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Hepatitis C virus induced changes in cellular lipids - identifying novel host antiviral drug target

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Declaration

I hereby declare that all work presented in this thesis describes original research work undertaken in the Storr Liver Unit, Westmead Millennium Institute, Sydney Medical School, The University of Sydney under the supervision of Dr Mark Douglas, Professor Jacob George and Professor Jens Coorssen. To the best of my knowledge these results have not been previously submitted for any degree, and will not be submitted for any other degree or qualification. The author performed all studies reported within this thesis, except where specifically stated.

Signed:.....

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August 29, 2014

Acknowledgment

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Abstract

Nearly 300,000 Australians and 200 million people throughout the world have been exposed to the hepatitis C virus (HCV). Furthermore, over 10,000 new infections are reported on an annual basis. HCV is associated with chronic disease leading to hepatic fibrosis that may progress to liver failure and cancer, and is a leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. Standard therapy using Pegylated interferon (PEG-IFN) and ribavirin leads to virus eradication in just ~50% of cases and is associated with significant toxicity; as such, more effective therapies are urgently required. Recently, licensed protease inhibitors that target viral proteins have been revealed to increase response rates to ~70%; however, a major challenge associated with the use of these inhibitors is that the fidelity of the viral replication machinery is notoriously low, thus enabling the virus to quickly develop resistant mutations. Strategies that target the host factors that support viral replication can potentially reduce the risk of these resistant mutations emerging. However, a major limitation associated with the design and development of such therapies is our limited understanding of the role the hepatocyte plays in virus replication. Understanding the molecular and biochemical pathways that are activated in infected cells will, therefore, provide a means of developing targets that can interrupt virus replication and thus ensure treatments are effective on a long-term basis. The process by which HCV modulates the global intracellular metabolism to create an environment that aids RNA replication and the production of progeny particles is currently unknown, especially at sub-cellular levels. Changes in the host lipoprotein expression levels can result in dramatic differences in the various fractions of the cells. The endoplasmic reticulum (ER) plays a central role in HCV replication: following infection, viral RNA is translated at the ER, where viral proteins induce membrane modifications, giving rise to the ‘membranous web’. This web acts as a platform for the synthesis of HCV

RNA. Additionally, lipid droplets (LDs) have recently attracted a considerable amount of attention because a link has been established between the accumulation of LDs and human diseases such as obesity, atherosclerosis and HCV-associated liver malfunctions. Thus, understanding the interaction of HCV/host lipoproteins at ER and LD levels can provide more enhanced insights than simply studying the whole infected cells.

The data described in this paper describes global changes in lipid abundance that can potentially impact the HCV life cycle and pathogenesis. These changes were more predominant at the ER. These efforts have led to several novel observations that have a potential translational application in hepatology research. This paper describes, for the first time, how PEMT pathways can have therapeutic efficacy in both inhibiting the replication of HCV and increasing interferon sensitivity. HP-TLC showed increased amounts of phosphatidylcholine (PC) in lipid extracts from whole cells and ER fractions. PC was the only phospholipid species detected in purified LDs and was significantly increased in infected cells. PCYT (CTP:phosphocholine cytidylyltransferase) is the rate-limiting enzyme of the major pathway of PC biosynthesis in hepatocytes and PEMT (phosphatidylethanolamine N-methyltransferase) is the rate-limiting enzyme in the minor PC pathway. Silencing PCYT by siRNA had no effect on HCV replication or infectivity. However, when PEMT was silenced, both viral replication and infectivity were decreased by more than 50%.

Accepted abstracts for oral and posture presentation:

1. Mosleh Abomughaid, Mohammed Eslam, Enoch Tay, Jens R. Coorssen, Jacob George, and Mark W. Douglas. (2014) *Lipidomics profiling identifies PEMT as a key enzyme in metabolic reprogramming in chronic hepatitis C infection*. 20th International Symposium on Hepatitis C Virus and Related Viruses (HCV meeting 2014).

2. Mosleh Abomughaid, Enoch Tay, Jens R. Coorssen, Jacob George, and Mark W. Douglas. *Detailed lipidomic analysis of hepatitis C virus infected cells to identify new drug targets*.10th Annual Scientific Workshop 2014. Australian Centre for HIV and Hepatitis Virology Research (ACH^2).

3. Mosleh Abomughaid, Enoch Tay, Jens R. Coorssen, Jacob George, and Mark W. Douglas. (2013) *Detailed lipidomic analysis of hepatitis C virus infected cells to identify new drug targets*. 2013 MBI Annual Colloquium 2013.

4. Mosleh Abomughaid, Enoch Tay, Jens R. Coorssen, Jacob George, and Mark W. Douglas. (2013) *Using lipidomics to identify new drug targets against the hepatitis C virus*.9th National Workshop Australian Centre for HIV &Hepatitis Virology Research (ACH^2).

5. Mosleh Abomughaid, Enoch Tay, Jens R. Coorssen, Jacob George, and Mark W. Douglas. (2013) *Detailed lipidomic analysis of hepatitis C virus infected cells to identify new drug targets*. 20th International Symposium on Hepatitis C Virus and Related Viruses (HCV meeting 2013).

6. Russell Pickford, Mosleh Abomughaid, Enoch Tay, Jens Coorssen and Mark W. Douglas. (2013) *Changes in the Lipodome during Hepatitis C Virus Infection measured with Mass*. 61st ASMS Conference on Mass Spectrometry and Allied Topics Spectrometry (2013).

7. Mosleh Abomughaid, Enoch Tay, Jens R. Coorssen, Jacob George, and Mark W. Douglas. (2012) *Detailed lipidomic analysis of hepatitis C virus infected cells to identify new drug targets*. SEIB Annual Colloquium 2012.

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Abbreviations

2-AG	2-Arachidonoylglycerol
2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
2’5’OAS	2’5’ oligo A synthestase
5’UTR	5’untranslated region
ACC	Acetyl coenzyme-A carboxylase-1
ACC1	acetyl coenzyme-A carboxylase-1
ACPA	Arachidonyl-cyclopropylamide
ACSL3	Acyl-coA synthetase 3
ADRP	adipocyte differentiation-related protein
AEA-d8	Arachidonoyl ethanolamide-d8
AECA	Arachidonyl-2-chloroethylamide
AMD	Automated Multiple Development
AMPK	AMP-activated protein kinase
apoB	Apolipoprotein B
APS	ammonium persulfate
BMI	body mass index
CAAX	COOH-terminal Cys-A-A-X sequence
cAMP	3'-5'-cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
CD81	LEL CD81 large extracellular loop
CE	cholesteryl esters
CHC	Chronic Hepatitis C
CK	choline kinase
CLDN1	Claudine 1
CMV	Cytomegalovirus
CPTA1	palmitoyl acyl-CoA transferase 1A

CTα	CTP:phosphocholine cytidylyltransferase
CYPA	cyclophilin A
DAA	Direct acting antiviral
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-phenylindole
DCI	dodecenoyl-CoA delta isomerase
DDX	DEAD-box RNA helicase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMVs	double membrane vesicles
DRM	detergent resistant membranes
eIF2	eukaryotic initiation factor
ER	endoplasmic reticulum
FAS	Fatty acid synthase
FC	free cholesterol
FCS	Foetal calf serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GWAS	Genome wide array studies
HADHB	Hydroxyacyl-CoA dehydrogenase beta subunit
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma HCC
HCV	Hepatitis C virus
HDL	High-density lipoprotein
HLM	Hypotonic lysis medium
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A

HPTLC	High performance thin layer chromatography
Huh-7	Human hepatocellular carcinoma
HVR1	Hyper variable region
IDUs	Injection drug users
IFN-α2	Interferon alfa-2a
IFN-β	Interferon Beta
IFN-λ2	Interferon Lambda-2
IFN-λ3	Interferon Lambda-3
IFN-λ4	Interferon Lambda-4
IKK1	I kappa B kinase
IL28B	Interleukin-28B
ILD	Intermediate-density lipoprotein
IR	Insulin resistance
IRES	Internal ribosomal entry site
IRF-3	Interferon regulatory factor 3
ISDR	Interferon sensitivity determining region
ISGs	Interferon-stimulated genes
ISRE	Interferon-stimulated response element
JFH1	Japanese fulfillment hepatitis-1
KO	Knockout
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
LD	linkage disequilibrium
LDL	Low-density lipoprotein
LDs	Lipid droplets
LPS	Lipopolysaccharides
LVPs	Lipo-viro particles
MAG	Monoacylglycerol

MAPK Mitogen-activated protein (MAP) kinase

MBS Modified Barth's saline

MHC Major Histocompatibility Complex class

MTP Microsomal triglyceride transfer protein

MW Membranous web

Mx1 Myxovirus (influenza virus) resistance 1

NAFLD Non-alcoholic fatty liver disease

NASH Non-alcoholic steatohepatitis

NKC Natural killer cells

OCLN Occluding

ORF Open reading frame

PA Phosphatic acid

PAMT1 Protein arginine methyl transferase 1

PBS Phosphate buffer saline

PC Phosphatidylcholine

PCYTCTP:phosphocholine cytidylyltransferase

PE Phosphatidylethanolamine

PEMT PhosphatidylethanolamineN-methyltransferase

PI Phosphatidylinositol

PI3K Phosphatidylinositol-3-kinase

PMSF Phenylmethanesulfonylfluoride

PPARα Peroxisome proliferator-activated receptor alpha

PS Phosphatidylserine

qPCR Real-time quantitative PCR

RIG-I Retonic acid-inducible gene I

RIPA Radio-immunoprecipitation assay

ROS Reactive oxygen species

SAMe	S-Adenosyl-Methionine
SDS	Sodium dodecyl sulfate
SGR-JFH1	Subgenomic replicon
SNPs	Single nucleopeptide
SOCS	Suppressor of cytokine signal
SR-BI	Scavenger receptor B type I
SREBP-1c	Sterol regulatory element-binding protein1-c
STAT 1/2	Signal Transducers and Activators of Transcription
SVR	Sustained virology response
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TBST	Tris-buffered saline and Tween
TCA	Trichloroacetic acid
TCID50	Tissue culture infectious dose 50
TCID50	Tissue Culture Infective Dose
TEMED	Tetramethylethylenediamine
TG	Triglyceride
TLRs	Toll-like receptors
VLDL	Very-low-density lipoprotein
Δ9-THC	Δ9-tetrahydrocannabinol

1. INTRODUCTION

1.1 Hepatitis C Virus

1.1.1 History of hepatitis C

In the mid-1970s, Harvey J. Alter et al., demonstrated that most post-transfusion hepatitis cases were not the result of hepatitis A or B viruses. Despite this discovery, international research efforts to identify the virus that caused so-called non-A, non-B hepatitis (NANBH), failed over the course of the following decade. In 1988, the presence of a virus was confirmed by Alter in a panel of NANBH specimens (Alter & Prince 1988). In April 1989, the virus was cloned and characterised, and named hepatitis C virus (HCV) (Choo et al. 1989; Kuo et al. 1989). Significantly, it was the first virus to be identified by molecular means alone, rather than by virus culture.

It is estimated that HCV has been associated with humans for over 1,000 years; however, there is little definitive evidence to support these claims. The estimated age of the most recent common ancestors of HCV was 1,500 years (95% CI: 1350-1700), suggesting that HCV was originally endemic in Central Africa, before spreading to other regions (Smith et al. 1997; Simmonds 2004; Ndjomou et al. 2003).

1.1.2 Epidemiology

HCV is recognized as a major threat to global public health. An estimated 170 million people worldwide have been infected, most of whom are chronically diseased and at risk of liver cirrhosis and hepatocellular carcinoma (HCC) (World Health Organization 2010). To be successful, HCV treatment, prevention and vaccination strategies must be

based on a sound understanding of the evolutionary and epidemiological behaviour of the virus, especially in those geographic regions that are most seriously affected (Figure 1.1).

Nearly 300,000 Australians and 200 million people worldwide have been exposed to HCV, and over 10,000 new infections are reported on an annual basis. Chronic disease can lead to hepatic fibrosis that may progress to liver failure and cancer (Centre for Epidemiology and Research PHD, Hepatitis C 2009).

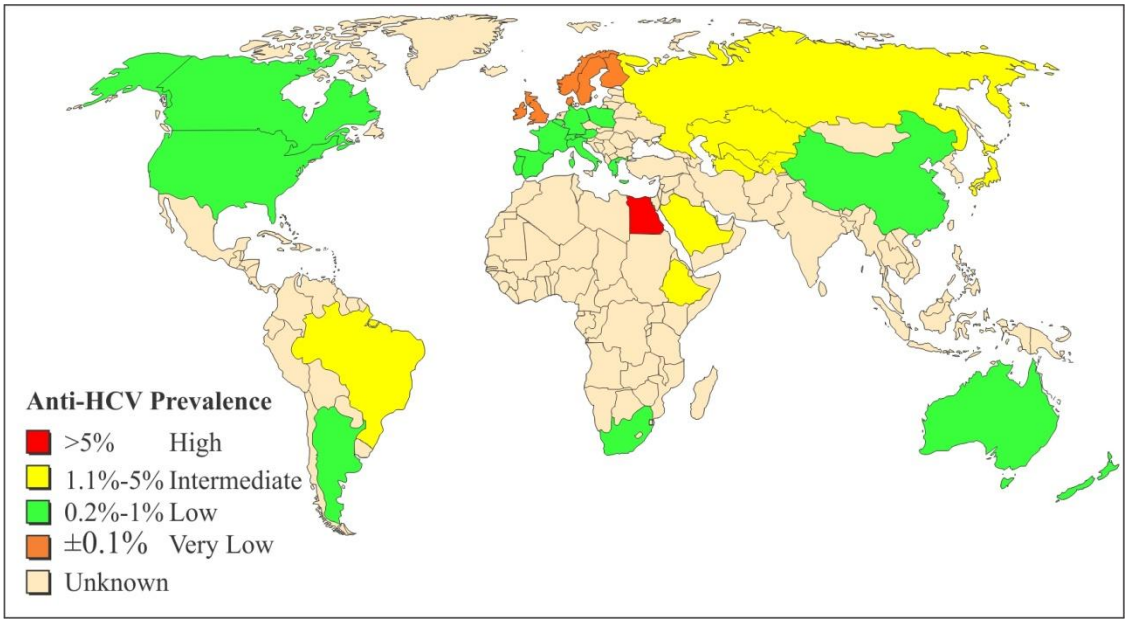

Figure 1.1 Estimated prevalence of HCV infection around the world (World Health Organization 2010)**.**

1.1.3 Natural history of hepatitis C virus

In people with chronic HCV infection, disease progression is variable; it leads to a slow but progressive deterioration of liver function in as many as 70% of affected individuals. Over time, 20% will develop cirrhosis and its related complications, and about

1% to 2% of subjects may develop hepatocellular carcinoma (HCC), after 2 to 3 decades of infection (Afdhal 2004; Alter et al. 1992; Seeff 2002)(Figure 1.2).

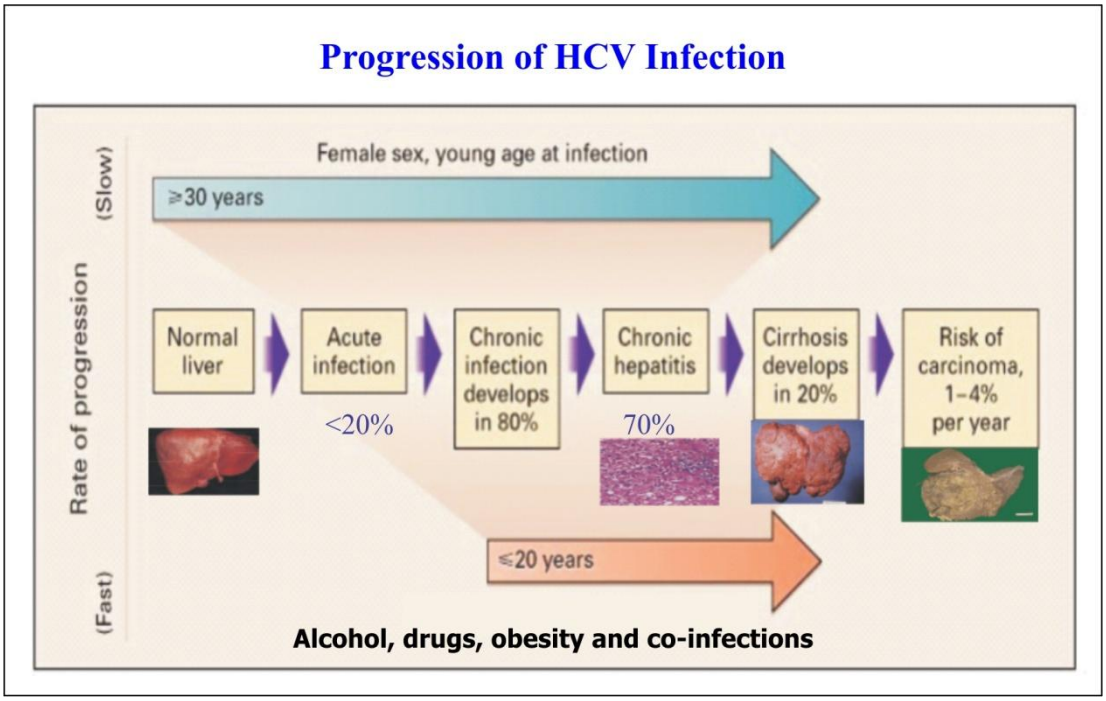

Figure 1.2 Natural history of hepatitis C virus (Lauer & Walker 2001)

The course of disease, either resolution or chronic infection, with varying degrees of hepatitis, seems to be controlled at the level of the host immune response to virus-infected hepatocytes. The hallmark of HCV infection is the establishment of persistent viral infection, but the mechanisms of how this occurs remain unclear.

Understanding how HCV establishes persistent infection is crucial for future strategies of controlling this disease. The mechanism of how this occurs remains unclear, but over the past decade, many mechanisms by which HCV may establish chronicity have been proposed (Bowen & Walker 2005). These mechanisms include virus-encoded anti-host immune strategies, virus-induced immunologic tolerance, viral escape mutations,

molecular mimicry, and decreased effectiveness of antiviral cytokines. In recent years, experimental evidence suggests that virus-encoded, anti-host strategies play an essential role in establishment of persistent viral infection (Bowen & Walker 2005).

Factors associated with development of chronicity:

The factors most consistently associated with development of chronicity are age at time of infection and immune status at time of exposure (Bowen & Walker 2005). Regarding mode of transmission, earlier studies of blood donors, transfusion recipients, and adults acquiring infection through injection drug use reported chronicity rates of 76% to 86%. In contrast, only 55% to 71% of children infected through contaminated blood were persistently infected 15 to 20 years later (Alter 1997; Bialek & Terrault 2006). Neither size of inoculum nor modes of HCV acquisition have been linked consistently with likelihood of achieving viral clearance after exposure or severity of chronic disease.

Racial differences in rate of spontaneous clearance of HCV are suggested by studies showing higher rates of HCV RNA positivity in anti-HCV-positive African Americans and Asian Americans than in white Americans (Busch et al. 2006; Piasecki et al. 2004). Recent findings indicate that polymorphisms near the interleukin-28B (IL28B) gene explains approximately half of the observed differences in spontaneous clearance rates and treatment response between African American and white patients (Thomas et al. 2009).

Studies investigating the impact of HCV genotype on the progression of chronic hepatitis C (CHC) have yielded conflicting results. Most investigators failed to identify genotype as an indicator of likely progression (Roffi et al. 1998; De Moliner et al. 1998), but a few reports suggest a possible association between genotype 1b and rapid disease

progression (Harris et al. 2007). These conflicting results reflect the difficulty in conducting long-term prospective or prospective/retrospective studies, using well-characterized cohorts, to assess the natural history of HCV infection, given the protracted course of disease progression.

Recent genome wide array studies (GWAS) have examined the influence of human genetic variations on clearance of HCV infection. These studies identified several polymorphisms near the IL28B gene that affected both natural and treatment-induced clearance of HCV infection. The two most commonly reported polymorphisms are rs12979860 and rs8099917 (Suppiah et al. 2009; Ge et al. 2009; Tanaka et al. 2009). These single nucleopeptide (SNPs) were in strong linkage disequilibrium (LD) with each other, that is, genotypes based on these SNPs were highly correlated and, as a result, provided very similar genetic associations. Subsequent reports confirmed these findings and showed that these SNP markers were also associated with the likelihood of spontaneous HCV clearance (Thomas et al. 2009; Rauch et al. 2010). A similar effect of the same genetic marker (rs8099917, located near the IL28B gene) for The lowest carriage frequency of the risk allele (24%) was observed among persons with spontaneous clearance and increased to 32% among chronically infected patients who responded to treatment and to 58% among chronically infected patients who failed to respond (Thomas et al. 2009; Rauch et al. 2010; Ge et al. 2009). Although both are probably really markers of the IFNL4 polymorphism (Prokunina-Olsson et al. 2013). The rs12979860 polymorphism is the most commonly used clinically (O'Brien et al. 2014), is known as the "Duke" SNP as it was identified in Ge *et al* paper.

1.1.4 Modes of transmission of HCV

1.1.4.1 Injecting drug use

Injection drug use is the most common mode of HCV transmission in the USA; the proportion of acute cases who reported injecting drug use increased from 31% in 1994 to 38% in 1999 to 45% in 2003 (Armstrong et al. 2006). The prevalence of HCV infection among injection drug users (IDUs) has been shown to be nearly 88% (Mateu-Gelabert et al. 2014). A recent meta analysis suggests that for patients who contracted HCV through injecting drug use, prognosis is poor (John-Baptiste et al. 2010). The key factors associated with an decreased rate of progression were the high proportion of male patients among IDU and the high proportion with excessive alcohol consumption (John-Baptiste et al. 2010).

1.1.4.2 Transfusion of blood and blood products

Historically, transfusion of blood and blood products played an important role in epidemiology of HCV infection. Transfusion associated hepatitis declined as a result of deferral of high risk blood donors and testing donated blood for anti-HCV antibodies (Alter et al. 1992)**.** In many countries HCV RNA is also tested for, further increasing the sensitivity of blood supply screening.

1.1.4.3 Sexual transmission

Medical opinion varies considerably regarding the likelihood of transmitting HCV through sexual contact; based on the study design, representativeness of the study population, and the methods used for case ascertainment. A recent review analyzed 80 qualifying reports regarding the evidence for or against sexual transmission (Terrault et al. 2013); regarding heterosexual transmission, the weight of evidence is that there is no

increased risk of sexual transmission of HCV among heterosexual couples in regular relationships. This risk increases among persons with multiple sexual partners (adjusted odds ratio [aOR] 2.2-2.9), however, this association may be confounded by increased likelihood of injection drug use with increased number of partners (Tohme & Holmberg 2010). Additionally, there is good evidence that HCV can be transmitted sexually among men who have sex with men (MSM) (Nguyen et al. 2010; L et al. 2013), and even higher rates in those who are HIV positive (Kouyos et al. 2014).

1.1.4.4 Perinatal transmission

The risk of HCV transmission to infants born to a high titer HCV RNA–positive mothers range from 4.6 to 10%. The risk increase two to seven fold for infants born to HCV-HIV coinfected mothers than for children born to mothers infected with HCV alone. Because maternal antibodies may remain detectable in the uninfected infant for more than 1 year, anti-HCV testing is not recommended before age of 18 months (Mast et al. 2005; Indolfi & Resti 2009).

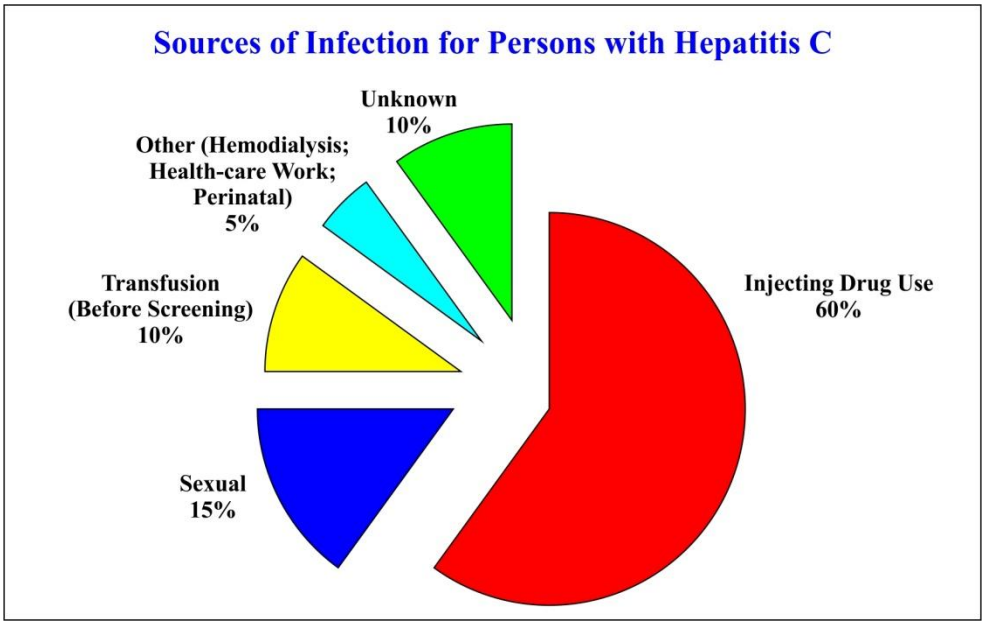

Figure 1.3 Mode of transmissionof hepatitis C virus (Aceijas & Rhodes 2007).However, the route of transmission varies from country to country, for example in some Middle East countries nosocomial spread is higher.

1.1.5 Virology

1.1.5.1 HCV genome organization

HCV was first isolated in 1989 and belongs to the Hepacivirus genus of the Flaviviridae family. The HCV genome is 9.6 kb of single-stranded positive sense RNA, which is packaged into an enveloped virus particle.. Its genome encodes one open reading frame flanked at each end by conserved untranslated regions (UTRs) (Choo et al. 1991). These UTRs are highly structured and critically important for viral replication (Moradpour et al. 2007).The 5' untranslated region (UTR) harbors an internal ribosome entry site (IRES) where translation of a 3000 amino acid precursor polyprotein of HCV coding region is initiated; which is both co- and post-transnationally cleaved at the endoplasmic reticulum (ER) membrane by host and viral proteases into 3 structural (core, E1, and E2)

and 7 nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Figure 1.4) (Otto & Puglisi 2004; Lindenbach & Rice 2005). Discussing the role of core protein is of relevance, as it is particularly interferes with cellular lipid distribution and metabolism. The core protein is a small protein of 21 kDa which is converted into its mature form by successive cleavages (Kato, Miyamoto, et al. 2003; McLauchlan et al. 2002). This process occurs by removal of Domain 3 by signal peptide peptidase (Kato, Miyamoto, et al. 2003); the mature core protein is thus composed of only two domains (1 and 2). The N-terminal region of the core (Domain 1) is highly basic and probably contributes to viral genome encapsidation, through interaction with the 3'-UTR (Ivanyi-Nagy et al. 2008).

Domain 2 targets the core protein to the cellular membrane and cytoplasmic lipid droplets (McLauchlan 2009). Domain 2 is organised into two amphipathic alpha-helices (Boulant et al. 2005) and contains a cysteine that is palmitoylated, leading to membrane targeting (Majeau et al. 2009).

The core protein also seems to be involved in HCV-induced liver dysfunction, through a series of molecular events (Giannini & Bréchot 2003). The core protein interacts with a variety of proteins implicated in signal transduction (e.g. NF-κB may be involved in the control of cell viability) (Giannini & Bréchot 2003; Rui & Goodnow 2006).

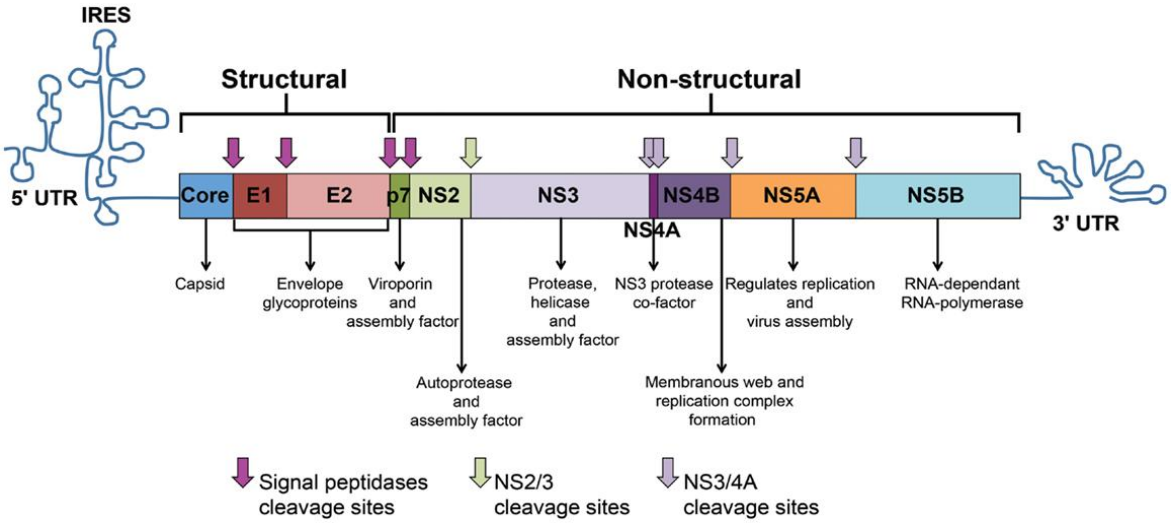

Figure 1.4 The HCV genome and expressed polyprotein.
The HCV genome is composed of an open reading frame (ORF) flanked by 5' and3' untranslated regions (UTRs). IRES-mediated translation of the ORF leads to the formation of a polyprotein that is processed into 10 viral proteins. Cleavage of the core protein from E1 involves cellular signal peptidases, which also cleave E1, E2, and p7 from the polyprotein (pink arrows). The NS2–NS3 protease auto-cleaves itself (green arrow). The NS3 protease located in the first one-third of NS3, assisted by its membrane-bound cofactor, NS4A, cleaves the remaining proteins NS3, NS4A, NS4B, NS5A, and NS5B (violet arrows). (adapted from: Abdel-Hakeem & Shoukry 2014).

1.1.5.2 Hepatitis C Virus Genotypes

Based on genome analysis HCV is divided among six genotypes with numerous subtypes (Figure 1.5)(Pawlotsky & Gretch 1998; National Institutes of Health Consensus Development Conference Statement 2002).These genotypes can differ up to 30% from each other in nucleotides(Sy & Jamal 2006). Within HCV genotypes, several subtypes (designated a, b, c and so on) that differ in their nucleotide sequence by 20–25%(Simmonds et al. 2005). Although HCV is endemic worldwide, there is a large degree of geographic variability in genotypes distribution (Table 1.1). Even though infection with all HCV genotypes can progress to chronic liver disease, genotype-specific differences in the natural history of the disease may exist. For example, type 2 diabetes mellitus is

strongly associated with HCV genotypes 1 (Douglas & George 2009) and genotype 3 have been found to induce steatosis (Cross et al. 2010; Hui, Kench, Farrell, Lin, D. E. V Samarasinghe, et al. 2002). Moreover the efficacy of the standard treatment with ribavirin and pegylated interferon depends on the genotype, with infection with either genotype 1 or 4 leading generally to a poorer treatment response than infection with genotype 2 or 3 (Eriksen et al. 2010).

Table 1.1: Geographic locations HCV genotypes.

Genotype	**Location**
Genotypes 1, 2 and 3	Australia, North America and Western Europe
Genotype 4	Africa, Egypt and the Middle East, but is increasingly seen in some parts of Europe
Genotype 5	Africa and the Middle East
Genotype 6	Southeast Asia
Genotype 7	Central Africa

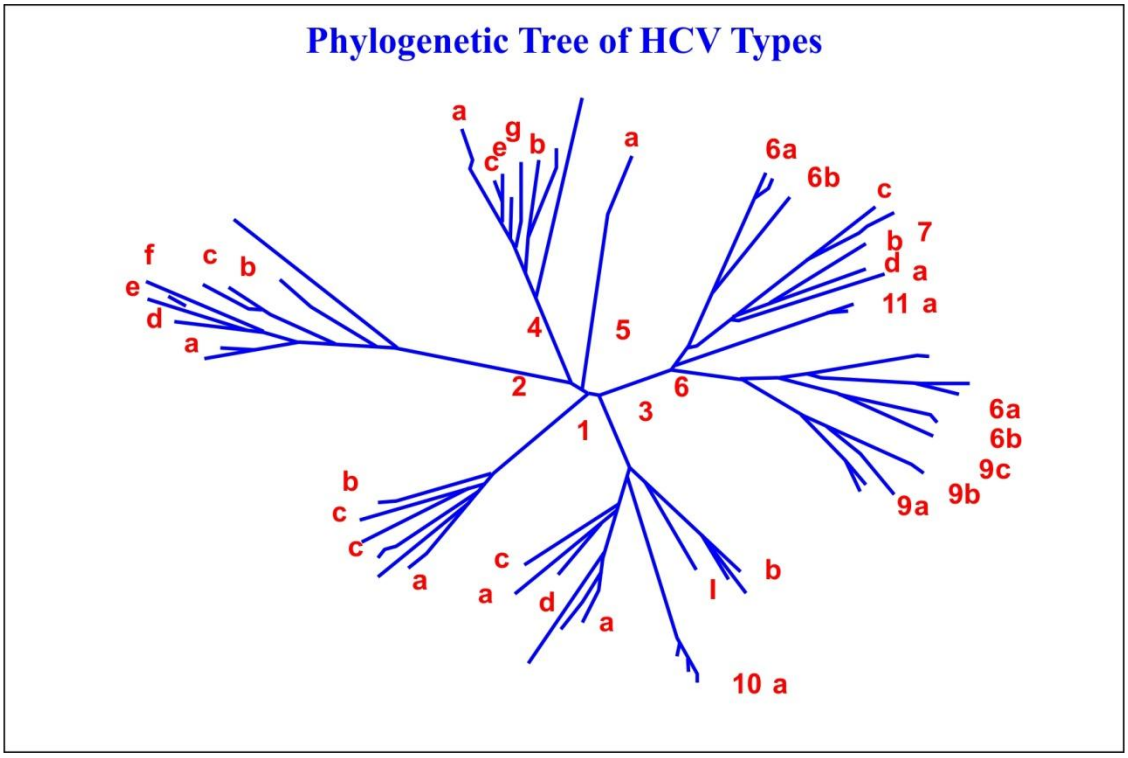

Figure 1.5 Phylogenetic tree of HCV types (Great Heterogeneity Is Now Appreciated) (Simmonds et al. 2005).

1.1.5.3 HCV life cycle

For a long time, it has been difficult to study HCV life cycle because of the difficulties in propagating this virus in cell culture. However, the development of a cell culture system that enables a relatively efficient amplification of HCV provides a powerful new tool for researching HCV (Kato, Date, et al. 2003; Zhong et al. 2005; Wakita, Pietschmann, Kato, Date, Zhao, et al. 2005). This system is based on the transfection of the human hepatoma cell line Huh-7 with genomic HCV RNA derived from a cloned viral genome of a HCV isolate from a Japanese patient with fulminant hepatitis (JFH). Data obtained with the culture system begin to shed light on the HCV life cycle.

The HCV structural proteins and the receptor molecules at the surface of target cells are involved in the early entry of viral particles. E1 and E2 glycoprotein are necessary for viral entry and fusion (B. Bartosch et al. 2003). Viral attachment is thought to be initiated via E2 interaction with one or more components of the receptor complex (Flint & McKeating 2000). In the other hand cell surface receptors like cell surface Glycosaminoglycans, Human CD81, Low density lipoprotein (LDL) receptors, Scavenger receptor B type I (SR-BI), Claudin-1, and different co-receptors molecules have been showed to mediate HCV binding to host cell (Barth et al. 2006; J. M. Pawlotsky et al. 2007).

Up on the infection of the host cell, HCV particles are taken up by receptor-mediated endocytosis and trafficked in endosomes, where the low pH of these compartments induces fusion of the viral envelope and pounding endosomal membrane. The nucleocapsid is then uncoated to release the virus genome into the cytoplasm to serve as a mRNA for syntheses of the viral proteins, where it can be directly translated into structural and non-structural proteins. In an early step of viral replication the IRES located in the 5' UTR of the viral genome regulates translation of HCV open reading frame that encodes a unique polyprotein by recruiting both cellular and viral proteins (Otto & Puglisi 2004), the post translated protein is targeted to the endoplasmic reticulum (ER) membrane where it is further processed by host and viral peptidases to yield out a 10 structural and non-structural proteins. Viral non-structural proteins and host factors assemble in cytoplasmic membrane-bound RNA replicase, which then recruits the HCV genome out of translation into replication (Pawlotsky et al. 2007). After RNA synthesis, new viral genome can be recycled back into translation and replication or packed by viral structural proteins into nascent viral particles. A hallmark of the HCV replication cycle is its extraordinary dependence on host cell lipids. Several studies have shown that viral RNA

replication is tightly linked to lipid synthesis pathways and sensitive to pharmacological intervention with statins and certain fatty acids (Figure 1.6)(Kapadia & Chisari 2005).

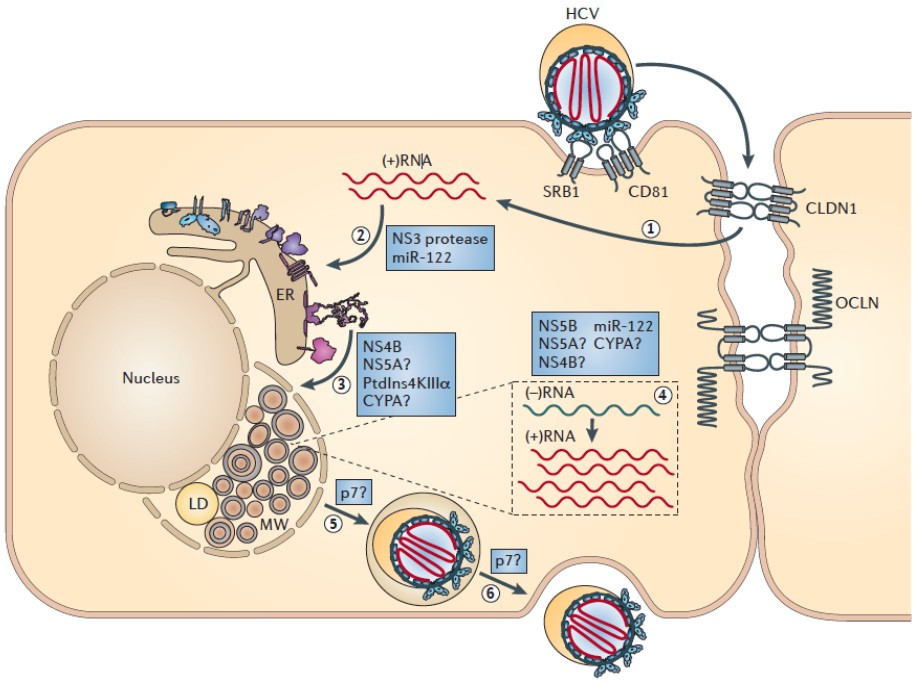

Figure 1.6 HCV life cycle.
Following initial binding of the hepatitis C virus (HCV) particle to scavenger receptor class B member 1 (SRB1) and CD81, the particle engages in further interactions with the tight junction proteins claudin 1 (CLDN1) and occludin (OCLN) and finally enters cells by receptor-mediated endocytosis (step 1). The viral RNA genome is released into the cytoplasm and translated at the rough ER (step 2). Viral proteins, in conjunction with host cell factors, induce the formation of a membranous web (MW)) composed of single-, double- and multi-membraned vesicles as well as lipid droplets (LDs) (step 3). RNA replication occurs at an unspecified site within the membranous web (step 4). Assembly of HCV particles probably initiates in close proximity to the ER and lipid droplets, where core protein and viral RNA accumulate. The viral envelope is acquired by budding through the ER membrane in a process that is linked to lipoprotein synthesis (step 5). HCV particles are released via the constitutive secretory pathway (step 6). CYPA, cyclophilin A; PtdIns4KIIIα,_phosphatidylinositol 4-kinase IIIα (adabted from: Bartenschlager et al. 2013).

