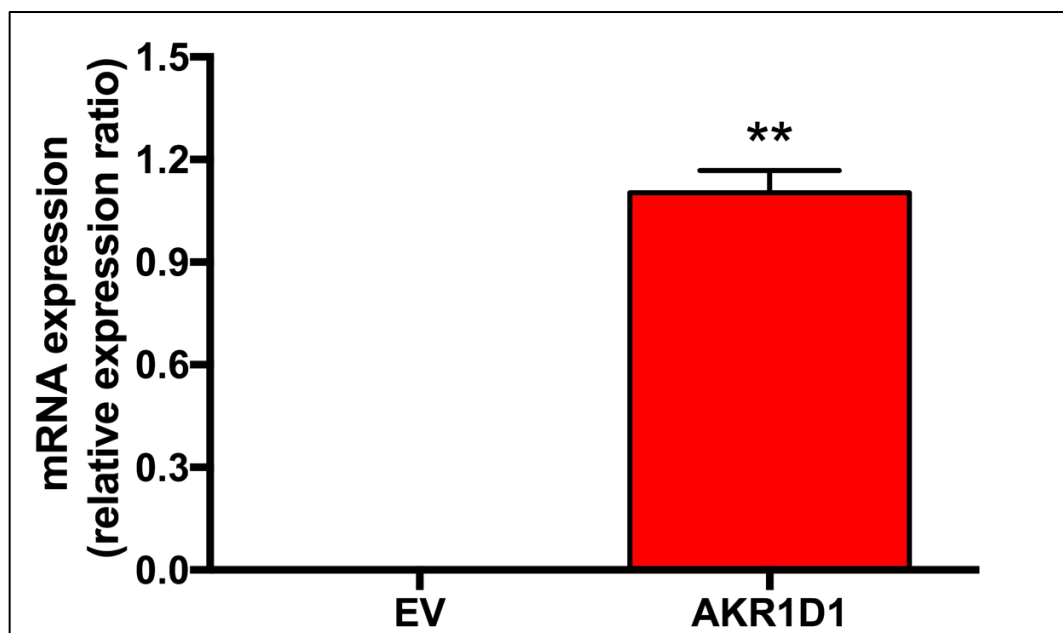


Figure 7.3.3-1 mRNA expression levels of AKR1D1 following over-expression in Huh7 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=3$ experiments (** $p<0.01$, compared to negative control).

In contrast to *AKR1D1* knockdown, *AKR1D1* over-expression had no effect on pro-inflammatory cytokine mRNA expression (Figure 7.3.3-2). Furthermore, no significant differences were observed in IL-8 secretion, consistent with the over-expression data in chapter 6 (Figure 7.3.3-3).

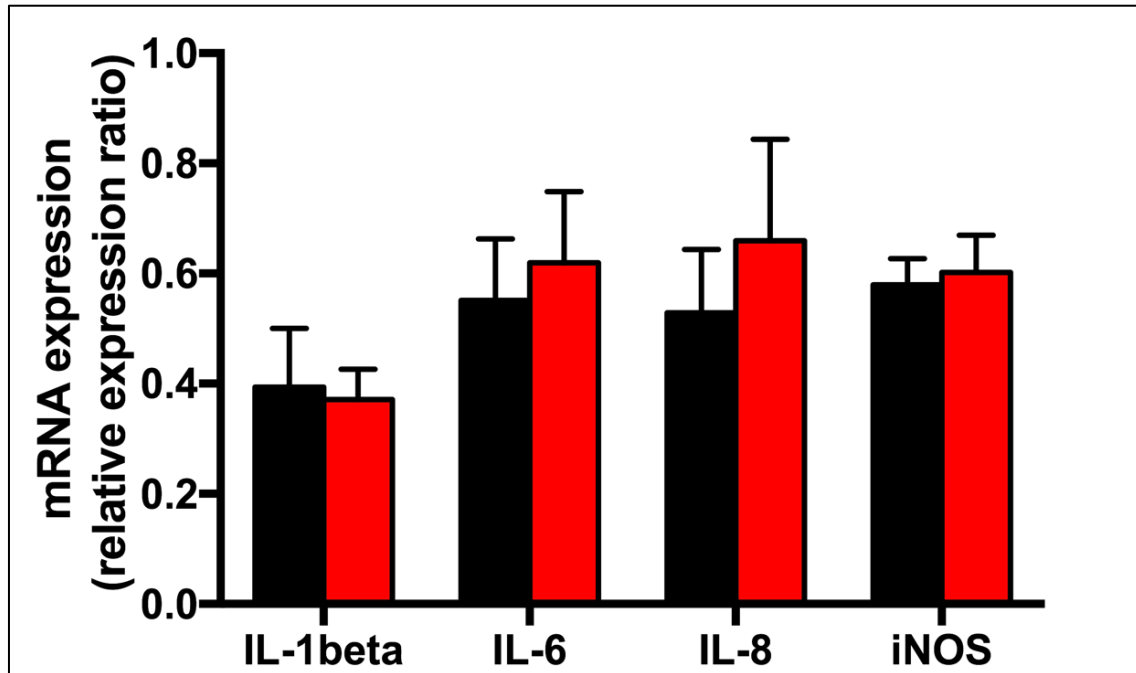


Figure 7.3.3-2 mRNA expression levels of IL-1 β , IL-6, IL-8 and iNOS, following AKR1D1 over-expression (red bars) in Huh7 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of n=5 experiments.

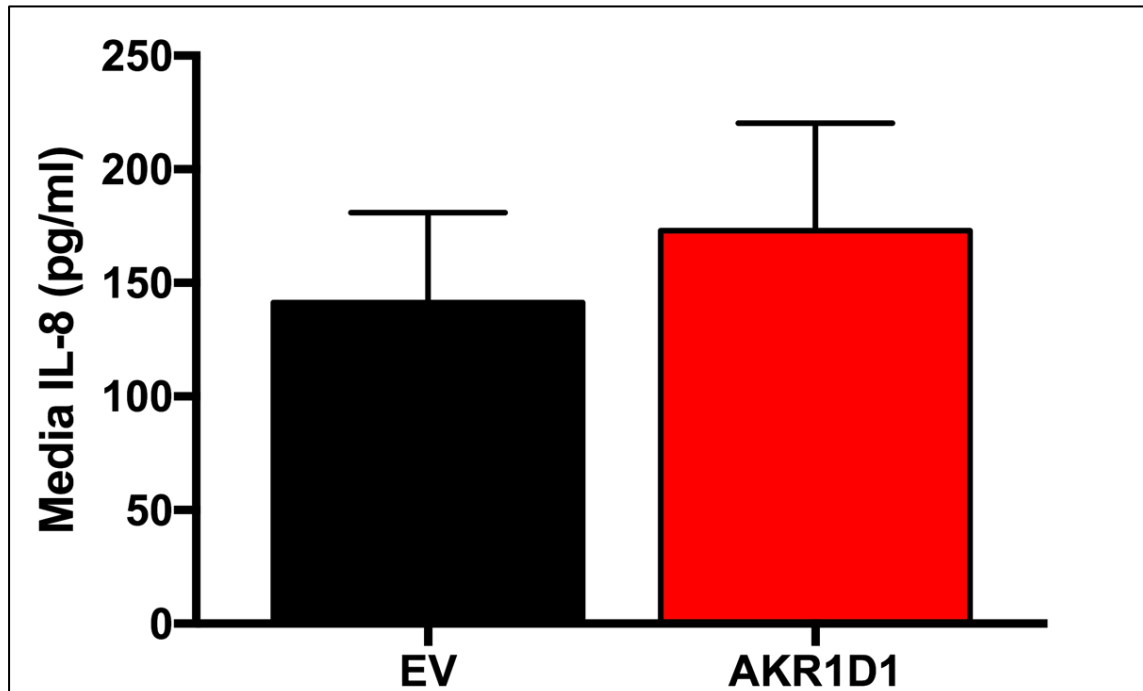


Figure 7.3.3-3 Media levels of IL-8 following AKR1D1 over-expression in Huh7 cells, as measured by ELISA. Data are expressed as mean $\pm$ se of n=3 experiments.

7.3.4. Impact of AKR1D1 knockdown on inflammatory response is not driven by glucocorticoids or FXR activation.

In order to try and identify the mechanisms that were responsible for the regulation of the inflammatory and ER stress response caused by AKR1D1 knockdown, Huh7 cells were treated with either cortisol (500nM, 24h) or GW4064 (5 μ M, 24h) and mRNA expression analysis was performed. GW4064 restored the expression levels of *IL-1 β* only, but had no impact on the induction of expression of *IL-6*, *IL-8* and *iNOS* caused by AKR1D1 knockdown (Figure 7.3.4-1).

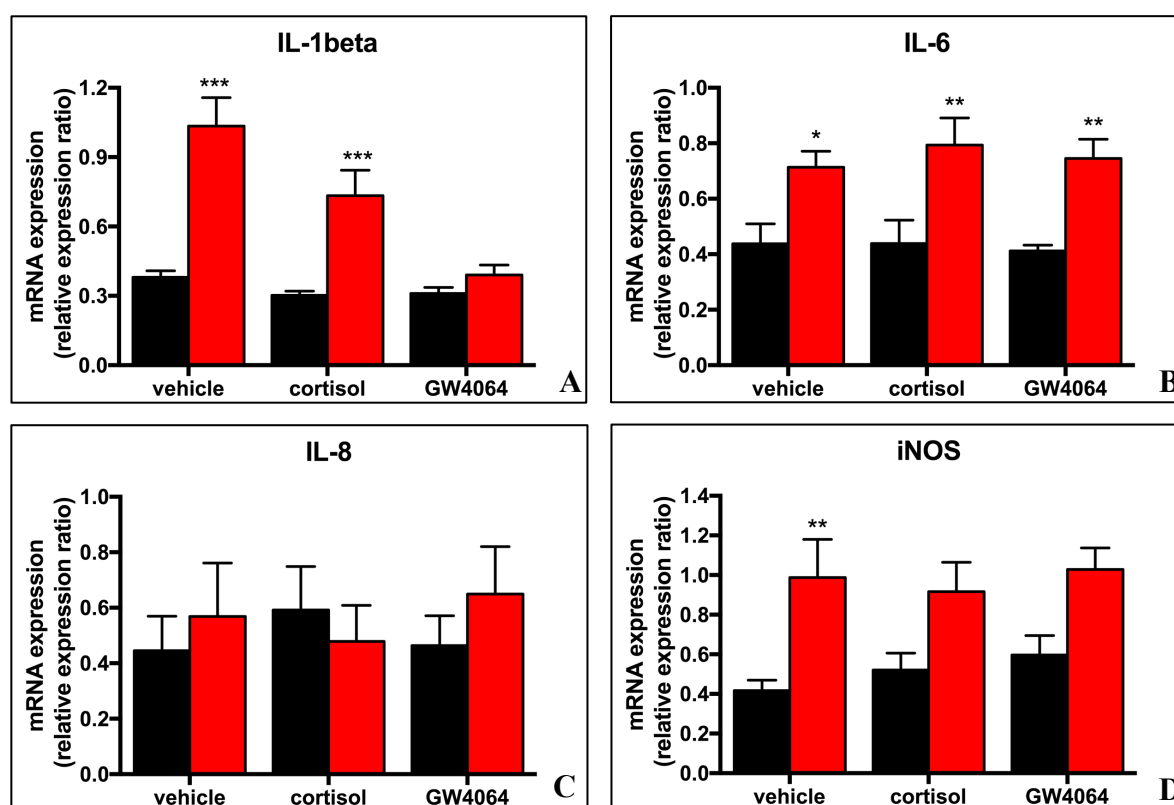


Figure 7.3.4-1 mRNA expression of (A). *IL-1 β* , (B). *IL-6*, (C). *IL-8* and (D). *iNOS* after treatment with 0.1% DMSO (vehicle), 500nM cortisol and 5 μ M GW4064, pre- and post- AKR1D1 knockdown (red bars), as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of n=4 experiments (* p <0.05, ** p <0.01, *** p <0.001, compared to negative control (black bars)).

In addition, HepG2 cells were knocked down, as previously described (section 5.2.2), and treated with the FXR agonist GW4064 (5 μ M, 24h), the LXR α antagonist 22-S hydroxycholesterol (22-S HC) (10 μ M, 24h) and with the LXR β antagonist GSK2033 (100nM, 24h).

FXR activation failed to restore mRNA expression and no differences in knocked down cells between vehicle and GW4064 were detected, suggesting that the inflammatory response is not FXR mediated. Importantly, qPCR analysis revealed increased expression of *IL-8* and *iNOS* after LXR α antagonism, indicative of the protective role of the LXR receptor in the development of hepatic inflammation. LXR β antagonism had no effect on either *IL-8* or *iNOS* expression (Figure 7.3.4-2).

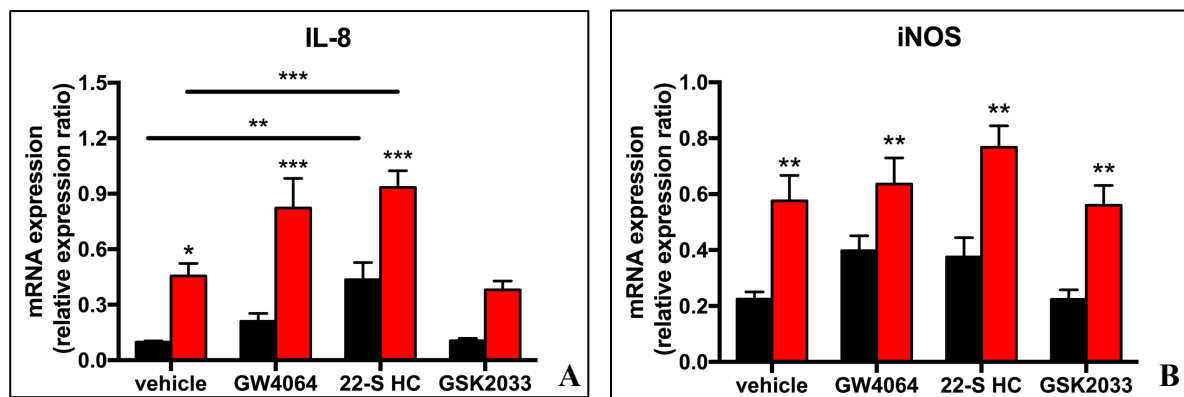


Figure 7.3.4-2 mRNA expression of (A). *IL-8* and (B). *iNOS*, following GW4064 (5 μ M), 22-S HC (10 μ M) and GSK2033 (100nM) treatment for 24h, pre- and post- *AKR1D1* knockdown (red bars) in HepG2 cells, as measured by qPCR. Data are expressed as relative expression ratio values $\pm$ se of $n=6$ experiments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to negative control (black bars)).

7.4. Discussion

I have demonstrated, for the first time, that manipulation of AKR1D1 expression and activity is also able to regulate inflammation and induce ER stress in human hepatocytes.

My data reveal that silencing of AKR1D1 in HepG2 cells led to increased IL-8 gene expression and secretion in the culture media. There is mounting evidence showing that IL-8 is elevated in patients with NASH, indicative of a potential role for this cytokine in the development of NAFLD (Kugelmas et al. 2003; Bahcecioglu et al. 2005). In chapter 6, I have shown that AKR1D1 knockdown led to increased palmitic acid synthesis in both the intracellular TAG and phospholipid fractions. Consistent with this, Joshi-Barve et al. (2007) demonstrated that treatment of HepG2 cells with pathologically high concentrations of palmitic acid increased IL-8 expression and secretion. IL-8 gene expression is also known to be regulated by NF- κ B, triggering JNK activation and I κ B α degradation in HepG2 and HEK293 cells (Wang et al. 2001; Joshi-Barve et al. 2007); my results did not show any regulation of JNK expression or I κ B α phosphorylation by AKR1D1 in HepG2 cells which may be due to phenotypic variation of this cell line. Alternatively, these findings may suggest an NF- κ B-independent impact on IL-8 expression (Kim et al. 2000).

AKR1D1 knockdown also increased inducible nitric oxide synthase (iNOS) expression in HepG2 cells. iNOS is known to play a crucial role in both metabolic disorders and immunity. Whilst *in vivo* experiments have revealed that small amounts of nitric oxide (NO) can have hepatoprotective effects, in particular during inflammation (Harbrecht et al. 1992; Harbrecht et al. 1994; Stanimirovic et al. 2015), chronic exposure or increased production of NO have cytotoxic effects and lead to liver injury through activation of NF- κ B (Nussler et al. 1995; Laszlo et al. 1995; Dallaire et al. 2008). In addition, since recent studies have demonstrated that increased lipid accumulation induces pro-inflammatory cytokine expression with

subsequent increase in iNOS expression in metabolic tissues (Charbonneau & Marette 2010; Perreault & Marette 2001; Fujimoto et al. 2005; Noronha et al. 2005), I would suggest that the increased expression in iNOS after AKR1D1 knockdown is the result of increased IL-8 expression caused by excessive lipid accumulation within AKR1D1 knocked down HepG2 cells compared to negative controls.

Extending my findings using Huh7 cells, I also observed increased pro-inflammatory IL-1 β , IL-8 and IL-8 cytokine mRNA expression and secretion as well as induced iNOS expression, after AKR1D1 knockdown. Although I did not measure intracellular TAG or fatty acid oxidation levels in this set of experiments, I would speculate, based on my findings in HepG2 cells, that increased lipid accumulation and impaired fatty acid oxidation due to AKR1D1 knockdown are responsible for the inflammatory response in Huh7 cells. A study from Chavez-Tapia et al. (2012) revealed that palmitic and oleic acid treatment on Huh7 cells resulted in increased intracellular fat accumulation with subsequent up-regulation of pro-inflammatory IL-6, IL-8 and TNF α cytokine mRNA expression and secretion. In contrast to HepG2 cells, in Huh7 cells, I observed activation of the NF- κ B signalling cascade, as measured by increased degradation of I κ B α , confirming previous changes in inflammatory pathways after fatty acid treatment (Maruyama et al. 2014).

AKR1D1 knockdown significantly increased IRE1 α protein expression in Huh7 cells, indicative of increased ER stress. The crucial role of the ER in cellular protein folding and trafficking is well known and failure to function properly results in unfolded protein accumulation, activation of the unfolded protein response (UPR) and finally ER stress (Hotamisligil 2010). Close examination of the ER stress and UPR pathways has revealed many connections between inflammation and stress signalling, including activation of the NF- κ B-IKK pathway (Hu et al. 2006), nitric oxide production (Gotoh & Mori 2006) and pro-inflammatory cytokine gene expression (Li et al. 2005). The relationship though, between ER

stress and inflammation is not likely to be one-sided and needs to be further explored; a study from Zhang et al. (2008) revealed that both ER stress and inflammation are able to activate each other, but the exact mechanisms underpinning these responses may depend on the cell type (Hotamisligil 2010). In addition, increased activation of the IRE1 α pathway by the UPR can also regulate hepatic lipogenesis and fatty acid oxidation (Basseri & Austin 2008). Whether these effects represent a protective response to ER stress or not, still needs to be investigated.

In chapter 5, I demonstrated that AKR1D1 knockdown altered BA synthesis and composition in both HepG2 and Huh7 cells. There is extensive literature on the role of BAs to drive inflammation and induce liver injury, cirrhosis and even hepatocellular carcinoma (Goldman et al. 1982; Trauner et al. 2008; Zhu et al. 2016). In HepG2 cells, as expected, AKR1D1 decreased total BA secretion into the cell culture media but, importantly, altered the BA pool composition with increased production of CDCA. A number of studies have highlighted the toxic effects of hydrophobic BAs, which, when accumulated, contribute to cholestatic liver injury (Higuchi & Gores 2003; Maillette de Buy Wenniger & Beuers 2010). Gong et al. (2016) have demonstrated that CDCA is an important mediator of NLRP3 inflammasome activation in macrophages, leading to cholestatic liver fibrosis. Since there is increasing evidence for induced pro-inflammatory cytokine expression in cholestatic livers (including IL-1 β , IL-6 and TNF α), it is plausible that the increased levels of CDCA in my HepG2 cells may have contributed to the increased IL-8 and iNOS expression, after AKR1D1 knockdown. In contrast to my findings in HepG2 cells, AKR1D1 knockdown decreased both CA and CDCA levels in Huh7 cells, indicative of lack to activate the alternative BA synthesis pathway, but also increased pro-inflammatory cytokine gene expression and ER stress. A significant number of studies have linked cholestasis and consequent liver inflammation and dysfunction (notably during infancy), with mutations in AKR1D1. It is thought that these

mutations lead to increased levels of BA precursors, *allo*-BAs (BAs produced via 5 α R activity) and low levels of CDCA (due to 27-hydroxylase activity and induction of the alternative pathway) (Drury et al. 2010; Lemonde et al. 2003; Palermo et al. 2008) that can then drive liver injury. A recent study from York et al. 2015 revealed that limiting cholesterol biosynthetic flux spontaneously induced type I interferon (IFN) production; although I did not measure differences in IFN expression or secretion, it is possible that the inflammatory phenotype observed in AKR1D1 knocked down hepatoma cells may be the result of disturbed cholesterol metabolism due to impaired BA synthesis.

There is increasing evidence on the role of FXR as a regulator of cellular inflammation and immune response; activation of the receptor induces the expression of anti-inflammatory genes (Zhao et al. 2003) as well as suppressing NF- κ B activation by negative regulation of inflammatory gene expression in the liver (Wang et al. 2008). The synthetic FXR agonist GW4064 has been extensively used as treatment for NAFLD, and has been reported to decrease hepatic inflammation, both *in vitro* and *in vivo*, by reducing the expression of pro-inflammatory cytokines (Yao et al. 2014; Ma et al. 2013). In my study, GW4064 treatment in HepG2 cells had no impact on the induction of expression of IL-8 or iNOS caused by AKR1D1 knockdown, suggesting that AKR1D1 knockdown increases inflammation via a NF- κ B independent pathway in HepG2 cells. Similar results were observed on the expression of IL-6 and IL-8 in Huh7 cells, where GW4064 treatment did not restore mRNA expression to levels seen with scrambled negative controls. Interestingly, the FXR agonist restored IL-1 β mRNA expression in Huh7 cells. This finding, in combination with the observed I κ Ba degradation caused by AKR1D1 knockdown, suggests that AKR1D1 regulates inflammatory response in a both NF- κ B-dependent and NF- κ B-independent action in Huh7 cells. In addition, the presence of another substrate able to drive the inflammatory phenotype in these

cells cannot be excluded. Similar to the findings in chapter 6, over-expression of AKR1D1 in Huh7 cells had no effect on pro-inflammatory gene expression.

In addition to FXR, there is accumulating evidence on the LXR receptors involvement in inflammatory signalling, especially in NAFLD. Numerous studies have described the anti-inflammatory effects of LXR activation in mice and in cultured macrophages leading to decreased expression of inflammatory mediators, such as IL-1 β , IL-6 and iNOS (Myhre et al. 2008; Joseph et al. 2003; Liu et al. 2012). In line with this, treatment of HepG2 cells with the LXR α antagonist 22-S hydroxycholesterol (22-S HC) aggravated inflammation, as observed by an increase in IL-8 and iNOS expression, and that was even enhanced after AKR1D1 knockdown. Treatment of HepG2 cells with the LXR β antagonist GSK2033 had no effect on inflammatory gene expression, indicating that the two LXR isoforms do not perform identical functions, not only in hepatic lipid metabolism, but also in inflammatory response in human hepatocytes.