1.2 Role of lipids in the HCV life cycle

There is increasing evidence that cellular lipid pathways are fundamentally involved in the replication cycle of HCV:

1- HCV circulates in plasma with lipoproteins as an infectious complex.

2- Lipoprotein hepatocyte receptors are involved in HCV entry.

3- Replication of HCV RNA in hepatic cells is inhibited by inhibitors of lipid metabolism.

4- HCV is released from hepatocytes with lipoproteins.

1.2.1 HCV circulates in plasma with lipoprotein as an infectious complex

HCV exhibits molecular heterogeneity and the characterization of HCV particles from the plasma of infected patients shows a highly heterogeneous population of viruses (buoyant density <1.03-1.25 g/ml). HCV circulates in the bloodstream in different forms; either free or in a complex with immunoglobulin or lipoprotein to form the so-called lipo-viro particles (LVPs). Implicated lipoproteins by density are very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), or low-density lipoprotein (LDL) (Monazahian et al. 2000). HCV RNA is always found in at least one of these fractions and represents 40% of the total plasma HCV RNA, but can reach up to 95% in some patients (Andre et al. 2002).

The low-density infectious particles are roughly spherical with a heterogeneous diameter of 50-150 nm. Their structure is rich in triglycerides, apolipoprotein B-100 (APOB), and apolipoprotein E (APOE), and physio-chemically resemble VLDL particles (André et al. 2002). The association of ApoC1 with infectious particles in the serum of experimentally infected chimpanzees has also been documented (Meunier et al. 2008).

Interestingly, these particles typically contain ApoB100 (Fisher & Ginsberg 2002), which is of hepatic origin and an integral part of VLDL (Zannis et al. 2001). However, the presence of ApoB48, normally produced by enterocytes, suggests that a proportion of circulating LVPs may originate not only from the liver but also from the intestine (Diaz et al. 2006).

The first report of an association between HCV core protein and lipid structures was made using an inducible expression system (Moradpour et al. 1996). Now, multiple studies have suggested that lipoproteins play a critical role in the infectivity of natural HCV, and also that apolipoproteins may be efficient regulators of HCV infectivity:

a) Most circulating HCV particles are of low density due to association with B-lipoproteins (Thomssen et al. 1992; Thomssen et al. 1993). Only the very low density fraction of serum-derived HCV (sHCV) was highly infectious in chimpanzees (Beach et al. 1992), tissue culture (Lindenbach et al. 2006), and in natural human infections (Diaz et al. 2008), in contrast to high-density populations of HCV which are poorly infectious (Agnello et al. 1999). Measurement of low density apolipoprotein B associated LVP in HCV genotype 1 infection was better correlated with more advanced fibrosis and poorer treatment response than total viral load. In these patients; insulin resistance and associated dyslipidaemia were the major determinants of low-density apoB-associated LVP in fasting plasma (Bridge et al. 2011).

b)In 2006 data by Lindenbach *et al*provided evidence that the specific infectivity of cell culture-produced HCV (HCVcc) produced *in vitro* and recovered from experimentally infected chimpanzees or uPA-SCID mice with human liver grafts was higher and buoyant density was lower than those of the same virus strain produced in cell culture (Lindenbach

et al. 2006). Thus, the association between virus particles and lipoproteins may confer higher viral infectivity (Lindenbach et al. 2006).

c) HCVcc infection *in vitro* could be efficiently inhibited not only by antibodies directed against the HCV envelope but also by antibodies against ApoB-containing lipoproteins (Andréo et al. 2007).

1.2.2 Lipoprotein hepatocyte receptors are involved in HCV entry

Two lipoprotein receptors on hepatocytes are known to be of the receptors components that mediate viral entry: SR-B1 and LDL-R (Scarselli et al. 2002). SR-B1 is a lipoprotein receptor, with the highest levels found in the liver and adrenal glands, which is responsible for the selective uptake of cholesteryl ester from high-density lipoprotein (HDL) (Out et al. 2004). As HCV particles have been reported to be complexed with lipoproteins, it is possible that HDL interacts with HCV particles, via protein/protein or lipid/protein interactions (Bartosch et al. 2005), suggesting an indirect interaction of virus with lipoprotein receptors (Maillard et al. 2006; Nielsen et al. 2006). Previous observations implicated SR-B1 as an important receptor for infection by different HCV subtypes and supporting this hypothesis is the fact that the same SR-B1 protein element is responsible for the recognition of different HCV E2 glycoproteins, despite the high level of variability in their amino acid sequences, especially in the hyper-variable region 1 (HVR1) previously shown to interact with SR-B1 (Birke Bartosch et al. 2003).

HCV appears to use SR-BI during cell entry not merely as an additional site for viral particle binding, but also by exploiting its physiological activity, i.e. the capacity to mediate lipid transfer from HDL, which is known to facilitate the entry of many different viruses, including influenza virus, HIV, and HCV (Voisset et al. 2005; Rhainds &Brissette

2004). However, HCV is many times more sensitive to HDL-mediated infection enhancement than other cholesterol-sensitive viruses (Voisset et al. 2005). Therefore, enhancement of HCV infection might be dependent on the lipid exchange activity of SR-B1 (Bartosch et al., 2005). Recently, a novel function of SR-B1 for viral antigen uptake and recognition has been suggested; SR-B1 may represent a cell-surface receptor for the recognition of viral antigens and has been implicated in trafficking exogenous viral antigens toward the Major Histocompatibility Complex (MHC) presentation pathway. The SR-B1-viral antigen interaction may represent a novel target for therapeutic or preventive strategies, aiming to induce an efficient antiviral immune response (Barth et al. 2008).Moreover, HDL with SR-B1 is the predominant enhancing factor in infectivity and the presence of HVR1 with HDL protects HCV from neutralizing antibodies as HDL can reduce the neutralizing effect of anti-HCV antibodies (Dreux et al. 2006; Voisset et al. 2006).This phenomenon might be responsible, at least in part, for the limited ability of the humoral immune response to control HCV infection in vivo, which raises concerns about the potential for using anti-HCV antibodies for active or passive immunotherapy (Dustin & Rice 2007)**.**

Thus, as an alternative to the development of anti- HCV antibodies, one could consider anti-SR-B1 human monoclonal antibodies capable of interfering with HCV infection as potential therapeutic leads (Catanese et al. 2007). Agents involved in modulating the normal hepatocellular processes of lipid transport have been reported to have pleiotropic effects on HCV infectivity. Antibodies to ApoB have been shown to have antiviral activity (Maillard et al. 2006; Andréo et al. 2007; Dreux & Cosset 2007).

1.2.3 Cellular Lipids and HCV replication

Replication of HCV, like all positive-strand RNA viruses, occurs in association with cytoplasmic membranous vesicles (Miller & Krijnse-Locker 2008). Proteomic

analysis of these membrane vesicles recently showed that they are enriched in several proteins involved in lipid metabolism and lipoprotein generation (i.e., apoB-100, Microsomal triglyceride transfer protein (MTP), apoE and Acyl-coA synthetase 3 (ACSL3) (Huang et al. 2007).

Acute HCV infection in chimpanzees is associated with an increased intrahepatic expression of genes involved in lipogenesis (Su et al. 2002) such as the ATP citrate lyase, an enzyme activated by the transcription factor sterol-responsive element binding protein (SREBP) (Su et al. 2002). This suggests possible links between viral replication and lipobiogenesis.

Cholesterol:

Viral RNA replication has been shown to be dependent on cholesterol, sphingomyelin and fatty acid synthetic pathways, which promote the formation of lipid rafts on which replicase complexes assemble (Kapadia & Chisari 2005; Amemiya et al. 2008; Tai et al. 2009). Cholesterol metabolism pathways are now known to impact on HCV replication, secretion, and entry (Ye 2007).

Cholesterol can be synthesized from acetyl-CoA via the mevalonate pathway (Goldstein & Brown 1990), which also generates several isoprenoids, including farnesyl and geranylgeranyl lipids that are covalently attached to the COOH-terminus of certain proteins (Zhang & Casey 1996). Cells can also acquire LDL–associated cholesterol in serum through LDL-receptor–mediated endocytosis (Goldstein et al. 1985).

The first clue that the mevalonate pathway is involved in HCV RNA replication was the finding that treatment of cultured cells with lovastatin inhibited HCV RNA replication (Ye et al. 2003). Lovastatin is a cholesterol-lowering drug that inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in

the entire mevalonate pathway (Goldstein & Brown 1990). These results were subsequently confirmed by others (Kapadia & Chisari 2005; Ikeda et al. 2006; S. S. Kim et al. 2007).

Geranylgeranyl lipid was found to be the essential product of the mevalonate pathway required for HCV RNA replication, as inhibition of HCV RNA replication by lovastatin was overcome by the addition of geranylgeraniol, but not farnesol or cholesterol (Kapadia & Chisari 2005; Ye et al. 2003). Geranylgeranyl lipid serves as a lipid substrate for protein geranylgeranylation, a post-translational modification that covalently attaches geranylgeranyl to various cellular proteins to facilitate their membrane association (Zhang & Casey 1996). Thus, it appears that one or more geranylgeranylated proteins are required for HCV RNA replication.

Recently; F-box and leucine-rich repeat-containing protein 2 (FBL2), which contains an F-box domain and a COOH-terminal Cys-A-A-X sequence (CAAX box) motif was identified as a cellular partner for the HCV nonstructural protein NS5A (Wang et al. 2005). FBL2 is localised to cellular membranes through geranylgeranylation by the geranylgeranyl transferase I; which is an enzyme of the mevalonate pathway, which governs cholesterol synthesis. This concept is further supported by the observation that HCV replication could be blocked by an inhibitor of geranylgeranyl transferase I (Ye et al. 2003), an enzyme that transfers geranylgeranyl groups to cellular proteins (Zhang & Casey 1996). Therefore, FBL2 could indirectly influence VLDL formation and promote the LVPs release. It should be noted that all of the aforementioned studies used genotype 1 HCV replicons. It is not known whether geranylgeranylation of host proteins is required for replication of other genotypes of HCV.

Unlike geranylgeranyl, the requirement of cholesterol per se for HCV RNA replication is still an area of debate due to the controversial available data. The observation that addition of cholesterol failed to rescue HCV RNA replication in the presence of lovastatin argues against cholesterol being directly involved in HCV RNA replication (Ye et al. 2003). On the other hand, inconsistent findings were obtained when cells were depleted of cholesterol by treatment with methyl-β-cyclodextrin, which directly extracts cholesterol from cell membranes (Kilsdonk et al. 1995). Under these conditions, HCV RNA replication is either not affected (Kapadia et al. 2007) or reduced by 50% (Aizaki et al. 2004). The inconsistency in these results could arise from a secondary effect of general cellular toxicity that occurs when cells are depleted of cholesterol, or from a difference in the HCV genotype used in these studies.

Fatty Acids:

In addition to being cholesterol precursors, fatty acids can also directly modulate (stimulate or inhibit) HCV replication. HCV infection takes control of cellular metabolism and drives, among other things, marked increases in fatty acid synthesis. Fatty acids can be used as prosthetic groups in exactly the same way as cholesterol intermediates. These prosthetic groups may facilitate viral non-structural protein anchoring to the specialised membrane structures that serve as the platform for HCV RNA replication and assembly. For example, Yu et al, have provided evidence that NS4B under goes a double palmitoylation on two cysteine residues (cysteines 257 and 261) located at the C-terminal end (Yu et al. 2006). Site-specific mutagenesis has shown that these lipid modifications, especially of the last cysteine residue (Cys261) have an additive effect on HCV replication and protein-protein interactions (Yu et al. 2006). Thus, palmitoylation may be a kind of post-translational modification essential to the HCV life cycle and may also play a role in

HCV pathogenesis. Moreover; inhibitors of palmitoylation also inhibited HCV replication (Yu et al. 2006).

Interestingly, the effect of fatty acids seems to depend on their degree of saturation (Kapadia & Chisari 2005). Saturated and monounsaturated fatty acids stimulate HCV replication (Kapadia & Chisari 2005). In contrast, polyunsaturated fatty acids, including arachidonic acid, docosahexaenoic acid and eicosapentaenoic acid, inhibit it (Leu et al. 2004). Inhibition of fatty acid synthase (FAS) by cerulenin (Su et al. 2002) or C75 (Yang et al. 2008) blocks HCV replication in a subgenomic replicon system and the secretion of virions in the JFH1 infectious system.

In patients with CHC even though there are many circumstances where the uptake of fatty acids by hepatocytes may be pathologically increased, such as in the insulin-resistant state (Browning & Horton 2004), there is clear experimental evidence that HCV can directly perturb lipid metabolism. Diamond and co-workers in a recent global proteomic and metabolomics profiling study provide important evidence that HCV directly reprograms numerous metabolic pathways and modulates accumulation of select lipid species in the infected human hepatocytes (Diamond et al. 2010). Specifically, they identified two novel proteins essential for HCV-induced metabolic reprogramming, the mitochondrial fatty acid oxidation enzymes dodecenoyl-CoA delta isomerase (DCI) and hydroxyacyl-CoA dehydrogenase beta subunit (HADHB) (Diamond et al. 2010). Interestingly, an altered abundance of these enzymes has also been observed in liver biopsies from CHC patients, suggesting that beta-oxidation, the major process by which fatty acids are oxidized influences HCV persistence not in the experimental level only but in the clinical setting, as well (Diamond et al. 2010). Recent studies have examined the mechanisms by which enzymes involved in fatty acid oxidation influence HCV replication in hepatocytes, either by targeted gene silencing or pharmacologic inhibitors. These studies

have confirmed that impairing fatty acid oxidation adversely impacts HCV growth (Rasmussen et al. 2011), providing a stimulus for new approaches to targeting host metabolic factors (Rasmussen et al. 2011).

In another evidence, during early stages of primary infection in chimpanzees, HCV activates genes involved in lipid metabolism, via SREBP (Su et al. 2002). Further, using a cell culture based model, Kim and coworkers (K. H. Kim et al. 2007) have shown that HCV is able to induce transcriptional activation, proteolytic processing and phosphorylation of SREBP-1, thereby causing the increase of fatty acid synthesis. Waris *et al* show that in vitro, in addition to SREBP-1c, HCV induces transcriptional activation of SREBP-2. SREBP-1c and SREBP-2 are, responsible for the transactivation of enzymes involved in the synthesis of fatty acids and cholesterol, respectively (Waris et al. 2007a). SREBP activity is stimulated in vitro by several viral proteins including the HCV core protein (Rasmussen et al. 2011; K. H. Kim et al. 2007) and the non-structural proteins 2 (Oem et al. 2008), 5A (Xiang et al. 2010) and 4B (Waris et al. 2007a; Park et al. 2009). Activation of SREBP and numerous enzymes implicated in lipidogenesis has also been reported in transgenic mice expressing various HCV proteins (Chang et al. 2008; Lerat et al. 2009).

It worth mention that in the subgenomic HCV replicon system it was shown that 25-hydroxycholesterol, which inhibits SREBP cleavage/translocation, causes not only a decrease in fatty acid biosynthesis but also in HCV replication (Su et al. 2002). In contrast to these findings, activation of the SREBP pathway by nystatin, which acts through cholesterol sequestration, caused a dose-dependent increase in replication levels by nearly 100% (Su et al. 2002).

Our group previously made the novel observation that cannabinoid receptor 1 (CB1) is widely expressed in the livers of patients with CHC and is associated with

advanced fibrosis and steatosis, providing another important link between HCV and lipids (van der Poorten et al. 2010). CB1 is an important regulator of lipid metabolism in the liver, and it has been show that treating mice with a CB1 agonist induces de novo fatty acid synthesis, via increased hepatic expression of SREBP-1c and its downstream targets fatty acid synthase and acetyl coenzyme-A carboxylase-1 (ACC1) (Osei-Hyiaman et al. 2005). We have now shown that CB1 antagonists inhibit HCV replication in the liver (Shahidi et al. 2014).

In contrast to the in vitro data, there is very little data available on the expression level of SREBP in the liver of CHC patients and liver histology. The available data suggest an association between the activation of SREBP-1c and the severity of inflammation and fibrosis, but not steatosis (McPherson et al. 2008). Similarly, intrahepatic levels of FAS mRNA were not correlated with steatosis (McPherson et al. 2008). However, in two more recent studies FAS mRNA has been reported to be increased (Diamond et al. 2010; Nakamuta et al. 2009). Further well designed large-scale studies are required, before we can elucidate a final conclusion.

Beside activating SREBP, the HCV core protein may also bind to and activate the DNA-binding domain of the retinoid receptor α, a transcriptional regulator that controls many cellular functions including cellular lipid synthesis (Fukasawa et al. 2006). Enzymes responsible for fatty acid synthesis such as acetyl-coA carboxylase and FAS are, indeed, strongly activated by the expression of viral proteins in vitro (Jackel-Cram et al. 2007).

1.2.4 Lipids and HCV Assembly and Secretion

The development of a cell culture system that supports not only HCV replication but also the production of infectious virus has revealed additional roles for lipid metabolism in viral particle assembly, secretion and infectivity. Circumstantial evidence

suggests that HCV virions and VLDL particles share a common route of secretion, and reagents that abrogate VLDL secretion also block HCV secretion. Triglyceride-rich VLDL, the main lipoprotein component of HCV in patient sera, is produced and secreted by hepatocytes. VLDL assembly occurs in two steps: the first step involves the co-translational lipidation of APOB-100 by MTP in the lumen of the ER which transfers triglycerides to nascent ApoB-100 (Hussain et al. 2003). Unlipidated APOB-100 is targeted for degradation by the ubiquitin proteasome machinery (Hussain et al. 2003; Shelness & Ledford 2005). This ApoB-containing VLDL precursor (pre-VLDL) subsequently fuses with triglyceride droplets during the second maturation step, a process also mediated by MTP (Shelness & Ledford 2005; Sellers & Shelness 2001). Although the exact mechanism and intracellular location (ER, post-ER or Golgi) of this second lipidation event are still uncertain, it appears to be dependent on the GTP binding protein ARF-1 (ADP ribosylation factor-1) and phospholipase D (Workman 2004).

Recent studies show that ApoB remains associated with infectious HCV particles after treatment of the virus with either deoxycholic acid or NP-40, providing evidence that HCV-associated lipoproteins are not simply adsorbed at the surface of virus particles circulating in patient sera, but that VLDLs are an integral part of HCV particles, with strong binding between HCV and ApoB-containing lipoproteins (Nielsen et al. 2006).

In addition, reagents that impair VLDL assembly (such as MTP inhibitors or siRNA targeting ApoB or ApoE) also abrogate HCV secretion, but do not affect HCV RNA replication per se (Nahmias et al. 2008; Yao & Ye 2008; Huang et al. 2007). These data provide further evidence that functional components of VLDL assembly and secretion are required by HCV for its secretion (Huang et al. 2007). Consistent with these observations, the density of intracellular virus particles in the HCV cell culture model was much higher than that of secreted virus (Gastaminza et al. 2008). As well as inhibiting virion secretion,

disruption of ApoB biosynthesis also resulted in reduced accumulation of intracellular infectious virions, suggesting that intracellular ApoB levels function as a major determinant of HCV virion morphogenesis (Gastaminza et al. 2008). Colocalization of HCV replication and VLDL assembly components indicate close proximity between the intracellular regions involved in virus genome replication and virion assembly (Huang et al. 2007). The property that makes the virus unique is that the entire virion is not exposed to serum during circulation and virus particles have to escape from VLDL-derived lipoprotein particles in endocytic vesicles.

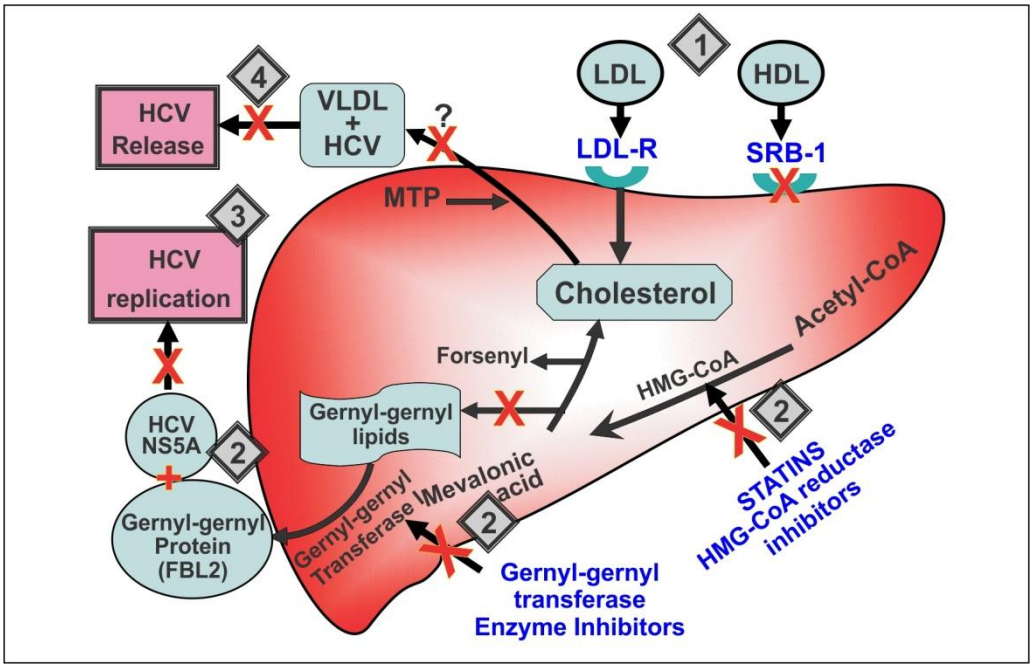

Figure 1.7Possible host factor sites to target as novel antiviral strategies
(1) Inhibition of HCV entry by anti-receptor antibodies; (2) Interference with the host metabolic factors involved in HCV replication; (3) Modulation of nuclear receptors involved in HCV replication; (4) Inhibition of HCV release. LDL-R: Low density lipoprotein receptor; HDL: High density lipoprotein; VLDL: Very low density lipoprotein; SRB-1: Scavenger receptor B1; FXR: Farnesoid X receptor; ER: Estrogen receptors; MTP: Microsomal triglyceride protein; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A. (Khattab 2009).

1.3 The role of the endoplasmic reticulum (ER) in HVC replication

The ER plays an essential role in multiple stages of HCV replication (Fig 1.8) including (I) initial translation of viral RNA; (II) formation of the membranous web; (III) production of lipid droplets on which virus is assembled and (IV) processing and export of mature virus. As with other positive strand RNA viruses (such as Dengue), HCV induces morphological membrane modifications to form double membrane vesicles (DMVs) that serve as the platform for virus replication (Shi et al. 2003; Hagemeijer et al. 2012). Although of crucial importance, little is known about how these vesicles are formed. HCV infection also induces lipid synthesis to produce larger lipid droplets, often leading to steatosis in patients with HCV infection (Kumar et al. 2005), and the ER is the primary

site for lipid synthesis. Hence, many of the factors involved in VLDL biogenesis and secretion through the ER pathway are also necessary for virus formation and export. Considering the importance of the ER in establishing the HCV replication complex and export of viral particles, we will quantitatively assess the changes induced in this organelle in response to HCV infection. Such information concerning these fundamental mechanisms is critical to the design of new therapeutic interventions.

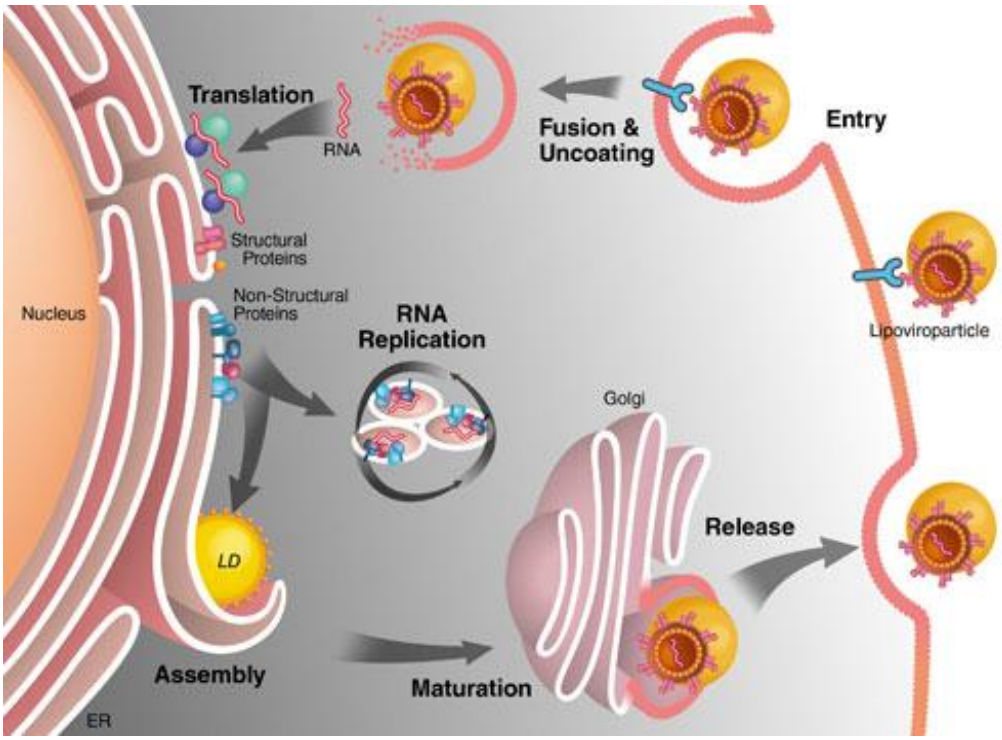

Figure 1.8 Life cycle of HCV showing the involving the ER(Herker & Ott 2012).

1.4 HCV and lipid abnormalities

1.4.1 HCV and fatty liver

Disruption of lipid metabolism during chronic HCV infection could contribute to the initiation of steatosis, or fatty liver (Mori et al. 2008).The prevalence of steatosis in patients with CHC is reported to be between 40 and 80% depending on the features of the population studied in terms of alcohol consumption, prevalence of overweight/obesity, diabetes and other risk factors for fatty liver (Poynard et al. 2003; Lonardo et al. 2006). However, even after all common causes of steatosis have been excluded, the prevalence of steatosis in CHC remains about 40%. This figure represents an approximately 2-fold increase compared to the prevalence of steatosis in other common chronic liver diseases such as hepatitis B (20%) (Rubbia-Brandt et al. 2000; Thomopoulos et al. 2006). Furthermore, the composition of FAs that accumulate in the livers of transgenic mice expressing HCV core protein is different from that seen in fatty liver due to simple obesity. Carbon-18 monounsaturated fatty acids (C18: 1) such as oleic or vaccenic acids are significantly increased with core protein. A similar difference is seen when comparing of liver tissues from patients with CHC and those with nonalcoholic fatty liver disease (NAFLD) (Moriya et al. 2001). This suggests that HCV may directly cause fatty liver, at least in subgroups of patients with CHC.

Hepatic steatosis in patients with CHC assumes importance clinically because it: (1) negatively impacts the response to antiviral therapy (2) increases the rate of fibrosis progression (Asselah et al. 2006) and (3) influences the development of HCC (Patton et al. 2004). The similarities between hepatitis C and NAFLD make it likely that these conditions often coexist. Thus, these conditions may both be present in many patients, and there is likely to be overlap in mechanisms.

1.4.2 Molecular mechanisms of hepatic steatosis in HCV patients

Multiple mechanisms may account for the development of steatosis in persons with CHC, including effects of HCV core protein expression and comorbid metabolic conditions; these interactions are summarized in Figure 1.9. Virus-specific alterations in host metabolism can induce insulin resistance (IR) and type 2 diabetes mellitus (T2DM)(Cargnel et al. 1999; Shintani et al. 2004), leading to hepatic steatosis, which in turn can exacerbate IR (Asselah et al. 2006; Péres et al. 2013).

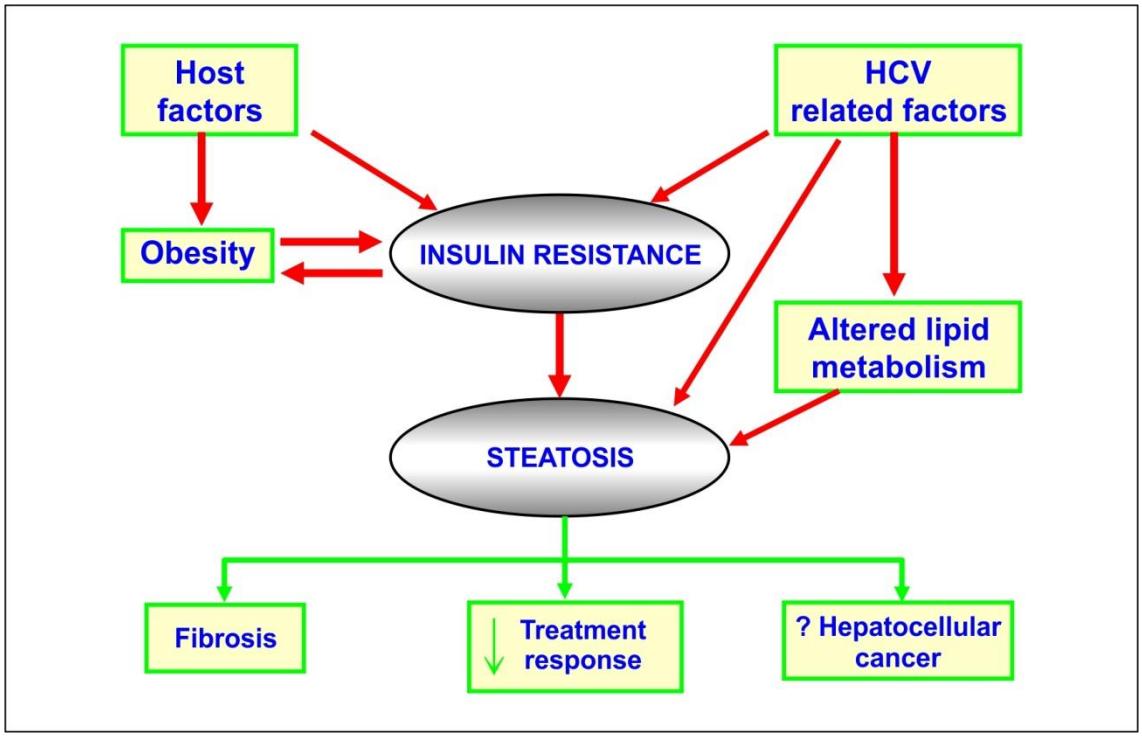

Figure 1.9 Virus and host interactions leading to steatosis in patient with HCV and clinical implication of those interactions(Puri & Sanyal 2006).

A recent Italian study looking at host genetics found that the PNPLA3 rs738409 genotype promotes steatosis development in CHC patients, and is independently associated with advanced fibrosis, cirrhosis, reduced response to antiviral treatment and development of hepatocellular carcinoma (Valenti et al. 2011).

The effect of HCV infection on steatosis is mediated in large part by the HCV core protein, which has significant effects on the transcription of genes involved in lipid metabolism, including palmitoyl acyl-CoA transferase 1A (CPT1A) and MTP, which correlates well with steatosis (Conjeevaram et al. 2007)(Figure 1.10). The association of core protein with mitochondria has significant effects on lipid accumulation, including decreased fatty acid degradation, disproportional increases in fatty acid uptake and changes in Ca^{2+} influx at the mitochondrial outer membrane (Li et al. 2007). These mechanisms also appear to be contribute to core protein induced generation of reactive oxygen species (ROS), effects on lipid peroxidation and hepatocyte damage (Machida et al. 2006). Several genes have been shown to be essential for HCV-related steatosis, including PA28-γ (Moriishi et al. 2007) and peroxisome proliferator-activated receptors-α (PPARα) (Dharancy et al. 2005; Tanaka et al. 2008).

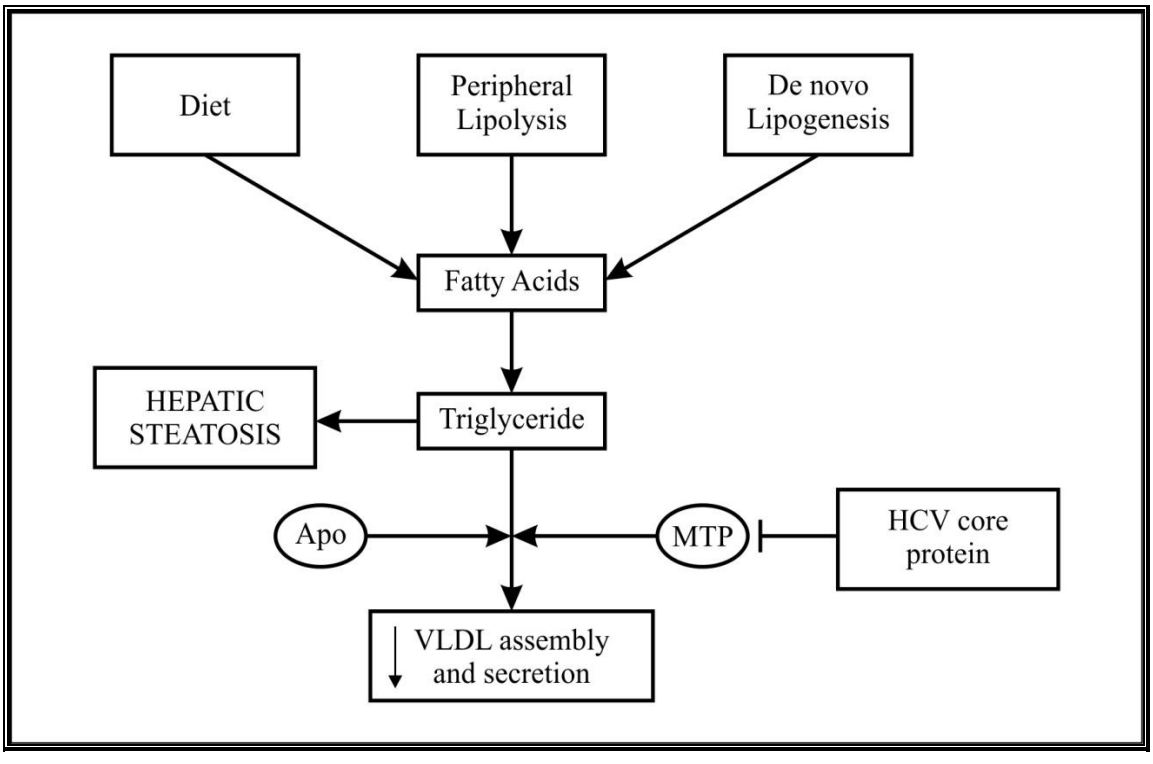

Figure 1.10 HCV core inhibits VLDL secretion, promoting steatosis.
It is postulated that HCV core protein inhibits microsomal triglyceride transfer protein (MTP) activity, thus inhibiting very low-density lipoprotein (VLDL) assembly and secretion(Puri & Sanyal 2006).

1.4.3 HCV Genotype-specific alterations in lipid metabolism

A genotype-specific alteration in lipid metabolism has been demonstrated for HCV. In patients infected with genotype 3, steatosis is mostly virus-induced and often severe, correlates with intrahepatic viral load, and resolves after successful antiviral therapy (Rubbia-Brandt, Leandro, et al. 2001; Hui, Kench, Farrell, Lin, D. Samarasinghe, et al. 2002). In contrast, in patients infected with genotype 1 or 4, steatosis is mainly associated with host metabolic factors and correlates with body mass index (BMI) and central adiposity (Conjeevaram et al. 2007).

In HCV genotype 3 infection, steatosis appears to be a direct consequence of viral protein expression, suggesting the presence of specific sequences across the genome of genotype 3 that are involved in fat accumulation within hepatocytes (Negro 2010). This observation is supported by two supplementary findings. First, the severity of steatosis in patients with genotype 3correlates with the level of HCV RNA, both in liver (Rubbia-Brandt et al. 2000) and in serum (Adinolfi et al. 2001). Second, steatosis may significantly decrease, or even completely disappear, following eradication of HCV genotype 3 with antiviral agents, which is not typically seen with non-3 genotypes (Rubbia-Brandt et al. 2000; Poynard et al. 2003). A relapse of HCV genotype 3 after the end of therapy may even result in the reappearance of steatosis in patients in whom it had disappeared during treatment (Rubbia-Brandt, Giostra, et al. 2001).

Although the mechanism by which HCV-3 induces steatosis more efficiently than others genotypes is not completely understood, some potential mechanisms have been postulated.

A) In hepatocytes, HCV interferes with cellular lipids through direct protein–protein or protein–lipid interactions, and also by influencing hepatic lipid metabolism. Indeed, it is important to recognize that HCV replication modifies fatty acid metabolism as well as the

cholesterol pathway. In general, HCV may interfere with lipid metabolism at three levels: impaired secretion, impaired degradation and increased neolipogenesis. (Syed et al. 2010).

B) Impaired secretion of lipoproteins from the infected hepatocyte was the first proposed mechanism of HCV induced steatosis. HCV core protein may cause hepatic steatosis by inhibiting the activity of MTP (Perlemuter et al. 2002), an enzyme that plays a key rate-limiting role in VLDL assembly, thereby inhibiting the secretion of VLDL from the liver. Although the effects of core on MTP were initially observed in transgenic mice, these findings have now been observed in humans. In a study of 58 HCV patients, a highly significant (P=0.0017) inverse correlation was found between liver MTP mRNA levels and the degree of hepatic steatosis, suggesting an important role for MTP in hepatic steatosis (Mirandola et al. 2006).

C) HCV-3 may also cause steatosis by either stimulating de novo synthesis of fatty acids as discussed above, or by decreasing FA oxidation. HCV may decrease FA oxidation through impairment of the expression and transcriptional activity of PPARα which regulates several genes responsible for FA degradation (Cheng et al. 2005). Expression of PPARα appears to be impaired with HCV infection (Dharancy et al. 2005; de Gottardi et al. 2006).In patients with CHC, expression of the PPARα gene in the liver was reduced by 86% compared with controls, and the expression of its target gene, CPT1A was coordinately reduced by 85%. Recent evidence of a direct role for PPARα activation in the pathogenesis of steatosis and HCC was obtained from studies that combined HCV core transgenic mice with PPARα knockout (KO) mice. These experiments found that PPARα KO mice expressing core protein had reduced expression of PPARα target genes, and only mild steatosis in the liver (Tanaka et al. 2008). In general, PPARα acts to ameliorate steatosis, but in the presence of mitochondrial dysfunction, induced by the core protein, core activated PPARα may exacerbate steatosis. Others have shown that PPARγ

expression is significantly lower in genotype 3 than genotype 1 HCV infection and that steatosis in genotype 1 HCV infection is also associated with decreased levels of PPARγ (de Gottardi et al. 2006).