In conclusion, I have demonstrated a novel role for AKR1D1 in human hepatocyte function and shown that manipulation of AKR1D1 expression and activity induces inflammation and ER stress within the liver. This may arise as a consequence, rather than as a result of increased lipid accumulation and decreased fatty acid oxidation, but also secondary to altered BA synthesis and secretion.

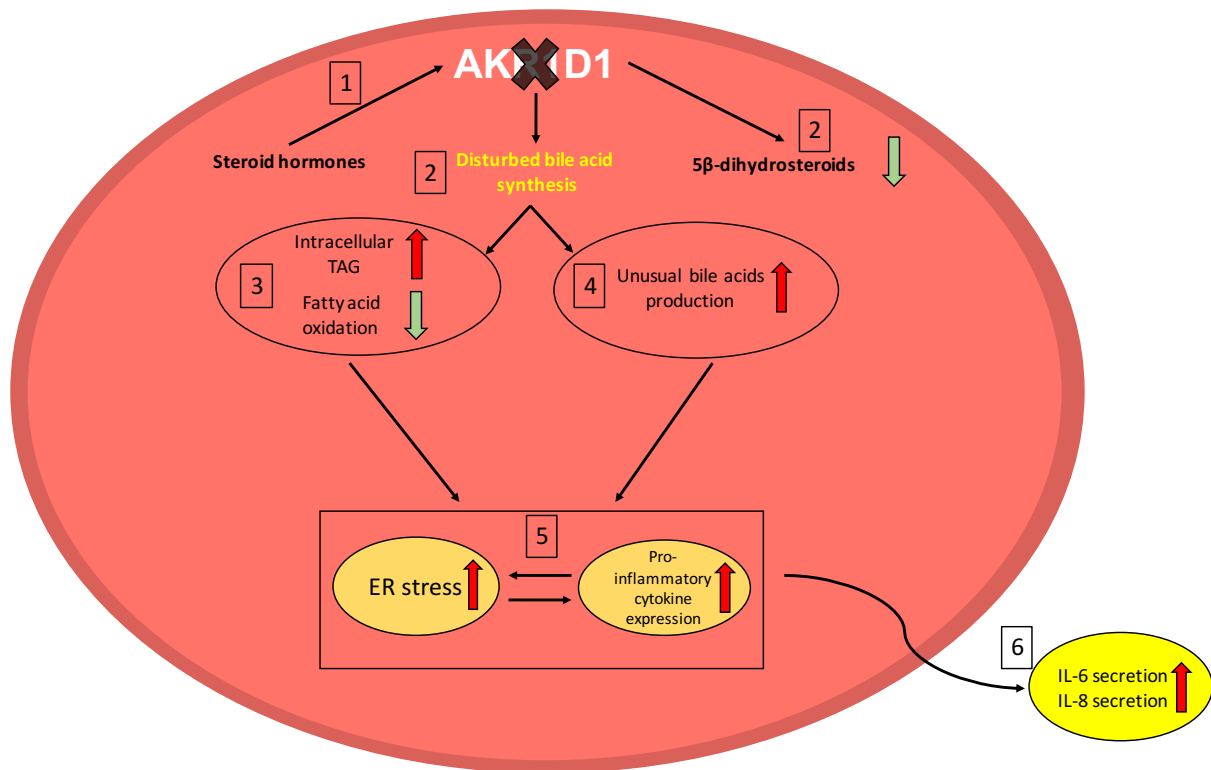


Figure 7.3.4-1 Proposed in vitro role of AKR1D1 knockdown in human hepatocytes. (1). AKR1D1 silencing resulted in (2). disturbed bile acid synthesis and decreased clearance of steroid hormones. Disturbed bile acid synthesis resulted in (3). Increased TAG accumulation, decreased fatty acid oxidation and (4). unusual bile acids production. The proposed mechanism suggests that increased TAG accumulation, unusual bile acids production or both resulted in: (5) increased ER stress and increased expression of pro-inflammatory cytokines, with a subsequent (6) increase in IL-6 and IL-8 secretion in the cell culture media.

Chapter 8 Final conclusions and future directions

8.1. Final conclusions

The rationale of my interest into the role of AKR1D1 in human hepatocytes was based upon the pathogenesis of NAFLD. Both steroid hormones and BAs have been implicated in NAFLD, therefore, considering the role of AKR1D1 in steroid hormone metabolism and BA formation, I hypothesised that this enzyme may represent a novel target for the study and treatment of metabolic liver disease. During my thesis, I characterised the enzymology of AKR1D1, I defined patterns of expression in human hepatocyte models and begun to define its role in cellular metabolism through genetic manipulation studies. I have demonstrated, for the first time that decreased AKR1D1 activity and expression limits glucocorticoid and BA availability and action in hepatic cell models. This has a profound impact on cellular phenotype, including increased intracellular lipid accumulation, impaired fatty acid oxidation and increased inflammation.

The ability of AKR1D1 to regulate cytosolic availability of a variety of steroid hormones in human hepatocytes indicates the presence of an intracellular complex system for steroid hormone metabolism; thus, the metabolic role of this enzyme is difficult to be elucidated. Access to functional cell-free models is, therefore, of importance, and these models can serve as clean platforms for the study of steroid hormone metabolism and could potentially explain discrepancies that might exist between cell-based, animal and human studies. In my work, I demonstrated the generation of human homogenous recombinant AKR1D1 protein that can metabolise steroid hormones and BA precursors as well as the development of a high-throughput fluorimetric screening assay, able to define the substrate specificity and the kinetic properties of AKR1D1.

To date, no specific pharmacological inhibitors for AKR1D1 are available. A previous study by Drury et al. (2009) reported that finasteride, a specific 5 α R2 inhibitor, partially inhibited

AKR1D1 activity and my data endorsed these findings. Finasteride is currently used for the treatment of benign prostatic hyperplasia and prostate cancer; therefore, the metabolic complications due to AKR1D1 inhibition should be now taken into consideration.

Through over-expression and knockdown experiments in liver and non-liver cell lines, I have shown that AKR1D1 represents a critical step in regulating the availability of glucocorticoids to bind and activate the GR. My findings also suggest that AKR1D1 expression may act as a protective mechanism against glucocorticoid excess by not only reducing cortisol within the liver, but also preventing generation of cortisol from cortisone. I have, therefore, demonstrated the important pre-receptor role of AKR1D1 and proved that this enzyme sits alongside other established pre-receptor enzymes, including the 11 β -HSD and the 5 α R isozymes. Genetic and pharmacological manipulations of these systems also alter steroid hormone availability in a tissue-specific manner that is independent of circulating steroid hormone levels. In addition, changes in activity and expression of 11 β -HSDs and 5 α R have been linked to the development of metabolic diseases including NAFLD, T2DM and IR (Ahmed et al. 2012; Tomlinson et al. 2008) Clinical studies using pharmacological inhibitors have shown improvements in clinical phenotype with selective 11 β -HSD1 inhibitors (Feig et al. 2011; Rosenstock et al. 2010; Shah et al. 2011); in contrast, the use of 5 α R inhibitors has detrimental effects, with decreased adipose tissue lipid mobilisation and increased hepatic lipid accumulation (Hazlehurst et al. 2016; Upreti et al. 2014), putatively through decreasing and increasing tissue-specific cortisol availability, respectively.

Manipulation of AKR1D1 expression was able to regulate activation of the GR in human hepatocytes. There is an emerging role for the GR in the regulation of carbohydrate metabolism; glucocorticoids perform their metabolic role through activation of the GR and

regulate the expression of major gluconeogenic enzymes such as PCK1, G6Pase and tyrosine aminotransferase (Moraitis et al. 2017). In line with this, hepatocyte specific GR knockout mice exhibit hypoglycemia after prolonged fasting, due to impaired gluconeogenic enzyme expression (Opherk et al. 2004). Furthermore, although there is compelling literature about the role of glucocorticoids in TAG synthesis and DNL in adipose tissue (Slavin et al. 1994; Gathercole et al. 2011; Xu et al. 2009b), their impact on human hepatic lipid metabolism has been less well characterised. A number of studies in rats and in primary cultures of rat hepatocytes have demonstrated increased activity of hepatic acetyl-CoA carboxylase (rate limiting step of DNL) and fatty acid synthase after glucocorticoid treatment (Diamant & Shafir 1975) as well as increased lipogenesis after glucocorticoid treatment in the presence of insulin (Amatruda et al. 1983). Consistent with this, cortisol treatment has been reported to increase lipogenesis following insulin stimulation in primary human hepatocytes (Nasiri et al. 2015). Moreover, decreased cortisol clearance via 5 α R1 deletion has also been associated with hepatic steatosis in mice (Joanna K. Dowman et al. 2013). These findings highlight, in total, the importance of GR activation in the regulation of hepatic carbohydrate and lipid metabolism, and abnormalities in AKR1D1 expression and activity can have subsequent effects in the activation of the GR. Further experiments using GR ChIP-Seq protocols could help to explore additional transcriptional targets of GR that may be affected by AKR1D1 activity and elucidate the role of this enzyme in glucocorticoid signalling.

In chapter 5 and 6, I showed that manipulation of expression and activity of AKR1D1 through gene silencing lead to TAG accumulation through a combination of increased fatty acid synthesis and decreased fatty acid oxidation, whilst changes in glucose transporter protein expression alongside alterations in the glycogen synthetic pathway lead to increased glycogen storage. In parallel, AKR1D1 silencing decreased total BA content and altered BA

composition. Plasma BAs have been associated with IR and T2DM (Cariou et al. 2011; Haeusler et al. 2013) and there is compelling evidence of their role in influencing NAFLD development and progression at multiple levels (Arab et al. 2016). BAs predominantly assess their metabolic actions through FXR activation, however, the role of FXR in the pathogenesis of metabolic disease and, in particular, NAFLD is still not fully understood. My data have shown that the changes in metabolic gene expression due to AKR1D1 knockdown are reversed by FXR activation, suggesting that this is the major pathway by which AKR1D1 is mediating its impact on metabolic phenotype in human hepatocytes.

If limiting FXR activation by AKR1D1 knockdown was the only mechanism responsible for our observations, some of our data would be in conflict with the published literature. Treatment of liver slices with OCA increased expression of both SLC51A and B (Ijssennagger et al. 2016), and this mirrors the observations that we made in our AKR1D1 knockdown experiments. A possible explanation for these findings may lie in analysis of the composition of the BA pool following AKR1D1 knockdown. The differing affinities of both primary and secondary BAs to activate (or antagonize) BA receptors are well described (Fiorucci & Distrutti 2015) and therefore this may offer an explanation as to why our observations are not rescued by simple FXR receptor agonism. Importantly, in patients with NAFLD, whilst total faecal primary BA levels are increased in comparison with healthy controls, there is a disproportionate increase in CDCA compared to CA (Mouzaki et al. 2016). Furthermore, human livers diagnosed as NASH demonstrated altered BA composition with a significant decrease in the levels of hydrophilic BAs, followed by increased hydrophobic BA production. In addition, mRNA expression analysis of genes implicated in BA synthesis revealed a shift from the classical to the alternative BA synthesis pathway highlighting, in total, the importance of BA metabolism in the development of NASH (Lake et al. 2013). Studies that have examined patterns of AKR1D1 expression and activity across

the histological spectrum of NALFD have not been performed, but are now clearly warranted.

There is an additional layer of complexity to the mechanisms that may underpin the role of AKR1D1 to regulate metabolic phenotype. FXR activation increases transcription of the glucocorticoid receptor (Renga et al. 2013) and the ability of FXR agonists to regulate gluconeogenic gene expression is dependent upon the glucocorticoid receptor (Renga et al. 2012). Therefore, reductions in AKR1D1 expression that would enhance glucocorticoid action through decreased cortisol clearance may have a relatively limited impact due to a concomitant reduction in the FXR-mediated increase in GR expression. Furthermore, there is an additional reciprocal interaction; glucocorticoids decrease FXR expression and its transcriptional activity (Lu et al. 2012; Rosales et al. 2013). Limiting AKR1D1 expression may therefore not only decrease FXR activation through decreased BA synthesis, but also through decreased receptor expression and activation as a consequence of increased glucocorticoid availability. The interactions *in vivo* are equally complex; CA supplementation to FXR null mice increased the concentration of urinary corticosterone and its metabolites (Cho et al. 2010). Mice treated with the FXR agonist, GW4064, have elevated circulating corticosterone (but not ACTH) levels suggesting an impact not only on adrenal steroidogenesis, but also on the sensitivity of the HPA axis (Hoekstra et al. 2012; Xing et al. 2009).

Inflammation and liver injury are also hallmarks in NAFLD/NASH; lipid accumulation within hepatocytes has also been proposed to trigger the activation of inflammatory pathways (Arrese et al. 2016). Further insight in NAFLD pathophysiology has implicated BAs in the prevalence of inflammation with increased generation of toxic hydrophobic BAs that result in cholestatic, inflamed livers. In this work, genetic manipulation of AKR1D1 expression and activity regulated the inflammatory response in human hepatocytes by enhancing the

expression and secretion of pro-inflammatory cytokines alongside increased intracellular ER stress. These results demonstrate, for the first time, a link between lipid accumulation and abnormal BA synthesis and highlight the importance of further investigation of this enzyme in the pathogenesis of the disease.

Although AKR1D1 is predominantly expressed in the liver, expression of this enzyme in the brain with subsequent formation of active 5β -dihydrosteroids involved in neurotransmission is also of interest. Considering my data and the role of AKR1D1 in hepatic carbohydrate and lipid metabolism, the potential of those active 5β -reduced steroids to act as appetite regulators cannot be excluded. It is also possible that hepatic and brain AKR1D1 expression and activity may act synergistically to control metabolic homeostasis; however, this role is entirely unexplored and should be elucidated.

In conclusion, I have shown that AKR1D1 is able to modulate glucocorticoid action and BA availability in human hepatocytes, and that decreased expression is associated with intracellular TAG, glycogen accumulation and inflammation. My data highlight the potential for AKR1D1 to have a significant role in the natural history and progression of NAFLD.

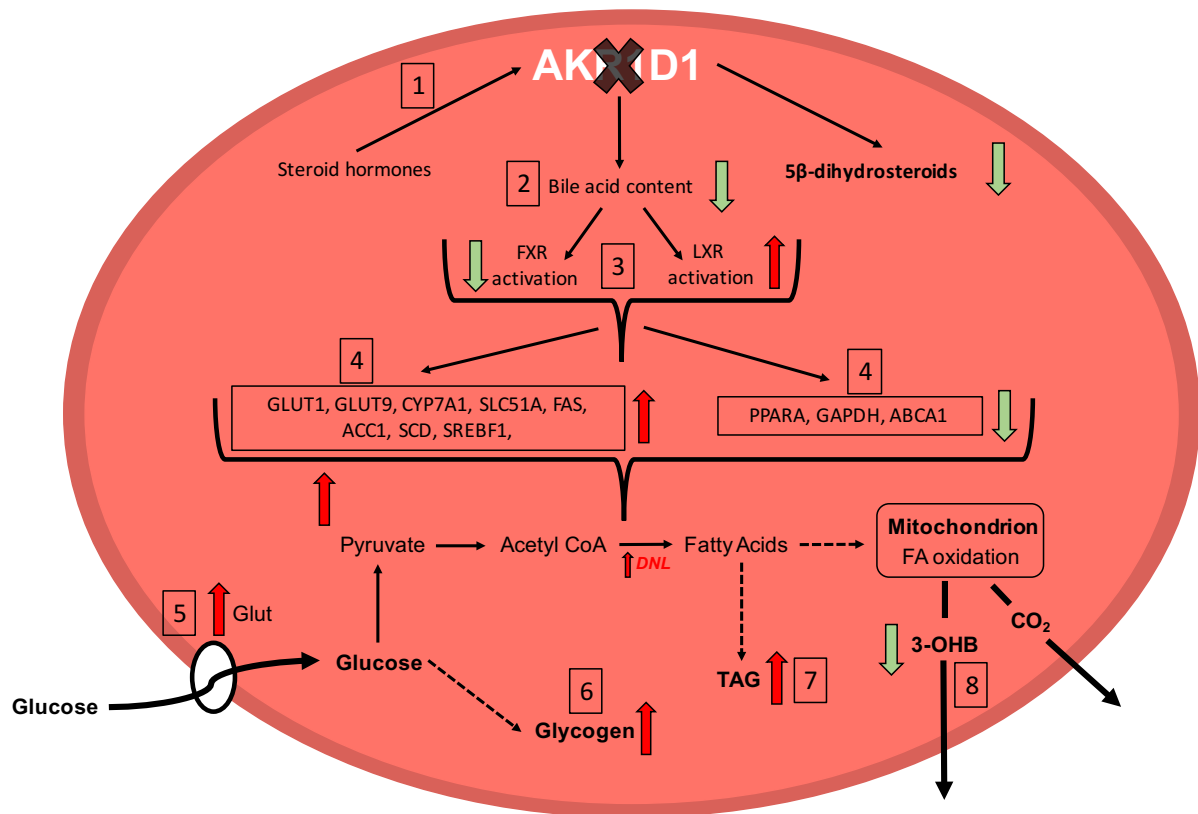


Figure 8.1-1 Proposed in vitro role of AKR1D1 in human hepatocytes. AKR1D1 silencing resulted in (1). decreased clearance of steroid hormones, and (2). decreased BA content and BA composition. (3). Lower BA content decreased activation of FXR with a parallel increase in the activation of LXR. (4). Alterations in the activation of the receptors FXR and LXR resulted in: increased mRNA expression of the glucose transporters GLUT1 and GLUT9, increased mRNA expression of the lipogenic genes FAS, ACC1, SCD and SREBF1, increased mRNA expression of the BA regulated genes CYP7A1 and SLC51A, and decreased expression of PPAR α , GAPDH and ABCA1. Subsequently, alterations in gene expression resulted in (5). increased glucose flux into the cells, (6). increased intracellular glycogen levels, (7). increased TAG generation and accumulation via DNL, and (8) decreased fatty acid oxidation, as measured by decreased 3OHB secretion in the cell culture media.

8.2. Future directions

8.2.1. AKR1D1 and insulin action

The work presented in this thesis has demonstrated important roles for AKR1D1 in carbohydrate and lipid metabolism within human hepatocytes. Although I have demonstrated that genetic manipulation of AKR1D1 is able to regulate glucose metabolism and drive DNL, it is of interest that all experimental studies have been performed in the absence of insulin. Insulin has a profound role in regulating hepatic glucose and lipid metabolism and data from the RNA sequencing analysis revealed significant differences in the expression of genes implicated in insulin signalling, including the insulin receptor substrate 1 (IRS1), AKT1, insulin receptor (INSR) and phosphoinositide-3-kinase regulatory subunit 1 (p85-alpha). Therefore, genetic manipulation of AKR1D1 in the presence of insulin and examination of the phosphorylation of known protein targets within the insulin signalling cascade, including AKT, are warranted; these assays will give further insight in the role of this enzyme in hepatic carbohydrate and lipid metabolism.

8.2.2. AKR1D1 regulation

In chapters 5 and 6, I demonstrated the ability of AKR1D1 to regulate steroid hormone availability, BA formation and carbohydrate and lipid metabolism. Whilst preliminary data from cortisol treatment studies did not reveal regulation of AKR1D1 expression by glucocorticoids, it would be of interest to explore the potential of other factors to regulate AKR1D1 expression and activity, including more steroid hormones (e.g. testosterone, androstenedione, aldosterone), fatty acids, BAs and glucose. Furthermore, as I demonstrated in chapter 7, AKR1D1 can drive inflammation and intracellular stress, therefore, treatment of human hepatocytes with pro-inflammatory cytokines could also be performed to investigate their potential role in the regulation of AKR1D1.

As previously mentioned, a number of studies have reported changes in the expression of pre-receptor enzymes across the NAFLD spectrum, including 11 β -HSD and 5 α R (Ahmed et al. 2012). Similarly, liver biopsies and urinary metabolite measurements could also help to explore potential differences in the expression and activity of AKR1D1 during the different stages of NAFLD.

8.2.3. AKR1D1 inhibitors

In chapter 4, I demonstrated the generation of an active and homogenous human recombinant AKR1D1 protein that can metabolise both steroid hormones and BA precursors. In addition, I showed that the 5 α R inhibitors finasteride and dutasteride are not able to block AKR1D1 activity, confirming previous attempts which failed to identify specific AKR1D1 inhibitors. Considering the role of AKR1D1 in regulating carbohydrate and lipid metabolism, it is evident that the development of a specific pharmacological inhibitor that could mimic the effects of AKR1D1 silencing is necessary. However, as this enzyme plays a crucial role in BA synthesis and decreased AKR1D1 activity has been linked to cholestatic livers in humans, the possible negative effects of AKR1D1 activity cannot be excluded. In collaboration with the European Lead Factory (ELF), a collaborative public-private partnership, 500,000 novel small molecules will be screened against our human recombinant AKR1D1 protein. The most potent molecules will be tested as potential *in vitro* inhibitors or allosteric activators of AKR1D1 using human hepatoma cell lines, including HepG2 and Huh7 cells. Upon characterisation of those inhibitors / activators, studies using healthy primary human hepatocytes, which represent the gold-standard for *in vitro* metabolic studies, could be performed to identify the exact role of AKR1D1 in hepatic carbohydrate and lipid metabolism. In addition, and in parallel with the already existing AKR1D1-KO animal model from our group, those compounds could be then used in animal studies. Although not

published, recent data from the AKR1D1-KO mouse model reveal similar findings to those observed in human hepatocytes, with decreased total BA content, alterations in BA composition and increased serum triglyceride levels in the KO animals, compared to wild type.

8.2.4. Cell cycle and proliferation

There is extensive literature on the protective role of FXR against hepatocellular carcinoma (HCC) (Guo et al. 2015; Yang et al. 2007; Liu et al. 2012; Su et al. 2012; Jiang et al. 2013; Degirolamo et al. 2015; Kim et al. 2007; Zhang et al. 2012). A study by Granata et al. (2009) has also reported decreased 5 β -reductase activity in cells derived from HCC cells compared to healthy liver tissue samples. During this thesis, I demonstrated that AKR1D1 knockdown resulted in impaired activation of FXR, possibly due to alterations in BA content and composition. Moreover, data from the RNA sequencing analysis between negative control and AKR1D1 knocked down cells revealed significant differences in genes implicated in cell cycle. Therefore, it would be of interest to explore the *in vitro* role of AKR1D1 in cell cycle and proliferation and investigate the potential of the expression of this enzyme to act as a biomarker in HCC. Stable AKR1D1 knockdown models as well as deletion of *AKR1D1* using CRISPR/Cas9 technology, followed by long term treatments, and could serve as a novel platform to elucidate this role.