1.4.4 Lipid induces insulin resistance

An additional mechanism leading to fatty liver may be related to the increased prevalence of IR among CHC patients. Insulin has a central role in lipid metabolism in the liver and IR increases the peripheral release and hepatic uptake of FAs, resulting in an accumulation of lipid in the liver (Browning & Horton 2004). In obesity, defects in insulin mediated lipid metabolism result in lipid metabolites being trafficked to the liver and skeletal muscle, instead of adipose tissue, producing "lipotoxicity". Trafficking of lipid from overloaded adipocytes to key metabolic tissue has been shown to specifically induce IR (Muoio & Newgard 2008). HCV induces inflammatory cytokines which potently activates the novel group of PKC isoforms δ, ε, θ and η (Sedaghat et al. 2002). Activation of PKCε in the liver and PKCδ and θ in muscle has been shown to induce IR, by increasing phosphorylation of IRS1 and IRS2 at inhibitory serine residues, while decreasing phosphorylation of activating tyrosines (Aguirre et al. 2002; Greene et al. 2006). (Figures1.10 and 1.11).

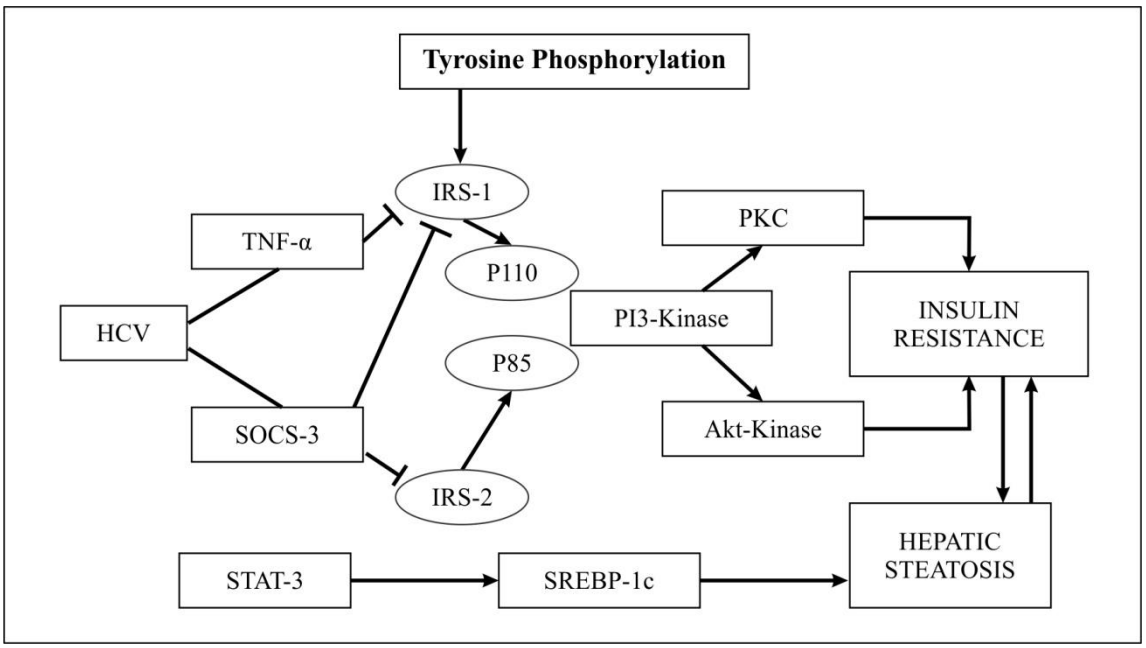

Figure 1.11 The interaction of putative mediators leading to IR and hepatic steatosis in HCV infection. Increased levels of TNF-α and suppressor of cytokine signalling-3 (SOCS-3) in CHC induce IR by interfering with tyrosine phosphorylation of insulin receptor substrate 1 and 2 (IRS 1&2). This process is associated with reduced phosphatidylinositol-3-hydroxykinase (PI3K) and downstream signals involving AKT-kinase and protein kinase C (PKC). SOCS-3 can also attenuate the activity of signal transducer and activator of transcripition-3 (STAT-3). This process can enhance SREBP-1c promoter activity, contributing to hepatic steatosis and IR (Quoted from: Buneet et al., 2006).

1.4.5 Lipid abnormalities and response to antiviral treatment

Many studies have reported a correlation between lipid profiles and virological response to antiviral treatment. A recent Japanese study of 44 patients with CHC found that high triglyceride (TG) content of VLDL was strongly predictive of a better response to treatment (Mawatari et al. 2010). In a large European cohort of 575 HCV genotype 1-infected patients, a low baseline level of cholesterol, but not TG, was an independent predictor of not achieving sustained virology response (SVR) (P= 0.084),. Despite a drop in the cholesterol level of patients while receiving antiviral treatment, cholesterol

rebounded to above baseline in patients with SVR, and or to baseline in nonresponders/relapsers (P = 0.02). Significant differences of TG levels in patients with and without SVR were only observed at follow-up (136 and 117 mg/dl, respectively, P=0.028) (Lange et al. 2010). A large German observational study enrolled 10390 patients treated with PEG-interferon alfa-2a and ribavirin and evaluated for cardiovascular and metabolic risk markers. In a prospective analysis , the authors concluded that elevated total cholesterol levels predicted higher SVR, but the presence of diabetes mellitus was a negative predictor of SVR (Jaeckel et al. 2010).

A recent study of the influence of race on the effect of lipid profiles on SVR analysed data from the Virahep-C study, a prospective study of treatment-naïve patients with genotype 1 HCV infection who received peginterferon (PEG-IN) alfa-2a plus ribavirin therapy for up to 48 weeks (Ramcharran et al. 2010). The study found that baseline TG levels were inversely correlated with SVR rate, but LDL-associated cholesterol (LDL-C) levels directly correlated with SVR. Albeit, baseline LDL-C was significantly higher in Caucasian Americans compared with African Americans, but the difference in these parameters did not explain the racial difference in treatment response (Ramcharran et al. 2010). In another study to provide a genetic analysis for the associations between serum lipid levels and hepatic steatosis in CHC patients.; Clark and co workers identified 4 SNPs in the IL28B gene region were significantly associated with pre-treatment LDL (top SNP rs12980275, P=10-17, good IFN response variant=higher LDL). In a logistic regression model, baseline LDL was strongly associated with SVR. The authors conclude that the poor response IL28B variant was strongly associated with lower pretreatment LDL levels and more hepatic steatosis (Clark et al. 2012). In accordance with these findings, in another recent trial from JapanSaito *et al*, show that the TT genotype of rs8099917 polymorphism near IL28B gene was associated with high LDL

cholesterol level this in addition to a high frequency of the mutations in interferon sensitivity determining region and a wild type of core aa 70 and high. Again; the rs8099917 polymorphism was the most significant factor to predict lack of the response for PEG-IFN-α/RBV in genotype 1b CHC patients with high viral load (Ito et al. 2011). These intriguing data may help to provide explanation to the series of clinical observations linking higher LDL, less steatosis and SVR.

1.4.6 Lipid abnormalities and liver fibrosis

The correlation between lipid profile and severity of liver fibrosis was assessed in some recent reports. In a recent study from Taiwan, the authors found advanced hepatic fibrosis was associated with an adjusted odds ratio of 0.03 (0.00–0.32) for total cholesterol level (Hsu et al. 2010). The same finding was elucidated by a retrospective analysis of the V ahep-C study; albeit, TG was significantly associated with steatosis (P=0.001). HDL (P=0.004) and total cholesterol (P=0.001) were significantly lower in those with fibrosis than in those without fibrosis (Ramcharran et al. 2011).

1.4.7 Lipid abnormalities and hepatocarcinogensis

A recent study tried to identify the signature genes involved in lipid metabolism, by characterizing normal liver vs. HCV infected liver vs. HCV associated HCC using gene microarray. Up regulation of gene-mediated FA de novo synthesis and down regulation gene-mediated FA oxidation were correlated with HCV associated HCC. Also, the down regulation of genes resulting in abnormal cholesterol metabolism in HCV infection was found to be intensified in the setting of HCC (Salomao et al. 2010).Steatosis and HCC developed in PPARα intact but not in PPARα heterozygous or PPARα null core gene

transgenic mice, indicating that not just the presence but the persistent activation of PPARα is important in hepatocarcinogenesis by HCV core protein.

1.5 HCV, interferon signalling and treatment response

1.5.1 Goals of therapy:

Primary goal:

- Eradicate HCV infection.

Secondary goal:

- Slow disease progression.
- Improve liver histology.
- Reduce risk of hepatocellular carcinoma.
- Improve health related quality of life.

Current recommended therapy for HCV (AASLD guidelines, January 2014):

Recommended regimen for treatment-naive patients with HCV genotype 1 who are eligible to receive IFN: Daily sofosbuvir (400 mg) and weight-based RBV (1000 mg [<75 kg] to 1200 mg [≥75 kg]) plus weekly PEG for 12 weeks is recommended for IFN-eligible persons with HCV genotype 1 infection, regardless of subtype.
Recommended regimen for treatment-naive patients with HCV genotype 1 who are not eligible to receive IFN: Daily sofosbuvir (400 mg) plus simeprevir (150 mg), with or without weight-based RBV (1000 mg [<75 kg] to 1200 mg [≥75 kg) for 12 weeks is recommended for IFN-ineligible patients with HCV genotype 1 infection,

regardless of subtype.
Recommended regimen for treatment-naive patients with HCV genotype 2, regardless of eligibility for IFN therapy: Daily sofosbuvir (400 mg) and weight-based RBV (1000 mg [<75 kg] to 1200 mg [≥75 kg]) for 12 weeks is recommended for treatment-naive patients with HCV genotype 2 infection.
Recommended regimen for treatment-naive patients with HCV genotype 3, regardless of eligibility for IFN therapy: Daily sofosbuvir (400 mg) and weight-based RBV (1000 mg [<75 kg] to 1200 mg [≥75 kg]) for 24 weeks is recommended for treatment-naive patients with HCV genotype 3 infection.
Recommended regimen for treatment-naive patients with HCV genotype 4 who are eligible to receive IFN: Daily sofosbuvir (400 mg) and weight-based RBV (1000 mg [<75 kg] to 1200 mg [≥75 kg]) plus weekly PEG for 12 weeks is recommended for IFN-eligible persons with HCV genotype 4 infection.
Recommended regimen for treatment-naive patients with genotype 4 who are not eligible to receive IFN. Daily sofosbuvir (400 mg) plus weight-based RBV (1000 mg [<75 kg] to 1200 mg [≥75 kg]) for 24 weeks is recommended for IFN-ineligible patients with HCV genotype 4 infection.
Recommended regimen for treatment-naive patients with HCV genotype 5 or 6. Daily sofosbuvir (400 mg) and weight-based RBV (1000 mg [<75 kg] to 1200 mg [≥75 kg]) plus weekly PEG for 12 weeks is recommended for IFN-eligible persons with HCV genotype 5 or 6 infection.

However, in Australia patients still have no access to either sofosbuvir or simeprevir. This will hopefully change soon, but it is not clear who will qualify for

sofosbuvir, given its high cost (US $84,000 for 12 week course). Generally, IFN is still the backbone of most recommended therapies for HCV; therefore IFN response is critical to treatment success. That will probably change in a few years as combination DAA treatment becomes available, but even then the host innate IFN response is important.

1.5.2 Interferons

Interferons (IFNs) are natural proteins produced by the cells of most vertebrates in response to challenges by foreign agents such as viruses, parasites and tumor cells. Interferons belong to the large class of glycoproteins known as cytokines. Interferons are produced by a wide variety of cells in response to the presence of double-stranded RNA, a key indicator of viral infection. Interferons assist the immune response by inhibiting viral replication within host cells (Figure 1.12), activating natural killer cells (NKC) and macrophages, increasing antigen presentation to lymphocytes, and inducing the resistance of host cells to viral infection. When the antigen is presented to matching T and B cells, those cells multiply and specifically eliminate the foreign substance, highlighting the importance of antigen presentation for the immune response (Liu 2005).

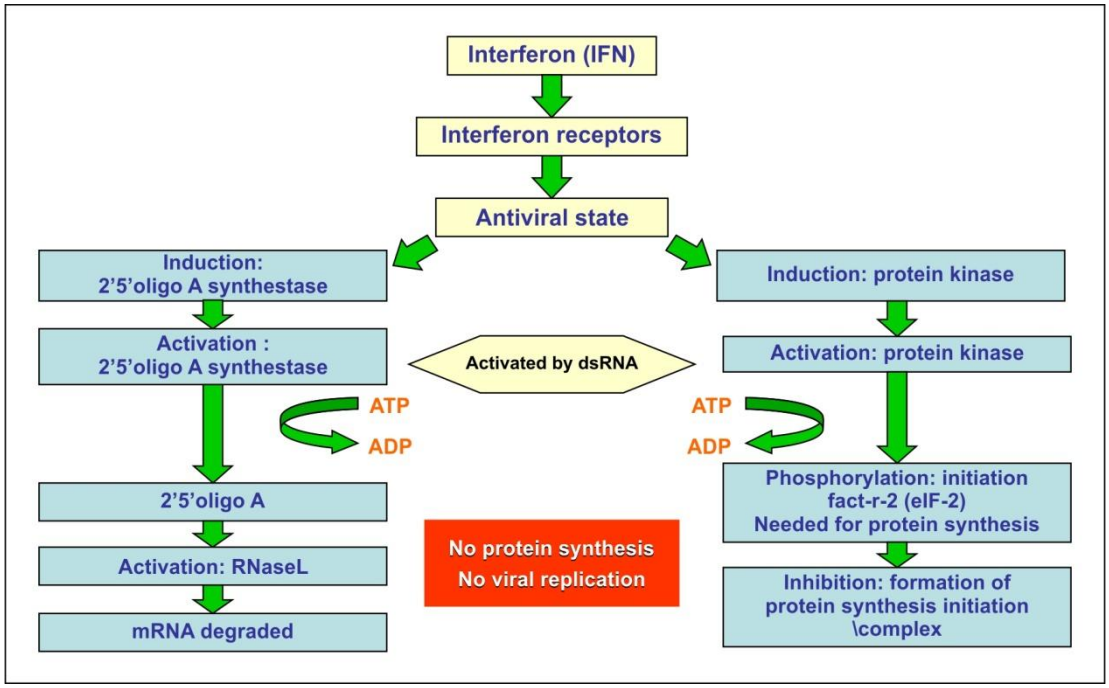

Figure 1.12. Cellular Events in the Action of Interferons.
The binding of IFN to its receptor results in the transcription of a group of genes that code for antiviral proteins involved in preventing viral replication in that cell. As a consequence the cell will be protected from infection with a virus until the antiviral proteins are degraded, a process which takes several days. The antiviral state in IFN-treated cells results from the synthesis of enzymes that result in the inhibition of protein synthesis, including 2'5' oligo A synthestase (2'5'OAS) and protein kinase (PK) (modified from: Goodbourn et al. 2000).
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1.5.2.1 Types of interferon

Three major classes of interferons have been described in humans, based on the receptors through which they signal:

1- Interferon type I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The type I interferons present in humans are IFN-α, IFN-β and IFN-ω(Liu 2005).

2- Interferon type II: Binds to the IFN-γ receptor (IFNGR). In humans this is IFN-γ.

3- Interferon type III: Signal through a receptor complex consisting of IFNLR1 and IL10R2. In humans four types of IFN-λ (IFNL1-4) have so far been recognised.

1.5.2.2 Natural function and synthesis

Interferons in general have several effects in common. They are antiviral and possess antioncogenic properties, induce macrophage and natural killer lymphocyte activation, and increase expression of major histocompatibility complexglycoprotein classes I and II, thus enhancing presentation of foreign (microbial) peptides to T cells. In a majority of cases, the production of interferons is induced in response to microbes such as viruses or bacteria and their products (viral glycoproteins, viral RNA, bacterial endotoxin, bacterial flagella, CpG sites). Interferons can also be induced by mitogens or other cytokines, such as interleukin 1, interleukin 2, interleukin-12, tumor necrosis factor or colony-stimulating factor, which are synthesised in response to various antigens. Metabolism and excretion of interferons take place mainly in the liver and kidneys; they rarely cross the placenta but can cross the blood-brain barrier(Tanabe et al. 2004).

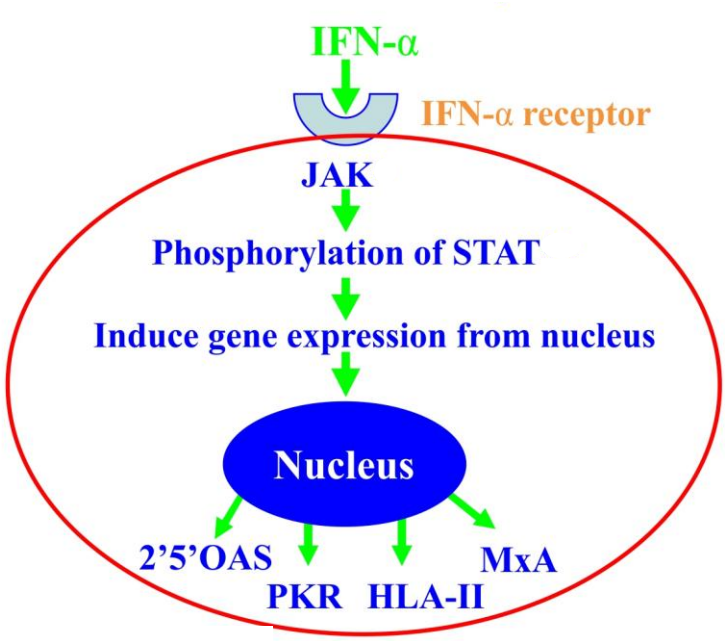


Figure 1.13. Intracellular signalling following the binding of interferon to cell surface receptors (modified from: Goodbourn et al. 2000)**.**

1.5.2.3 Viral induction of interferons

All classes of interferon play a role in fighting RNA virus infections, but their presence also accounts for many of the host symptoms, such as sore muscles and fever. Interferons are secreted when abnormally large amounts of dsRNA are detected in a cell .dsRNA is normally present in very low quantities, but increased amounts are present in infected cells, due to replication of viral RNA. Retonic acid-inducible gene I (RIG-I) detects cytoplasmic dsRNA, while Toll Like Receptor 3 (TLR3) detects dsRNA in endosome(Yoneyama et al. 2004).The dsRNA acts like a trigger for the production of interferon via RIG-I and TLR 3,, a pattern recognition receptor of the innate immune system which leads to activation of the transcription factor IRF3 and late phase NF (kappa Beta) (Chang et al. 2006). The gene that codes for this cytokine is switched on in an infected cell, and the interferon synthesized and secreted to surrounding cells.

When an infected cell dies, either because of a cytolytic RNA virus or immune lysis, thousands of viruses will be released that could infect nearby cells. However, if these cells have received interferon from the infected cell, this essentially warns the other cells that there's a wolf in the flock of sheep. They then start producing large amounts of a protein known as protein kinase R (or PKR). If a virus infects a cell that has been “pre-warned” by interferon, the PKR is indirectly activated by the dsRNA (actually by 2'-5' oligoadenylate, produced by 2'-5' oligoadenylate-synthetase which is induced following TLR3 activation), and begins transferring phosphate groups (phosphorylating) to a protein known as eIF-2, a eukaryotic translation initiation factor. After phosphorylation, eIF-2 has a reduced ability to initiate translation, the production of proteins coded by cellular mRNA. This impairs translation of viral proteins, preventing viral replication and inhibiting normal cell ribosome function, killing both the virus and the host cell if the

response is active for a sufficient amount of time. All RNA within the cell is also degraded, preventing the mRNA from being translated by eukaryotic initiation factor (eIF2), in case some if the eIF2 failed to be phosphorylated(Proud 2005; Jackson et al. 2010).Furthermore, interferon causes up regulation of MHC Class I and therefore to increased presentation of viral peptides to cytotoxic CD8 T cells, as well as a change in the proteasome (exchange of some beta subunits by b1i, b2i, b5i - then known as the immunoproteasome), which leads to increased production of MHC I compatible peptides.

Interferon can cause increased p53 activity in virus infected cells, by inducing increased production of the p53 gene product. This promotes apoptosis (programmed, non-lytic cell death), limiting the ability of the virus to spread. Increased levels of p53 transcription are observed even in cells which are not infected, but only infected cells show increased apoptosis. This increased transcription may serve to prepare susceptible cells so they can respond quickly in the case of infection. When p53 is induced by viral infection, it behaves differently than it usually does. Some p53 target genes are expressed under viral load, but others, especially those that respond to DNA damage, aren't. One of the genes that is not activated is p21, which can promote cell survival, so leaving this gene inactive would help to promote apoptosis. Interferon enhances the apoptotic effects of p53, but it is not strictly required. Normal cells exhibit a stronger apoptotic response than cells without p53 (Takaoka et al. 2003).

1.5.3 Interferon resistance

Both host and viral factors are likely involved in determining the antiviral efficacy of IFN.

A- Viral factors:

HCV has evolved important interactions with IFN induction and signalling that allow it to evade host immune mechanisms, causing persistent virus infection and IFN resistance (Figure 1.14A and B). IFN is rapidly synthesized after virus infection, rapidly triggering intracellular signaling events. The subsequent expression of IFN-stimulated (ISGs) is central to these antiviral responses. Several HCV proteins are suggested to interact in the IFN signaling pathway (Rani & Ransohoff 2005). HCV NS3/4A serine protease blocks phosphorylation and the effecter action of IRF-3, a key cellular antiviral signaling molecule (Foy et al. 2003). RIG-I has been shown to bind to the secondary structured HCV RNA efficiently to confer IFN-β induction (Saito et al. 2007). HCV NS2 and NS3/4A proteins are potent inhibitors of host cell cytokine/chemokine gene expression (Kaukinen et al. 2006). Furthermore, Both the full polyprotein and the HCV core protein have been shown to inhibit the JAK–STAT pathway in cell culture and transgenic mice (Heim et al. 1999; Blindenbacher et al. 2003).

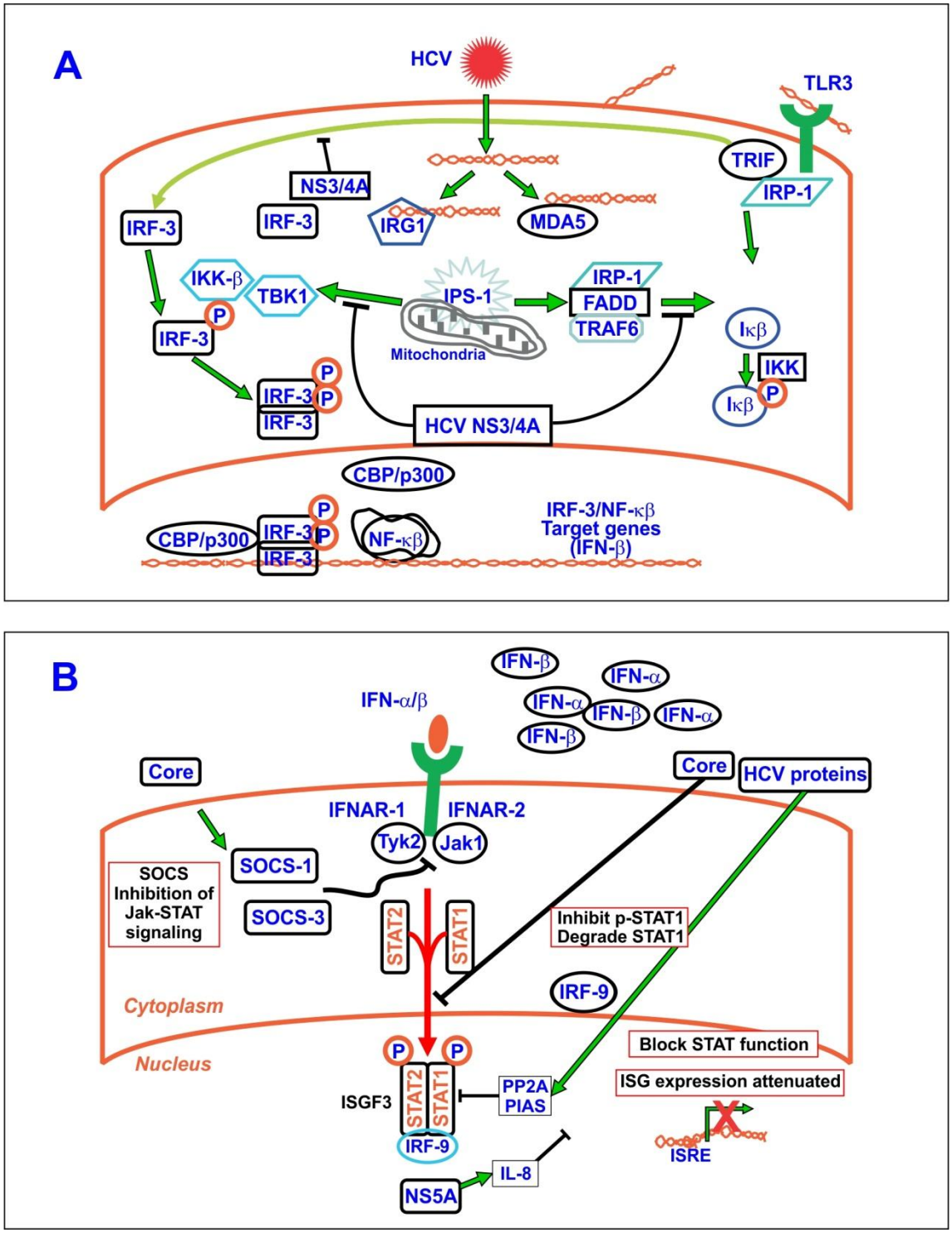


Figure 1.14 HCV subversion of IFN activation and signal transduction.
(A) HCV NS3-4A blocks IFN induction at multiple levels and (B) HCV blocks IFN signal transduction(modified from: Goodbourn et al. 2000)..

B- Host factors:

Age, sex, race, body weight, hepatic steatosis, insulin resistance (Figure 1.15), hepatic fibrosis and other comorbidities influence interferon efficacy. For example, Body fat mass and hepatic steatosis have been recognized as independent risk factors for a poor response to IFN-α therapy(McCullough 2003), older individuals have a lower response to IFN-α treatment than younger individuals (Hayashi et al. 1998), and African American HCV patients respond poorly to IFN-α therapy (Reddy et al. 1999). Furthermore, Several inhibitory pathways are identified that negatively regulate IFN-α–activated signal cascades. The suppressor of cytokine signaling (SOCS) family has been studied the most extensively and also is the most notably important negative factor for IFN-α signaling (Krebs & Hilton 2000). Interaction between Insulin Resistance and SOCS3 is one, which interacts with body weight and steatosis (Douglas & George 2009b).

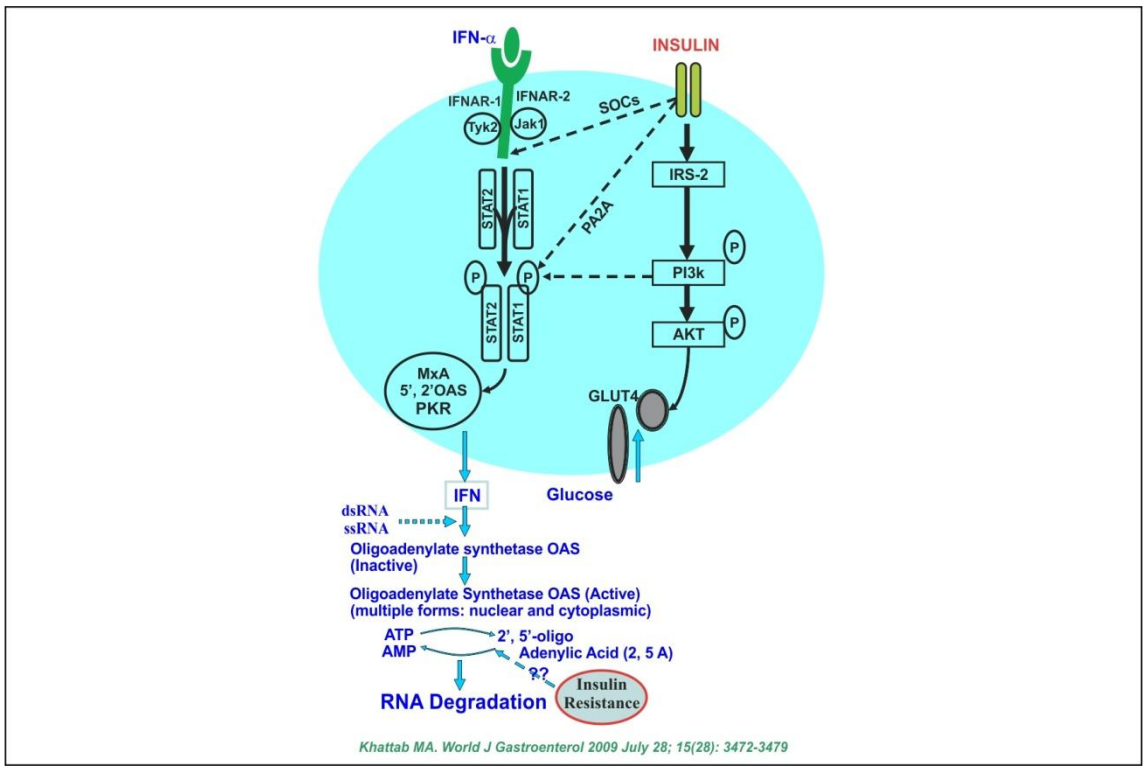


Figure 1.15 Interaction between insulin and interferon-α signaling pathways (Khattab 2009).

1.5.4 Interferon preparations

Several different types of interferon are now approved for use in humans.

1- Conventional interferon:

Conventional interferon-α2a (Roferon A) and 2b (intron-A) are given as subcutaneous injection of 3MU of INF-α 3 times/week combined with ribavirin 10.6mg/kg/day.

2- Pegylated interferon:

Pegylated interferon provides greatly improved pharmacokinetics over conventional interferon, and now forms the backbone for current standard of care treatments for CHC.

To make pegylated INF-α, polyethylene glycol (PEG) is added to the IFN-α molecule, reducing elimination from the body and increasing its serum half life (Manns et al. 2001). This allows pegylated interferon to be injected once weekly, rather than three times per week for conventional interferon-alpha, and provides more sustained serum levels. Two pegylated formulation of IFN are currently available: Pegylated interferon-alpha-2b (Peg-Intron) (12 kd) was approved in January 2001; pegylated interferon-alpha-2a (Pegasys) (40 kd) was approved in October 2002. The dose of peginterferon α-2b is weight-based: 1.5μg/kg/wk, while the dose of peginterferon α--2a is fixed at 180 μg/wk.

1.5.5.Adverse effects

The most frequent adverse effects of interferon treatment are flu-like symptoms (increased body temperature, feeling ill, fatigue) headache, muscle pain, chills, dizziness, hair thinning, and depression. Erythema, pain and induration (hardness) at the injection site are also frequently observed. Paradoxically, interferon therapy also causes immunosuppression, in particular though neutropenia, and can result in some infections manifesting in unusual ways (Larrey et al. 2007).

Most adverse effects are reversible and disappear a few days after the therapy has finished.

1.6 Genetic markers and antiviral responses

1.6.1 HCV genetic variability and antiviral responses

The inherent genetic variability of HCV may have important clinical consequences. Comparisons of different HCV genomes have identified amino acids that differed consistently by response to treatment (Torres-Puente et al. 2008). However, it now seems that the outcome of antiviral treatment might depend not only on variations of one or a few

independent positions, but more likely on the combination of amino acid sequences at several positions along the HCV genome.

Interferon sensitivity determining region (ISDR) is a 40 amino-acid section in the center of NS5A protein (aa2209-2248). It was the first position implicated in determining the response to IFN therapy for HCV-genotype 1b (Enomoto et al. 1995). In addition to the ISDR, hypervariable regions (HVR1, HVR2 and HVR3) of the E2 glycoprotein show tendency to be correlated with antiviral therapy outcome (Osei-Hyiaman et al. 2005) (Figure 1.16). More recently, a relationship has been found between poor response to peg-INF plus ribavirin combination therapy and substitutions at amino acid 70 and 91 in the core protein (Enomoto & Maekawa 2010).

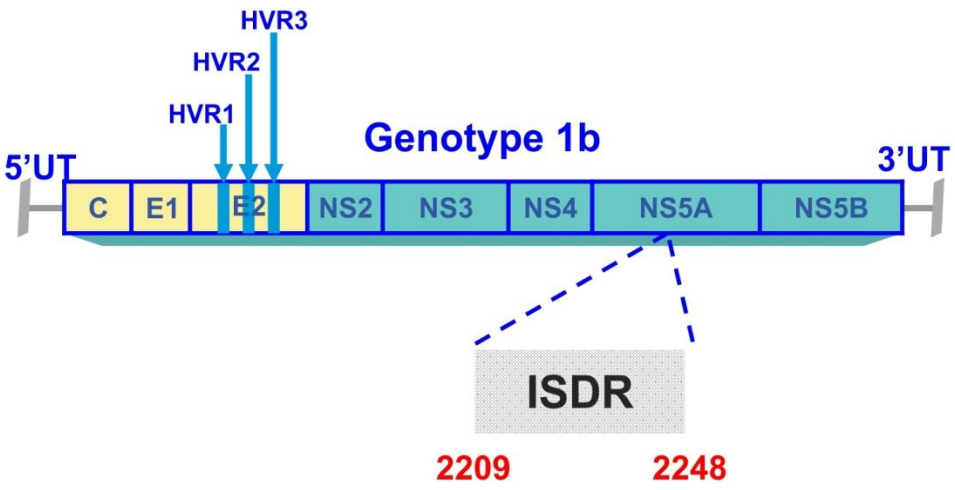


Figure 1.16 HCV genetic Variability and antiviral responses.

1.6.2 Host genetic markers and antiviral responses

Host genetic factors may play also a role in determining disease outcome and response to therapy in CHC. In 2009, three different studies showed that single nucleotide polymorphisms (SNPs) near the interferon lambda 3 [IFNL3] gene predict the response to interferon-based treatment of hepatitis C (at SNP rs12979860, CC connotes the responder genotype, CT or TT connotes the non responder genotype; at rs8099917, TT is the responder genotype, while GT or GG is the non-responder (Suppiah et al. 2009; Tanaka et al. 2009; Ge et al. 2009), an observation of major translational impact.

1.6.3 Ribavirin (RBV)

Ribavirin is a guanosine nucleoside analogue, first synthesized more than 35 years ago. Although it shows no direct antiviral activity against HCV when used alone, it is highly effective in treatment of CHC when used in combination with IFN-α, and remains an important part of triple therapies containing new protease or polymerase inhibitors.

The molecular mechanisms by which RBV enhances early and sustained virological response rates during IFN-based antiviral therapy are still unknown. Several mechanisms have been proposed including:

i. Immunomodulatory properties, altering the host immune response from Th2 to Th1.

ii. Inhibition of the inosine monophosphate dehydrogenase (IMPDH), the NAD-dependent enzyme that cintrols de novo synthesis of purine nucleotides (Pankiewicz & Goldstein 2003). Inhibition of IMPDH results in an interruption of DNA and RNA synthesis (Jayaram et al. 1982).

iii. Direct inhibition of the HCV-encoded NS5B RNA polymerase.

iv. Induction of lethal mutagenesis ("error catastrophe").

v. Modulation of ISGs expression (Feld et al. 2010).

The dose of RBV ranges from 800-1400 mg/day according to body weight (15 mg/kg body weight/day).

RBV treatment is associated with many side effects, most commonly haemolytic anemia and rash.

1.7. HCV Therapy: What will the future look like?

1.7.1 Targeting anti-HCV therapies

Advances in the understanding of HCV biology have identification of a large number of compounds, collectively known as Direct Acting Antivirals (DAAs), which target HCV proteins, or Host Targeting Agents. Boceprevir and telaprevir were approved in 2011 in many countries including Australia, and are now standard of care for HCV genotype 1. Sofosbuvir and simeprevir have been approved in USA, Europe and very recently in Australia, although still being finalised here. They are now standard of care in USA according AASLD guidelines. Pivotal studies in New England Journal of Medicine earlier this year showed extremely high cure rates with IFN-free treatment. Either sofosbuvir plus NS5A inhibitor (daclatasvir or ladipasvir), or multi-drug combinations in Abbvie study (Poordad et al. 2014). DAAs have been selected to target several critical steps in viral replication including cell infection, RNA transport to the endoplasmic reticulum, HCV translation, transcription, and assembly (Figure 1.17).

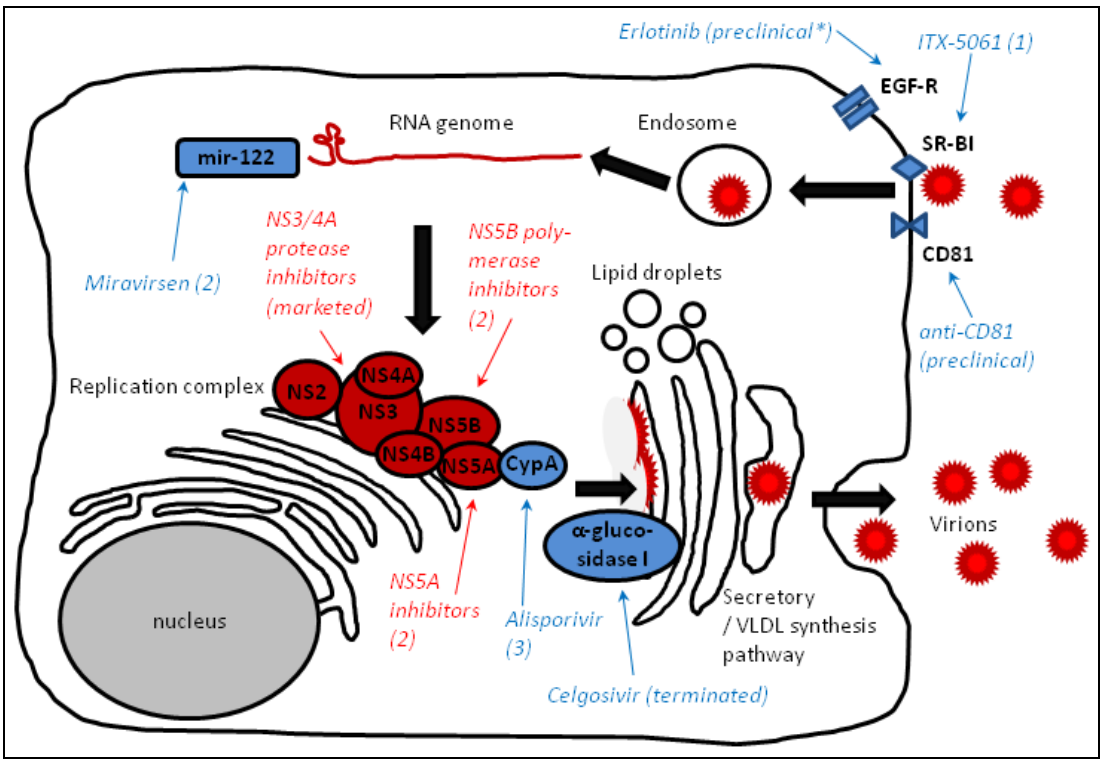


Figure 1.17 HCV life cycle and targets for Direct Acting Antivirals (DAAs)(Hahn et al. 2011).