8.2.5. BA composition and inflammation

Although I have demonstrated that AKR1D1 silencing decreased total BA content and altered BA composition using ELISA assays, there were some limitations, including increased cross-reactivity; in addition, I was not able to determine the appearance of BA intermediates and *allo*-BAs (5 α -reduced BAs) in cell culture media samples. Further experiments using LC/MS

technology should be therefore performed to explore the role of AKR1D1 in BA synthesis and composition within human hepatocytes. Moreover, LC/MS analysis of cell culture media samples from primary hepatocyte cultures, after treatment with AKR1D1 inhibitors and/or activators, should be also assessed to identify differences in BA synthesis between healthy primary hepatocytes and hepatoma cells. Considering the elevated expression and secretion levels of the pro-inflammatory cytokines IL-1 β , IL-6 and IL-8 in human hepatoma cells, it would be of interest to explore the role of AKR1D1 in cell types that produce much higher quantities of these cytokines, e.g. macrophages, dendritic cells or lymphocytes. The role of this enzyme in these cells is unknown and It would also be interesting to explore whether genetic manipulation of AKR1D1 alters the response to inflammation.

Chapter 9 References

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Chapter 10 Publications

ORIGINAL RESEARCH

Optimizing human hepatocyte models for metabolic phenotype and function: effects of treatment with dimethyl sulfoxide (DMSO)

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Abstract

Primary human hepatocytes are considered to be the “gold standard” cellular model for studying hepatic fatty acid and glucose metabolism; however, they come with limitations. Although the HepG2 cell line retains many of the primary hepatocyte metabolic functions they have a malignant origin and low rates of triglyceride secretion. The aim of this study was to investigate whether dimethyl sulfoxide supplementation in the media of HepG2 cells would enhance metabolic functionality leading to the development of an improved in vitro cell model that closely recapitulates primary human hepatocyte metabolism. HepG2 cells were cultured in media containing 1% dimethyl sulfoxide for 2, 4, 7, 14, and 21 days. Gene expression, protein levels, intracellular triglyceride, and media concentrations of triglyceride, urea, and 3-hydroxybutyrate concentrations were measured. Dimethyl sulfoxide treatment altered the expression of genes involved in lipid (FAS, ACC1, ACC2, DGAT1, DGAT2, SCD) and glucose (PEPCK, G6Pase) metabolism as well as liver functionality (albumin, alpha-1-antitrypsin, AFP). mRNA changes were paralleled by alterations at the protein level. DMSO treatment decreased intracellular triglyceride content and lactate production and increased triglyceride and 3-hydroxybutyrate concentrations in the media in a time-dependent manner. We have demonstrated that the addition of 1% dimethyl sulfoxide to culture media changes the metabolic phenotype of HepG2 cells toward a more primary human hepatocyte phenotype. This will enhance the currently available in vitro model systems for the study of hepatocyte biology related to pathological processes that contribute to disease and their response to specific therapeutic interventions.

Introduction

The liver is the major metabolic visceral organ in the human body and performs over 500 different functions (Naruse et al. 2007) including bile and albumin production and secretion, foreign and metabolic poison detoxification, and metabolism of lipids, carbohydrates, and vitamins (Sherlock 1978). Diseases of the liver are increasing in prevalence; for example, nonalcoholic fatty liver disease (NAFLD), which encompasses a spectrum ranging from simple steatosis to fibrosis, cirrhosis, and

hepatocellular carcinoma (Trauner et al. 2010) is now one of the most common liver diseases in Western countries (de Alwis and Day 2008). Investigating the mechanisms that underpin the development and progression of NAFLD, as well as potential treatments, requires in vitro cellular models that mimic human hepatocyte metabolism and functionality.

The liver comprised parenchymal (hepatocytes) and nonparenchymal cells (including Kupffer cells, hepatic stellate cells), with hepatocytes contributing approximately 80% of cellular liver mass. Primary human

hepatocytes are therefore the ideal *in vitro* cellular model to investigate aspects of liver function and metabolism as they most closely recapitulate the *in vivo* liver (Green et al. 2015). Although human primary hepatocytes are considered the gold standard, there are significant challenges in their use, such as availability of cells, donor variability, inability to proliferate, and a short life span (Green et al. 2015). Therefore, a variety of human hepatoma cell lines have been generated to investigate human hepatocyte metabolism and function, including the HepG2, Hep3b, and Huh7.0 cell lines. The HepG2 cell line has been extensively used in cell-based metabolic studies; however, because of their malignant origin, they tend to present with an abnormal metabolic phenotype compared to human primary cells. As an example, HepG2 cells have increased rates of *de novo* lipogenesis and glycolysis (Pereira da Silva et al. 2009; Daniels et al. 2014).

The amphipathic molecule dimethyl sulfoxide (DMSO) has multiple effects on cellular function (Santos et al. 2003; Pal et al. 2012). A number of previous studies have examined whether DMSO treatment alters hepatoma cell line functionality. However, no study published to date has examined in detail the impact of DMSO treatment of carbohydrate and lipid metabolism. DMSO treatment of HepG2 and Huh7.0 cells has been reported to enhance drug metabolism (Choi et al. 2009) and reduce lipid accumulation (Song et al. 2012), although the mechanisms underpinning these observations remain to be elucidated. Moreover, it remains unclear whether DMSO treatment of hepatoma cell lines affects carbohydrate metabolism. The aim of this study was therefore to determine whether DMSO treatment could enhance HepG2 cell metabolic functionality leading to the development of a novel and improved *in vitro* model that may more closely resembles primary human hepatocytes.

Methods and Materials

C3A and HepG2 cell culture and treatments

HepG2 cells were a gift from Dr. Karl Morten (University of Oxford) and were cultured in Dulbecco's minimum essential medium (DMEM) (Zen Bio Inc., Durham, NC), containing 4.5 g/L glucose, and supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% non-essential amino acids. The C3A human hepatocyte cell line was purchased from LGC Standards (ATCC – CRL-10741, Middlesex, UK), and cultured in Eagle's minimum essential medium (EMEM) (Sigma-Aldrich, Dorset, UK) containing 4.5 g/L glucose, and supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% nonessential amino acids. For all treatments, cells were seeded in six-well Cell Bind plates (Appleton Woods,

Birmingham, UK) at a cell density of 3.5×10^5 cells per well. At 90% confluence, culture medium was replaced with 2 mL complete medium containing 1% DMSO (Sigma-Aldrich, Dorset, UK). Based on a previously described protocol (Sainz and Chisari 2006), cultures were incubated for 2, 4, 7, 14, and 21 days, during which time complete DMSO-containing medium was replenished every 2 days. Non-DMSO-treated cells were also seeded in six-well Cell Bind plates at the same cell density until 90% confluence (approximately 5 days), then medium was replenished for 2 days and samples were collected. An important methodological consideration is that it is not possible to have control cells that were "time matched" in non-DMSO-supplemented media. Both HepG2 and C3A cells will continue to proliferate and it is well established that there is increased spontaneous cell loss in the postconfluent stage (Hosick 1976). We observed increased cell death in HepG2 cells after 9–10 days in cell culture without passage. For biochemical measurements using media, cells were cultured in complete media without phenol red $\pm$ DMSO for 2, 4, 7, 14, and 21 days.

Primary human hepatocytes

Liver was obtained from patients undergoing surgery who had consented (NRES Committee South Central; Berkshire B 11/SC/0443) to the use of excess tissue (resection surplus) for research. Hepatocytes were dissociated from whole liver tissue via a two-stage collagenase digestion as described previously by Green et al. (2015). Cells were diluted to 1 million cells per mL in William's E buffer containing hepatocyte supplements before plating on Type I collagen coated plates. Cells were collected 24 h postseeding for RNA isolation.

Fatty acid treatments

In media lacking phenol red, cells were treated with 0.25% fatty acid-free BSA alone or conjugated to 45 μ mol/L oleic, 30 μ mol/L palmitic, and 25 μ mol/L linoleic acid (OPL; physiological ratio 45:30:25%) for 24 or 48 h (Green et al. 2015).

RNA extraction and reverse transcription

Total RNA was extracted from cells using the Tri-Reagent system (Sigma-Aldrich, Dorset, UK). Concentration was determined spectrophotometrically at OD260 on a Nanodrop spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). In a 20- μ L volume, 1 μ g of total RNA was incubated with 10 $\times$ RT buffer, 100 mmol/L dNTP mix, 10 $\times$ RT random primers, 50 U/ μ L MultiScribe reverse transcriptase, and 20 U/ μ L RNase inhibitor. The

reverse transcription was carried out at 25°C for 10 min, 37°C for 120 min, and then the reaction was terminated by heating to 85°C for 5 min.

Real-time PCR

All real-time PCR experiments were conducted using an ABI 7900HT sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). Reactions were performed in 6 μ L volumes on 384-well plates in reaction buffer containing 3 μ L of 2 $\times$ KlearKall Master Mix with standard ROX (LGC Genomics, Middlesex, UK). Life Technologies supplied all primers and reactions normalized against the housekeeping gene GAPDH, provided as a pre-optimized control probe. All target genes were labeled with FAM. The reaction conditions were as follows: 95°C for

10 min, then 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The Ct (dCt) of each sample using the following calculation (where E is reaction efficiency – determined from a standard curve): $\Delta Ct = E^{[min\ Ct - sample\ Ct]}$ (Pfaffl 2001), using the 1/40 dilution from a standard curve generated from a pool of all cDNAs as the calibrator for all samples. The relative expression ratio was calculated using the following: $Ratio = \Delta Ct_{[target]} / \Delta Ct_{[ref]}$ (Pfaffl 2001) and expression values were normalized to 18 sec.

Protein extraction and immunoblotting

Total protein was extracted from cells using RIPA buffer (150 mmol/L NaCl, 1.0% IGEPAL[®] CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris, pH 8.0) (Sigma-Aldrich, Dorset, UK), and protease

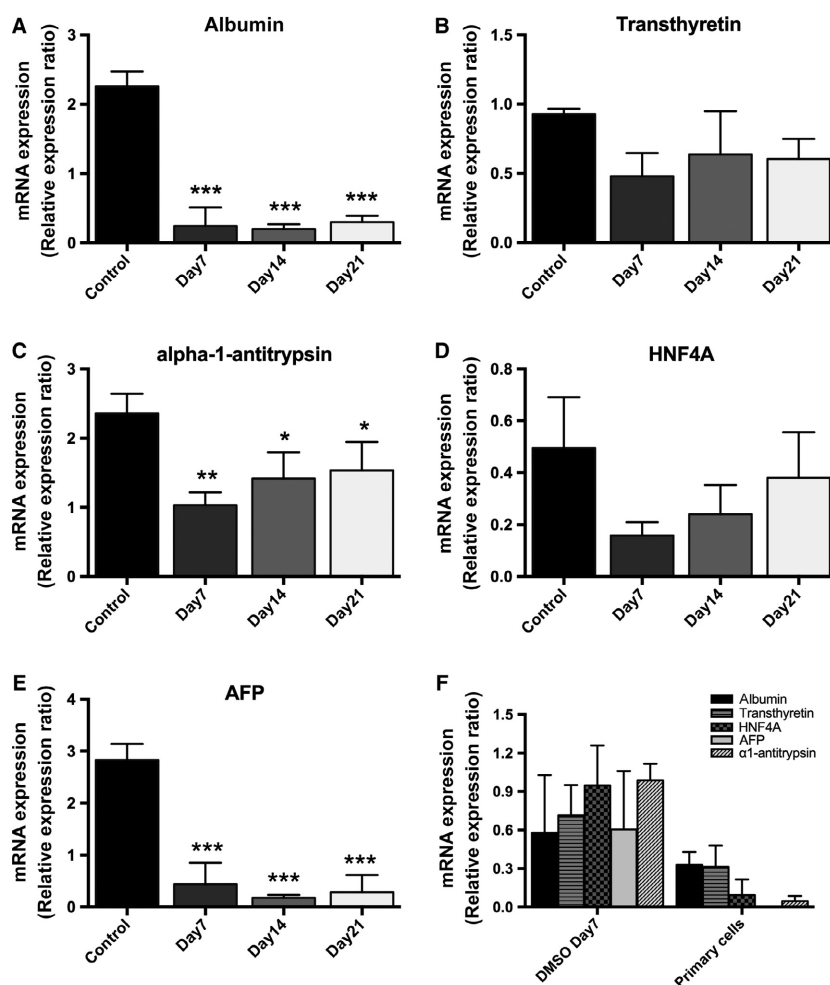


Figure 1. DMSO treatment causes a time-dependent change in mRNA expression of liver function markers albumin, transthyretin, alpha-1-antitrypsin, HNF4A, and AFP in HepG2 cells (A–E). Furthermore, DMSO treatment of HepG2 cells for 7 days reduces the mRNA expression of these markers to levels that are similar to those seen in primary human hepatocytes (F). Data presented are mean $\pm$ SD of $n = 3$ experiments performed in triplicate, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, control versus day of DMSO treatment. The relative expression ratio was calculated using the following: $Ratio = \Delta Ct_{[target]} / \Delta Ct_{[ref]}$ (Pfaffl 2001) and expression values were normalized to 18 sec.

Table 1. The impact of DMSO to modulate gene expression as measured by qPCR in human hepatoma C3A cells.

C3A	Control	Day 7	Day 14	Day 21
Albumin	3.59 ± 0.66	1.04 ± 0.17***	0.42 ± 0.03***	0.77 ± 0.1***
α1-Antitrypsin	2.18 ± 0.25	1.29 ± 0.13***	1.51 ± 0.12**	2.19 ± 0.21
Transthyretin	2.86 ± 0.49	1.39 ± 0.13***	0.93 ± 0.1***	1.31 ± 0.26***
HNFA4A	0.98 ± 0.25	0.45 ± 0.12*	0.44 ± 0.12*	0.8 ± 0.24
AFP	4.08 ± 0.17	1.58 ± 0.08***	0.33 ± 0.04***	0.51 ± 0.1***
FAS	3.98 ± 0.73	1.63 ± 0.45**	1.76 ± 0.17**	2.7 ± 0.78
ACC1	5.44 ± 1.37	2.06 ± 0.43**	1.31 ± 0.17***	2.08 ± 0.27**
ACC2	2.6 ± 0.43	1.82 ± 0.46*	1.54 ± 0.09**	2.21 ± 0.08
SCD	2.04 ± 0.34	1.75 ± 0.3	1.01 ± 0.09*	2.04 ± 0.45
DGAT1	4.77 ± 0.5	1.8 ± 0.32***	1.66 ± 0.27***	2.14 ± 0.06***
DGAT2	3.05 ± 0.56	0.82 ± 0.15***	0.47 ± 0.04***	0.88 ± 0.27***
PEPCK	0.01 ± 0.002	0.19 ± 0.03*	0.31 ± 0.04**	0.88 ± 0.14***
G6PC	0.03 ± 0.01	0.03 ± 0.006	0.08 ± 0.009**	0.12 ± 0.03***

Data are presented as mean ± SD of relative expression ratio, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The relative expression ratio was calculated using the following: Ratio = $\Delta Ct_{\text{target}} / \Delta Ct_{\text{ref}}$ (Pfaffl 2001) and expression values were normalized to 18 sec.

inhibitor cocktail (ThermoFisher Scientific, Loughborough, UK). Protein concentrations were measured using a commercially available assay (Bio-Rad Laboratories Inc., Hercules, CA). Protein levels were determined using the Bio-Rad assay kit, according to the manufacturer's protocol (Bio-Rad, Hemel Hempstead, UK). Primary (G6PC [Abcam plc, Cambridge, UK], PEPCK, IRE1, CHOP, β -tubulin [Cell Signalling, Danvers]) and secondary antibodies from Dako were used at a dilution of 1/1000 and 1/2000, respectively. To ascertain equal gel loading, proteins were normalized to β -tubulin. Bands were visualized with ECL (Pierce Thermo Fisher Scientific) and Chemi-DocXS imager (Bio-Rad). Western blots were quantified by densitometry analysis using ImageJ (NIH, Bethesda, MD, <http://rsb.info.nih.gov/ij>).

Oil red O staining

Cells were washed twice in PBS and fixed using 10% formalin for 1 h. Cells were then washed in 60% isopropanol and left to dry. Staining was carried out by adding Oil red O (6 mmol/L) (Sigma-Aldrich, Dorset, UK) for 15 min at room temperature. Cells were then counterstained using hematoxylin and eosin and mounted onto slides using aqua mount.

Urea, triglyceride (TG), and 3-hydroxybutyrate (3OHB) concentrations

Urea and TG (intracellular and media) and 3OHB were analyzed using Instrumentation Laboratory kits on an ILab 650 Clinical Chemistry analyzer as described previously (Green et al. 2015; McNeil et al. 2014). Complete medium-containing FBS or lysis buffer were used as background controls.

Statistical analysis

For multiple comparisons, statistical analysis was performed using ordinary one-way analysis of variance (ANOVA) and control versus different time points were compared. Statistical analysis on qPCR data was performed on mean relative expression ratio values (Ratio = $\Delta Ct_{\text{target}} / \Delta Ct_{\text{ref}}$) (Pfaffl 2001). Data analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla) and considered statistically significant at $P < 0.05$.

Results

DMSO treatment of human hepatoma cell lines alters the expression of liver function markers

Expression levels of four hepatic function markers (albumin, HNF4A, transthyretin, and alpha-1-antitrypsin) were measured in confluent untreated and DMSO-treated HepG2 and C3A cells. mRNA expression levels of many genes were higher in the untreated compared to DMSO-treated HepG2 cells (Fig. 1A–D). Importantly, mRNA expression of the tumor marker alpha-fetoprotein (AFP) decreased after DMSO treatment, with a significant decrease observed after 7 days that persisted up to 21 days of treatment (Fig. 1E). Urea secretion, a measure of hepatic cell function, was not compromised by DMSO treatment in HepG2 cells (0.037 ± 0.01 [control] vs. 0.023 ± 0.007 [day 7], 0.039 ± 0.01 [day 14], 0.036 ± 0.02 $\mu\text{mol/mg}$ protein [day 21]). To further confirm the effect of DMSO on human hepatoma cell lines, C3A cells were also used as an additional cellular model. The C3A cell line is a derivative of the

HepG2 cell line and has also used for a number of metabolic studies (Iyer *et al.* 2010; Nasiri *et al.* 2015). Similar observations were made in C3A cells treated with DMSO and absolute changes in mRNA expression levels are presented in Table 1.

In order to ascertain if DMSO treatment of HepG2 cells induced a phenotype more reflective of primary human hepatocytes, the mRNA expression profile of liver function markers at day 7 of DMSO treatment was compared with that of primary cultures of human hepatocytes. At day 7, DMSO-treated cells had reached 100% confluence and demonstrated decreased proliferation rate. DMSO treatment (7 days) reduced mRNA levels of liver function makers to levels similar to those seen in primary human hepatocytes (Fig. 1F).

DMSO treatment of HepG2 cells decreases the expression of ER stress markers

ER stress has the potential to influence hepatocyte functionality and metabolism and therefore markers of convergent ER stress pathways were measured across the time course of the experimental protocol. IRE-1 protein expression decreased after 7 days of DMSO treatment, while there was no significant change in CHOP protein levels (Fig. 2A–C).

DMSO treatment changes the lipid metabolic phenotype of HepG2 cells

The fatty acid synthetic and oxidation genes, acetyl-CoA carboxylase alpha (ACC1), acetyl-CoA carboxylase beta (ACC2), diacylglycerol O-acyltransferase 1 (DGAT1), diacylglycerol O-acyltransferase 2 (DGAT2), fatty acid synthase (FAS), and stearoyl-coA desaturase (SCD) mRNA expression were significantly decreased in HepG2 cells after DMSO treatment in a time-dependent manner (Fig. 3A–F). Observations were similar in C3A human hepatoma cells (Table 1).

Fatty acids within the hepatocyte can be partitioned into esterification (to synthesize TG) or oxidation (resulting in the generation of 3OHB) pathways. Compared with untreated control cells, DMSO-treated HepG2 cells had significantly lower concentrations of intracellular TG at all time points (209.7 ± 17 [control] vs. 95.7 ± 21.4 [day 7], 93.0 ± 16.9 [day 14], and 93.7 ± 12.3 [day 21] nmol/mg protein; $P < 0.05$) (Fig. 4A). These observations were mirrored by a decrease in intracellular neutral lipid (TG) staining with oil red O, after 7 days of DMSO treatment (Fig. 4D).

Removal of fatty acids from the hepatocyte can occur either by secretion of TG from the cell or through oxidation pathways. 3OHB concentrations were significantly

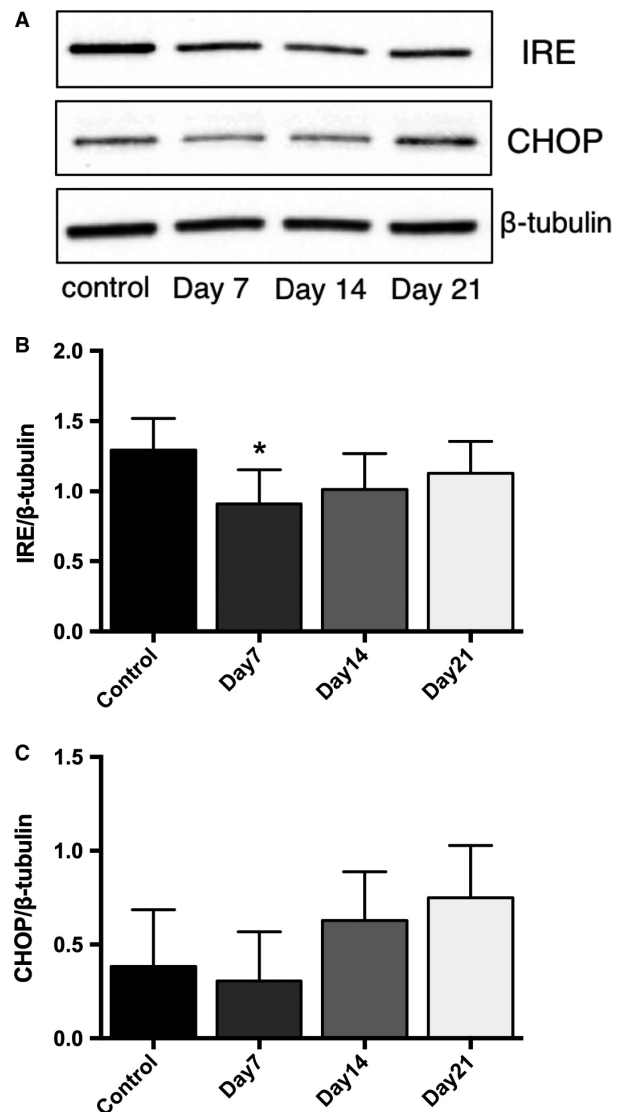


Figure 2. DMSO treatment decreases the expression of the ER stress markers IRE and CHOP in HepG2 cells, as shown by western blotting experiments. (A). Protein expression levels of IRE and CHOP were normalized against β -tubulin (B–C). Data presented are mean $\pm$ SD of $n = 3$ –5 experiments, * $P < 0.05$, control versus day of DMSO treatment.

increased in HepG2 cell medium after treatment with 1% DMSO for 7 and 14 days (8.4 ± 2.7 [control] vs. 13.4 ± 1.7 [day 7], 12.12 ± 2.4 [day 14] μ mol/mg protein; $P < 0.05$) (Fig. 4B) in comparison with untreated control cells. Unlike primary hepatocytes, HepG2 cells secrete very low levels of TG (Wang *et al.* 1988). While DMSO treatment appeared to increase the TG concentration in the medium from HepG2 cells in a time-dependent manner, this did not achieve statistical significance (Fig. 4C).