Table 1.2: Steps in HCV replication and potential mechanisms for inhibition

Step of HCV replication (target)	Option for inhibition
Transport of RNA to ER	Ribozymes Antisense-oligonucleotides Short interfering RNAs
Translation and post-translational processing	Protease inhibitors
RNA transcription	Polymerase inhibitors
Viral assembly	Glucosidase inhibitors

1.7.2 Therapies that target host factors

A major obstacle in combating HCV infection is that the fidelity of the viral replication machinery is low, enabling the virus to quickly develop mutations that resist compounds targeting viral enzymes (Bartenschlager et al. 2013). Targeting host factors essential for viral replication provides an alternative strategy that should minimise the risk of viral resistance and treat all HCV genotypes. [Most NS3 inhibitors and non-nucleos/tide NS5B inhibitors are relatively specific for genotype 1, with little or no activity against genotype 3 (Eltahla et al. 2014). However it is essential to identify treatments that have minimal side effects on patients.

1.7.2.1 Antireceptor antibodies

The rationale for antireceptor antibodies as antiviral drugs is based on: that is not being prone to the problems of viral variability and HDL attenuation of neutralizing

activity; we could consider anti-SR-B1 and anti-CD-81 antibodies capable of interfering with HCV infection an alternative to the development of anti-HCV antibodies (Drummer 2014; Lacek et al. 2012).

Recent data refer to the critical role of CD81 in HCV infection in vivo and offer a new perspective for the prevention of allograft reinfection after orthotopic liver transplantation in chronically infected HCV patients (Fofana et al. 2013). However, the efficacy of targeting CD81, SR-BI or CLDN1 may be limited by receptor-independent modes of virus transmission.

1.7.2.2 Targeting host metabolism

1.7.2.2.1 Cyclophilin B inhibitors

A key host cell factor involved in HCV RNA replication is the human protein cyclophilin B, which interacts with the C-terminal region of NS5B and appears to stimulate its RNA binding activity. The cyclophilin B inhibitor Debio-025 potently suppresses genotype 1 HCV replication in vivo (Quarato et al. 2012), but its development is currently suspended due to concerns about possible toxicity.

1.7.2.2.2 Insulin sensitizers

Insulin resistance has emerged as a new target for treatment of CHC patients. The rationale for increasing insulin sensitivity in patients with CHC is that the insulin resistant state 1) directly or indirectly inhibits the antiviral action of IFN; 2) increases viral fitness, making it more resistant to therapy, or both (Serfaty et al. 2012). Interestingly, intracellular factors deregulated by HCV and responsible for the insulin resistant phenotype may have promiscuous effects, as they are also involved in regulating IFN-α signaling (Irshad et al. 2013).

The available clinical data on the use of insulin sensitisers reveals conflicting results (Cheng et al. 2014).

1.7.2.2.3 Host lipid biosynthesis inhibitors

HCV increases cellular lipids metabolism (Perlemuter et al. 2002). So, existing anti-obesity drugs may represent a safe way to block these metabolic changes and inhibit viral infection as one of the potential novel approaches that may improve response rates to treatment. There are at least 2 different molecular mechanisms providing novel targets for treatment of HCV: either through the modulation of cellular TG or cholesterol metabolism.

In vitro data suggest that statins, the widely used cholesterol-lowering drugs, may inhibit HCV RNA replication by depletion of geranylgeranyl lipids (Kapadia & Chisari 2005; Ye et al. 2003). It was demonstrated that dose-dependent strong antiviral effects exist for all the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-coA) reductase inhibitors, except for pravastatin, in vitro. Fluvastatin exhibited the strongest antiviral activity, followed by atorvastatin and simvastatin (Ikeda et al. 2006). Several clinical trials have used statins as monotherapy or in combination with the standard of care (SOC). O'Leary et al. undertook a human pilot study and treated 10 CHC patients with hypercholesterolaemia with atorvastatin (at the conventional daily dose of 20 mg) for 12 weeks. They reported no statistically significant change in HCV RNA levels compared with pretreatment levels at 4 and 12 weeks, despite improvement in lipid parameters (O'Leary et al. 2007). Bader et al reported that fluvastatin inhibited HCV RNA replication in patients with CHC; fluvastatin (20-320 mg/day) was well tolerated in patients with CHC and was effective at relatively low doses (Bader et al. 2008). Milazzo et al reported disappointing results in a pilot trial of 42 patients coinfected with HCV and HIV and receiving fluvastatin 80 mg daily, in whom the expected decrease in serum cholesterol and LDL was paralleled by a paradoxical

increase in HCV RNA levels (Milazzo et al. 2009). A more recent open label randomized controlled trial in HIV/HCV genotype 1 co-infected patients found that the addition of Fluvastatin (80 mg/day) to SOC therapy significantly improved the rate of rapid virological response (RVR, HCV PCR negative after 4 weeks), but did not significantly increase the SVR rate (Milazzo et al. 2010). In another small uncontrolled trial, 20 mg/day fluvastatin was added to pegylated IFN-α/ribavirin in a group of 21 patients. Among the 15 patients who received a 48-week course of therapy the SVR was 67% (Sezaki et al. 2009). In a large retrospective trial Harrison et al (Harrison et al. 2010), showed that administration of a statin was associated with a significant increased rate of SVR; interestingly this effect was noted to be independent of the baseline lipid profile. In subset analysis, statin users had higher chances of SVR when aged >48 years, of non-African American ethnicity and female. These data were recently supported by a very large cohort of diabetic CHC patients, in which use of statin was associated with improved SVR (Rao & Pandya 2011).

The mechanisms by which statins interfere with HCV replication are not completely understood. Tiedemann et al recently suggested that statins my have multifactorial effect on chronic HCV infection. They may interfere with HCV replication by induction of the enzyme heme oxygenase 1 (HO-1), with subsequent reduction of cellular levels of ROS (Tiedemann et al. 2010). This effect was associated with induction of endogenous antiviral defense mechanisms by induced expression of IFN alpha 2 and 17 and several IFN stimulated genes (e.g. OAS 1 and 2, PKR and HRI).

All together, these findings suggest that statin use is safe in CHC patients and support ‘proof-of-concept’ for trials combining statins with standard pegylated interferon plus ribavirin therapy. However, the choice of statin may be vital in order to achieve

antiviral effect, and further studies are needed to investigate the mechanism of action of statins against HCV.

In addition to the antiviral effects of statins, HMG-CoA-reductase inhibitors(Fluva- and simvastatin) are able to selectively induce apoptosis of hepatoma cells. The underlying mechanism seems to include insufficient prenylation of Ras and Rho proteins, resulting in a lack of cellular integrity. Therefore HMG-CoA-reductase inhibitors, which have been shown to interfere with HCV replication, might also have beneficial effects in reducing the occurrence of HCC (Pathil et al. 2006).

Other classes of drugs designed for treating hypercholesterolemia block the assembly and secretion of VLDL

These drugs may also be effective in treating HCV infection because they inhibit release of HCV particles from infected cells (Huang et al. 2007). In this regard, antisense RNA drugs targeting apoB and several microsomal triglyceride protein (MTP) inhibitors (Amemiya et al. 2008; Tai et al. 2009) have already been tested in clinical trials because of their ability to block VLDL secretion, thereby lowering the plasma levels of VLDL triglycerides and LDL cholesterol. Long-term treatment with MTP inhibitors led to the toxic accumulation of fat in livers (Amemiya et al. 2008; Tai et al. 2009), thus hampering the approval of these drugs for the treatment of hypercholesterolemia on a long-term basis. However, short-term treatment (up to several weeks) reduced the plasma level of VLDL with only minor adverse effects, which disappeared after drug discontinuation (Tai et al. 2009). It will be interesting to examine whether short-term treatment with MTP inhibitors is beneficial in treating HCV infection (Khattab 2009). Furthermore, DGAT1 is required for the trafficking of core protein to lipid droplets. Inhibition of DGAT1 activity or RNAi-mediated knockdown of DGAT1 severely impairs infectious virion production (Herker et

al. 2010). Lastly, a recent studies by our group showed that acyl-CoA cholesterol acyltransferase (ACAT) inhibitors and cannabinoid 1 (CB1) antagonist are an effective strategies against HCV (Read et al. 2014; Shahidi et al. 2014).

1.8 Summary and Research Objectives

Understanding the molecular and biochemical pathways activated in HCV-infected cells will provide targets to interrupt virus replication, and facilitate the development of more effective treatments. A unique aspect of HCV is that the entire viral life cycle is associated with host lipoproteins: (1) HCV circulates in plasma with lipoprotein as an infectious complex (lipoviral particle); (2) Hepatocyte lipoprotein receptors are involved in HCV entry; (3) Replication of HCV RNA in hepatic cells is inhibited by inhibitors of lipid metabolism; (4) HCV particles released from hepatocytes are attached to lipoproteins; and (5) Serum lipid profiles (LDL-C, HDL-C and triglycerides) are associated with rates of spontaneous or treatment-induced HCV clearance (Negro 2010). Despite the fact that the importance of cellular lipid for HCV infection has been proven (Diamond et al. 2010), the extent to which HCV modulates global cellular metabolism to create a favourable environment for RNA replication and the production of progeny particles is currently unknown, especially at the sub-cellular level. Changes in host lipoprotein expression can dramatically affect the lipid content of different sub-cellular compartments. As a result, measuring metabolite concentrations in different cellular fractions can identify changes in metabolic pathways more accurately than quantifying the relevant enzymes or mRNAs that encode them (Kell et al. 2005b; Tan et al. 2009). The endoplasmic reticulum (ER) plays a central role in HCV replication: following infection, viral RNA is translated at the ER, where viral proteins induce membrane modifications, giving rise to the ‘membranous web’. This web acts as the platform for the synthesis of HCV RNA. Viral structural

proteins are then packaged with RNA, assembled into 'lipoviral' particles and exported through the ER-Golgi pathway (Gastaminza et al. 2008b). Thus, understanding HCV/host lipoprotein interactions at the level of the ER should provide novel insights into the HCV life cycle that would be missed by studying the whole infected cell in isolation.

Research objectives:

1) Examine the effect of HCV infection on the hepatocyte lipidome by comparing global lipidomic profiles between HCV-infected (JFH1) and uninfected Huh-7 cells using a multi-analytical approach.

2) Examine the effect of HCV infection on the lipid content of specific Huh7 cell fractions (LDs and ER), using the same multi-analytical approach, to determine the mechanism of HCV-induced ER membrane modifications.

3) Investigate the potential translational applications of these findings for the development of novel HCV therapies.

Some previous studies have performed fairly crude analysis of specific lipids, such as triacylglycerol using biochemical assays (Negro & Sanyal 2009), but have not examined all major lipid species in a true quantitative way. Nor have they analysed the FA composition of lipids. Other studies have performed global mass spectrometry analysis of whole lipid extracts (Diamond et al. 2010), but this approach provides limited quantitation and can be biased by strong signals from polar lipids.

This study combined high performance thin layer chromatography (HPTLC) separation of lipids with automated multiple development (AMD) technology for the first time to analyse changes in the lipid content of HCV-infected cells (Figures 1.18 A and B). HPTLC-AMD technology provides consistent, optimized extraction and fractionation systems to fractionate lipid extracts before more detailed analysis is performed. The silica

gel plate is developed with a reproducible stepwise elution gradient from polar to unpolar, allowing separation over a wide range of polarity and making it possible to separate substances that exhibit few differences in polarity. Software-controlled gradient ensures a high reproducibility. The advantages of using HPLTC technology are as follows:

1. HPTLC identifies major lipid species and provides accurate, reproducible quantitation. This shows which lipid classes are increased or decreased in infected cells.

2. Cell fractionation allows a more detailed analysis of the relative increase/decrease of lipids, as in point 1, but in individual sub-cellular compartments.

3. Lipid mass spectrometry can be used downstream to determine the individual FA composition of lipid species that are altered (up or down). The purpose of this approach is to identify which enzymes are responsible for the change.

Figure 1.18 HPTLC-AMD instruments.
(A) Automatic thin layer chromatography (TLC) sampler. (B) high performance thin layer chromatography (HPTLC) development chamber (http://www.camag.com).

2. Materials and Methodology

2.1 Materials

2.1.1 Chemical reagents

Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Lonza. Foetal calf serum (FCS), penicillin, Geneticin (G418), streptomycin, Opti-MEM media and GlutaMAX™ were purchased from Gibco BRL. An Ultraclean® tissue and cells RNA isolation kit was purchased from Mol Bio Laboratories. A RNeasy miniprep kit was purchased from Qiagen. A Superscript III First Strand cDNA Synthesis kit, RNAiMax™ transfection reagent, Taq DNA polymerase, random hexamers, deoxynucleotides, a SYBR green master mix and DTT were purchased from Invitrogen. MMLV reverse transcriptase and the T7 RiboMAX™ Express Large Scale RNA Production System were purchased from Promega. Real-time PCR buffer premix D was purchased from Epicentre. Primers were purchased from Geneworks, and TaqMan® primer probes were purchased from Applied Biosystems.

Tween 20, tetramethylethylenediamine (TEMED), ammonium persulfate (APS), Triton X- 100, bovine serum albumin (BSA) and all horseradish peroxidise (HRP) conjugated secondary antibodies were purchased from Sigma-Aldrich. Nitrocellulose paper,a DC™ protein assay kit, and 30% acrylamidewere purchased from Bio-Rad. SuperSignal West pico-chemiluminescent and SuperSignal West femtochemiluminescent substrates and a PageRuler™ Plus pre-stained protein ladder were purchased from Thermo Scientific.

Chloroform, hexane, isopropanol and ethylacetate were purchased from VWR BDH Prolabo. Ethanol, methanol, dichloromethane and copper (II) sulfate heptahydrate, cholesterol and cholesteryl oleatewere purchased from Sigma. Phospholipid standards,

including1,2-Dioleoyl-sn-Glycerol-3-[phosphor-L-Serine]1-Oleoyl-2-Hydroxy-sn-Glycero-3-Phosphocholine; 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; and 1,2-Dioleoyl-sn-glycero-3-[Phospho-rac-(1-glycerol)], were purchased from Avanti Polar Lipids.All neutral lipid standards, including 1-Oleoyl-glycerol; 1,3Diolein; cardiolipin; cholesterol and cholesteryl linoleate, were purchased from Sigma. Silica gel 60 HPTLC plates and solvents were purchased from EMD Chemicals.

HCV rabbit anti-core (308) and sheep anti-ADRP antibodies were kindly provided by Dr. John McLauchlan (University of Glasgow, UK). HCV sheep anti-NS5A was provided by Prof. Mark Harris (University of Leeds, UK). STAT1, phosphor-STAT1, eIF-2α and Erlin 1 Rabbit polyclonal antibodies were purchased from Cell Signaling. Alexa 488-conjugated Donkey-anti-Rabbit secondary antibody, Alexa 594-conjugated Donkey-anti-sheep secondary antibody, BODIPY 493/503 (4,4-difluoro-1,3,5,7,8- pentamethyl-4-bora-3a,4a-diaza-s-indacene; 10 μg/mL) and Prolong GOLD mounting medium were purchased from Invitrogen. Human S-Adenosyl-Methionine (SAMe) ELISA kit was purchased from MyBioSource.

2.1.2 Immunological reagents

The monoclonal antibodies (mAbs) and polyclonal antisera (pAbs) that were used in this study are detailed below.

Table 2.1: List of antibodies and Western blot conditions used for protein quantification.

1° Antibody	Raised in	Working dilution	2° Antibody	Working dilution
PEMT (LSBio)	Rabbit	1:1000 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
STAT1α (Santa Cruz)	Rabbit	1:1000 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
pSTAT1 (Cell signaling)	Rabbit	1:1000 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
HCV NS5A	Sheep	1:1000 in 5% skim milk/TBST	Anti Goat-IgG (Sigma)	1:10000 in 5% skim milk/TBST
HCV Core	Rabbit	1:1000 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
DDX3X (Santa Cruz)	Rabbit	1:500 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
Erlin-1	Rabbit	1:500 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
ADRP	Sheep	1:1000 in 5% skim milk/TBST	Anti Goat-IgG (Sigma)	1:10000 in 5% skim milk/TBST
eIF2-α (Cell signaling)	Rabbit	1:1000 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
NFKβ (Cell signaling)	Rabbit	1:1000 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
AKT (Cell signaling)	Rabbit	1:1000 in 5% skim milk/TBST	Anti Rabbit-IgG (Sigma)	1:10000 in 5% skim milk/TBST
B-actin (Sigma),	Mouse	1:10000 in 5% skim milk/TBST	Anti Mouse-IgG (Sigma)	1:20000 in 5% skim milk/TBST

Table 2.2: Primary and secondary antibody dilutions and incubation times for immunofluorescence labelling

1° Antibody	Raised in	Working dilution	2° Antibody	Working dilution
Anti HCV (core)	Rabbit	Core 1:1000 in 1% FBS/PBS	Alexa Fluor 488 antiRabbit IgG	1:1000 in % FBS/PBS
Anti NS5A	Sheep	1:1000 in 1% FBS/PBS	Alexa Fluor 594 antiSheep IgG	1:2000 in 1%FBS/PBS

2.1.3 Cell lines

All replicating viruses were grown in the Huh-7 hepatoma cell line that was originally extracted from a liver tumour found in a 57-year-old Japanese male. Huh-7 cells were the kind gift of Dr. John McLauchlan (University of Glasgow, UK). The cells were cultured in Dulbecco's minimal essential medium (DMEM) that contained 4.5 g/L D-Glucose and L Glutamine (Walkersville MD USA) supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (Gibco-Life Technologies).

2.1.4 Plasmid

Plasmid DNA encoding JFH1 (pJFH1) were the kind gift of Prof. Takaji Wakita (National Institute of Infectious Disease, Tokyo, Japan). HCV RNA was prepared according to the method described by Wakita et al. (Wakita, Pietschmann, Kato, Date, Zhao et al. 2005) (see Section 2.2.2.1).

2.1.5 Solutions

Table 2.3 Standard solutions used in this project.

Solution	Contents
SDS-PAGE sample loading buffer	100 mM Tris-HCl pH 6.9, 2 % SDS, 10 % glycerol, 5 % β mercaptoethanol, 1 µg/ml bromophenol blue
SDS-PAGE running buffer (10X)	30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O
Western blot transfer buffer	24 mM Tris, 194 mM glycine, 20% methanol, 0.1% SDS
Tris-buffered saline with Tween (TBST) buffer	20mM Tris-HCl, pH 7.4, 500 mM NaCl and 0.1% Tween
Protease inhibitors solution	20 mM AEBSF, 100 mM Benzamidine, 50 mM EDTA, 100 µg/ml Apronitin, 100 µg/ml Leupeptine
Coomassie stain	0.2 % Coomassie brilliant blue dye R200, 50 % methanol, 7 % acetic acid
Destain solution	5 % (v/v) methanol, 7 % (v/v) acetic acid (in H_20)
MTE buffer	10 mM Tris-base, 2 mM MgCl2, 1 mM DTT, 270 mM D-mannitol, 0.1 mM EDTA, protease inhibitor mixture, pH to7.5 with 6 M HCl
Modified Barth's saline (MBS) buffer	0.15 M NaCl, 25 mM, Mes, pH 6.5
Hypotonic lysis medium (HLM) buffer	40 mM Tris, pH 7.4, 2 mM EDTA, 20 mM NaF, protease inhibitors

2.2 Methods

2.2.1 Molecular virology

2.2.1.1 Tissue culture

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% v/v foetal bovine serum (FBS), 100 U/ml penicillin, 100 □ g/ml streptomycin and 2 mM L-glutamine, and incubated at 37 °C in a humidified incubator with 5% CO_2. Culture medium for Huh-7 cells was additionally supplemented with 1% v/v non-essential amino acids (Cambrex).

Cells were passaged by washing in phosphate-buffered saline (PBS) pH 6.8 prior to incubation with trypsin-EDTA solution pH 8 (Sigma-Aldrich) to disassociate cells from the growth surface. Cells were mechanically resuspended in culture medium, quantified using a haemocytometer and seeded into fresh culture medium at the desired density.

2.2.1.2 Transfection of cells with JFH1 RNA

Huh-7 cells were grown in T150 flasks up to ~80% confluence. Cells were harvested by a process of trypsinisation. 4×10^6 cells were washed twice by centrifugation at 1500 rpm for five min in 40 ml of cold phosphate-buffered saline (PBS) and resuspended in 1.6 ml cold PBS. Cell suspension was then combined with 4 µl of transcribed JFH1 viral RNA, mixed gently and transferred in two electroporation cuvettes of 800 µl each. Electroporation was performed with a single pulse at 0.34 kV, 974 µF. Electroporated cells were mixed with 10 ml complete media (DMEM+ 10% FCS) in two T25 flasks. Cells were then incubated at 37 °C for 3-5 days. Viral protein expression was confirmed by immune fluorescence microscopy, using antibody against viral core or NS5A

proteins. JFH1 virus-infected cells were allowed to grow for no longer than six passages to limit viral mutations.

2.2.1.3 Harvesting cells

Following removal of supernatant, Huh-7 cells or JFH1-infected cells were washed once with PBS and then detached by trypsinisation. Identically treated cells were pooled from replicate flasks, if necessary, to obtain 2×10^7 total cells per time point, per condition. Cells were washed twice in PBS, pelleted and stored at −80 °C until sample preparation.

2.2.2 Preparation, manipulation and analysis of HCV and cellular RNA

2.2.2.1 pJFH1 plasmid linearization for in vitro transcription

DNA plasmids encoding the HCV genotype 2a strain JFH1 (pJFH1-pUC) were linearized at the 3′ end of the HCV DNA with the restriction enzyme XbaI (Promega). According to the manufacturer's protocol, plasmids encoding the full-length of the JFH1 isolate were linearized by XbaI digestion overnight at 37 °C. XbaI was heat-inactivated at 65 °C for 20 minutes, after which samples were cooled to RT then incubated for 10 minutes on ice. The four terminal nucleotides produced by linearization were removed by treating the samples with 1 μl Mung Bean Nuclease (New England Biolabs) for 45 min at 30 °C, resulting in the correct 3'-end of the HCV DNA. Mung Bean Nuclease was inactivated with the addition of 0.1 % w/v SDS and the DNA was purified by phenol/chloroform extraction and ethanol precipitation before being resuspended in RNase-free water.

2.2.2.2 In vitrotranscription of HCV RNA

After precipitation and purification of the plasmid DNA, RNA transcription was performed using a T7 RiboMAX™ Express Large Scale RNA Production System kit (Promega). The reaction mixture, which consisted of 1x RiboMAX™ Express T7 buffer, 1 µg of linearized template DNA and 2 µl of T7 express enzyme mix, was then increased to a volume of 20 µl using nuclease-free water. The mixture was made in accordance with the manufacturer's instructions. After combining the mixture gently, it was incubated at 30°C for 30 min. One unit of RQ 1 RNase-free DNase was added to the mixture and incubated for 15 min at 37 °C. To remove protein, an equal volume of phenol: chloroform: isoamylalcohol (125:24:1) pH 4.0 (Sigma) was added, the mixture was vortexed for one minute, followed by centrifugation at 16,100 × g for two minutes. The aqueous phase of the extracted mixture was transferred to a new tube and then mixed with an equal quantity of chloroform, vortexed for one minute and then centrifuged for two more minutes at 16,100 × g. The aqueous phase was transferred to a new tube where the RNA was precipitated by adding 1/10th volume v/v of 3 M sodium acetate pH 5.2 and the same volume of isopropanol. The extracted samples were vortexed thoroughly, then incubated on ice for five min. The precipitated RNA was then converted into pellets through centrifugation. Centrifugation was performed at 16,100 × g for 10 min. The pellets were washed with 70 % v/v ethanol, then pelleted again. The pellets were air-dried, then dissolved in 20 µl RNase-free water. RNA was quantified using a NanoDrop ND1000 spectrophotometer and RNA integrity was checked by agarose gel electrophoresis.

2.2.2.3 Extraction of total RNA from cells

Total RNA was extracted from cell culture monolayers using the Qiagen RNeasy Kit, according to the manufacturer’s instructions. In brief, cells in six-well plates were

lysed in 350 µl of RNeasy Lysis buffer and transferred to a new RNase-free tube. Next, 350 µl of 70% ethanol was added to each cell lysate and mixed thoroughly. 700 µl of sample solution was applied to an RNeasy spin column in a collection tube and centrifuged for 30 seconds at 10,000 RPM. The flow-through was discarded, and 700 µl wash buffer RW1 was added to each spin column and centrifuged for 30 seconds at 10,000 RPM. After transferring the spin column to a clean tube, 500 µl of second wash buffer, RPE was applied to each column and the tubes centrifuged as per the process described above. This step was repeated, RNA was eluted from the spin column with 50 µl of RNase-free water and centrifuged for one minute at 10,000 RPM into a clean, RNase-free tube. Eluted RNA was quantified using a NanoDrop ND1000 spectrophotometer and stored at -80 °C for subsequent experiments.

2.2.2.4 Extraction of RNA from extracellular HCV virions

RNA was extracted using the FavoPrep^{TM} kit (Favorgen Biotech corp). In brief, 140 µl of supernatant from JFH1-infected Huh-7 cells was mixed with 560 µl lysis buffer containing carrier RNA and incubated at room temperature for 10 minutes. After the addition of 100% ethanol, the mixture was transferred to QIAamp Mini columns and tubes were centrifuged at 8000 RPM for one minute. RNA was then washed twice using AW1 and AW2 washing buffers and eluted from mini columns with 30 µl buffer AVE. The extracted RNA was stored at -80 °C for future experiments.

2.2.2.5 First-strand cDNA synthesis

cDNA was reverse transcribed from total RNA using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. In brief, 0.5 µg RNA and 250 ng/µl random hexamer primers (Invitrogen) were added to a sterile RNase-free microcentrifuge tube in a total volume of ≤ 15 µl water. The mixture was incubated at 70

°C for five minutes to melt the mRNA secondary structure. The tubes were immediately cooled on ice for at least one minute. A reaction mixture containing 1x MMLLV reaction buffer (Promega), 0.5 μM each dNTPs (Invitrogene), 40 units RNaseOUT™ Ribonuclease (RNase) Inhibitor (Invotrogen) and 200 units M-MLV RT was prepared and added to the primer/template tube. Samples were mixed gently and incubated for 60 minutes at 37 °C. The synthesized cDNA was stored at -20 °C for further experiments.

2.2.2.6 Real-time PCR

Real-time quantitative PCR (qPCR) was performed using the Rotor-Gene 6000 (Corbett Research, Sydney, NSW, Australia) with SYBR Green or Taqman protocols. mRNA levels were normalized to 18S ribosomal RNA or GAPDH, which produced comparable results. Real-time qPCR reaction conditions comprised initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 45 seconds. A melt curve analysis was performed at the end of each run of the SYBR Green protocol to confirm the generation of specific PCR products. QPCR results were analysed using Rotor-gene 6000 Corbett software. The fold change of each gene was calculated using the Delta-delta threshold (ΔΔCt) data analysis method.

Table 2.4 List of primer sequences used for real-time qPCR.

Gene	Forward	Reverse
PEMT	*5'GGATGAAGAGATCTGGGAACC3'*	*5'ATGACGGCAGCCACAAAG3'*
PYCT 1A	*5'TCCCGAATTCATTGGAAG3'*	*5'TGAAGCGACAGGTTTCTTCTG3'*
ISG15	*5'CGCAGATCACCCAGAAGATC 3'*	*5'GCCCTTGTTATTCCTCACCA 3'*
OAS3	*5'GTCAAACCCAAGCCACAAGT 3'*	*5'GGGCGAATGTTCACAAAGTT 3'*
SREBP-1c	*5'GCCATGGATTGCACTTT3'*	*5'CAAGAGAGGAGCTCAATG3'*
ABCA1	*5'GCACTGAGGAAGATGCTGAAA 3'*	*5'AGTTCCTGGAAGGTCTTGTTCAC 3'*
GAPDH	*5'CCATTCAATGACCCTTGTTG-3'*	*5'CTGGGATTTCCATTGATGACAAG-3'*

Table2.5 List of Taqman gene expression assays used for real-time qPCR.

Gene symbol	Gene name	Supplier	Gene ID
HCV 5'UTR	PATHOGEN	Applied Biosystems, Foster City,CA	Pa03453408_s1
18S	Eukaryotic, 18S	Applied Biosystems, Foster City,CA	Hs03003631_ml
MTP	Microsomal triglyceride transfer protein	Applied Biosystems, Foster City,CA	Hs00165177_ml
LDL-R	Low density lipoprotein receptor	Applied Biosystems, Foster City,CA	Hs00181192_ml

Table 2.6 Real-time qPCR reactions content using forward and reverse Primers.

Component	Volume (µl)	Final concentration
Fast SYBER Green qPCR Master Mix (Invitrogen)	10	1X
Forward Primer (10 µM)	0.5	0.25 µM
Reverse primer (10 µM)	0.5	0.25 µM
Diluted cDNA Template	2	
RNase free H2O	7	
Total Reaction volume	20	

Table 2.7 Real-time qPCR reaction content using Taqman gene expression assay.

Component	Volume (µl)
MasterAMPTM2X PCR PreMix D (Epicenter)	10
Taqman Gene expression assay	0.5
Taq DNA Polymerase Recombinant 5U/µl (Invitrogen)	0.4
Diluted cDNA Template	2
RNase free H2O	7.1
Total Reaction volume	20

2.2.2.7 siRNA knockdown

PYCT1A and PEMT siRNA (Origene) were transfected into JFH1-infected Huh-7 cells using Lipofectamine transfection. Transfections were carried out using the Lipofectamine RNAiMAX (Invitrogen, CA, USA) kits according to the manufacturer's guidelines. Briefly, JFH1-infected Huh-7 cells were seeded at 1×10^5 cells per well into a 12-well plate in P/S free media for one day before transfection. siRNA-RNAiMax complex was made up as follows: 10 nmol siRNA was added to 85 µl of Opti-MEM (Invitrogen) and mixed gently. 1.4 µl RNAiMAX was added to 80 µl of Opti-MEM and mixed gently. Diluted siRNA and diluted RNAiMAX were combined and incubated at room temperature for 30 min to allow the formation of complexes. Complexed RNAi was added to wells containing 1 ml of P/S free DMEM. Transfections were carried out at 37 °C. Expression efficiency was assessed 48 h post-transfection using qPCR.

2.2.3 Concentration of JFH1 viruses (PEG precipitation)

JFH1-infected Huh-7 cells were cultured in T150 tissue culture flasks, in 200 ml of DMEM media containing 10% FBS. After ten days, the culture media was removed and filtered through a 0.45 µm MillEX-HV (MILLIPORE) membrane to remove all cellular debris. 40 mL of 40% w/v polyethylene glycol (PEG) (PEG 8000, PROMEGA) was added to the culture media to produce an 8% w/v mixture. The mixture was incubated overnight at 4^oC with gentle agitation. All the tubes were then centrifuged at 8000 x g for 20 minutes at 4 ^oC. Pellets were collected and resuspended, either in TNE buffer or complete medium, then processed for sucrose density analysis.

2.2.4 Tissue culture infective dose (TCID50)

Tissue culture infectious dose assay (TCID50) was performed to determine the concentration of infectious particle in the culture media (Shavinskaya, Boulant et al., 2007). Each well of a 96-well plate was seeded with 10^4-Huh-7 cells and incubated overnight at 37 °C. Culture media was drained out of the wells, and 200 µl fresh DMEM was introduced in each well. Next, 50 µL of filtered culture media from JFH1-infected cells was added to each of the eight wells in the first row, diluting it by 1:5. A multichannel pipette was used for further serial dilutions: 50 µl was pipetted out from these wells, introduced into adjacent wells in the next row and mixed gently. This step was usually repeated six times (determined by the experiment requirements), to dilute infectious particles and provide accurate infectious titres.

After 72 h incubation at 37 °C, cells were washed with PBS buffer, then fixed with 100% methanol. PBS was supplemented with 2% FCS for antibody blocking, added to cells for 30 min at room temperature then washed two times with PBS. PBS containing 0.3% hydrogen peroxide was added to the cells to inactivate endogenous peroxides. Cells were washed in PBS two more times then incubated with anti-NS5A primary antibody (1:10,000 dilution) in PBS buffer containing 2% FCS at room temperature for 2 hr. Cells were washed in PBS as per the process above, then secondary antibody conjugated to horseradish peroxidase was added, diluted 1:100 in PBS buffer containing 2% FCS and incubated for 1 hr at room temperature. After the antibody was removed, cells were washed thrice in PBS, then horseradish peroxidase substrate (50 µl) was added to the cells (VECTOR NovaRED^{TM} Substrate Kit) for 5 min at room temperature. Finally, the wells were examined with an inverted microscope at 10 × magnification to detect JFH1 infection. Infected cells were identified by the presence of a red stain surrounding the nucleus. Each well was scored as either positive (at least one infectious centre) or negative,

and the TCID50 was calculated using the formula described in the Spearman–Karber method (Carrère-Kremer et al. 2004).

2.2.5 Immunofluorescence

Viral infection was confirmed by growing cells on glass coverslips in 24-well plates after nearly two passages following electrophoresis of JFH1 RNA. PBS buffer was used to wash the cells, and the cells were fixed with 100% methanol at -20 °C for 20 min. Alternatively, cells were fixed with 4% paraformaldehyde/PFA along with 0.01% Triton ×-100 for 5 minutes. Next, as a blocking step, cells were incubated in PBS containing 2% FCS at room temperature for 1 hr. After washing, cells were next incubated for 1 h at room temperature with anti-core or anti-NS5A antibody (Table 2.2) at 10× Western blotting concentrations in 2% FCS (see Table 2.2). Cells were washed thrice with PBS and incubated with fluorescent secondary antibodies, diluted 1:2000 in 2% FCS. Coverslips were again washed thrice with PBS and examined at 40 x magnification using the Zeiss Axiovert 200 M live cell imaging microscope.

2.2.6 Statistical analysis

Raw data was first analysed using Microsoft Excel. Graphs were generated, and statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Student's t-test was used as appropriate to determine statistical significance between groups, with $p < 0.05$ deemed significant (*), $p < 0.01$ very significant (**) and $p < 0.001$ extremely significant (***).

2.3 Proteomics

2.3.1 Cellular protein extraction

Cellular protein was extracted from cell culture monolayers using RIPA (Radio Immunoprecipitation Assay) buffer (Sigma). First, DMEM media was removed from six-well plates, and the cells were washed with ice-cold PBS. Cells in each well were scraped, and 600 µl of cell suspension in PBS transferred to Eppendorf tubes that were then centrifuged to pellet the cells. RIPA buffer containing a mix of Phosphatase Inhibitor Cocktail 2 (Sigma), Complete Protease Inhibitor Cocktail (Roche), Phenylmethanesulfonyl fluoride (PMSF) and sodium fluoride was added to the cell pellet in each tube. Cell pellets were resuspended in lysis buffer by pipetting, and incubated on ice for 30 minutes. The lysates were then centrifuged at 14,000 RPM for 10 minutes at 4 °C, and supernatant containing extracted protein was transferred to a clean tube. Protein samples were then aliquoted and stored at -80 °C for future experiments.

2.3.2 Protein precipitation protocols

2.3.2.1 Trichloroacetic acid (TCA)

One volume of TCA stock was added to four volumes of protein sample, incubated for 10 min at 4 °C, centrifuged at 14,000 rpm for 5 min, then supernatant was removed, leaving the protein pellet intact. The pellet was washed twice with 200 µl cold acetone then dried by placing the tube in 95 °C heat block for 5-10 min to drive off acetone. For SDS-PAGE, 2× or 4× sample buffer was added (with or without β-ME), and the sample was heated for 10 min at 95 °C before being loaded onto polyacrylamide gel.

2.3.2.2 Acetone precipitation

Proteins from detergent-insoluble fractions were precipitated with acetone, 10% trichloroacetic acid, 0.07% β-mercaptoethanol for 1 h at −20 °C. Following centrifugation at 4000 × g for 10 min, pellets were washed with acetone, 0.07% β-mercaptoethanol.

2.3.3 Protein quantification

Protein concentrations were determined using the Bio-Rad DC assay kit with purified bovine serum albumin (Sigma) as standard. Briefly, 20 µl of reagent S was added to 1 ml of reagent A. Then, 5 µl of standard and samples were transferred to a clean microtitre plate, and 25 µl of reagent A+S was added to each well. Next, 200 µl reagent B was combined with the content of each well and, after 15 minutes incubation at room temperature, the absorbance was read at 750 nm using a Wallac Victor 1420 Multipliable Counter Microplate Reader.

2.3.1 Cellular protein extraction

Cellular protein was extracted from cell culture monolayers using RIPA (Radio Immunoprecipitation Assay) buffer (Sigma). First, DMEM media was removed from six well plates and cells were washed with ice-cold PBS. Cells in each well were scraped and 600 µl of cell suspension in PBS transferred to Eppendorf tubes that were then centrifuged to pellet the cells. RIPA buffer containing a mix of Phosphatase Inhibitor Cocktail 2 (Sigma), Complete Protease Inhibitor Cocktail (Roche), Phenylmethanesulfonyl fluoride (PMSF) and sodium fluoride was added to the cell pellet in each tube. Cell pellets were resuspended in lysis buffer by pipetting, and incubated on ice for 30 minutes. The lysates were then centrifuged at 14,000 RPM for 10 minutes at 4 °C, and supernatant containing

extracted protein was transferred to a clean tube. Protein samples were then aliquoted and stored at -80 °C for future experiments

2.3.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The concentrations of separating and stacking gels were 10% and 5% respectively, and gel casting was performed in a Bio-Rad Mini PROTEAN® Tetra Cell gel tank. The volume of protein sample that was introduced to each well was 20 to 50 µl, preparation was performed in a 4× SDS-PAGE sample buffer and the mixture was boiled for five minutes at 95 ^{o}C prior to separation by SDS-PAGE. Gels were placed in 1× running buffer and samples loaded into wells. A 100 V potential was used for electrophoresis. The protein size was estimated by comparing to commercial standards, PageRulerTM Plus Pre-stained Protein Ladder (Thermo Scientific). Gels were either stained with Coomassie Brilliant Blue G250 stain or proteins were transferred to membranes for Western blot analysis.

2.3.5 Western Blot Protein Analysis

Proteins separated on polyacrylamide gels were transferred to Nitrocellular (Bio-Rad) or Polyvinylidene fluoride (Millipore) membrane using a BioRad Trans-Blot SD blotting device. Transfer was carried out at 35 V overnight at a temperature of 4 °C. Skim milk powder (10%) dissolved in TBST buffer (containing 20mM Tris-HCl, pH 7.4, 500 mM NaCl and 0.1% Tween) was used to block membranes after protein transfer by incubating for 1 hr at room temp with gentle agitation. Membranes were incubated overnight with primary antibodies diluted in 5% skim milk/TBST at a temperature of 4 ^{o}C, then washed three times in TBST buffer. Secondary antibody conjugated to horseradish peroxidase, diluted in TBST/10% skim milk, was added for one hour at room temperature,

with gentle agitation, then rinsed off as previously described. Visualisation of the bound antibody was facilitated by the use of a Super Signal West Pico Chemiluminescence Kit (Thermo Scientific) and membranes were exposed to Kodak BioMax Film for different time intervals. Table 2.6 lists the different primary and secondary antibodies used in the Western blot analysis.

2.3.6 Densitometry analysis

Autoradiographs of Western blots were scanned and analysed using ImageJ software. A boxed area of fixed size was selected around each band, images were colour inverted, then the band density for each protein species was calculated. Mean density values for each sample area were calculated and then the background density value for each box was subtracted.