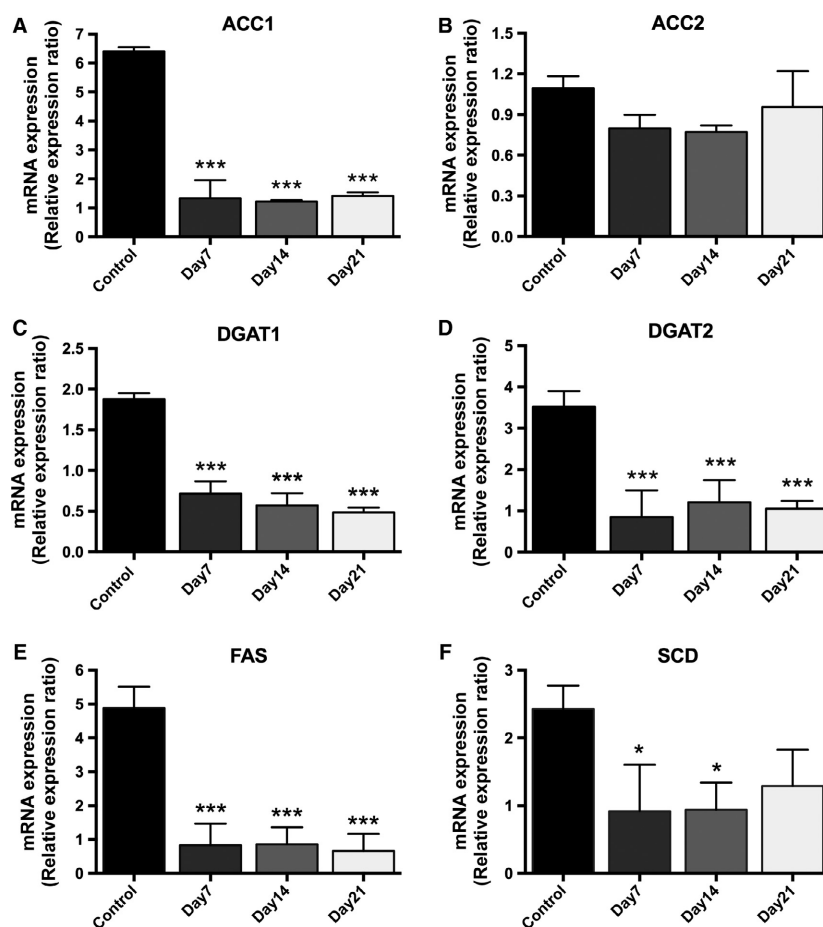


Figure 3. DMSO treatment decreases the mRNA expression of the fatty acid synthetic and oxidation genes acetyl-CoA carboxylase alpha (ACC1), acetyl-CoA carboxylase beta (ACC2), diacylglycerol O-acyltransferase 1 (DGAT1), diacylglycerol O-acyltransferase 2 (DGAT2), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD) in a time-dependent manner in HepG2 cells. Data presented are mean $\pm$ SD of $n = 3$ experiments performed in triplicate, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, control versus day of DMSO treatment. The relative expression ratio was calculated using the following: Ratio = $\Delta C_{t[\text{target}]} / \Delta C_{t[\text{ref}]}$ (Pfaffl 2001) and expression values were normalized to 18 sec.

In order to determine whether fatty acid supplementation in addition to DMSO treatment had any further impact upon the lipid metabolic phenotype, control and DMSO-treated cells (7 days) were treated with 45 $\mu\text{mol/L}$ oleic acid, 30 $\mu\text{mol/L}$ palmitic acid, and 25 $\mu\text{mol/L}$ linoleic acid (OPL; physiological ratio 45%:30%:25%) for 24 or 48 h. We observed no further effect of fatty acid treatment for either 24 or 48 h on all markers of lipid metabolism (Fig. 5A–D).

DMSO treatment changes glucose metabolism in HepG2 cells

In untreated control HepG2 cells, the mRNA expression of genes driving glucose production, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) was low (Fig. 6A and B). DMSO treatment significantly

increased the mRNA expression of both genes in a time-dependent manner (PEPCK: 0.003 ± 0.0008 [control] vs. 0.56 ± 0.35 [day 7], 1.64 ± 1.32 [day 14], 4.0 ± 1.38 [day 21], $P < 0.05$; G6Pase: 0.004 ± 0.0009 [control] vs. 0.48 ± 0.33 [day 7], 1.54 ± 0.16 [day 14], 3.42 ± 0.53 [day 21], $P < 0.05$, data expressed as relative expression ratio). Changes in mRNA expression were mirrored by changes at the protein level (Fig. 6C and D). While glucose concentration in the media did not change significantly (Fig. 6E), DMSO treatment (21 days) decreased media lactate concentrations (3.93 ± 0.4 [control] vs. 3.18 ± 0.36 [day 21] $\mu\text{mol/mg}$ protein; $P < 0.05$) (Fig. 6F).

Discussion

Liver diseases are major causes of mortality and morbidity in humans (Younossi *et al.* 2013), therefore in order for

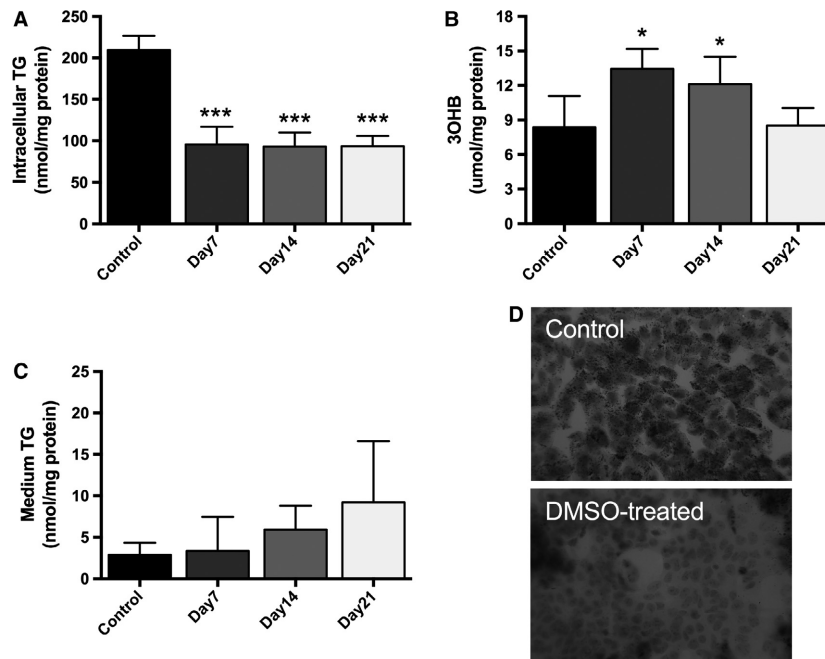


Figure 4. DMSO treatment reduces the intracellular TG content in a time-dependent manner in HepG2 cells (A). These observations were mirrored by a decrease in intracellular neutral lipid (TG) staining with oil red O, after 7 days of DMSO treatment (D). Furthermore, 3OHB levels are significantly increased in HepG2 cell medium after treatment with 1% DMSO for 7 days (B). In addition, DMSO treatment appears to increase the TG concentration in the medium from HepG2 cells in a time-dependent manner (C). Data presented are mean $\pm$ SD of $n = 3$ –5 experiments, * $P < 0.05$, *** $P < 0.001$, control versus day of DMSO treatment.

effective treatment strategies to be developed, the cellular mechanisms involved in their etiology need to be defined. As a consequence, there is a necessity for *in vitro* cellular models that recapitulate human hepatic metabolism. Primary cultures of human hepatocytes still represent the gold standard, but their limitations including availability, inability to proliferate, and donor variability hamper their utility. In addition, in many circumstances the primary culture tissues that are available are from individuals with underlying liver disease and therefore their physiology and function may already be altered. Alternative models of human hepatocytes, including cell lines that have been derived from hepatomas (e.g., HepG2, Huh7.0, Hep3b), offer the potential to be useful for metabolic studies, but have limitations, principally in understanding how closely they reflect human metabolism. Previous reports have suggested that DMSO treatment induces differentiation and drug metabolism in human hepatoma cell lines (Isom *et al.* 1985; Choi *et al.* 2009), although the molecular mechanisms are not fully understood.

By supplementing 1% DMSO to cell culture medium of HepG2 cells, we observed metabolic functionality over a time course of 21 days, with optimal functionality occurring at 7 days. Earlier time points were also tested (2 and 4 days of treatment), but little effect was observed (Fig. 7A–F). In particular, we have shown that DMSO

treatment reduces intracellular TG accumulation which appears to be mediated through the suppression of lipogenic gene expression. In contrast, we observed a significant increase in the expression of key genes involved in intracellular glucose metabolism.

Hepatocyte functionality has previously been assessed by the measurement of markers involved in cell differentiation and viability. The most frequently measured markers include albumin, alpha-1-antitrypsin, transthyretin, and HNF1A (Sainz and Chisari 2006; Nikoozad *et al.* 2014). We measured markers of liver functionality that have been shown to be key in human hepatocyte physiology and found robust changes in the expression of these genes that more closely resembled primary human hepatocytes. Although we did not directly assess cell viability, previous reports have demonstrated DMSO treatment does not cause cytotoxicity in concentrations up to 2% (Wang *et al.* 2012), double the concentration used in our experiments. One of the advantages of using hepatoma cell lines is their ability to proliferate reflecting their origin from hepatocellular carcinoma. Among the reported effects of DMSO is its ability to decrease proliferation and induce or maintain differentiation (Sainz and Chisari 2006). Following DMSO treatment, we observed a reduction in the malignant phenotype as evidenced by a significant decrease in AFP mRNA

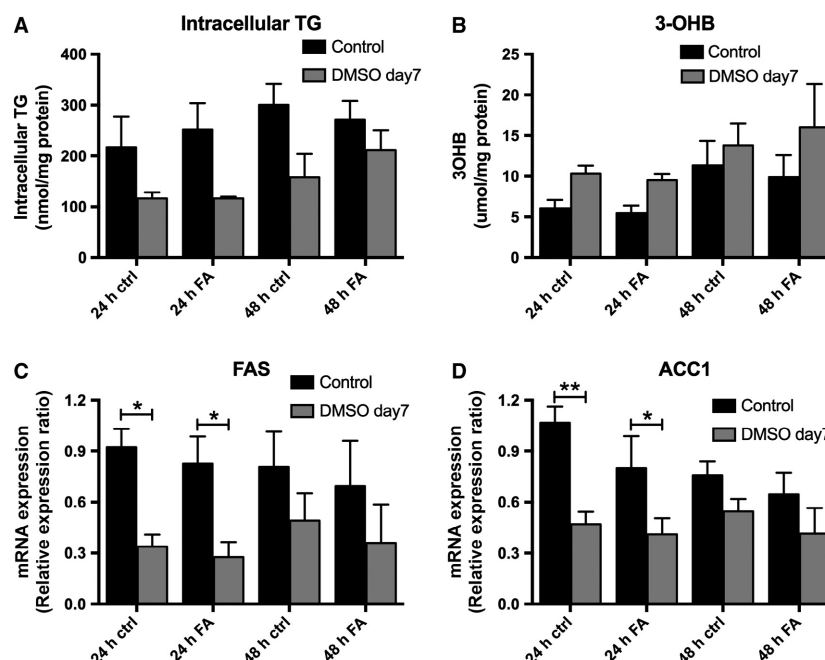


Figure 5. Additional exogenous fatty acid treatment (45 $\mu\text{mol/L}$ oleic acid, 30 $\mu\text{mol/L}$ palmitic acid, and 25 $\mu\text{mol/L}$ linoleic acid), does not significantly change intracellular TG and media 3OHB levels in HepG2 cells after 7 days of DMSO treatment (A and B). Furthermore, exogenous fatty acid treatment has no additional effect in the mRNA expression of FAS and ACC1 after 7 days of DMSO treatment (C and D). Data presented are mean $\pm$ SD of $n = 3$ experiments. The relative expression ratio was calculated using the following: Ratio = $\Delta\text{Ct}_{\text{target}}/\Delta\text{Ct}_{\text{ref}}$ (Pfaffl 2001) and expression values were normalized to 18 sec.

expression. DMSO is known to upregulate tumor suppressor PTEN expression in leukemic HL60 cells (Lee et al. 2005) and tumor suppressor protein HLJ1 in lung cancer cell lines (Wang et al. 2012). Taken together, treatment with 1% DMSO in cell culture medium has the ability to enhance hepatocyte functionality of hepatoma-derived cell lines to be more closely resemble human primary hepatocytes.