2.3.7 2-Dimension polyacrylamide gel electrophoresis (2-D PAGE) for proteomics

The first dimension isoelectric focussing was carried out using 8 cm immobilized pH gradient dry strips (IPG) with a linear pH 4–7 gradient. For rehydration, the sample in an appropriate volume of rehydration buffer (Urea 7 M, Thiourea 2 M, Tris 30 mM and CHAPS 4%. up to 50 ml Milli-Q H_2O) was passively rehydrated overnight at room temperature in IPGPhor cassettes. Proteins were separated by the Ettan IPGphor II using a programmed voltage gradient at 20 °C, under the following conditions: 4 h at 400 V, 8000 V linear gradient 1 h, rapid 8000 V to 20000 V hrs. After IEF, the IPG strips were equilibrated in SDS equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 1% DTT) and buffer two containing 2.5% iodoacetamide instead of DTT in each case for 20 minutes.

Second dimension separation was performed using the PROTEAN® II (Bio-Rad) Cell system. The equilibrated strips were applied to the top of 12% SDS-PAGE gels and

sealed with 1% agarose prepared in SDS-Tris-glycine buffer, with traces of bromophenol blue as a tracking dye to monitor electrophoresis. Electrophoresis was performed at a constant voltage (150 V) at 20 °C until the dye front reached the bottom of the gel. Following electrophoresis, the gel was stained using Coomassie Brilliant Blue G250 stain. Briefly, gels were fixed in 10% methanol and 7% acetic acid for at least 20 minutes, and then immersed in colloidal staining solution overnight. To remove background staining, gels were washed in 1% methanol.

2.3.8 In-gel trypsin digestion of gel bands

Protein bands were excised, and gel pieces were destained using 35 mM ammonium bicarbonate, 40% (v/v) acetonitrile, before dehydrating with 100% acetonitrile. Dehydrated gel pieces were completely dried in a vacuum centrifuge then 15 µl trypsin (12 ng/µl in 50 mM ammonium bicarbonate) was added. After being incubated on ice for 1 h, all excess trypsin was removed, and 15 µl of 50 mM ammonium bicarbonate was added before incubating the tubes overnight at 37 °C.

2.3.9 Protein mass spectrometry

Tryptic peptides were analysed on an AB SCIEX TOF/TOF 5800 (MALDI) mass spectrometer using alpha-cyano-4-hydroxycinnamic acid as matrix. Mass spectrometry data were processed with ProteinPilot software at The Biomedical Proteomics Facility and the Australian Cancer Research Foundation Centre for Kinomics (ACRF CFK), Children's Medical Research Institute, Westmead, Australia.

2.4 Lipidomics

2.4.1 Cell fractionation

A sonicator (Probe Sonicator) was used to homogenize the cell pellet. The lysate was also centrifuged at 800 × g for 5 min to remove nuclei. The cytosolic fraction (supernatant) and the membrane fraction (precipitate) were separated from the post-nuclear fraction by centrifugation at 14,000 × g for 20 min. BSA was used as the standard to determine the concentration of protein used in the preparation for normalisation. The concentration was set in accordance with BCA protein assay reagents (Pierce Biotechnology, Rockford, IL, USA). The membrane fractions used for the experiment were re-suspended in a buffer and purified again through a process of ultracentrifugation.

2.4.2 Isolation of Endoplasmic Reticulum (ER)

Virus infected and control Huh-7 cells were plated in 150 cm^2 flacks until 90% confluent. They were then washed two times with PBS and harvested with 0.05% trypsin. The cells were pelleted by undergoing centrifugation at 1500 × g for 5 min. The cell pellet was resuspended in 1 ml of MTE buffer (10 mM Tris-base, 2 mM MgCl2, 1 mM DTT, 270 mM D-mannitol, 0.1 mM EDTA, protease inhibitor mixture, pH to7.5 with 6 M HCl) and incubated at 0 °C for 5 min. Unless stated, the following procedures were performed on ice. The cells were disrupted using sonication at low power (3 × 5 sec, amplitude 10%), separated by 10-sec rest intervals. The lysate was clarified of unbroken cells by 10 min centrifugation at 8,000 x g. The supernatant was transferred on top of a three-step sucrose gradient: bottom to top, 1 mL 2M sucrose, 3 mL 1.5 M sucrose, and 5 ml 1.3 M sucrose. All sucrose gradients were prepared using an MTE buffer. The gradients were spun for 1 h

at 32000 rpm in a Beckman SW 41 rotor, and the large band (ER gradient) at the interface of the 1.3 M sucrose gradient was collected. The extracted band was transferred to 14 × 89-mm ultra clear SW41 tubes and filled up to the top with ice-cold MET buffer, mixed and ultracentrifuged for 1 h at 32000 rpm. Supernatant was discarded, and the pellet was resuspended in 500 µl PBS, labelled as ER, and stored immediately at -80 $^{\circ}$C.

2.4.3 ER lipid rafts isolation using detergent-free purification

To allow the purification of ER-lipid rafts without the use of detergent, Triton ×-100 was replaced with sodium carbonate (Song et al. 1996). The pellet from ER isolation (Section 2.4.2) was resuspended in 2 ml of 0.5 M Na_3CO, pH 11.0. The suspension was adjusted to 45% sucrose by adding 2 ml of 90% sucrose prepared in MBS (0.15 M NaCl, 25 mM Mes, pH 6.5) and laid at the bottom of an ultracentrifuge tube. 5% and 35% sucrose solutions, both in MBS containing 250 mM Na_3CO, were added carefully to form a 5-35% discontinuous sucrose gradient and centrifuged at 39,000 rpm for 18 h in an SW41 Ti Beckman rotor. ER-Lipid rafts were collected from the light scattering band confined to the 5-35% sucrose interface.

2.4.4 Isolation of lipid droplets (LDs)

LDs were isolated by subcellular fractionation following published protocols (Brasaemle & Wolins 2006). Cells were trypsinised from culture flasks, pelleted by centrifugation at 2000 RPM for 5 min and suspended in 1 ml hypotonic lysis medium (HLM) buffer (40 mM Tris, pH 7.4, 2 mM EDTA, 20 mM NaF, protease inhibitors). After incubation on ice for 10 min, the cells were sonicated (2 × 10 sec, amplitude 10%), then centrifuged at 4000 × g for 10 min, the resulting supernatant with the floating fatty layer

was collected as the non-nuclear fraction into a separate 13.2 ml ultracentrifuge tube for a SW41 Ti rotor. 1/3 volume of ice-cold HLM containing 60% sucrose was added and mixed by gentle pipetting (final 20% sucrose). To form discontinuous gradients, 2.5 ml ice-cold HLM containing 15% sucrose and 4 ml ice-cold HLM containing 5% sucrose consecutively were slowly layered over the sample, then 5 ml ice-cold HLM was layered over the gradients to fill the tube. Gradients were spun for 45 min at 28000 x g at 4 °C. Four discrete fractions were collected sequentially from top to bottom. The lipid droplet fraction was collected from the top layer (fraction 1). The protein content of each fraction was analysed using DC Protein Assay (Bio-Rad). A small aliquot of the lipid droplet fraction was removed and confirmed to contain LDs by SDS-PAGE separation and immunoblotting with antibody against the lipid droplet associated protein, adipocyte differentiation-related protein (ADRP).

2.4.4.1 LDs protein extraction

Lipid droplet fractions contain a large amount of neutral lipids, including triacylglycerols, cholesterol esters, and phospholipids, but a low relative amount of protein. The high lipid content of the samples interfered with the resolution of proteins by SDS-PAGE. To overcome this obstacle, fresh or frozen lipid droplet fractions were delipidated using solvents, and the precipitated proteins were solubilised in concentrated detergent solutions through warming and sonication (Brasaemle and Wolins, 2006). In a fume hood, 10 volumes of cold acetone that had been stored at −80 °C were added to the thawed or fresh lipid droplet fraction in 15 ml falcon tubes. Samples were mixed thoroughly by inversion and incubated overnight at −20 °C. The precipitated protein samples were pelleted by centrifugation at 4,300 × g for 10 min at 4 °C, then acetone was carefully removed from the loose pellet with a glass pipette and discarded into a suitable

container. 50-100 µl of 2 × SDS sample buffer was added to the dry pellet, mixed, and incubated for 4 to 6 h at 60 °C in a sonicating water bath with vigorous agitation every 15 min using a vortex mixer. After adding β-mercaptoethanol (10% final), the samples were loaded onto an SDS-PAGE gel in 2 × SDS sample buffer without dilution.

2.4.5 Lipid extraction

Cellular lipids were extracted using a Bligh-Dyer protocol (Bligh & Dyer 1959) with modifications (Pettitt et al. 2006). Fresh or frozen cell pellets were resuspended in PBS to achieve a final volume equal to five times the volume of the cell pellet (i.e., a 20% cell suspension). The cell suspensions were homogenized in a mixture of methanol and chloroform. The ratio of methanol, chloroform and saline solution in the mixture was 2:1:0.8. One volume of 1M NaCl/ 50 mM HCl (1:1 v/v) was then added to the mixture, then one volume of chloroform was introduced to induce phase separation. The ratios of methanol, chloroform and water in the final mixture were 2:2:1.8 respectively. The extract was left for 5 min at room temperature and then centrifuged at 800 × g for 10 min. The lower (chloroform) phase was collected and evaporated under a stream of gaseous nitrogen. Lipid extracts were stored under N_2 (−80 °C) until required. 1 ml of the upper (aqueous) phase was collected in Eppendorf tubes and dried under a vacuum with heating for protein estimation.

2.4.6 High-performance thin layer chromatography (HPTLC)

Extracted lipids were dissolved into chloroform/methanol (2:1, v/v) then loaded onto HPTLC plates with a parallel dilution series of lipid standards. For neutral and

phospholipid separations, plates were pre-washed with methanol/ethyl acetate (6:4, v/v) and activated at 110 °C for at least 30 min before loading. Dilution series of both native extract and lipid standards in chloroform/methanol (2:1) were loaded onto HPTLC plates under N_2 using the Automatic TLC Sampler 4 (ATS4) and developed in the CAMAG AMD2 (CAMAG, Wilmington, NC, USA) (Churchward, Brandman, Rogasevskaia & Coorssen 2008; Churchward, Rogasevskaia, Höfgen, Bau et al. 2005). For neutral lipids and sterols, HPTLC plates were developed twice with dichloromethane/ethyl acetate/acetone (80:16:4, v/v/v) to 40 and 55 mm, then sequentially three times with hexanes/ethyl acetate to 68 mm (90:10, v/v), 80 mm (95:5), and 90 mm. Phospholipids were developed according to Weerheim (Weerheim, Kolb, Sturk & Nieuwland 2002) with modifications (Churchward, Brandman, Rogasevskaia & Coorssen 2008), using two-step separation. HPTLC plates were first developed with dichloromethane/ethyl acetate/acetone (80:16:4, v/v/v) to 90 mm, dried under a vacuum for 6 min, then developed again to 90 mm with chloroform/ethylacetate/acetone/isopropanol/ethanol/methanol/water/acetic acid (30:6:6:6:16:28:6:2, by volume).

2.4.6.1 Chromatogram staining and visualization of HPTLC plates

After development, plates were sprayed uniformly with 10% cupric sulfate in 8% aqueous phosphoric acid (Daugherty 1987; Churchward, Brandman, Rogasevskaia & Coorssen 2008), allowed to dry for 10 min at room temperature, and then heated in an oven at 145 °C for 10 min.

Imaging of cupric-sulfate-charred plates was carried out using the Luminescent Image Analyser LAS-4000 (Fuji Film, Japan). All images were analysed using Multi Gauge V3.0 software.

2.4.7 Lipid mass spectrometry

Samples were analysed using both positive and negative electrospray ionisation on a Thermo Fisher Scientific QEXactive Plus mass spectrometer.

Samples were dissolved in 200uL solvent A, centrifuged to remove TLC particles and transferred into glass LC-MS vials. 10 uL was injected using an ULTIMATE 4000 system (Dionex) and chromatographed on a Waters CSH 2.1 x 100 mm C18 column according to the method described by Castro-Perez et al. (2010). Mass spectrometer settings were based on those published by Bird et al. (2011). The instrument was run at an 140,000 resolution full scan followed by six lower resolution DDA scans on the six most intense peaks observed in the full scan. Data was interrogated using Lipid Search software (Thermo Fisher Scientific). Samples were analysed multiple times to allow statistically significant comparisons to be made.

3. HCV infection induces global Changes in the Host Cell Lipidome

3.1 Introduction

Chronic hepatitis C (CHC) can be considered to be both a viral and a metabolic disease. The hepatitis C virus (HCV) interacts with and changes the host's lipid metabolism to its advantage, supporting the virus lifecycle through changes in membrane transport and sorting, and in cell signalling. To examine HCV-induced changes in the lipid metabolism, cellular lipidomics must not only determine which lipids are present, but also the concentration of each lipid at each specific intracellular location in time and the lipid's interaction partners (van Meer 2005). A better understanding of the relationship between lipid metabolism and the key stages involved in the production of infectious HCV will improve our understanding of the pathophysiology of HCV infection and may provide new antiviral drug targets.

There are basically two groups into which the lipids can be subdivided: non-polar or neutral lipids, like acylglycerols and wax esters; and polar lipids, which mostly consist of phospholipids (Makula et al. 1975). Lipids perform key functions in all living beings (Athenstaedt & Daum 2006). Neutral lipids mostly consist of triacylglycerols and steryl esters, and are considered to be hydrophobic molecules because they are deficient in charged groups (Devaux 1991). In eukaryotic cells, neutral lipids are the final form in which cholesterol and free long-chain fatty acids are stored when cells are exposed to excess amounts of nutrients. It is not possible to incorporate these storage lipids into biomembranes, so they are separated from the cytosolic environment in membrane-coated LDs or lipid particles (Walther & Farese 2009).

Phospholipids are an essential part of many processes in the body and comprise different membranes and layers of cells. Phospholipids have been classified into various groups according to their dimensions, geometry, amount of charge and organic structure.

Phospholipids of different classes help to build up the plasma sheath of the cells; their uneven distribution within the membrane can aid the performance of a number of functions. They also constitute the outer surface of the lipoproteins, which aids the transfer of neutral lipids around the body. They help to absorb lipids from the gut, following secretion into the bile. Phospholipids also help to monitor the function of various molecules, such as arachidonic acid, phosphatidate and inositol trisphosphate, and prompt them to function properly.

Phospholipids are formed when two fatty acyl molecules are ester-linked to the sn-1 and sn-2 positions of glycerol, leaving a phosphate residue at the sn-3 position (Figure 3.1). This is the "head position", which allows linking of the phospholipid head group via the phosphate residue. Because the head group is typically charged (hydrophilic), while the fatty acyl side chains are hydrophobic, this causes the phospholipid molecule to be polar, forming biological membranes and leading to compartmentalization of the cell.

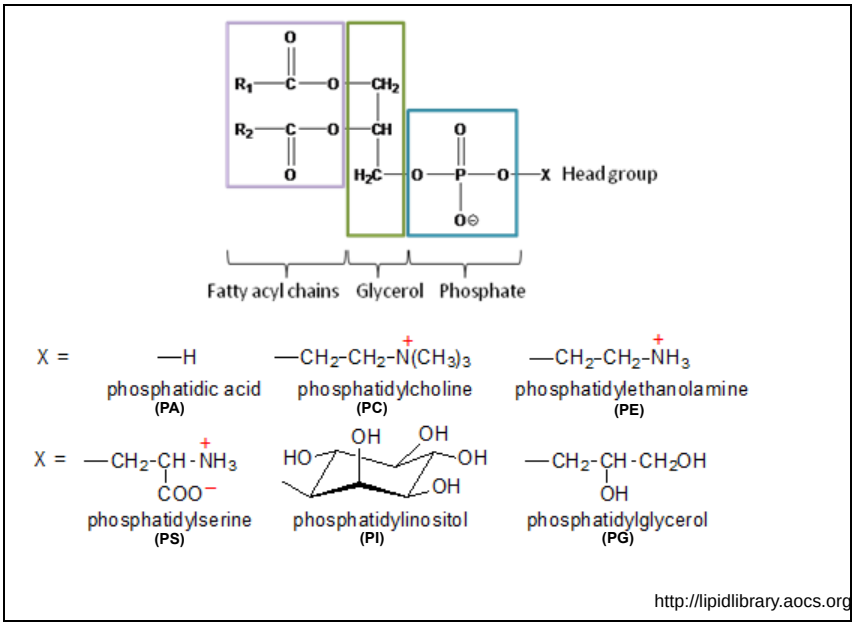


Figure 3.1 Structure and major classes of phospholipids (http://lipidlibrary.aocs.org)

Replication of HCV depends on the virus modulating lipid metabolism, thereby reconfiguring the endomembrane system. These alterations create an environment that is lipid-rich, and allows the virus to replicate efficiently. Analysis of the blood of an HCV-infected patient reveals that the lipoviral particle biochemically closely resembles the VLDL particle (Bassendine et al. 2011). Host cell lipids are a major focus in the HCV life cycle, with the intracellular levels and composition of fatty acids, including cholesterol being key factors in HCV RNA replication.

Liver steatosis, or fatty liver disease, occurs when there is an accumulation of LDs in hepatocytes. HCV interacts with the metabolic syndrome, resulting in insulin resistance and obesity in infected patients. HCV genotype 3 is associated with increased steatosis, while genotypes 1 and 4 are associated with insulin resistance and diabetes (Hui et al. 2003; Cua et al. 2008). The results from these studies highlight the clinical importance of up-regulated lipid metabolism for HCV replication and offer the potential for new targets for antiviral therapy.

The effects of HCV infection on lipid homeostasis and turnover in hepatic cells will be examined in this chapter, together with the redistribution of cellular lipids. The experimental outline is summarised in Figure 3.1.

For these experiments, the JFH-1 HCV cell culture model was used, as this is the only HCV model that produces infectious virus particles in vitro (Wakita, Pietschmann, Kato, Date, Miyamoto, et al. 2005).

3.2 HCV infection and lipid extraction

JFH1 is the first full-length HCV viral genome capable of replicating and producing intact virus in cell culture (Huh-7 cells). Huh-7 hepatoma cells were transfected (by electroporation) with full-length viral RNA (JFH1). Uninfected and JFH1-infected Huh-7 cells (2×10^6 cells/flask) were cultured in 3 T150 cell culture flasks until >90% of cells were infected (typically 10-14 days). Infection was confirmed by the immunofluorescence of the fixed cells that grew on glass coverslips, using antibodies against HCV core and NS5A proteins.

After media had been removed, the cells were harvested and adjusted with cells number (2×10^7 cells). Total lipids from both Huh-7 and JFH-1 infected cells were extracted using a modified Bligh-Dyer protocol (Bligh & Dyer 1959) and the protein in all samples was measured as an internal control. Extracted lipids were subjected to separation by high-performance thin layer chromatography-automated multiple development (HPTLC-AMD) technologies (Churchward et al. 2008). This was followed by mass spectrometry analysis to identify alterations in the host cell lipidome that occurred in response to infection with HCV (JFH1).

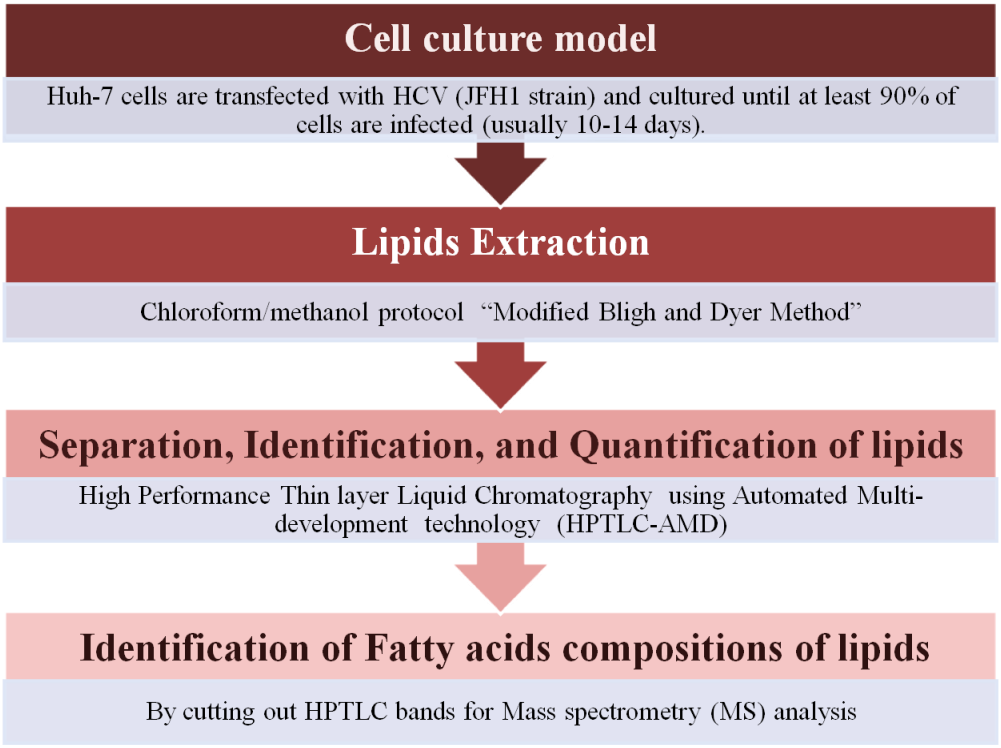


Figure 3.2An overview of the experimental outline for lipidomic analysis of HCV infected Huh-7 cell line.

3.3 Analysis of cellular lipid distribution by fluorescence microscopy

As an initial approach, the cellular distribution of lipids following HCV infection was analysed using the JFH1 HCV cell culture model. HCV-infected and mock-infected cells were labelled for NS5A using specific antibodies. Neutral lipid was stained with BODIPY® 493/503 and nuclei was stained with 4'6-Diamidino-2-phenylindole (DAPI). The cells were examined using fluorescence microscopy to determine changes in the accumulation and distribution of intracellular lipids in response to the HCV infection. As expected, a significant accumulation of lipids were observed only in cells that stained positively for HCV NS5A (Figure 3.3) Interestingly, there was little co-localisation between lipids and NS5A.

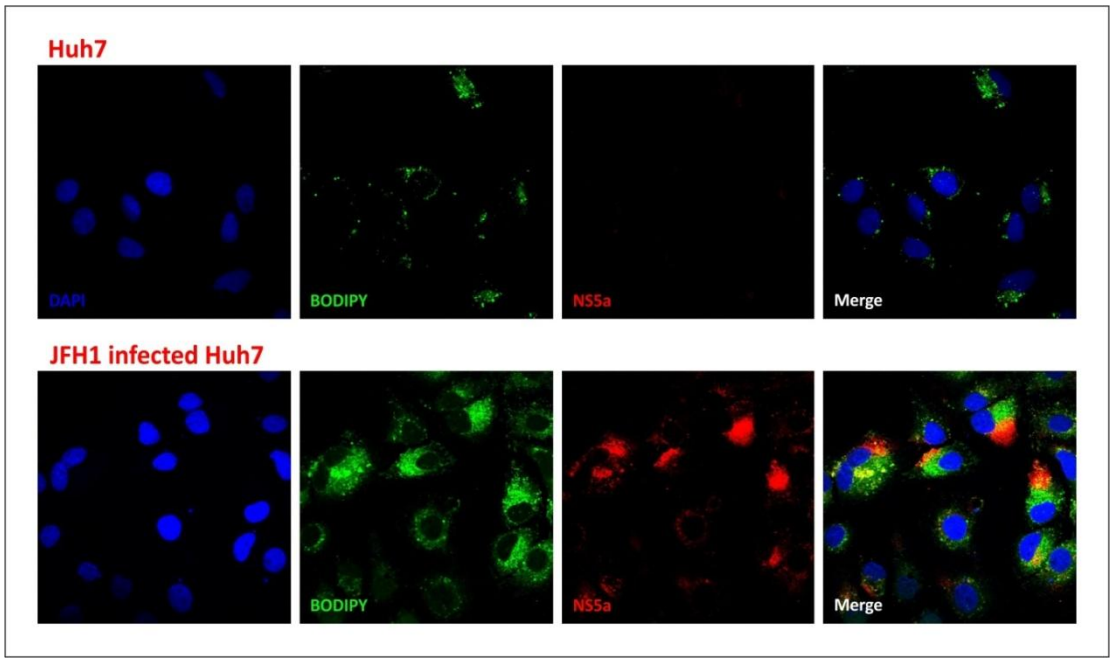


Figure 3.3 Lipids accumulation in Huh-7 cells chronically infected with JFH1.
Huh-7 cells were labeled with immunofluorescence, using antibodies against NS5A (red), BODIPY® (green) and DAPI (blue), before (A) and (B) after a minimum of 2 weeks infection. JFH1 infected Huh-7 cell show a significant cholesterol accumulation compared to uninfected cells.

The observed increase in cellular lipid supports a role for lipids in forming a cellular niche for HCV replication. These data are consistent with previous reports of increased lipid accumulation in HCV-infected cells (Abid et al. 2005; Hofer et al. 2002). However, these studies only used microscopy to measure the number and size of LDs, and simple biochemical assays to show increased triglyceride content (Yamaguchi et al. 2005; Negro & Sanyal 2009). The change in the cellular content of other lipid species following HCV infection is not known; as such, detailed lipidomic analysis of the infected cells was performed using HP-TLC and mass spectrometry.

3.4 High-performance thin chromatography separation of lipids using automated multiple development (AMD) technology

To further study lipid accumulation in HCV-infected Huh-7 cells, lipids from Huh-7 cells that had been infected with JFH1 strain and mock Huh-7 cells were extracted, andseparation and quantification of the lipid and sterol composition was performed using HPTLC-AMD and a fluorescent detection method based on the classic cupric sulfate charring techniques. The optimized protocol yielded the reproducible detection of a wide range of phospholipids and neutral lipids with higher sensitivity than standard TLC protocols.

The technique involved developing neutral and polar lipids with a reproducible stepwise elution gradient from polar to nonpolar using different solvent systems. An automatic TLC sampler was used because this offers fully automatic sample application for qualitative and quantitative analyses as well as for preparative separations. It is suitable for routine use and in studies involving high sample throughput in mass analysis. The whole system is fully automated and is software controlled to ensure reproducibility.

Lipids were extracted using the modified Bligh and Dyer method for each sample, and were then subjected to HPTLC separation, using separate plates and solvents for neutral lipids and phospholipids. After development, separated lipid classes were detected by spraying plates uniformly with 10% cupric sulphate, placing them into a 145 °C oven for 10 minutes, and performing densitometric analysis of cupric-sulfate-charred plates using a Luminescent Image Analyser LAS-4000 (Fuji Film, Japan).

3.5 Chronic hepatitis C infection induces global changes in host cell neutral lipid

Using a high-resolution HPTLC, a global increase in neutral lipids in JFH1 (HCV) infected Huh-7 cells compared to the mock-infected Huh-7 cells was observed. This effect

was dose-dependent and consistent at two different loading volumes (5 and 10 µl). All major lipid classes present in the sample were well separated (Figure 3.4).

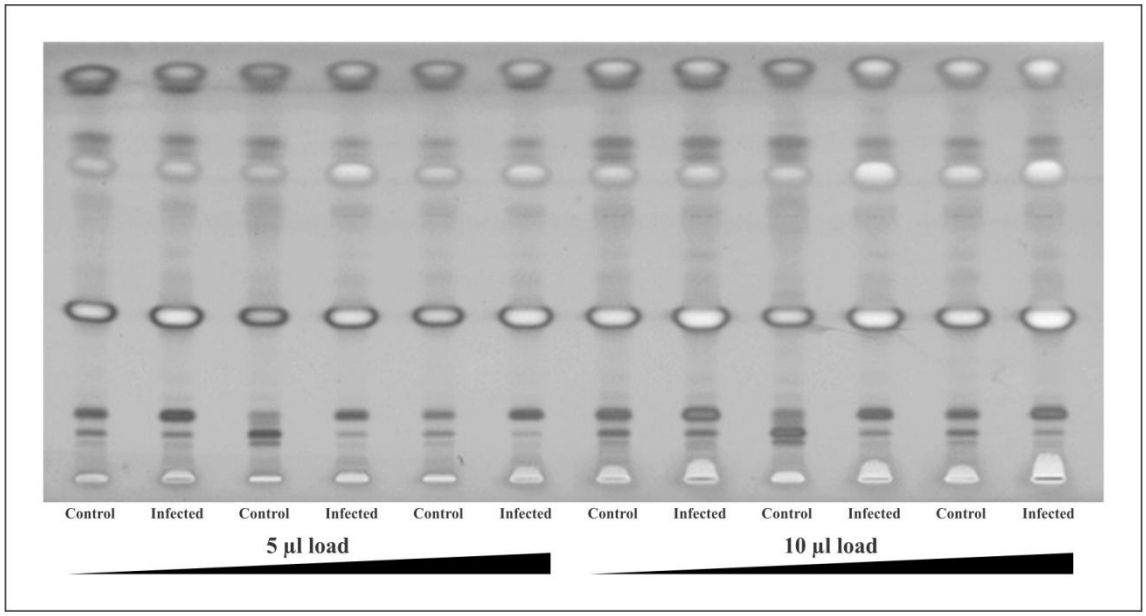


Figure 3.4 HPTLC separation of neutral lipids in HCV infected hepatic cells.
JFH1 (HCV) infected Huh-7 cells compared to uninfected Huh-7 using two different loading concentrations (5 and 10 µl). JFH1 (HCV) infected Huh-7 cells demonstrated a global increase in neutral lipids compared to mock infected Huh-7.

3.5.1 Identification of the neutral lipids composition in the chronic hepatitis C infected cells

Next, we analysed the effect of HCV infection on individual classes of neutral lipid, using commercial standards for monoacylglycerol (MAG), diacylglycerol (DAG), free cholesterol (FC), cardiolipin (Car), triacylglycerol (TAG), and cholesteryl esters (CE)(Figure 3.5).

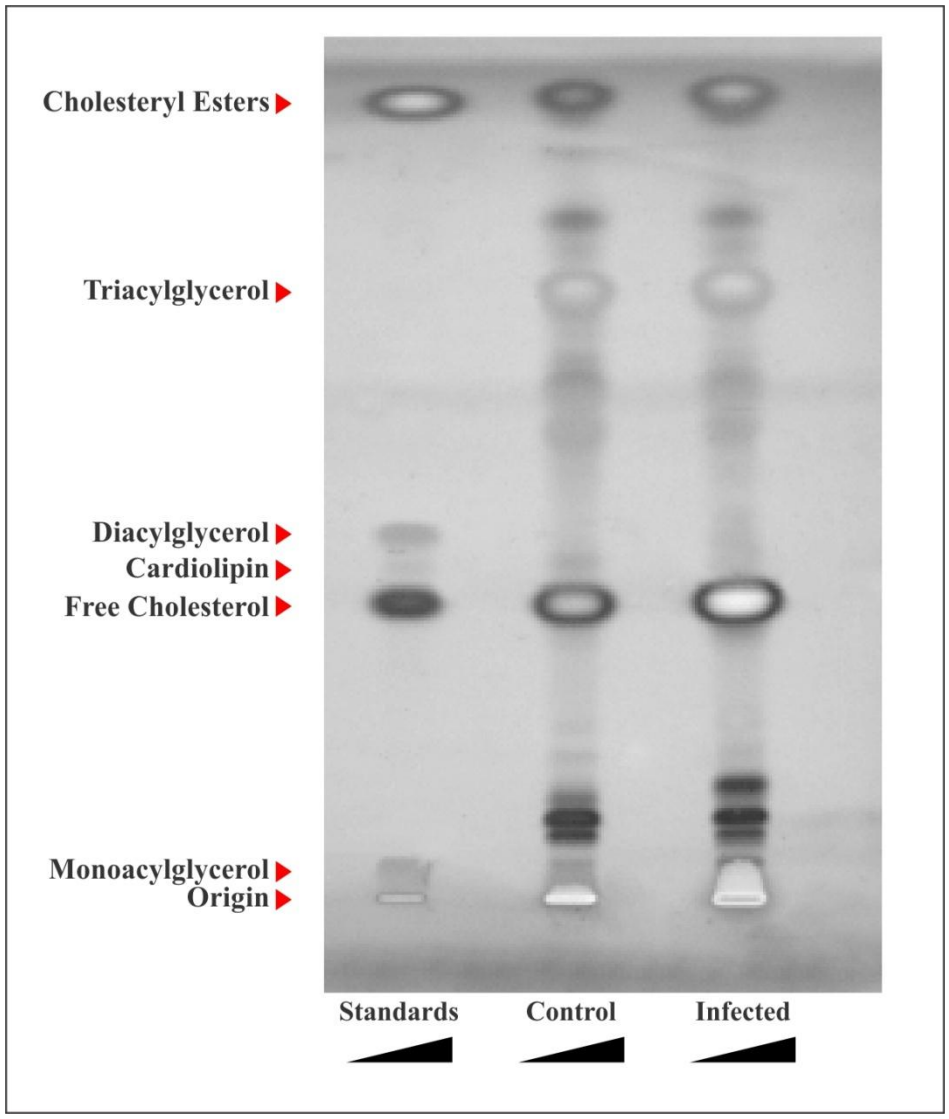


Figure 3.5Identification of neutral lipid bands using purified standards. Purified standards for monoacylglycerol (MAG), diacylglycerol (DAG), free cholesterol (FC), cardiolipin (Car), triacylglycerol (TAG) and cholesteryl esters (CE) were used to characterize the specific effects of chronic hepatitis C infection on different neutral lipid classes

Following HPTLC, identified bands of neutral lipid species were scraped off to be analysed by mass spectrometry analysis (MS).The major neutral lipid species such as cholesteryl esters, triacylglycerol, free cholesterol, diacylglycerol and monoacylglycerol were first identified by HPTLC then all identified bands were scraped off for MS confirmation. Furthermore, some major bands with consistent changes between JFH1 infected and mock Huh-7 cells that could not be identified by HPTLC were identified by MS as diacylglycerol species **(**Figure 3.6).

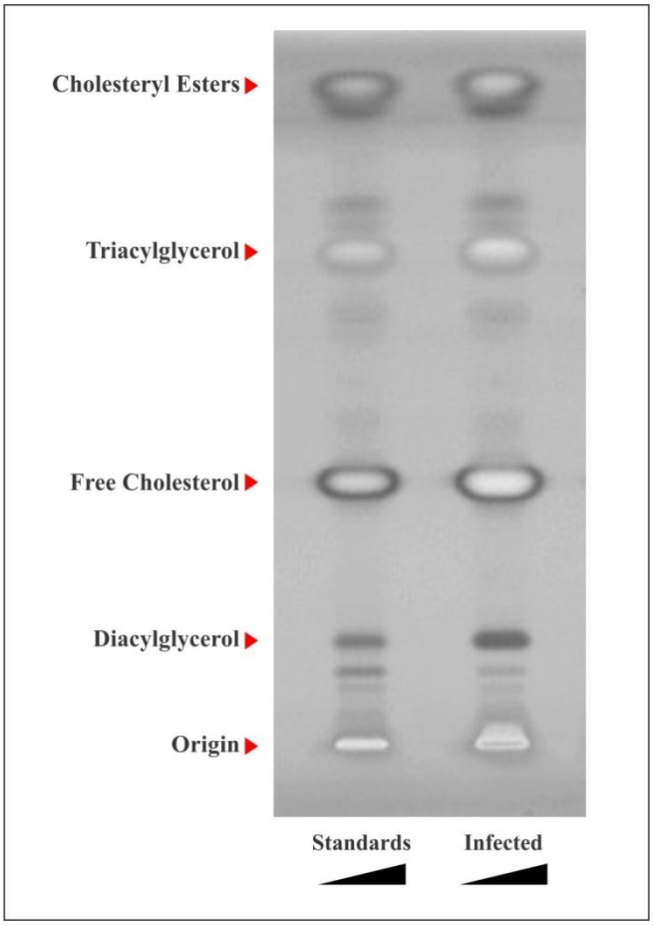


Figure 3.6 Major neutral lipids bands after separation by HPTLC and confirmed with MS.

All major neutral lipid bands, either identified or unidentified which showed consistent change in JFH1 infected Huh-7 cells were cut out for MS analysis. Cholesteryl esters, triacylglycerol, and free cholesterol bands were reconfirmed by MS analy.

3.5.2 The effect of HCV infection on neutral lipids

To examine the effects of chronic HCV infection on neutral lipids in host cells,Huh-7 cells were transfected with JFH1 RNA, cultured until >90% of cells were infected (confirmed by IF), then lipids from JHF1 infected and mock Huh-7 cells were extracted and separated by HPTLC. Densitometry was performed on the HPTLC plate for TAG, FC and CE bands

HCV increases Triacylglycerol

As expected we identified a significant increase in TAG in JFH1 (HCV) infected Huh-7 cells compared to mock infected Huh-7 (P=0.03) (Figure 3.7). This is consistent with accumulated data in the literature pointing to the role of triglycerides in HCV life cycle and clearance.

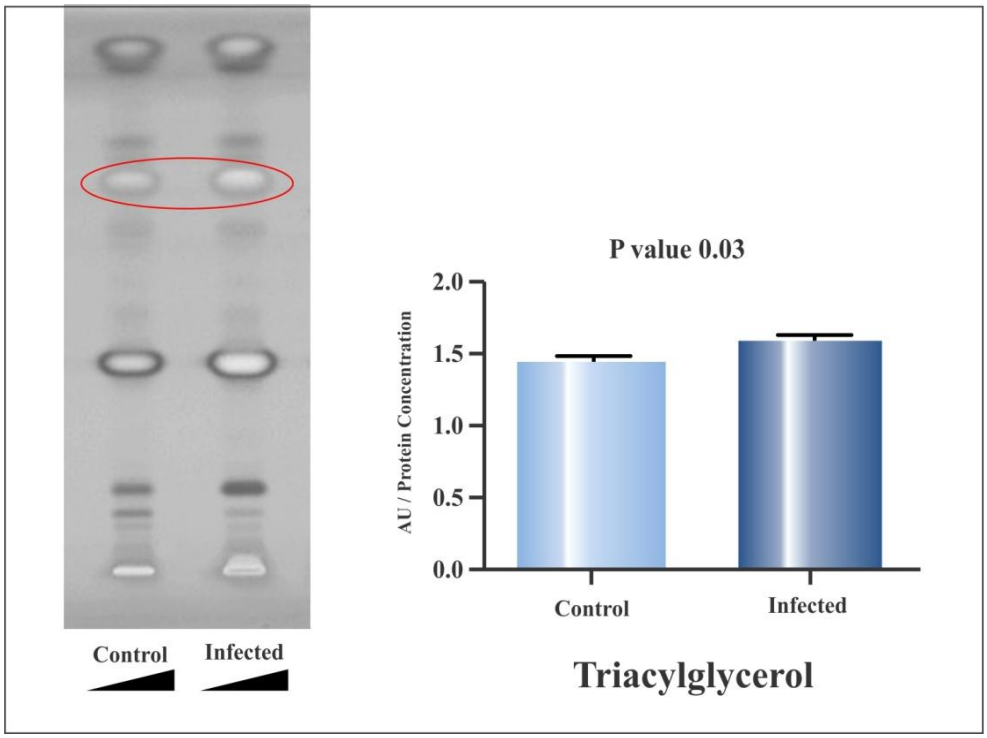


Figure 3.7Chronic hepatitis C infection induces increase ontriacylglycerol in the host cells.
Triacylglycerol bands were identified against known standards and densitometric detection of cupric-sulfate-charred plates was carried out using Luminescent Image Analyser LAS-4000. Chronic JFH1 (HCV) infected Huh-7 cells has a significant higher levels of triacylglycerol compared to mock infected Huh-7.

HCV increases cholesteryl esters

After HPTLC separation, cholesteryl ester (CE) bands were identified using known standards. We observed a significant increase in cholesteryl esters in JFH1 (HCV) infected Huh-7 cells compared to mock infected Huh7 (P=0.02) (Figure 3.8).

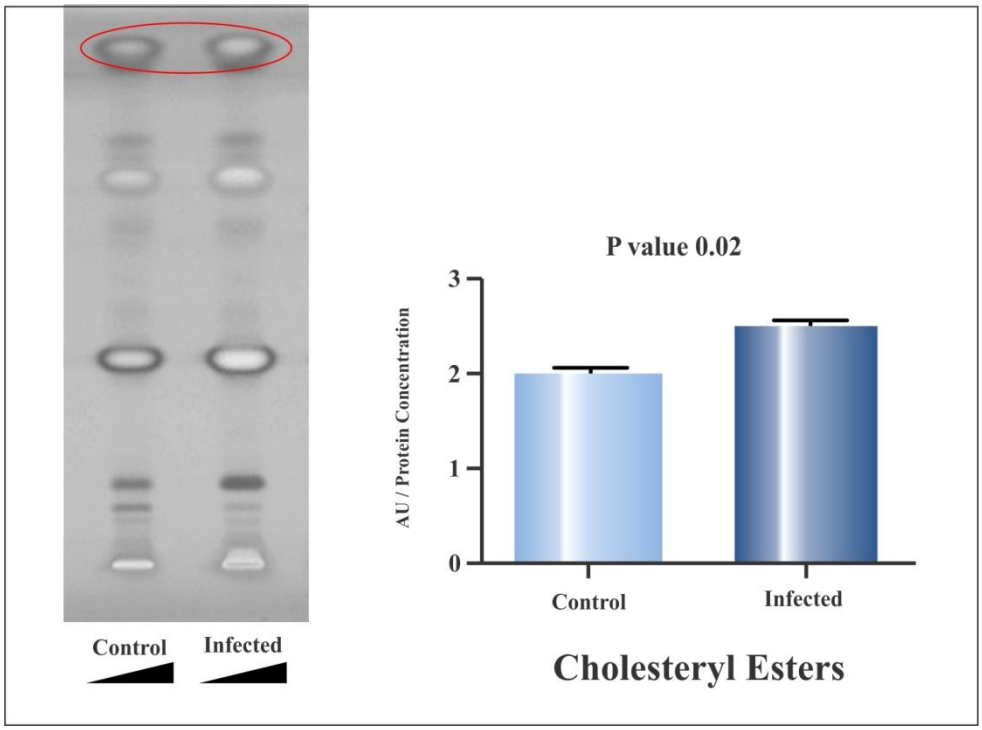


Figure 3.8HCV infection induces increase oncholesteryl esters in the host cells.
Cholesteryl esters bands were identified against known standards and compared between (JFH1 (HCV) infected Huh-7 cells and mock infected Huh-7). Chronic JFH1 (HCV) infected Huh-7 cells has a significant higher levels of cholesteryl esters compared to mock infected Huh-7 (P= 0.02).

HCV increases cellular free cholesterol

Bands representing free cholesterol (FC) wereidentified using known standards. We observed a significant increase in FC in JFH1 (HCV) infected Huh-7 cells compared to mock infected Huh-7 (P=0.04) (Figure 3.9).

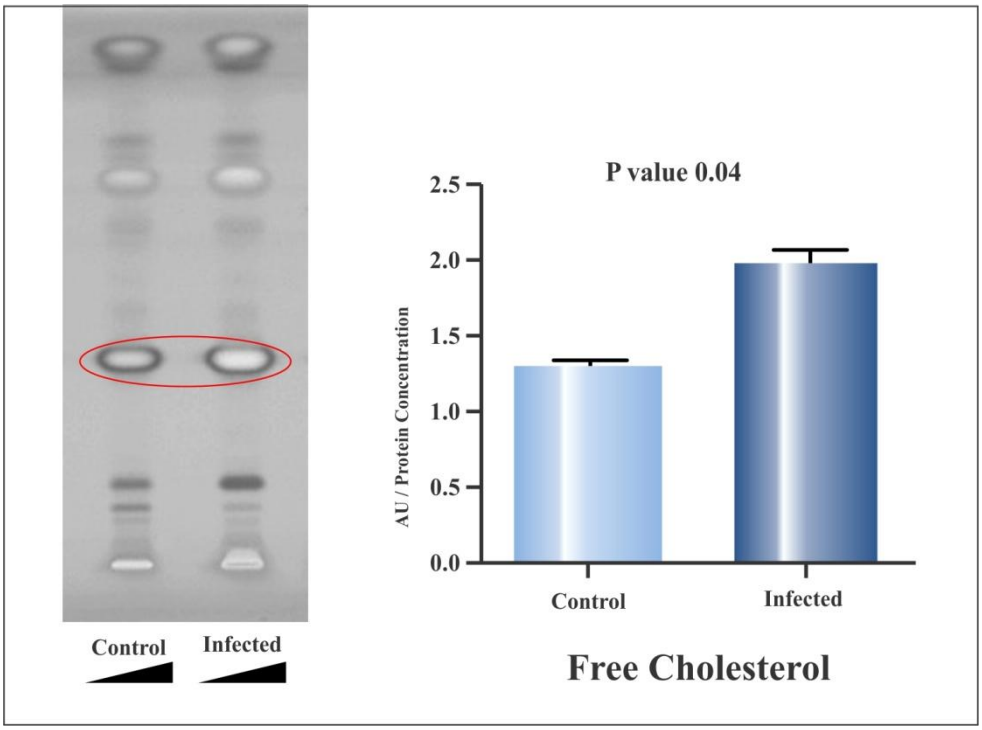


Figure 3.9 HCV induces increase onfree cholesterol in the host cells.
Free cholesterol bands were identified against known standards and compared between JFH1 (HCV) infected Huh-7 cells and mock infected Huh-7. Chronic JFH1 (HCV) infected Huh-7 cells has a significant higher level of free cholesterol compared to mock infected Huh-7.

3.6 HCV infection induces global changes in cell phospholipid composition

The major components of cell membranes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), so we next investigated the effect of HCV infection on cell phospholipids. Consistent with the increased in neutral lipids in HCV infected cells, HPTLC lipidome analyses revealed a significant accumulation of several phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species. Representative examples are presented in **(**Figure 3.10), showing global increase in phospholipids in JFH1 (HCV)-infected Huh-7 cells compared to mock infected Huh-7.

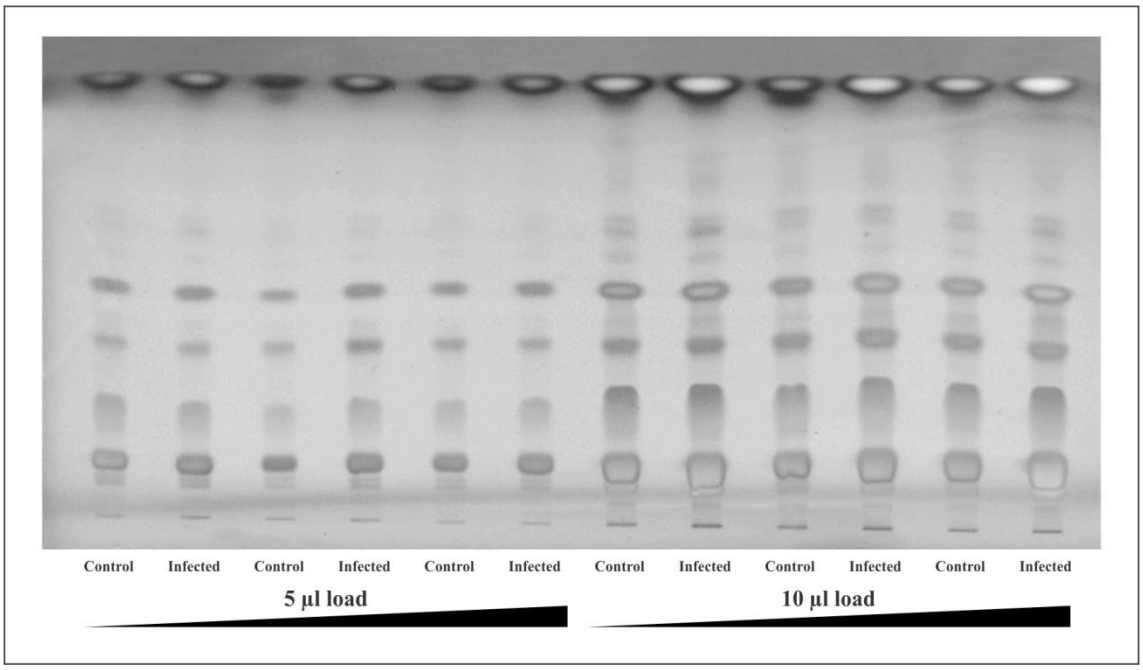


Figure 3.10HPTLC separations ofphospholipid classes. Phospholipids from JFH1 infected Huh-7 cells (Infected) and mock-infected Huh-7 (Control) were separated by HPTLC using different loading concentrations (5 and 10 µl).

3.6.1 Identification of altered phospholipid species in HCV-infected cells

Having demonstrated that the main neutral lipid classes were up-regulated in chronically JFH1-infected cells, we further sought to identify the effect that chronic HCV infection had on phospholipids. Whole cell lipid extracts were run on HPTLC plates using a protocol optimised for separating phospholipids. To identify bands, the samples were run in parallel with purified commercial standards for phosphatic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylserine (PS) (Figure 3.11).

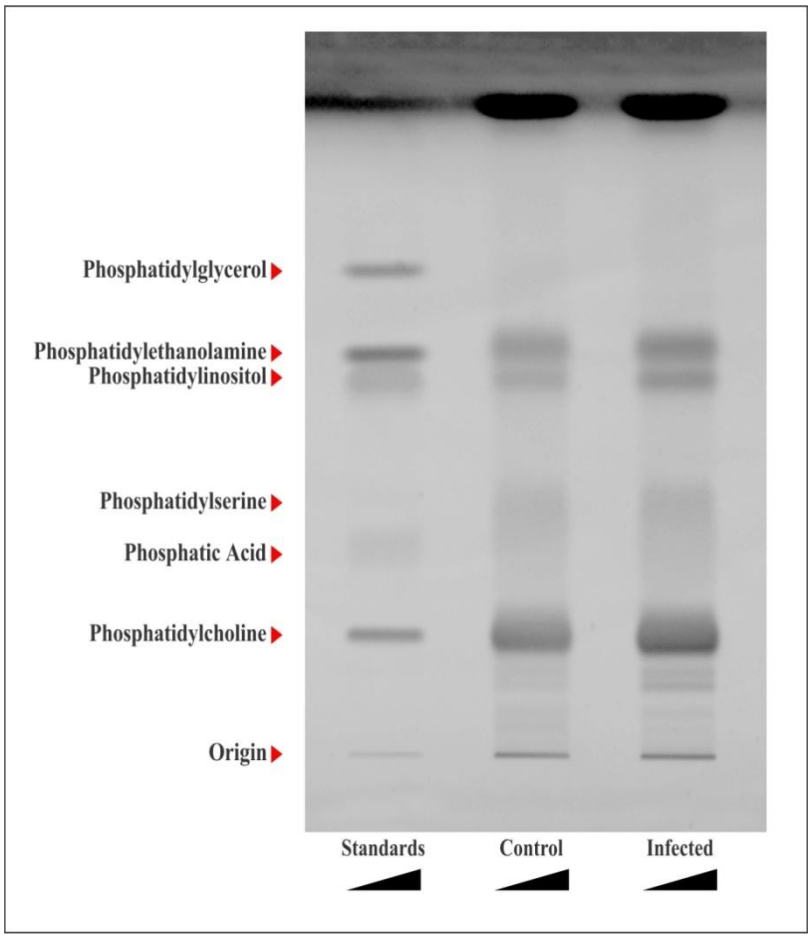


Figure 3.11Identification of phospholipids species bands against known standards. Known standard bands for phosphatic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylserine (PS) were utilized to characterize the specific effects of chronic hepatitis C infection on phospholipids.

The major phospholipids species were identified by HPTLC and confirmed by mass spectrometry analysis.

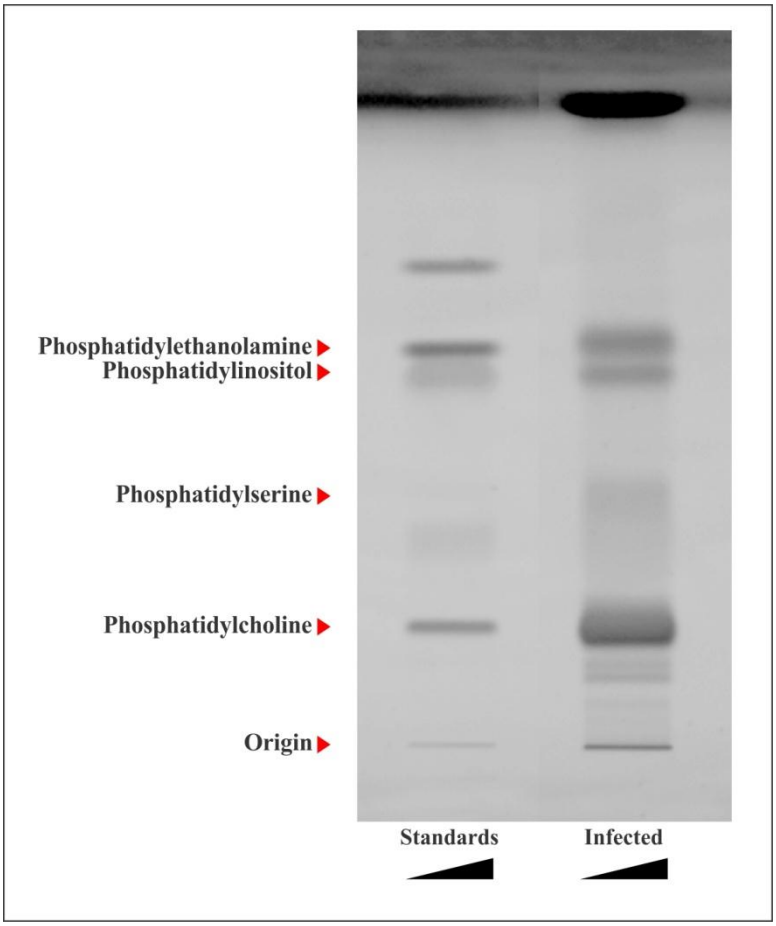


Figure 3.12Identification of phospholipids species bands against known standards. Huh-7 cells were infected with HCV (JFH1) for two weeks then total lipids was extracted and separated by HPTLC. PC, The most abundant phospholipids species as detected by HPTLC were PE, PI and PS respectively.

3.6.2 HCV induces a significant increase in global host phospholipids

To examine the effects of chronic HCV infection on phospholipids in host cells, Huh-7 cells were transfected with JFH1 RNA, cultured until >90% of cells were infected (confirmed by IF), then lipids from JHF1-infected and mock-infected Huh-7 cells were extracted and separated using HPTLC. Densitometry was performed on the phospholipid bands. Figures 3.13 A and B demonstrate that PC and PE were significantly increased in JFH1 HCV-infected Huh-7 cells compared to mock-infected Huh-7, by approximately 30% and 20% respectively. Furthermore, PI showed significant increase in JFH1-infected Huh-7 cells by approximately 20% (Figure 3.14A). In contrast, the apparent increase in PS in JFH-infected Huh-7 cells was not significant (Figure 3.14B).

Together PC, PE and PS HPLTC findings indicate a role for lipid species in the viral life cycle. An increase of phospholipids suggests that host cell phospholipids play a crucial role in the formation of cytosolic LDs and modified membrane compartments for the promotion of viral replication and infectious virus assembly.

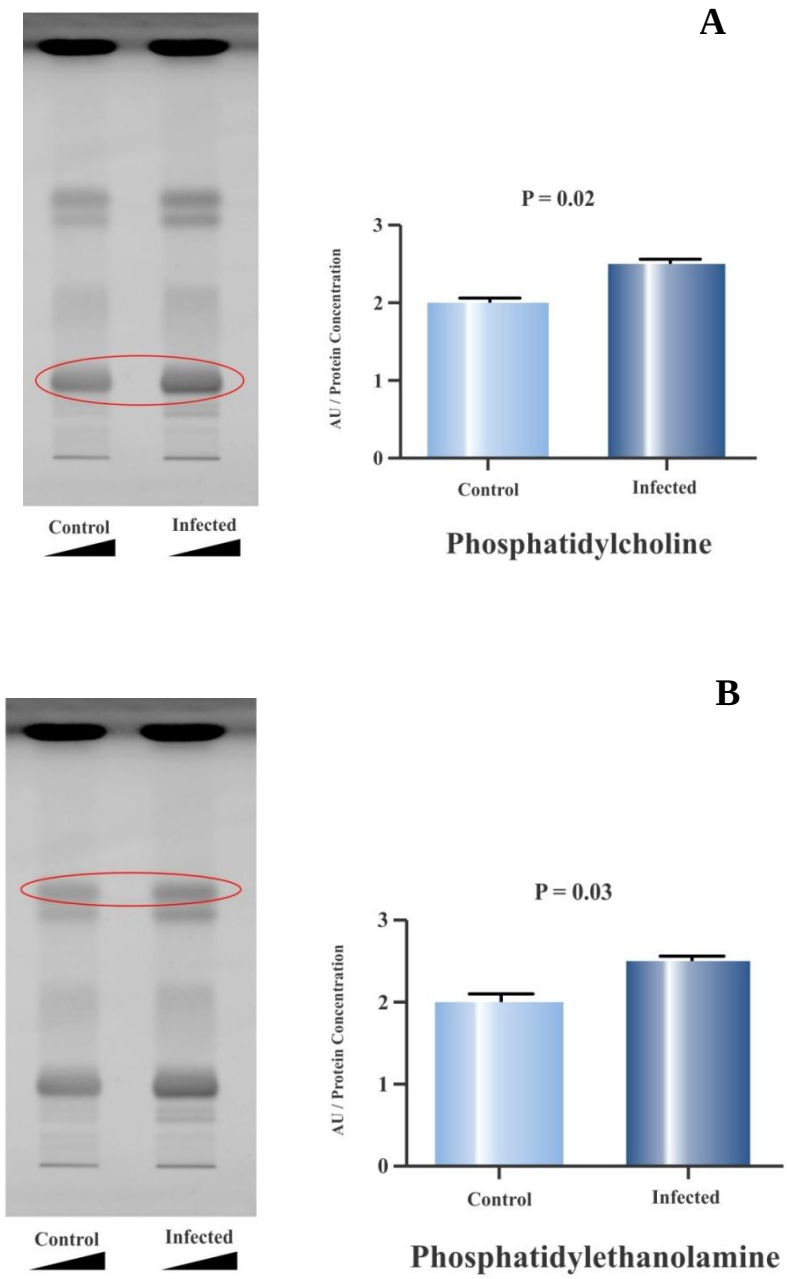


Figure 3.13 HCV induces increase onPC and PE in the host cells.
PC and PE bands were identified against known standards and compared between (JFH1 (HCV) infected Huh-7 cells and mock infected Huh-7). Chronic JFH1 (HCV) infected Huh-7 cells has significant higher levels of PC (A) and PE (B) compared to mock infected.

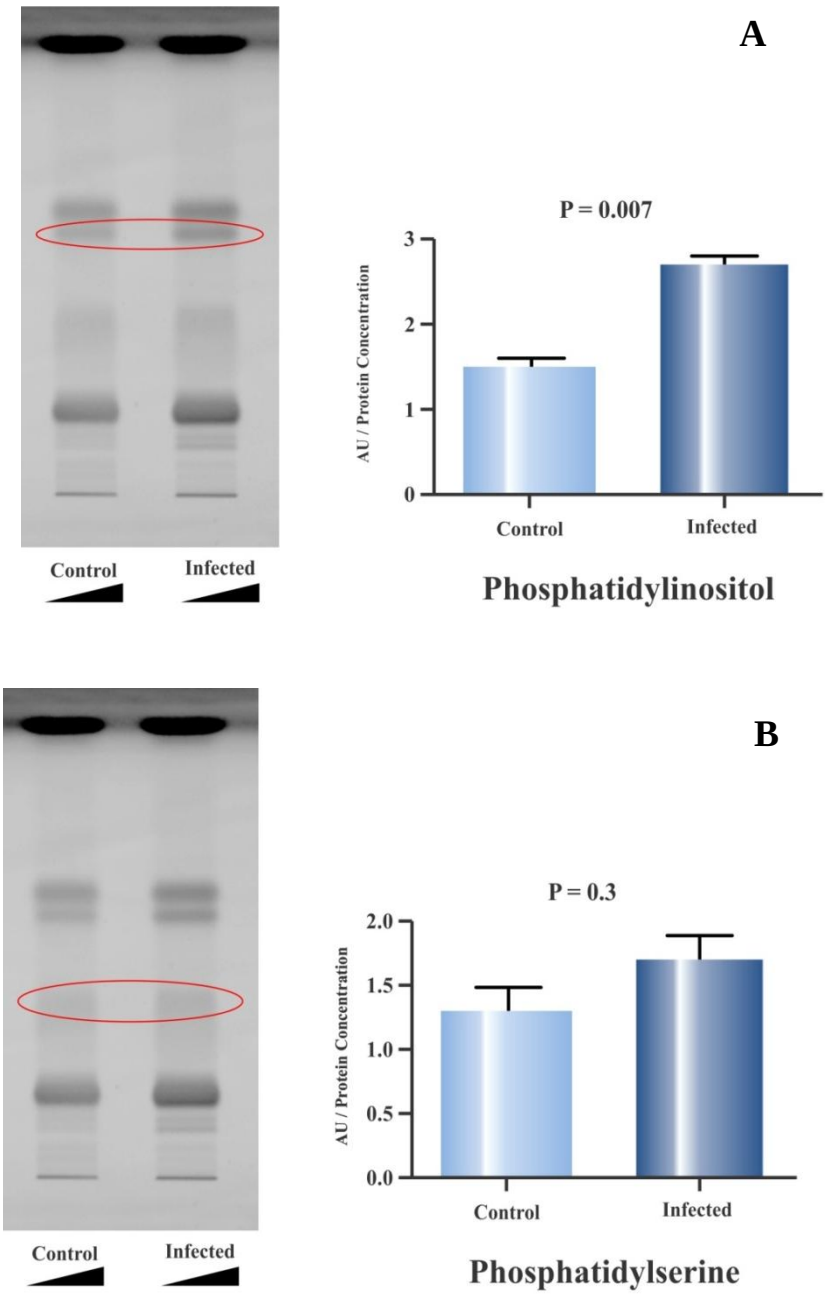


Figure 3.14HCV induces increase on PC and PE in the host cells.
PI and PS bands were identified against known standards and compared between (JFH1 (HCV) infected Huh-7 cells and mock infected Huh-7). (A) Chronic JFH1 (HCV) infected Huh-7 cells has significant higher levels of PI. (B) a non-significant higher levels of PS compared to mock infected Huh-7 (*p=0.3).

3.7 Mass spectrometry analysis supported the finding that chronic HCV infection induces global changes in the host cell lipidome

To further characterize changes in lipid content, the detailed fatty acid composition of some lipid classes that showed significant increase in HCV-infected cells, such as TAG and PC, were analysed individually using mass spectrometry. This was initially performed directly on whole lipid extracts, using liquid chromatography and tandem mass spectrometry (LC-MS/MS). An alternative technique was to separate lipids by HP-TLC as above, and then analyse individual lipid species using mass spectrometry. Both of these techniques were available through established collaborations at the University ofWestern Sydney and University of New South Wales.

The excellent sensitivity and molecular specificity offered by modern mass spectrometry has made it arguably the method of choice for lipid analysis. No other analytical method allows for both the simultaneous and differential detection of individual lipid variants and the acquisition of the detailed structural information that is required by the lipid researcher (Ellis et al. 2013).

Generally, the typical methods used to analyse lipids can be divided into two categories: liquid chromatography (LC)- and mass spectrometry (MS)-based methods (Li et al. 2011), and shotgun lipidomics (Han & Gross 2005). LC-MS-based methods are limited as it is time consuming to perform the experiment and analyse the data. Additionally, the ionization efficiency of lipids may vary with elution order (where mobile phase compositions are different over time), creating challenges for quantitation studies (Han et al. 2012). In contrast, shotgun lipidomics, which is performed by direct infusion, is faster and may be more suitable for the purposes of analysing a large number of samples. However, shotgun lipidomics has a limited ability to detect nonpolar lipids due to the

overwhelming signal effect of polar lipids. Furthermore, downstream bioinformatics analysis is particularly challenging due to the large amount of data acquired.

HPTLC-AMD technology was used to overcome the limitations of both the LC-MS and shotgun MS methods. The HPTLC system represents a good alternative to conventional TLC and is a fast, inexpensive, robust and reliable method for lipid analysis. Additionally, HPTLC-AMD is capable of separating lipids that exhibit few differences in polarity and over a wide range of polarity. It is easier to compare the relative abundance of different species of the same class or subclass of lipids within samples when using the MS lipidomic approach, as the solution composition is constant during the analysis (Han et al. 2012), overcoming one of the main challenges associated with the use of shotgun lipidomics.

3.7.1 Mass spectrometry analysis shows that chronic HCV infection induces global changes in the fatty acid composition of triacylglycerol

As can be seen in Figure 3.15, which is consistent with HTPLC data, there was a global increase in the fatty acid components of triacylglycerol in JFH1 HCV-infected Huh-7 cells than there was in uninfected Huh-7.

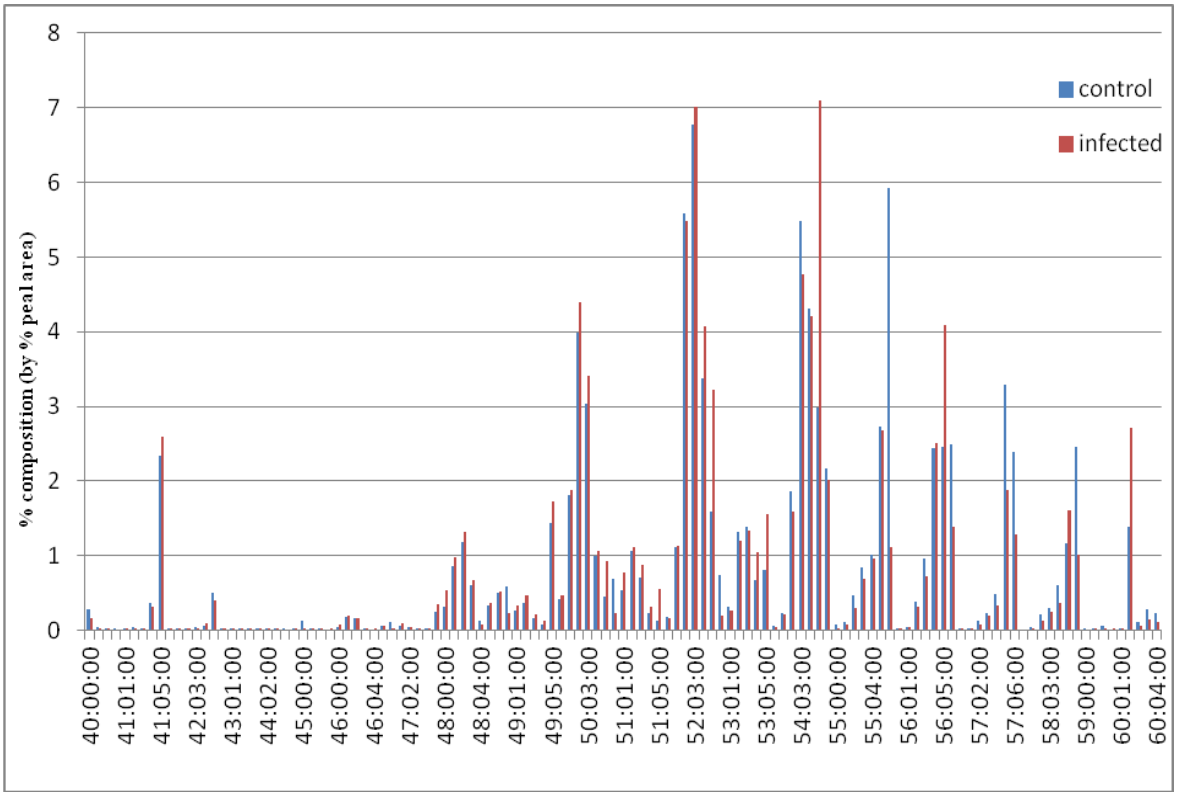


Figure 3.15Mass Spectrometry lipidomic analysis of TAG fatty acids composition. Lipids were extracted from JFH1 infected Huh-7 and mock infected Huh-7cells and separated by HPTLC. TGA bands were scraped off for MS analysis.

Representative examples are presented in (**Figure 3.16**) where the triacylglycerol species TAG 52:05, TAG 54:05, TAG 56:05 and TAG 60:12, exhibit increased abundance in the JFH1 (HCV) infected Huh-7 samples, relative to mock infected samples. In contrast, the triacylglycerol species TAG 55:06, TAG 57:05, TAG 57:06 and TAG 58:06 are shown to exhibit decreased abundances in the JFH1 (HCV) infected Huh-7 samples relative to mock-infected samples (**Figure 3.17**). Overall, these changes suggest an intimate link between lipids and HCV life cycle.

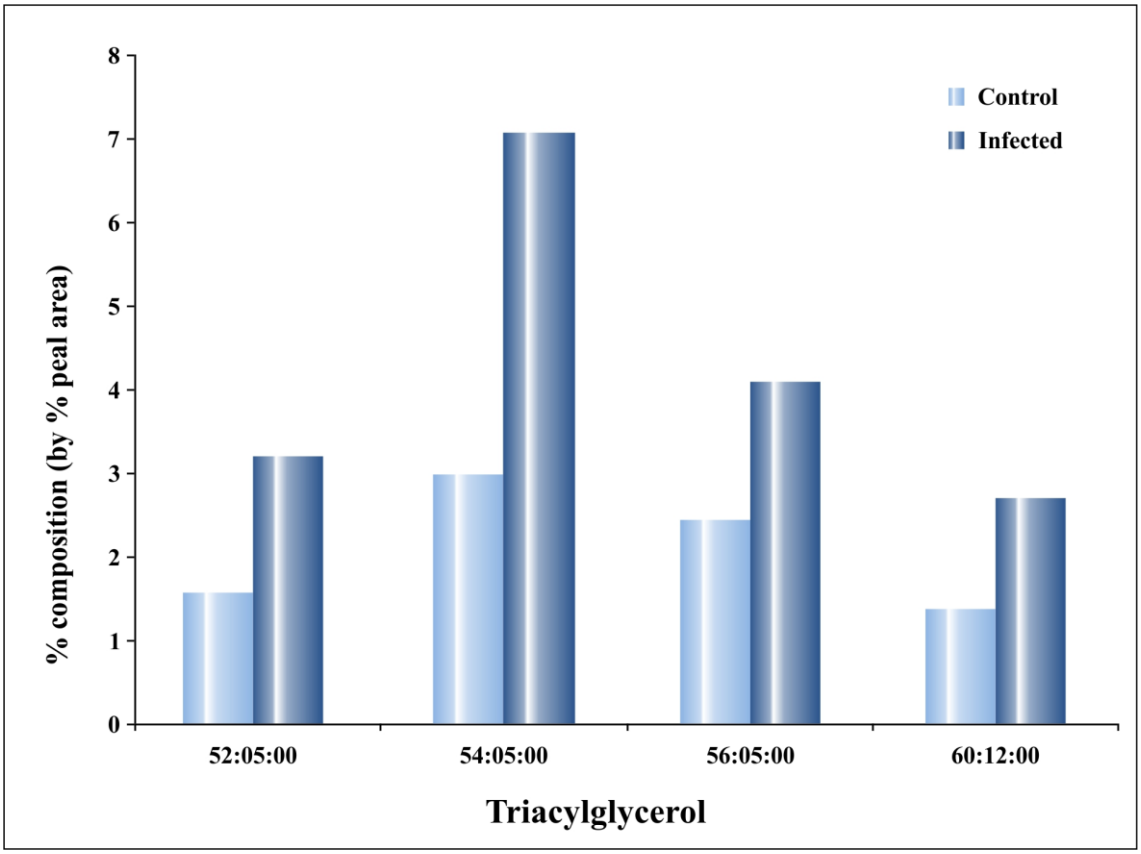


Figure 3.16 Representative examples of triacylglycerol fatty acid chains differentially regulated during chronic HCV infection.
The relative ion intensity is plotted for various TAG in the JFH1 infected Huh-7 and mock infected Huh-7 as a reference. Panels A-D (lipid species TAG 52:05, TAG 54:05, TAG 56:05 and TAG 60:12) provide examples of triacylglycerol fatty acid chains exhibiting notable changes in abundance during chronic HCV infection.

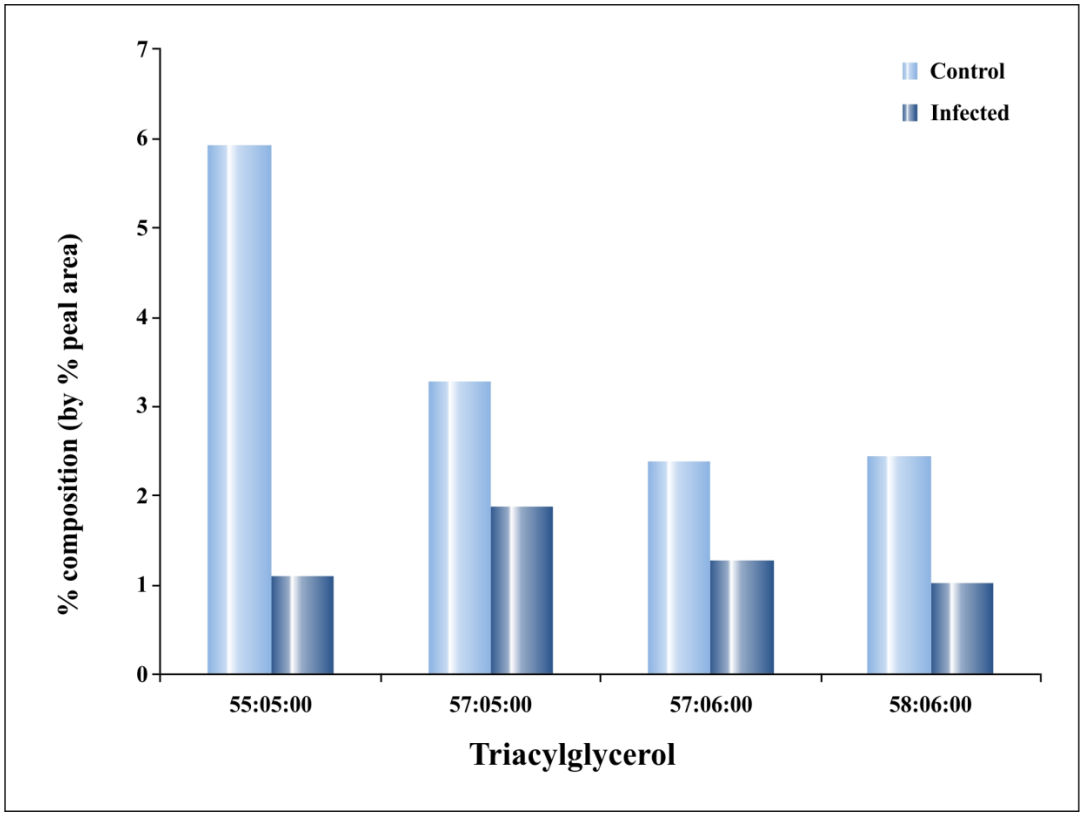


Figure 3.17 Representative examples of triacylglycerol fatty acid chains differentially down regulated during chronic HCV infection.
The relative ion intensity is plotted for various TAG in the JFH1 infected Huh-7 and mock (conditioned media) infected Huh-7 as the reference. Panels A-D (lipid species TAG 55:06, TAG 57:05, TAG 57:06 and TAG 58:06) provide examples of triacylglycerol species exhibiting notable decrease in abundance during chronic HCV infection.

3.7.2 Mass Spectrometry Analysis shows that chronic HCV infection induces global Changes in the fatty acid composition of phosphatidylcholine

In addition, as shown in Figure 3.18**,** consistent with HPTLC data there was a global increase in the fatty acid composition of phosphatidylcholine (PC) in JFH1 (HCV) infected Huh-7 cells compared to mock infected Huh-7.

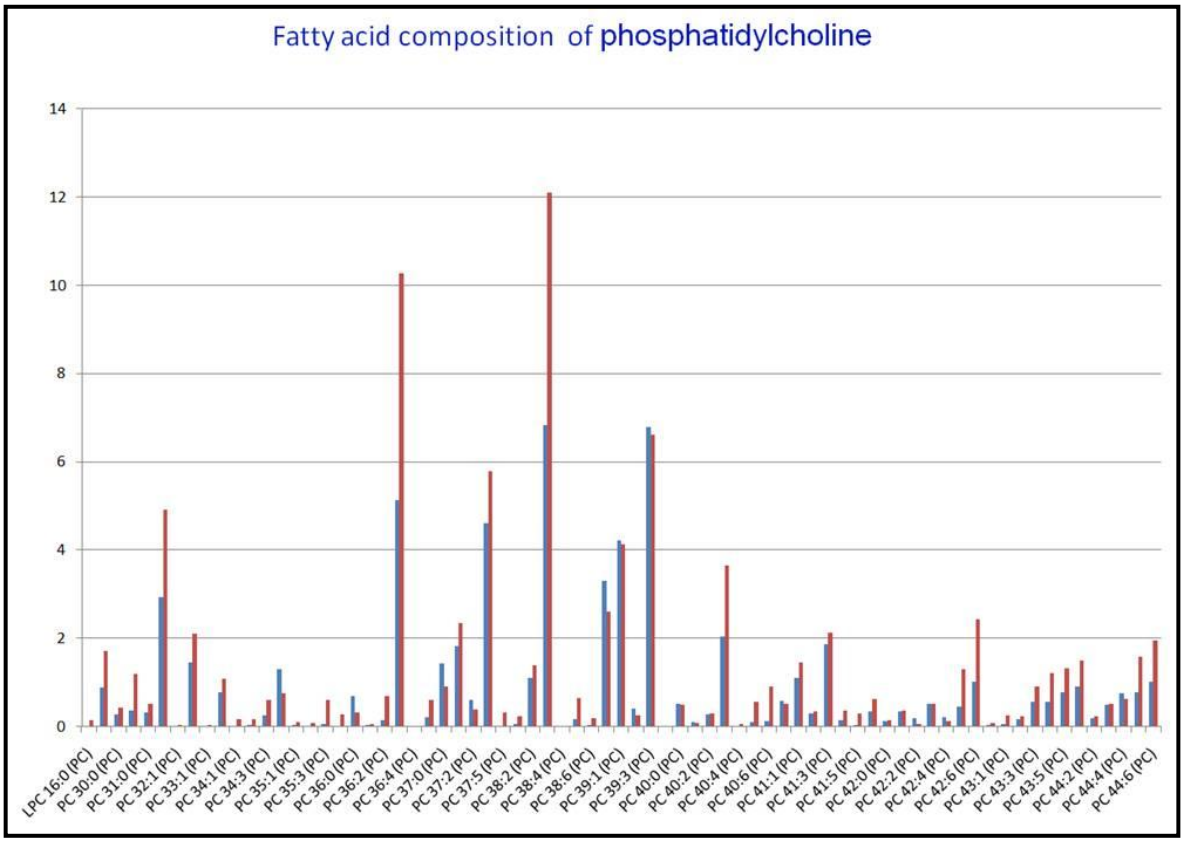


Figure 3.18 Mass Spectrometry lipidomic analysis of PC fatty acids composition. Lipids were extracted from JFH1 infected Huh-7 and mock infected Huh-7cells and separated by HPTLC. PC bands were scraped off for MS analysis. PC; phosphstidylcholine.