Lipid synthesis in the context of hepatocellular carcinoma (HCC) has been reported to be associated with increased HCC survival allowing it to survive at times of metabolic stress (Wang et al. 2016). The decrease in lipid synthesis that we observed provides further evidence that we have reduced the malignant phenotype of the HepG2 cells. Furthermore, rat primary hepatocytes have been reported to have lower levels of intracellular lipid content compared to HepG2 cells. In our study, we have clearly demonstrated that DMSO treatment affects lipid metabolism. The mRNA expression of genes involved in de novo lipogenesis (FAS, ACC1, ACC2) and TG synthesis (DGAT1, DGAT2) was decreased with a consequent reduction in intracellular TG accumulation. Song et al. (2012) demonstrated that low-dose DMSO (0.01%) treatment of HepG2 cells decreased palmitate-induced TG accumulation; however, in the absence of palmitate supplementation and in contrast to our findings, intracellular TG content

did not differ between untreated and DMSO-treated cells. It is possible that the difference between these data and our observations are a reflection of the different doses of DMSO used in the respective protocols.

Fatty acid oxidation is impaired in HepG2 cells compared to parenchymal hepatocytes, as measured by carnitine palmitoyl-transferase activity (Agius 1988). Consistent with this, Gibbons et al. (1994) failed to detect any ketone body formation in olate-treated HepG2 cells. In our study, fatty acid oxidation in DMSO-treated HepG2 cells was increased compared to untreated cells, even in the absence of exogenous fatty acids and this may have contributed to the observed decrease in intracellular TG accumulation.

Previous studies have reported TG secretion from HepG2 cells to be impaired when compared to primary rat hepatocytes (Wang et al. 1988), although culture conditions may also have an effect (e.g. glucose concentrations, variability in fetal serum) (Clavey et al. 1999). In our study, we demonstrated that DMSO-treated cells had increased TG levels in the cell medium compared to untreated controls, in a time-dependent manner, which again suggests the adoption of a more primary hepatocyte-like phenotype.

The liver plays an important role in glucose metabolism regulating the balance between exogenous glucose

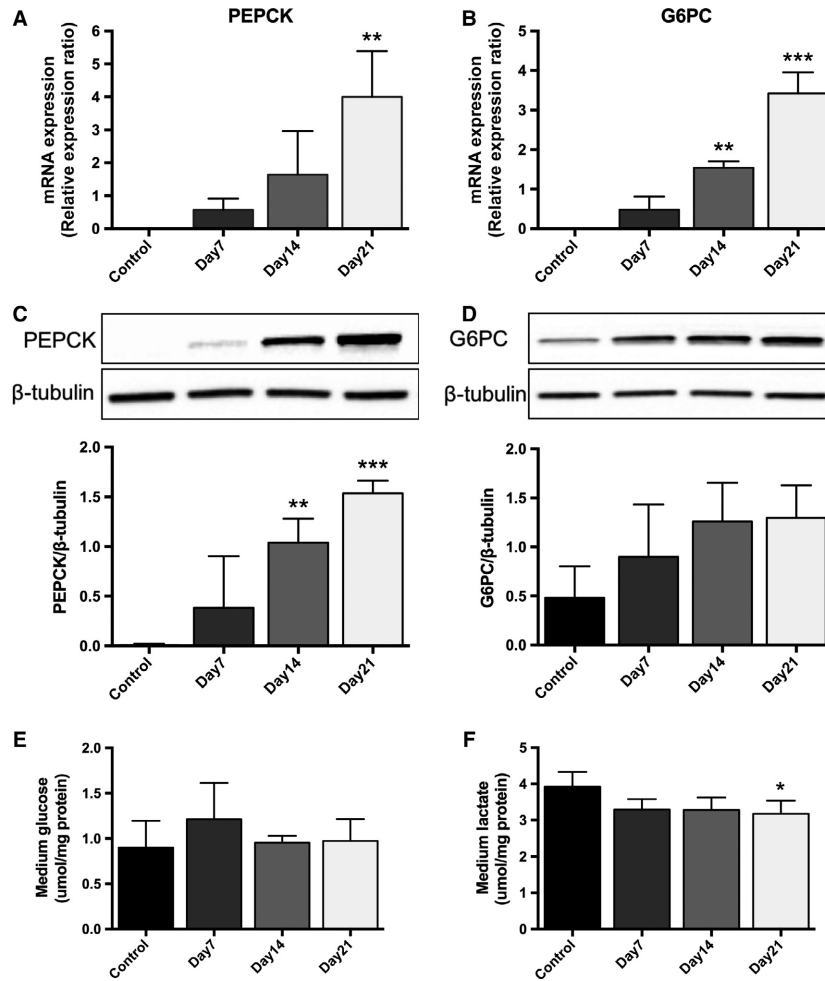


Figure 6. DMSO treatment significantly increases the mRNA expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in a time-dependent manner in HepG2 cells (A and B). Changes in mRNA expression were mirrored by changes at the protein level and the protein expression levels of PEPCK and G6Pase were normalized against β -tubulin (C and D). Furthermore, while glucose concentration in the media did not change significantly (E), DMSO treatment decreased media lactate concentrations in HepG2 cells. Data presented are mean $\pm$ SD of $n = 3$ –5 experiments, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, control versus day of DMSO treatment. The relative expression ratio was calculated using the following: Ratio = $\Delta Ct_{\text{target}}/\Delta Ct_{\text{ref}}$ (Pfaffl 2001) and expression values were normalized to 18 sec.

utilization and endogenous production through gluconeogenesis. Indicative of their HCC origin, HepG2 cells have impaired glucose production, as demonstrated by down-regulated gluconeogenic gene expression (Wang et al. 2013). In our study, DMSO treatment induced gluconeogenesis in a time-dependent manner, as indicated by increased PEPCK and G6Pase expression. Carbohydrate metabolism differs in hepatoma cells compared to primary hepatocytes as the former maintain energy production through anaerobic glycolysis (Hugo-Wissemann et al. 1991). We observed a significant decrease in lactate production after 7 days of DMSO treatment, again suggestive of a more hepatocyte-like phenotype.

While we have demonstrated robust findings, there are some limitations to our experiments. They were

undertaken using standard culture medium containing 25 mmol/L glucose (4.5 g/L). These levels of glucose are nonphysiological and indeed may induce cellular stress and inflammation (Green et al. 2015). This needs to be considered when interpreting our data, although all relevant control cells (and primary hepatocytes) were cultured in the same glucose concentrations. We did not directly assess cell proliferation directly, although others have reported that DMSO at concentrations up to 0.1% enhances proliferation in HepG2 cells (Song et al. 2012); however, at concentrations exceeding 0.5% proliferation rates decrease, though viability is not impaired at concentrations below 2% (Wang et al. 2012). While we have robustly demonstrated the impact of DMSO treatment, cellular confluence is a natural phenomenon and

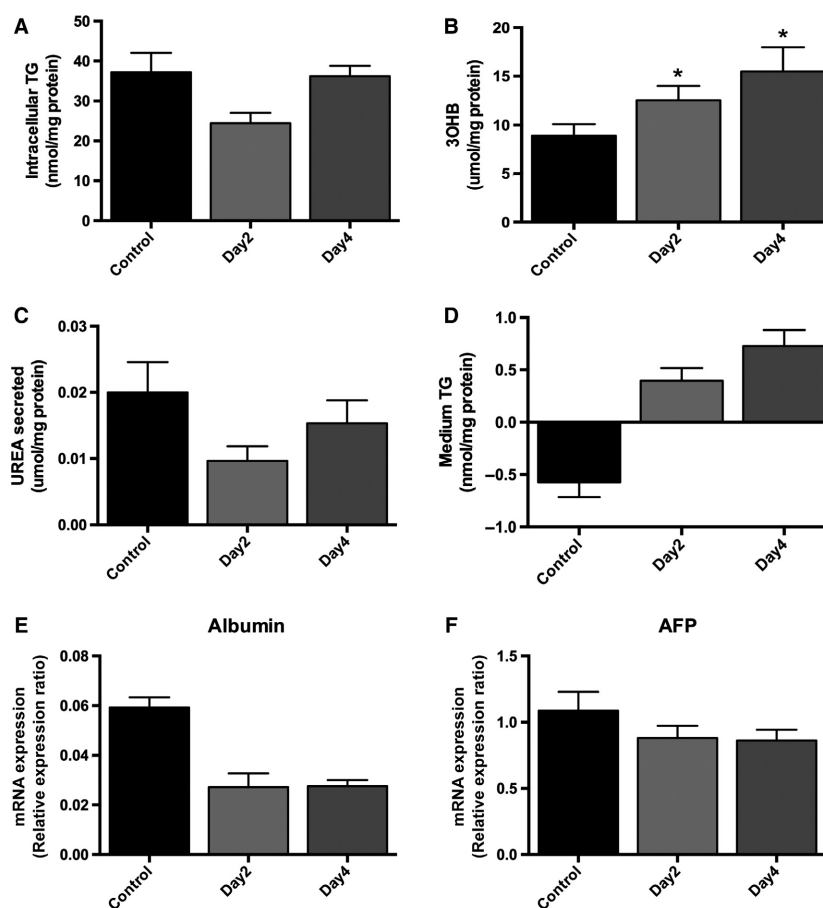


Figure 7. DMSO treatment significantly increases 3OHB levels (B), but it is without effect on intracellular TG content (A), secreted urea (C), or media TG (D) in HepG2 cells after 2 and 4 days of treatment. Furthermore, DMSO treatment does not significantly change the mRNA expression of albumin (E) and AFP (F) after 2 and 4 days of treatment. Data presented are mean $\pm$ SD of $n = 3$ experiments, $*P < 0.05$, control versus day of DMSO treatment. The relative expression ratio was calculated using the following: Ratio = $\Delta C_{t[\text{target}]} / \Delta C_{t[\text{ref}]}$ (Pfaffl 2001) and expression values were normalized to 18 sec.

untreated (control) HepG2 cells continue to proliferate rapidly, resulting in aberrant monolayer and increased cell death in the postconfluent stage, as measured by extensive floating cells in the cell medium (data not shown). As a consequence, it was not possible to match experimental treatment times with non-DMSO-treated control cells.

In conclusion, we have shown that 1% DMSO treatment attenuates lipid accumulation, induces fatty acid oxidation and TG secretion, and augments gluconeogenesis in HepG2 cells such that they more closely resemble primary human hepatocytes. This represents a significant development and enhancement of the currently available in vitro model systems to study hepatocyte biology and has important implications, not only for the study of the pathological processes that contribute to disease, but also their response to specific therapeutic interventions.

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Conflict of Interest

We confirm that this manuscript has not been presented or published elsewhere. N. N., C. J. P., P. J. G., L. H., and J. W. T. do not have any conflict of interest to declare.

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