3.8 Summary

In summary, using two complementary approaches (high-resolution HPTLC and mass spectrometry) we demonstrated that infection of Huh-7 cells with JFH1 HCV induces global cellular changes in both neutral lipids and phospholipids. These findings provide useful insights into the metabolic reprogramming induced by chronic HCV infection aimed at benefiting both energetic and biosynthetic needs for supporting the viral life cycle.

The increased abundance of some phospholipids potentially reflects their role as crucial constituents in the various structural entities that support viral replication, including the lipid droplet and membranous replicase compartments. In contrast, the decline of some species of other lipid classes (triacylglycerol) probably reflects their consumption following incorporation into lipoprotein-associated viral particles, and this potentially plays an important role in infectivity. However, the analysis of whole cell lipid extracts did not identify changes of lipid content in different cellular organelles. This level of detail is crucial to understanding the effects of HCV on cell lipid metabolism and is explored in more detail in Chapter 4, where cellular organelles were separated using centrifugal fractionation for individual lipid analysis.

Identification of the metabolic pathways during HCV infection and the key enzymes mediating this effect could serve as candidates for novel antiviral targets against hepatitis C virus, offering higher barriers against drug resistances than current DAA drugs.

In conclusion, this study of HCV-infected cells provides a valuable reference pertaining to the use of HPTLC and mass spectrometry to dissect the role of various host cell lipid species in the viral life cycle. The observations presented open the door to several new and exciting avenues of investigation, which should improve existing understanding

of HCV-associated pathogenesis and lay the groundwork for the development of novel antiviral therapies.

4. Lipidomic Analysis of Purified ER From HCV Infected Hepatocytes

4.1 Introduction

The previous chapter described how chronic HCV infection modulates global intracellular lipidomics to create an environment that aids RNA replication and the production of progeny virus particles. The complementary techniques of HP-TLC and mass spectrometry were employed.

This chapter will describe the influence of HCV infection on lipidomic changes at the sub-cellular level. It is likely that changes in host lipid gene expression levels will cause variable effects in different fractions of the cell. Furthermore, measuring downstream metabolite concentrations in different fractions can determine the activities of metabolic pathways more accurately than just quantifying lipid enzyme concentrations or mRNA expression (Kell et al. 2005; TAN et al. 2009).

Among the various subcellular compartments, the endoplasmic reticulum (ER) is a prime candidate for HCV infection. The ER plays a central role in HCV replication: following infection, viral RNA is translated at the ER, where viral proteins induce membrane modifications, giving rise to the 'membranous web'. This web acts as the platform for the synthesis of HCV RNA. Viral structural proteins are then packaged with RNA, assembled into 'lipoviral' particles and exported through the ER-Golgi pathway (Gastaminza et al. 2008). Thus, understanding HCV/host lipid homeostasis and turnover in the ER should provide novel insights, over and above examining changes in the whole infected cell.

These experiments commenced by optimising a protocol to isolate the ER subcellular fraction, using ultracentrifugation over a sucrose gradient (described in detail in the Methodology section, Chapter 2). The complementary techniques of HPTLC and mass spectrometry were then employed to define the lipid profiles in the purified ER of

JFH1-infected Huh-7 cells, compared to uninfected Huh-7. Three replicates for each sample were analysed and statistical analysis was performed to identify the main lipids with differential expression across conditions. A particular emphasis was placed on identifying the differences between HCV-infected and uninfected cells in the lipid classes that are known to be important for HCV replication, including neutral lipids and phospholipids.

4.2 HCV infection induces diffuse changes in neutral lipids purified from host cell ER

To examine how the lipid content of the ER organelle is impacted by chronic HCV infection, Huh-7 cells were transfected with HCV (JFH1) RNA, then cultured for two weeks until >90% of cells were infected, confirmed by immunofluorescence using anti-NS5A protein. Cells were harvested and an equivalent number of cells (2×10^7 cells) were used for each sample. The ER fraction was isolated using discontinuous sucrose gradient centrifugation. Validation of the purity of the ER fraction was determined by examining "house-keeper" (HK) protein markers using standard SDS-PAGE analysis (Figure 4.1). Lipid was extracted from the isolated ER fractions by a chloroform-methanol method then separated and quantified by HPTLC. To allow a valid comparison among samples, protein concentrations were measured post-lipid extraction and used to normalise the amount of lipid loaded onto each HPTLC lane.

First, the effect that chronic HCV infection had on neutral lipids in the ER was examined. Commercial standards for the major neutral lipid classes were used to identify bands in ER extracts from both JFH1-infected Huh-7 and uninfected Huh-7 cells. All identified bands were scraped off and analysed by MS to further confirm their identity (data not shown). A dramatic increase in free cholesterol in ER from JFH1-infected Huh-7 cells relative to uninfected Huh-7 cells was observed (Figure 4.2). In contrast, no significant change in triacylglycerol and cholesterol esters was exhibited.

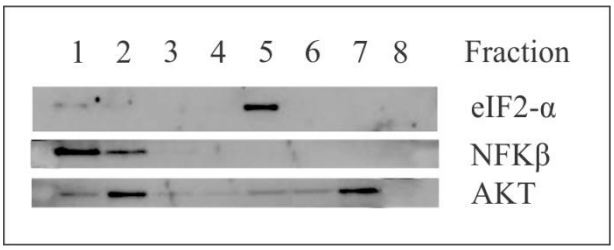


Figure 4.1 Immunoblot analysis of subcellular marker proteins to verify the purity of isolated ER.
Subcellular fractionation ofJHF1 infected and control Huh-7 cells was performed by discontinuous sucrose gradient centrifugation. Protein (20 µg) from each fraction was separated by 10% SDS PAGE and transferred onto PVDF membranes.Blotted proteins were probed for markers of ER (eIF2-α), nucleus (NF-κβ) and cytosol (AKT).

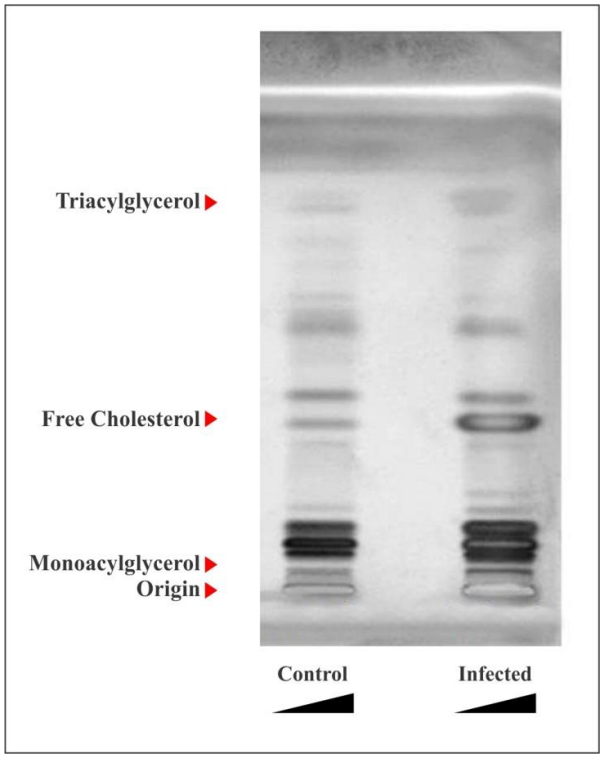


Figure 4.2 HPTLC separation of neutral lipids extracted from ER.
ER fractions were isolated from JHF1 infected and control Huh-7 cellsby discontinuous sucrose gradient centrifugation, followed by lipid extraction and HPTLC analysis. ER from JFH1 infected Huh-7 cells demonstrated a dramatic increase in free cholesterol (FC) compared to uninfected Huh-7 cells ($P<0.05$).The other major neutral lipid classes (monoacylglycerol, diacylglycerol and tiyacylglycerol) showed some slight but not significant changes.

4.2.1 HCV-induced significant increase in free cholesterol (FC) in ER fraction

Because of its key roles in HCV RNA replication and virus export, it was anticipated that more profound lipid changes would be observed in the ER of HCV-infected cells than would be evident in whole cell extracts. To investigate, the changes observed in the neutral lipids in the whole cell were compared with those exhibited in purified ER (Figure 4.4). There was a significant difference in the lipid profile of purified ER, relative to the whole cells. In particular, the HCV-induced increase in free cholesterol (FC) was more profound in the ER than it was in the cell overall (Figure 4.5). This further suggests that the ER plays a crucial role in the HCV life cycle.

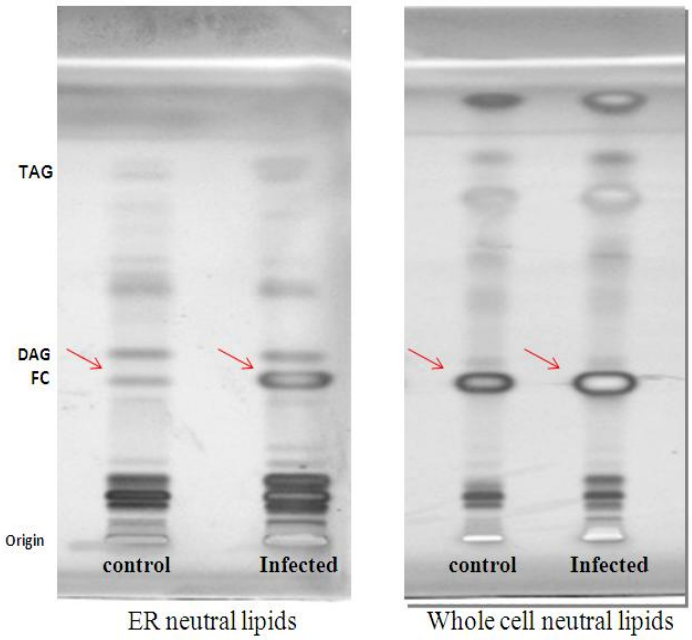


Figure 4.4 Neutral lipid composition of purified ER from HCV infected cells, relative the whole cell.
In ER fraction the effect of HCV infection on neutral lipids was more profound for FC, the relative increase in FC is more in ER than in whole cell. DAG, TAG and CE showed no significant difference between HCV infected and uninfected samples of ER fractions. FC; free cholesterol, DAG; diacylglycrol, TAG; triacylglycerol and CE; cholesterol esters

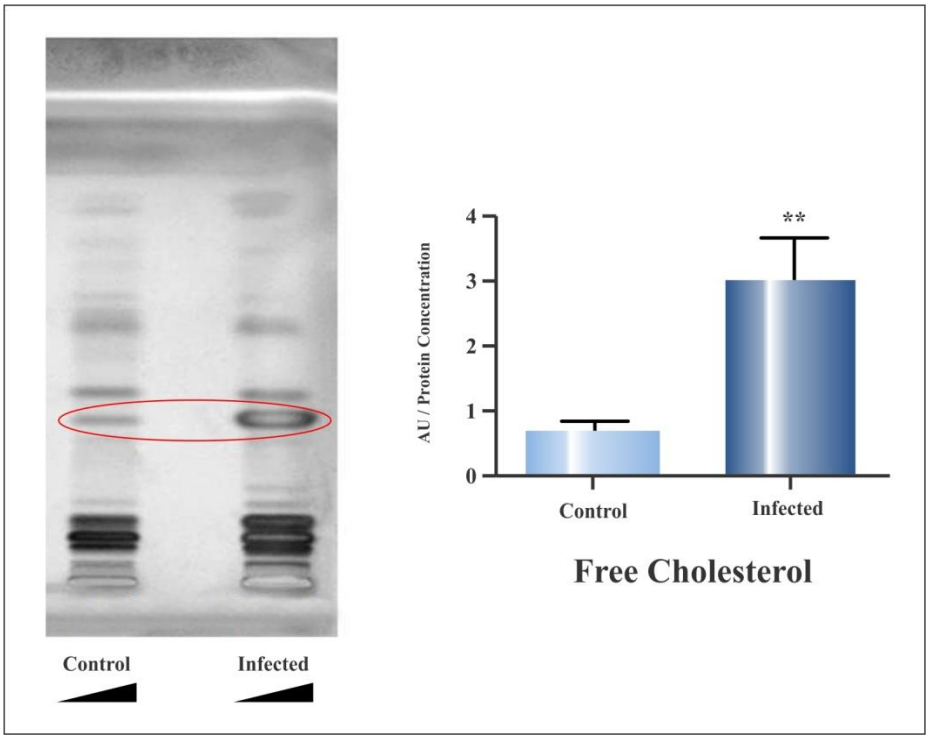


Figure 4.5 HCV increases free cholesterol in the ER of infected cells.
FC bands were identified against known standards (red oval) and densitometric detection of cupric-sulfate-charred plates was carried out using Luminescent Image Analyser LAS-4000. Chronic JFH1 (HCV) infected Huh-7 cells has a significant higher levels of FC compared to mock infected Huh-7 (**p<0.01).

4.2.2 HCV infection induces diffuse Changes in the Host Cell purified ER phospholipids

We next investigated the effect of chronic hepatitis C infection on phospholipids in ER. Consistent with the overall increase in neutral lipids we observed in purified ER from HCV infected cells, compared to uninfected cells, HPTLC lipidomic analysis revealed a significant up regulation of several phospholipid species (Fig 4.6). Using commercial phospholipid standards, the phospholipids classes, phosphatidic acid (PA), phophatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylserine (PS) were identified (Figure 4.6A). Densitometry analysis demonstrated that JFH1 infection resulted in a significant increase in phospholipid, including PC, PE, and PI (Figure 4.6B).

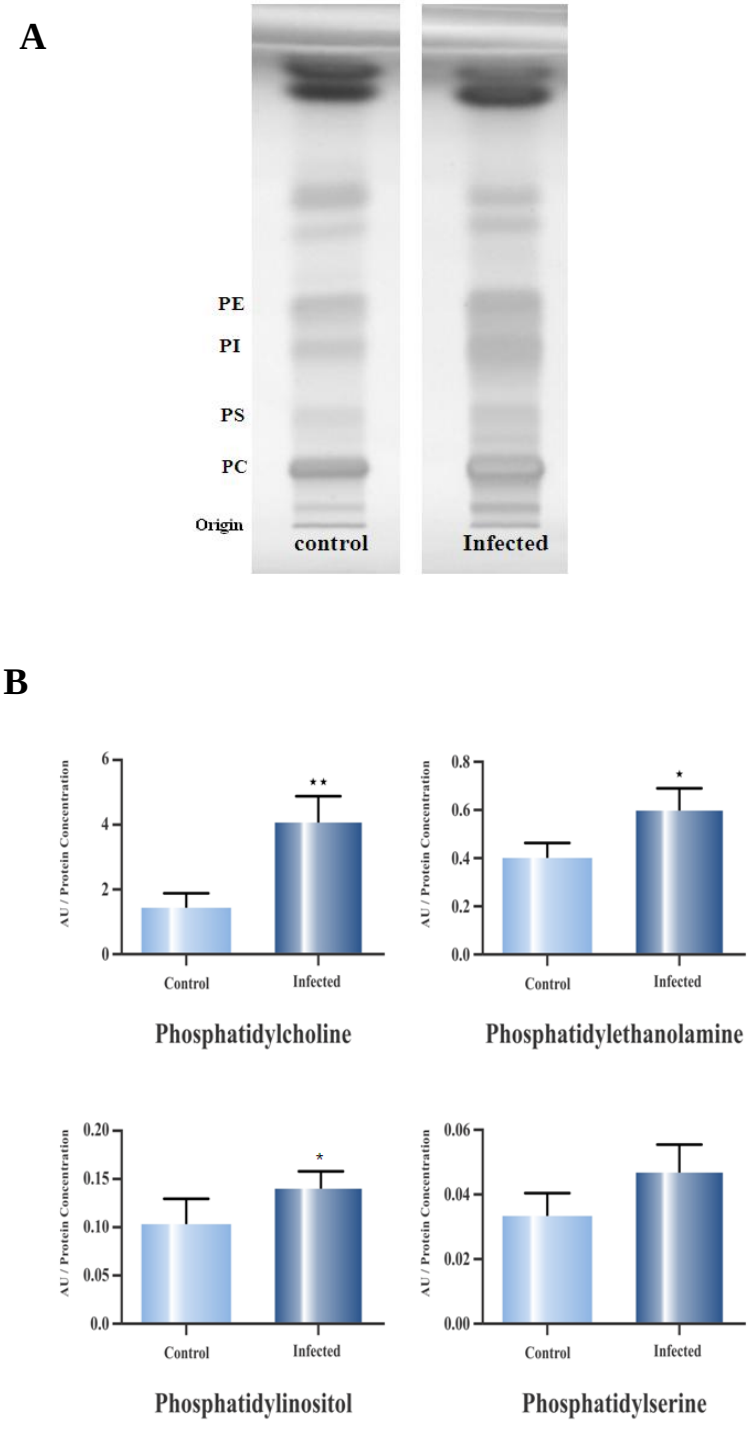


Figure 4.6 HCV increases phospholipids in the ER of infected cells.
Huh-7 cells were infected with HCV (JFH1) for two weeks then total lipid was extracted from purified ER fractions and separated by HPTLC. The major phospholipid classes PC, PE, PI, and PS were identified against known standards (A). Densitometric detection of cupric-sulfate-charred plates to characterize the specific effects of chronic hepatitis C

infection on phospholipids classes (B). There was a significant increase in PC, PE and PI. (PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.) (*p<0.05, **p<0.01)

4.2.3 Comparison of the changes in the neutral and phospholipids composition in the purified ER from HCV-infected cells relative to the whole cell

As described above, the hypothesis applied to the research was that HCV-induced lipid changes would be more profound in the ER organelle than they would be in the whole cell. Therefore, the relative changes in lipid content of purified ER fractions were compared with those of whole-cell extracts. As expected, the overall changes in neutral lipids (Figure 4.7A) and phospholipids (Figure 4.7B) were greater in the ER than they were in the whole cell. The most striking differences were the relatively greater HCV-induced increase in phosphatidylcholine (150% vs. 30%, $p < 0.001$) and free cholesterol (200% vs. 50 %, $p < 0.01$) in ER. This suggests that chronic HCV infection causes the profound accumulation of phosphatidylcholine in the ER fraction. This emphasises the role of the ER in HVC replication, suggests ER is an attractive target for anti-viral therapies and reveals the vital role phosphatidylcholine plays in HCV replication in the ER. Modulation of the enzymes that control phosphatidylcholine pathways may represent novel host-targeted anti-HCV therapies.

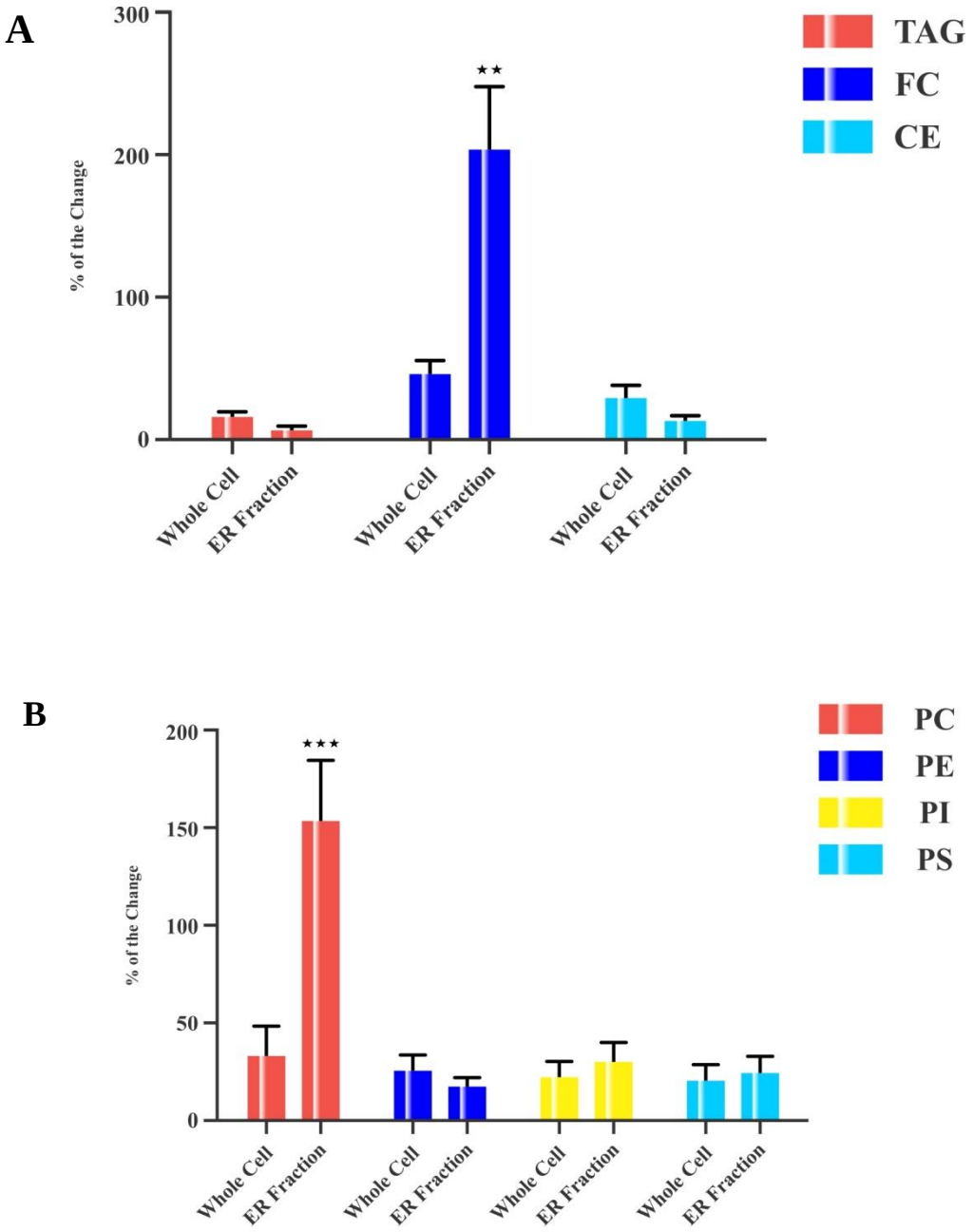


Figure 4.7 Relative changes in major lipid classes in purified ER, compared to whole cell extracts, following HCV infection. Comparison of the changes of the neutral lipids composition in the purified ER from chronic JFH1 infected cells relative the whole cell (A). Comparison of the changes of the phospholipids composition in the purified ER from chronic JFH1 infected cells relative the whole cell (B).

4.2.4 HCV induces diffuse changes in the host cell purified ER lipid rafts

Thus far, the research demonstrated the following: 1) The effect of HCV infection on lipids was more profound in the purified ER than it was on the whole cell; this was consistent with the vital role of the ER organelle in HCV replication; and 2) HCV infection induced a significant accumulation of several PC and PE species, which are essential components of cell membranes. Therefore, more detailed analysis of the effect that chronic HCV infection have on ER lipid rafts was performed. Recent studies suggest that the HCV replication complexes are formed on lipid rafts (which are detergent insoluble micro domains of intracellular vesicular membranes that are rich in cholesterol and sphingolipid). Lipid rafts are small platforms in the outer exoplasmic leaflet of the lipid bilayer, and consist of sphingolipids and cholesterol, which are connected to phospholipids and cholesterol in the inner cytoplasmic leaflet. These assemblies are fluid, but are more ordered and tightly packed than the surrounding bilayer. Lipid rafts were first discovered as a detergent-insoluble fraction (REF), and the concept of lipid rafts has changed our understanding of cell membrane organization. In mammalian cells, lipid rafts are first assembled in the Golgi complex (Simons & Ehehalt 2002), and are involved in cell signalling and intracellular trafficking (Helms & Zurzolo 2004). Some viruses utilize these subcellular membrane domains for cell entry, virion assembly, or budding (Chazal & Gerlier 2003; Salaün et al. 2004). Viruses can also perturb cell signalling pathways by interfering with proteins in lipid rafts (Krautkramer et al. 2004; Avota et al. 2004). Moreover, different studies have indicated that HCV replication is associated with ER lipid rafts (Shi et al. 2003; Aizaki et al. 2004). Therefore, lipidomic analysis of HCV–lipid raft interaction may have the potential to partially explain the abnormality of lipid metabolism that is associated with HCV infection.

4.2.4.1 Purification of lipid rafts from the ER of HCV-infected cells

To study the effects of HCV on lipid rafts in the model, a protocol to purify ER-lipid rafts was first established. Detergent-free buffer and discontinuous sucrose gradients were used (described in detail in Methods Section 2.4.3) that were adapted from a published protocol for total lipid raft isolation (Song et al. 1996). To allow the purification of ER-lipid rafts without the use of detergent, Triton ×-100 was replaced with sodium carbonate. The pellet resulting from ER isolation was resuspended in 2 ml of 0.5 M Na_3Co and adjusted to sucrose gradient. All sucrose gradients were prepared in a detergent-free buffer. Validation of the purity of the ER-lipid rafts fraction was performed by examining "house-keeper" (HK) protein markers, Erlin-1 (endoplasmic reticulum lipid rafts protein) using standard SDS-PAGE analysis (Figure 4.8A). Erlin-1 is found in the detergent-insoluble low-density fraction of cell lysates of many cell types, and was observed to be localized to the endoplasmic reticulum (ER) and highly enriched in detergent-resistant membranes (DRM) fractions. HCV infection was further confirmed by Western blot, using specific antibodies against NS5A protein (Figure 4.8B). The high level of NS5A protein in the ER-lipid rafts fraction (F4) was consistent with previous data, thus demonstrating that the membranous web, where HCV-RNA synthesis and replication take place in hepatocytes, is a lipid raft membrane structure (Gao et al. 2004; Aizaki et al. 2004).

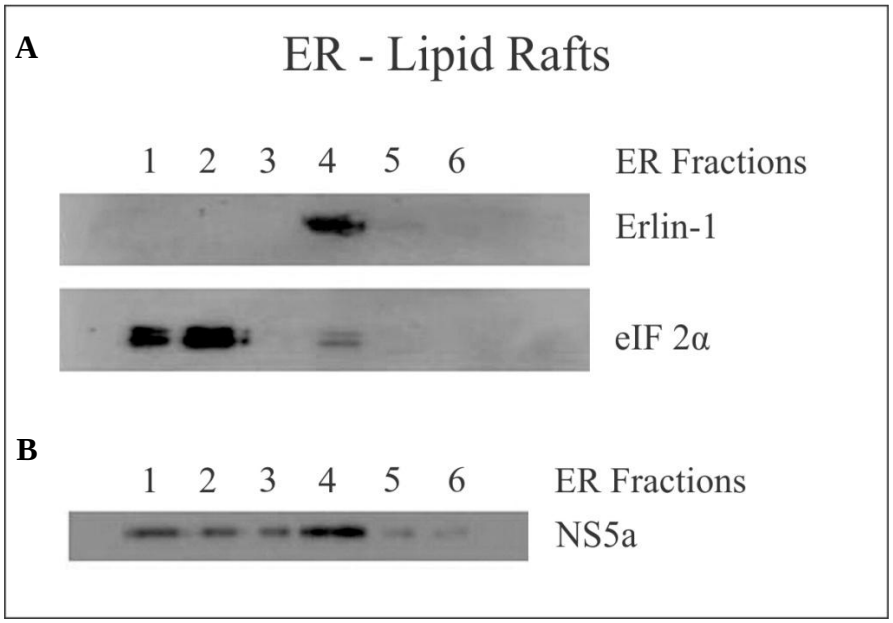


Figure 4.8 (a) Detergent-free purification of ER-lipid rafts using discontinuous sucrose gradients.
Protein extracts were analysed for the presence of Erlin-1 (lipid raft marker) and eIF2 α (ER marker) by western blot. The experiment was repeated three times independently with the same results.

(b) Confirmation of HCV protein in the purified ER-lipid rafts.
Total cellular protein was extracted from JFH1 infected cells. Protein was separated by gel electrophoresis, transferred to a PVDF membrane and examined by western blot. HCV NS5A protein was detected using specific antibodies.

4.2.4.2 Identification of the neutral lipids composition in the purified ER-lipid rafts

Next, the effect that HCV infection had on the composition of neutral lipids in ER-lipid rafts was examined. JFH1 HCV-infected and uninfected Huh-7 cells were lysed, and ER-lipid rafts isolated, as per the process described above (4.3.1). Total lipids were extracted, and the neutral lipids were analysed using HPTLC. Using commercial standards, neutral lipid bands were identified in ER-Lipid raft extracts from both JFH1-infected Huh-7 cells and uninfected Huh-7 cells (Figure 4.9). Free cholesterol (FC) was the most prominent neutral lipid class observed in ER-lipid rafts and appeared to be up-regulated in HCV-infected cells (Fig 4.9A). Densitometry analysis of HPTLC plates confirmed that HCV infection resulted in a significant increase in FC in ER-lipid rafts (Fig 4.9B).

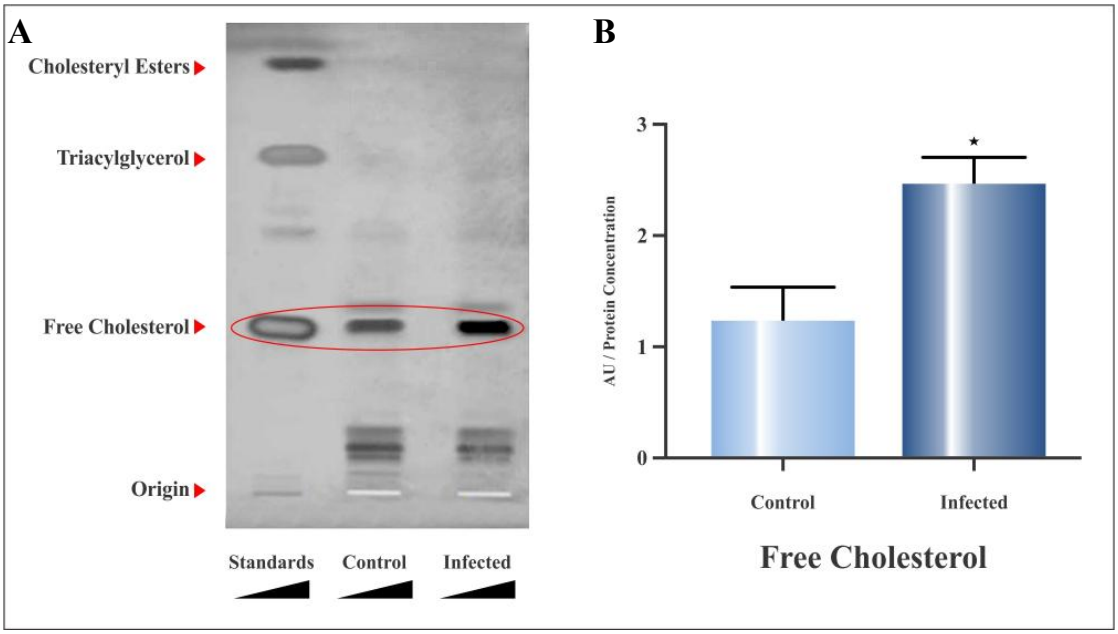


Figure 4.9 Identification of neutral lipids classes in ER-lipid rafts against known standards by HPTLC.
ER-lipid rafts were isolated from HCV (JFH1) infected and uninfected Huh-7 cells, lipids extracted and analysed by HPTLC **A)** Commercial standards for FC, TAG and CE were used to characterize the specific effects of HCV infection on the composition of neutral lipids in ER-lipid rafts.**B)**Densitometry analysis demonstrated that JFH1 infection significantly increased FC in ER-lipid rafts.* demonstrates significant difference between Huh-7 and infected cells ($p<0.05$).

4.2.4.3 The increase of FC in ER, as induced by HCV infection, is not global, but ER-lipid rafts are specific

After the fractionation and isolation of the ER organelle from the JHF1-infected and control Huh-7 cells, ER fraction was further fractionated to isolate lipid rafts using the protocol described in Chapter 2. Next, using the same protocol for ER-lipid rafts isolation, fractions were individually analysed to clarify whether FC up-regulation in the ER was specific to lipid rafts or ER global. All fractions obtained from each ER sample were analysed using HPTLC against commercial standards for FC. The ER-lipid rafts fraction (F2) exhibited in Figure 4.10 showed the highest band density, thus indicating that the increase of the level of FC in ER organelles in HCV infection is lipid rafts specific**.**

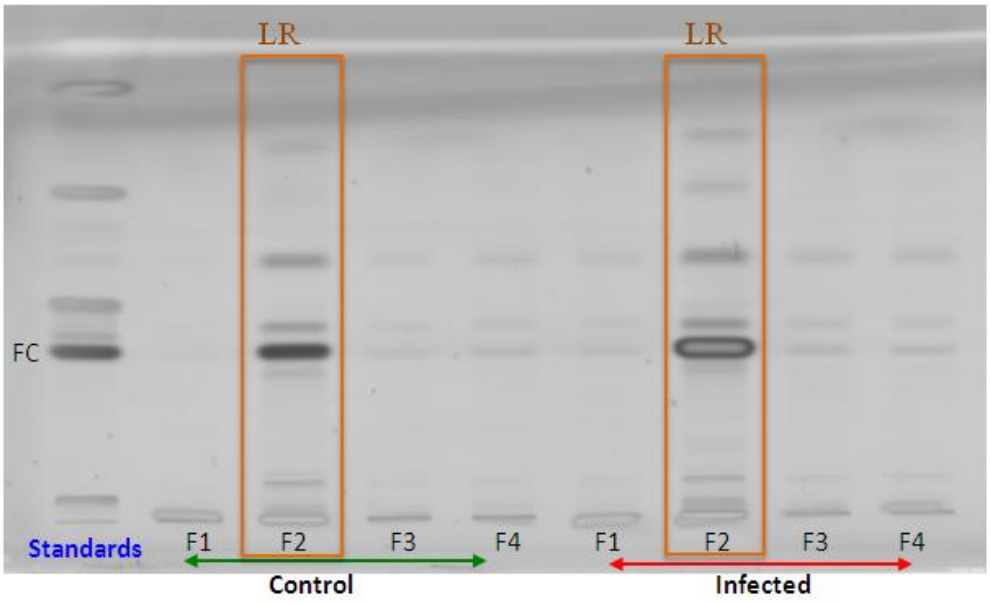


Figure 4.10 HPTLC separation of purified ER lipid rafts neutral lipids.
ER fractions isolated from JFH1 HCV infected and mock Huh-7 cells were further fractionated using discontinuous sucrose gradient into four fractions. Neutral lipids in all fractions were analysed by HPTLC. ER-lipid rafts Fraction (F2) from JFH1 infected cells showed dramatic increase in FC. F1, F2 , F3 and F4 are ER fraction numbers.

4.2.4.4 Comparative proteomics analysis of detergent-insoluble fractions by 2-D PAGE

To identify novel candidate biomarkers for HCV infection, ER-lipid rafts isolated from JFH1-infected and uninfected Huh-7 cells were analysed as per the process described previously. The proteins from ER-lipid rafts fractions were precipitated with acetone, 10% trichloroacetic acid, 0.07% β-mercaptoethanol for 1 h at −20 °C, following centrifugation, pellets were washed with acetone, 0.07% β-mercaptoethanol. Changes in protein expression in ER-lipid rafts were analysed using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (Figure 4.11).

Tens of protein spots were visualized on 2-DE gels, and differences in spot intensities were compared visually. No significant changes were induced by HCV infection on ER-lipid rafts proteins.

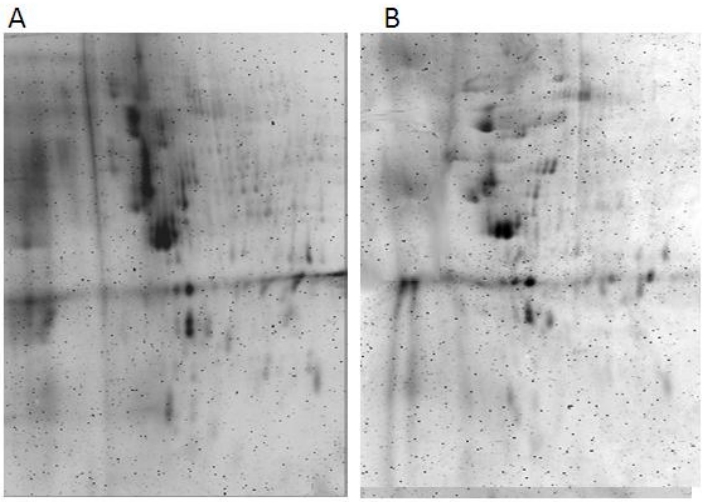


Figure 4.11 Two-dimensional polyacrylamide gel electrophoresis separation of ER-lipid rafts proteins. Proteins from ER-lipid rafts fraction of JFH1 infected Huh-7 (A) and uninfected Huh-7 (B) cells were subjected to IEF in a 17-cm IPG strip spanning the pH range of 4-to-7. The strip containing focused proteins was transferred to an 18 x 20 cm SDS-PAGE gel (8-16%T) and subjected to electrophoresis. Proteins in the gel were stained with coomassie brilliant blue stain and the image shown was captured with a laser-based instrument.

4.3Lipidomic analysis of LDs isolated from HCV-infected cells.

Sections 4.1 and 4.2 confirmed that broad changes in lipid content were induced in the whole infected cell. Section 4.3 showed that this effect was more profound in the ER, the organelle that plays a crucial role in HCV replication and membrane formation.

Next, there was a requirement for further detailed analyses of other sub-cellular compartments. In particular, studying cytoplasmic LDs (LDs) was deemed to be of potential significant value. The LD is an organelle that is used for the storage of neutral lipids. It dynamically moves through the cytoplasm, interacting with other organelles, especially the endoplasmic reticulum (ER) (Martin & Parton 2006; Blanchette-Mackie et al. 1995; Vock et al. 1996). These interactions are thought to facilitate the transport of lipids and proteins to other organelles throughout the cell.

An association between the HCV capsid protein (Core) and the LDs has been reported, mediated by lipophilic interactions with two amphipathic helices in the core D2

domain (Boulant et al. 2006). The association of core with LDs is thought to form part of the assembly complex, and is essential for the production of infectious virus particles (Boulant et al. 2007; Miyanari et al. 2007). The envelope proteins E1 and E2 reside in the ER lumen, and the viral replicase is assumed to localize on ER-derived membranes in association with NS5A protein (Penin et al. 2004).

Briefly, HCV core protein is cleaved from the viral polyprotein by cellular proteases, thereby producing the 191 amino-acid (aa) immature form of core. This form (MW 23 kDa) remains anchored to the ER. It is then cleaved by a signal peptide peptidase, which removes the signal peptide to generate the mature form of core (MW. 19–21 kDa), which is 173–179 aa long and is trafficked from the ER membrane to LDs (McLauchlan et al. 2002). The association of the mature core protein with LDs is directly related to the intracellular transport of this protein to the perinuclear area, the site of assembly of infectious HCV particles. HCV particles are then secreted through the VLDL-secretory pathway (Gastaminza et al. 2008; Chang et al. 2007).

LDs have recently attracted considerable attention because of the link between the accumulation of LDs and human diseases such as obesity, atherosclerosis and HCV-associated liver malfunctions. However, the actual role of LDs in the complexity of the HCV life cycle, especially in terms of HCV assembly and release, is still not clearly understood. Therefore, this research sought to clarify this issue by investigating the way HCV virus particles interact with LDs, as understanding this relationship may lead to the development of new therapeutic strategies that protect against HCV.

First, a protocol to isolate LDs using methods adapted from published protocols was developed (Brasaemle & Wolins 2006). To measure the recovery efficiency and purity of LDs obtained using this protocol, equal volumes of the recovered fractions were analysed on a 12% SDS-PAGE, followed by immunoblotting with antibodies against

adipose differentiation-related protein (ADRP), a LD-resident protein found in most tissues (Strey et al. 2005) (Figure 4.12A). The purity of the isolated LDs was assessed using the relative enrichment of ADRP and by considering the absence of markers that correspond to other cellular organelles (Figure 4.12B).

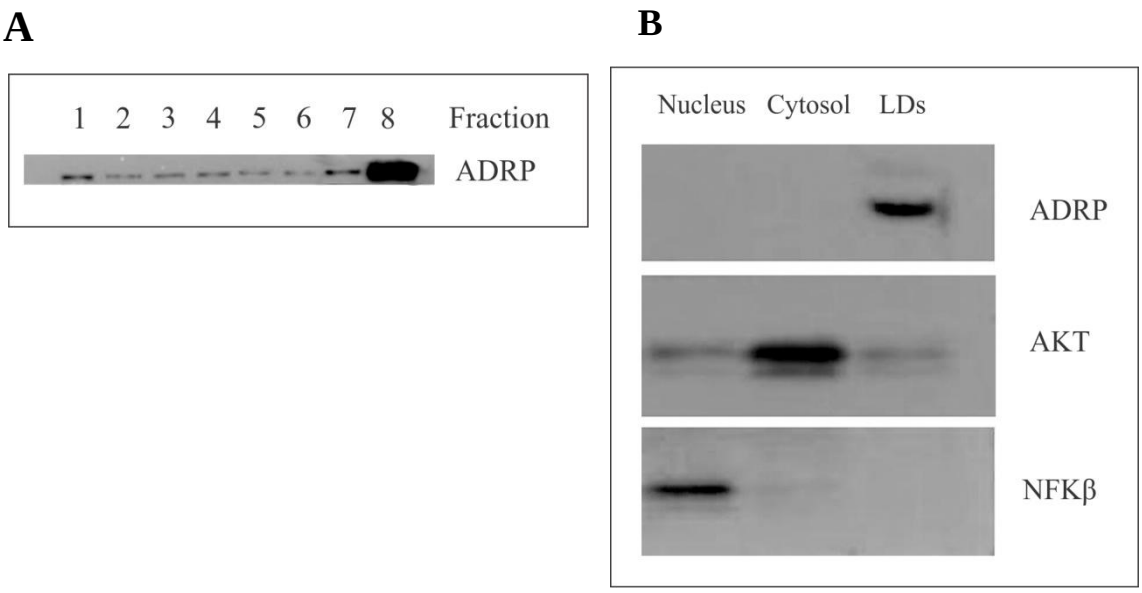


Figure 4.12Sub cellular distribution of ADRP, analysed by sucrose-density-gradient centrifugation. A) Cells were solubilised with 1% Triton X-100, 0.5% DDM, 1% CHAPS or 1% Brij99 and separated on discontinuous sucrose density gradients. Fractions collected from the bottom of the gradient were analysed by western blotting.
B) Immunoblot analyses of sub cellular marker proteins ADRP (lipid droplet), AKT (cytosol) and NFKβ(nucleus), confirming the purity of the LD fraction.

4.3.1 TAG content in LDs increased by HCV infection

In order to study the effects of HCV infection on different classes of neutral lipids, LDs were isolated using discontinuous sucrose gradient (Section 2.4.4). The LDs fraction was isolated from both JFH1-infected Huh-7 cells and uninfected Huh-7, lipid was extracted then separated and quantified by HPTLC. The separated lipid bands were identified using commercial standards (Figure 4.13A). TAG was the most prominent among the neutral lipid classes and was significantly up-regulated in HCV-infected cells. Different bands with different lengths of fatty acyl chains were identified as TAG (Figure

4.13B). This finding was consistent with existing data pertaining to the role of triglycerides in the HCV life cycle (André et al. 2005; Negro 2010).

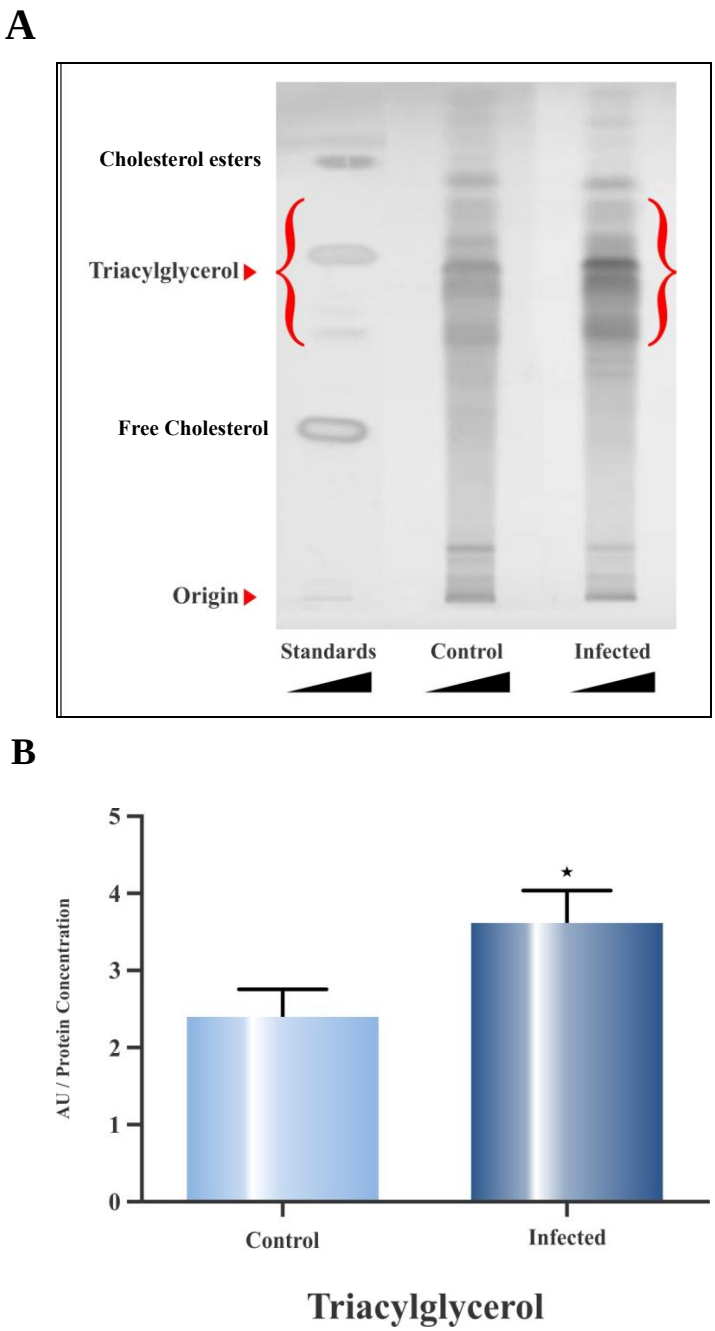


Figure 4.13 Identification of neutral lipid bands against known standards. Commercial standards for FC, TAG and CE were utilized to characterize the effects of HCV infection on the composition of neutral lipid classes in both Huh-7 cells (control) and HCV (JFH1) infected Huh-7 cells (infected) (A).Densitometry analysis demonstrated that HCV infection significantly increased TAG in LDs (B).
* demonstrates significant difference between Huh-7 and infected cells. FC free cholesterol, TAG triacylglycerol and CE cholesterol esters. (*p<0.05).

4.3.2 HCV induces a significant increase in phospholipids in the purified LDs relative to the uninfected cells

Next, the effect of HCV infection on the phospholipid composition of LDs was investigated. PC was the only detectable phospholipid class in lipid extracts from purified LD fractions. An accumulation of PC in LDs purified from HCV-infected cells relative to uninfected cells was observed; this effect was statistically significant, as shown in Figure 4.14.

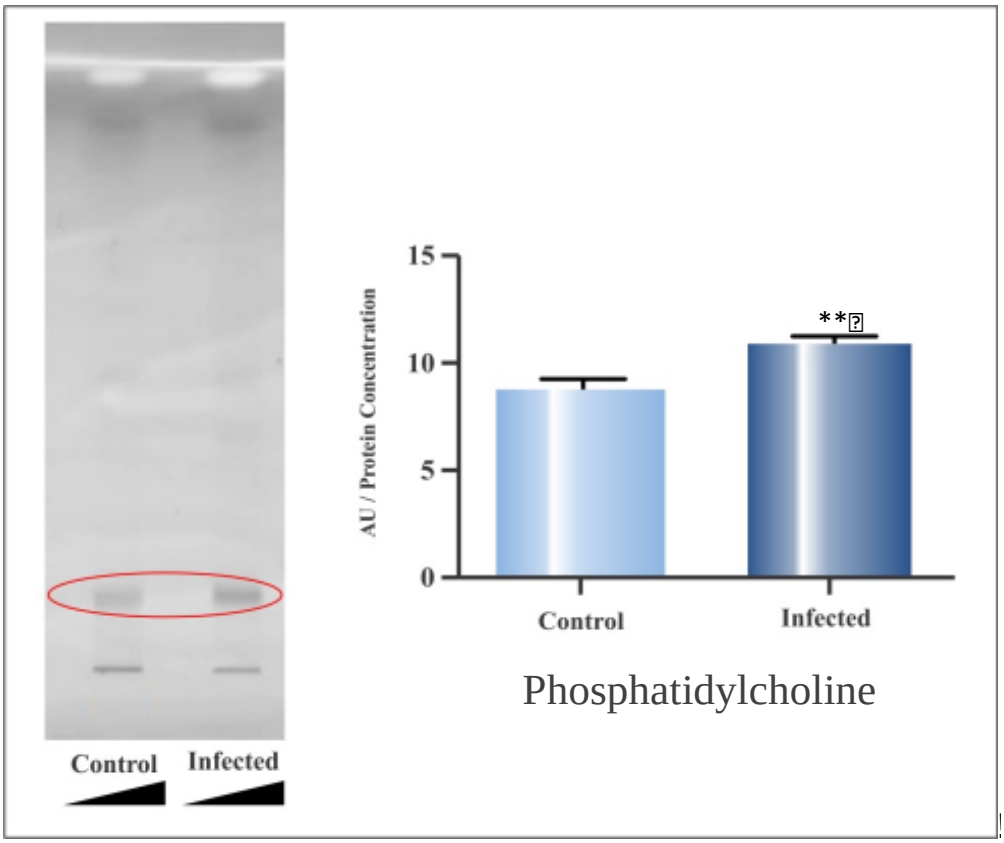


Figure 4.14 HPTLC analysis of phospholipids in LDs isolated from HCV infected and uninfected cells.
Phosphatidylcholine bands were identified against known standards and compared between JFH1infected Huh-7 cells and mock infected Huh-7.Densitometry analysis showed purified lipid droplets from chronic JFH1 infected Huh-7 cells has a higher levels of Phosphatidylcholine compared to mock infected Huh-7. (**p<0.01)

Finally, the relative HCV-induced increases in PC levels in whole cells, ER fraction and LDs fraction was compared to determine whether the increase in PC is global, or more specific to some subcellular organelles. Figure 4.15A shows that ER fractions from HCV-infected cells contained a higher level of PC than whole cell extracts and LD fractions. On the other hand, there was no significant difference in PC increase between whole cell and LD fractions. Again, this emphasizes the vital role that ER plays in HCV replication and suggests that PC plays an important role in HCV replication in the ER.

Another comparison was performed for the changes in PL and neutral lipid in the LDs were compared to the whole cells changes. The LDs neutral lipids analysis indicated that TAG was the predominant lipid class detected by HPTLC, with up to 40% significant increase in JFH1-infected Huh-7 cells LDs samples, compared to less than 20% increase in whole cell samples. Whereas, in the case of the PL analysis, PC was only detected among PL classes in LDs, with no significant difference in increase from the whole cell (Figure 4.15B).

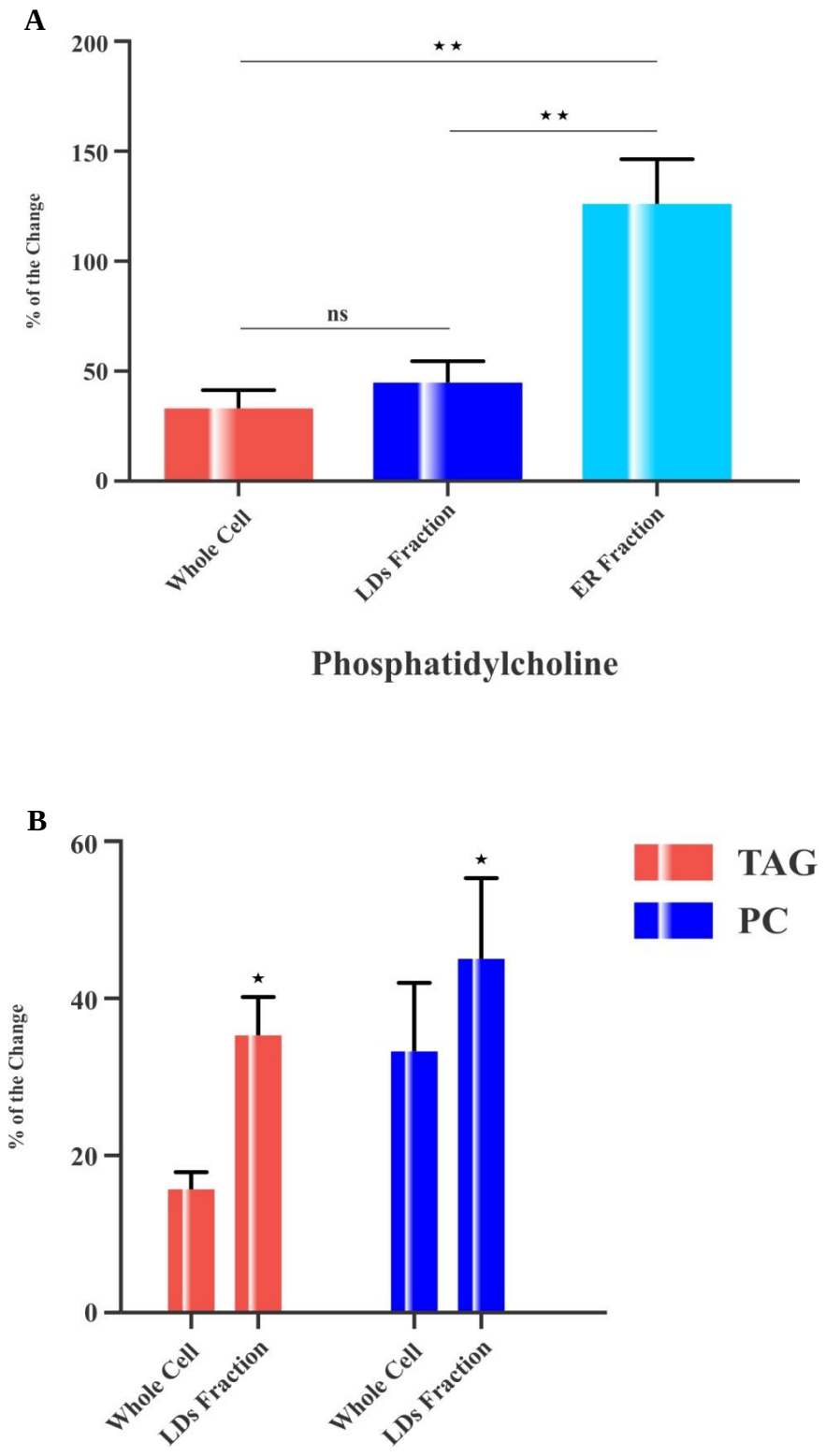


Figure 4.15 Relative increase of lipid classes in subcellular fractions.
(A) Comparison of HCV-induced changes in the phosphatidylcholine composition of whole cells, ER and LDs fractions in Huh-7 cells. (B) Comparison of the HCV-induced changes in the triacylglycerol (TAG) and phosphatidylcholine (PC) composition of purified LDs, relative to the whole cell. All lipids were extracted from chronically JFH1 infected Huh-7 cells. (ns, non significant; * $p<0.05$, ** $p<0.01$).

4.3.3 Identification of DDX3 in the purified LDs from HCV-infected Huh-7 cells

We sought to gain further insights into the effects of HCV on cellular LDs by comparing the protein content of purified LDs from JFH1 HCV-infected Huh-7 cells compared to uninfected Huh-7 cells. Protein was precipitated using cold acetone protocol and quantified using a BioRad colorimetric kit. One-dimensional protein gel electrophoresis (1-DE) of purified LDs was used to examine the protein composition of LD fractions. Three independent experiments were performed with very consistent results. Tens of protein bands were visualized on 1-DE gels using Coomassie stain, and differences in the intensity of the bands were compared visually. Bands with molecular masses of approximately 110, 90 73, and 66 kDa were more prominent in JFH1 HCV-infected cells than they were in uninfected Huh-7 cells, suggesting possible roles in the HCV replication cycle. To identify the proteins, the bands were excised, digested with trypsin and analysed using mass spectrometry by TOF/TOF-MALDI- MS. The 110, 90 73, and 66 kDa protein bands were identified as human ATP dependant RNA helicase, DEAD-box RNA helicase (DDX3), and insulin-like growth factor 2 respectively (Figure 4.16).

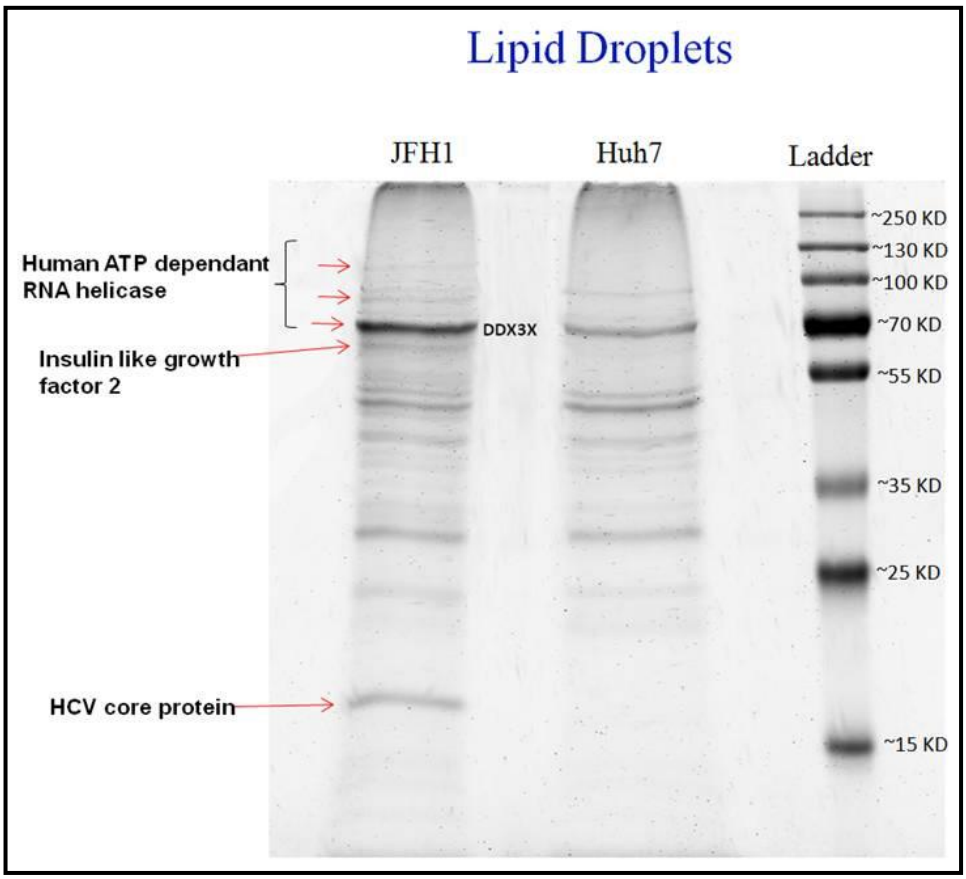


Figure 4.16One D gel protein electrophoresis (1-DGE) of purified lipid droplets. Lipid droplets were purified from HCV (JFH1) infected and uninfected Huh-7 cells and proteins extracted by acetone precipitation. Proteins from of each sample (20 µg total protein) were separated using a 12% SDS–polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue. Bands with increased intensity were excised and identified by MALDI-TOF mass spectrometry. The cellular proteins DEAD-box RNA helicase (DDX3), Human ATP dependant RNA helicase and Insulin like growth factor 2were increased in lipid droplets purified from JFH1 infected Huh-7 cells compared to uninfected Huh-7.

The HCV-induced increase in DDX3 in LDs was confirmed using Western blot. As shown in Figure 4.16, there was strong labelling of DDX3 protein in LDs purified from JFH1 HCV-infected Huh-7 cells, which was undetectable in LDs from uninfected Huh-7 (Figure 4.17A). This is consistent with recent reports that the cellular DEAD-box protein DDX3 is essential for HCV replication (Angus et al. 2010). Furthermore, the role of DDX3 in the life cycle of other viruses has been previously documented for human immunodeficiency virus-1 (HIV-1) (Yedavalli et al. 2004) and brome mosaic virus

(Noueiry et al. 2000). In contrast, DDX3 has been reported to exhibit antiviral activity against the hepatitis B virus (HBV), as it inhibits transcription following incorporation into virus nucleocapsids (Wang et al. 2009). Thus, there is increasing evidence that DDX3 is important in the life cycle of several diverse viruses.

4.3.3.1 DDX3 interacts with CRM-1/exportin in shuttling of HCV core protein

The data presented in Figure 4.16 indicates that HCV results in the accumulation of DDX3 protein in LDs, where HCV assembly takes place, suggesting that the interaction between DDX3 interaction and HCV core on LDs is potentially important for HCV replication. As such, understanding the viral strategies for manipulating or co-opting DDX3 in functional and molecular detail can provide valuable insights that can aid the development of strategies to therapeutically target DDX3.

The data described above indicates that LDs are vital for HCV replication, this is consistent with the published data (Boulant et al. 2006; Miyanari et al. 2007) where HCV core accumulate DDX3 (Angus et al. 2010). To test this hypothesis, uninfected Huh-7 cells and JFH1 HCV-infected cells were treated with 10 nM leptomycin B (LMB, Sigma), a specific inhibitor of CRM-1/exportin, or mock treated. After 24 h, cells were harvested, LDs isolated and Western blots were performed, using specific antibodies against DDX3, ADRP and core protein. As shown in Figure 4.17B, leptomycin B significantly reduced the accumulation of DDX3 in purified LDs from chronic JFH1 (HCV).

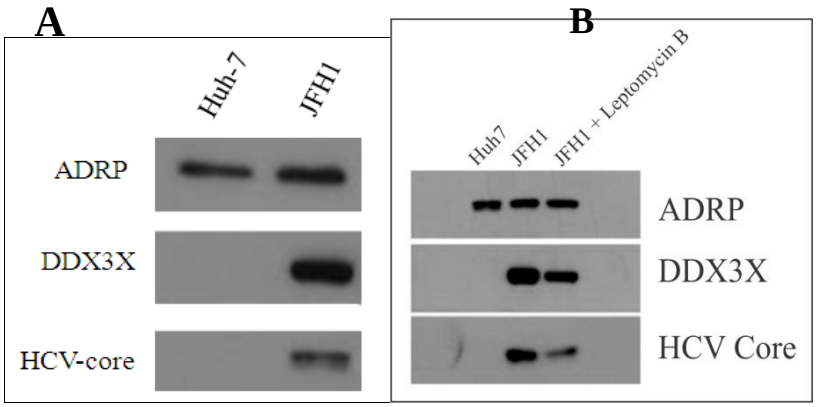


Figure 4.17 Association of DDX3 with LDs in response to HCV-JFH1 infection. The LD fraction was collected from uninfected cells (Huh-7) or HCV-infected cells (JFH1) at 12 days postinfection. (A) The results of Western blot analyses of DDX3 and the HCV core protein, as well as the LD marker ADRP are shown. (B) Protein extracts were evaluated for DDX3 compared ADRP after. The accumulation of DDX3 in the purified lipid droplets decreased significantly after treatment with leptomycin B as assessed by western blot. Confirmation of the infection of the purified LDs was done using antibodies against HCV core protein. This figure is a representative figure of three times independently, all yielded the same results.

4.4 Summary

These findings collectively suggest that HCV infection impacts hepatic lipid metabolism broadly, with these changes being more evident in ER fractions and LDs. In ER fraction, a significant difference in the lipid profile of purified ER, relative to the whole cells was observed. In particular, the HCV-induced increase FC. Moreover, the most prominent neutral lipid class seen in ER-lipid rafts, and appeared to be up-regulated in HCV-infected cells is FC. On the other hand, phospholipids analysis showed a significant increase in PC, PE, and PI classes in the ER fraction of HCV-infected cells. The most striking difference was the relatively greater HCV-induced increase in PC (150% vs. 30%, $p<0.01$) and free cholesterol (200% vs. 50 %, $p<0.01$) in the ER, compared to whole cell extracts. This suggests that chronic HCV infection results in the profound accumulation of PC in the ER fraction and emphasizes the role ER plays in HVC replication. Furthermore, it suggests that ER is an attractive target for anti-viral therapies and that PC plays a vital role in the HCV replication in the ER. The modulation of the enzymes that control PC pathways may represent novel host-targeted anti-HCV therapies.

In LD fractions, TAG was the most prominent among neutral lipid classes and was significantly up-regulated in HCV-infected cells. For phospholipids, PC was the only detectable class by HPTLC in LDs with a significant up-regulation in infected cells.

Lastly, proteomic analysis of ER-lipid rafts and LD fractions was performed. No significant change was induced by HCV infection on ER-lipid rafts proteins. However, DDX3 protein was the most significant up-regulated protein in LDs proteomic analysis. The treatment of HCV-infected cells with leptomycin B (CRM-1/exportin inhibitor) significantly reduced the accumulation of DDX3 in purified LDs from chronic JFH1, which suggests that targeting DDX3/CRM-1/exportin pathway in LDs represents a potential novel host-targeted agent against HCV.

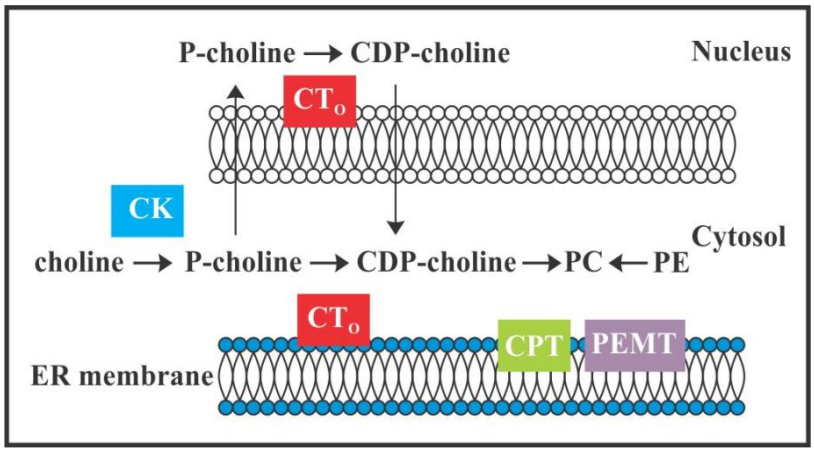
HCV induced the accumulation of DDX3, a helicase that is important in the HCV life cycle. On the other hand, HCV also induced a shuttling protein, CRM-1/exportin. The research thus far has shown that both DDX3 and Exportin 1 interact together in the process of shuttling of HCV core protein between the nuclear and cytoplasmic compartments of the cell. Leptomycin B (a specific CRM-1/exportin) pre-treatment reduces HCV RNA. This suggests that targeting the DDX3/CRM-1/exportin pathway in LDs may result in a potential novel host-targeted agent that can protect against HCV.

5. PEMT a novel pathway for host-targeted agents against hepatitis C

5.1 Introduction

The previous two chapters described how chronic HCV infection modulates the global intracellular lipidome to create an environment that aids RNA replication and the production of progeny particles. This effect was more prominent in the endoplasmic reticulum (ER) and cytoplasmic LDs (LDs), where HCV protein translation, RNA replication and virus assembly take place. As well as the known effects on neutral lipids and cholesterol, HCV induced a significant accumulation of phosphatidylcholine (PC).

PC and PE are the major phospholipids found in mammalian cell membranes (Kent 1995; Li et al. 2006). In hepatocytes, PC is mainly synthesized from choline via the CDP-choline pathway, phosphate cytidylyltransferase 1 alpha (PCYT1A), known also as CTP:phosphocholine cytidylyltransferase (CTα) is the rate-limiting enzyme in this pathway. PCYT1A facilitates the conversion of phosphocholine to CDP-choline, the addition of the phosphocholine moiety to diacylglycerol completes the synthesis of PC. The alternative phosphatidylethanolamine N-methyltransferase (PEMT) pathway accounts for 20-30% of total PC synthesis (Figure 5.1) (Bremer & Greenberg 1961). PEMT catalyses the synthesis of PC from PE, as shown in Figure 5.2A, by the stepwise transfer of methyl groups from S-adenosylmethionine (SAMe) to the amino head group of PE. This reaction consumes three molecules of SAMe for each molecule of PC being formed (Sundler & Akesson 1975).



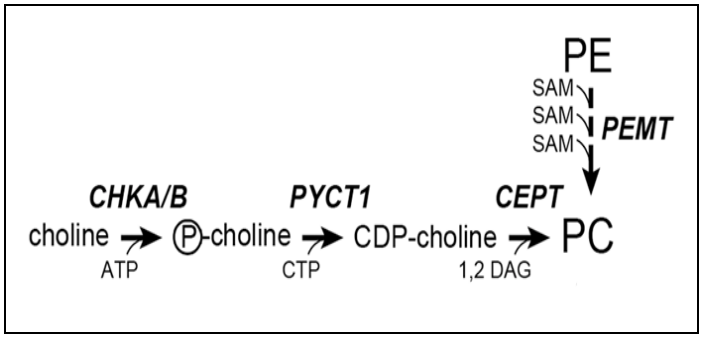


Figure 5.1 Phosphatidylcholine biosynthesis pathways in human hepatocytes.PC, phosphatidylcholine; PEMT,phosphatidylethanolamineN-methyltransferase;CT, CTP:phosphocholine cytidylyltransferase; CPT,CDP-choline:1,2- diacylglycerol cholinephosphotransferase; CK, choline kinase; CT, CTP:phosphocholine cytidylyltransferase; cholinephosphotransferase; PEMT, phosphatidylethanolamine N-methyltransferase; P-choline, phosphocholine; CDP-choline, cytidine diphosphate-choline;PE, phosphatidylethanolamine;

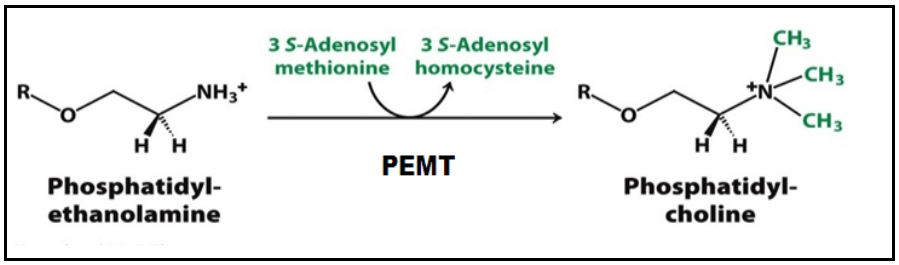


Figure 5.2 S-Adenosyl methionine (SAMe) dependent methylation reactions.
Phosphatidylethanolamine N-methyltransferase (PEMT) pathway: PEMT catalyses the synthesis of phosphatidylcholine (PC) by sequential addition of methyl groups from SAMe to the amino head group of phosphatidylethanolamine (PE).

According to this hypothesis, metabolic reprogramming could help HCV to establish and maintain persistent chronic infection, as inhibiting STAT1-induced ISG activation would reduce the effectiveness of an important early component of the anti-viral immune response, Type I interferon. Furthermore, this could help to explain the development of interferon resistance, which is frequently observed during HCV anti-viral therapy. To this end, a series of experiments was designed with the goal of investigating whether the inhibition of PEMT in HCV-infected cells: (1) affects lipid accumulation; and (2) enhances IFN-α induced expression of antiviral ISGs.

5.2 HCV infection activates phosphatidylcholine (PC) biosynthesis pathways in Huh-7 cells

To examine the effect that HCV infection has on PCYT1A and PEMT expression, the key enzymes in PC biosynthesis, JFH1 HCV-infected and uninfected-Huh7 cells were seeded in 6-well plates, with 1 $\times 10^6$ cells per well, and incubated overnight at 37 °C. The

next day, the cells were subjected to RNA extraction, using the process described in Chapter 2 (Section 2.2.2.3). Real-time quantitative PCR analysis (qPCR) using PEMT or PCYT1A specific primers revealed significant up-regulation of expression for both enzymes in HCV-infected cells, compared to uninfected controls (Figure 5.3)

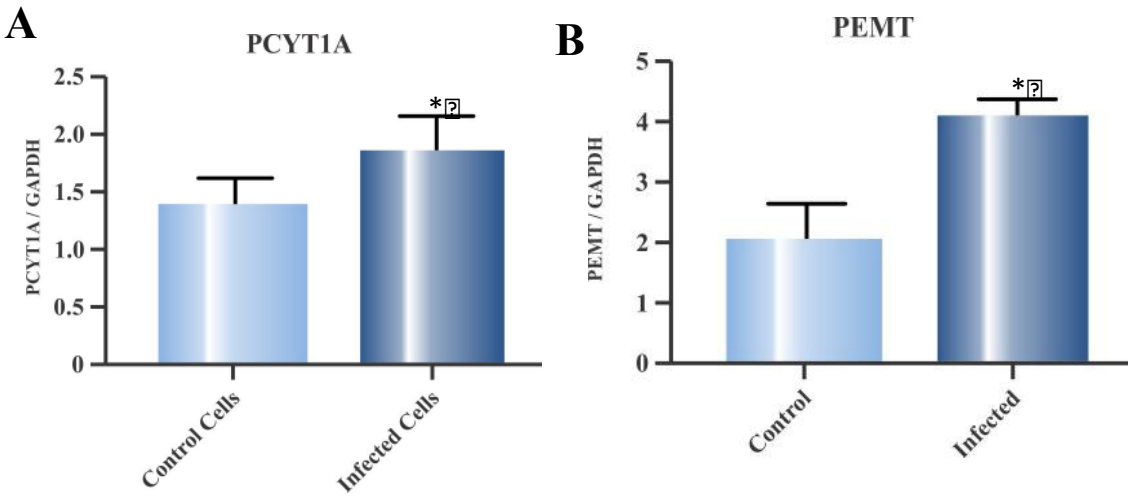


Figure 5.3 PEMT and PCYT1A mRNA expression is increased in HCV infected Huh7 cells.
Huh7 cells were transfected with HCV (JFH1) then cellular RNA was extracted and relative mRNA expression was quantified using qPCR for A) PCYT1A and B) PEMT. * Significant change from uninfected control ($p<0.05$).

Having confirmed up-regulation of PCYT1A and PEMT expression in JFH1-infected cells, the next step was to investigate the effect of PCYT1A and PEMT inhibition on HCV replication. To test this, three small interfering RNAs (siRNA) that target different regions of PCYT1A or PEMT mRNA were transfected into JFH1-infected Huh-7 cells to silence gene expression. In cells, siRNAs are produced by enzymatic cleavage of long dsRNAs by the RNase-III class endoribonuclease Dicer. The siRNAs associated with the RNA-induced silencing complex (RISC) in a process that was facilitated by Dicer. The siRNA-27 contained three Dicer-Substrate 27-mer duplexes targeting a specific gene that were selected from a predesigned set of duplexes from the RefSeq collection of Genbank.

OriGene's siRNA collection of 27-mer sites were chosen by a rational design algorithm that integrated both traditional 21-mer siRNA design rules as well as new 27-mer design criteria (Kim et al. 2005). The efficiency of PCYT1A and PEMT knockdown was confirmed using qPCR, as shown in Figure 5.4. Forty-eight hours post-transfection with siRNA, mRNA expression was reduced by approximately 90%. As such, this time point was selected for subsequent HCV infections. Two days following HCV infection, intracellular HCV RNA levels were determined using qPCR.

In cells where ePYCT1A expression was knocked down, there was no significant reduction in HCV RNA levels, suggesting that PYCT1A is not important for HCV replication. In stark contrast, knockdown of PEMT resulted in more than a 50% decrease in infection, as quantified by qRT-PCR (Figure 5.5). This suggests that PEMT-dependent PC synthesis is important for HCV replication.

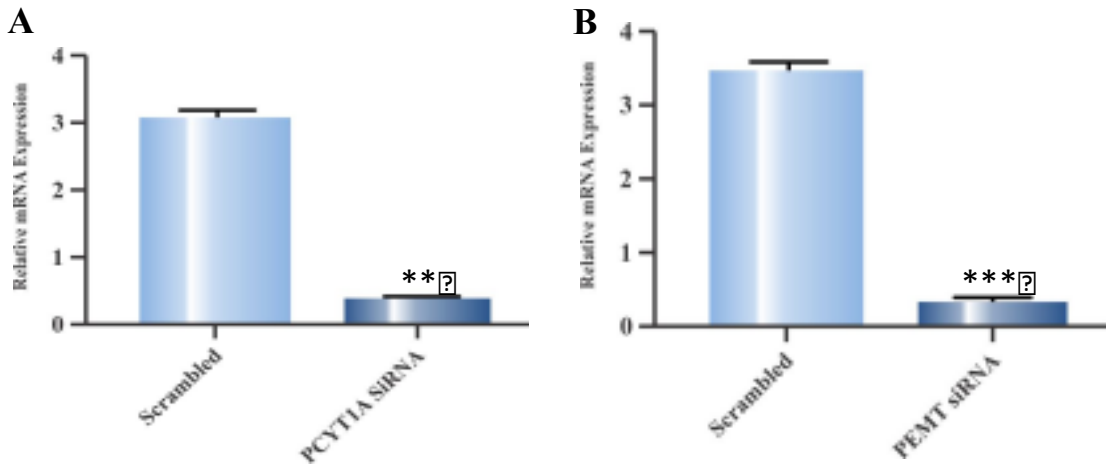


Figure 5.4 siRNA mediated PCYT1 and PEMT knockdown efficiently reduces their expression.
Treating Huh-7 cells with siRNA targeting (A) PCYT1 or (B) PEMT reduced gene expression by approximately 85% or 90% respectively at 48 hr.compared with scrambled controls (** $p<0.01$, ***$p<0.001$).

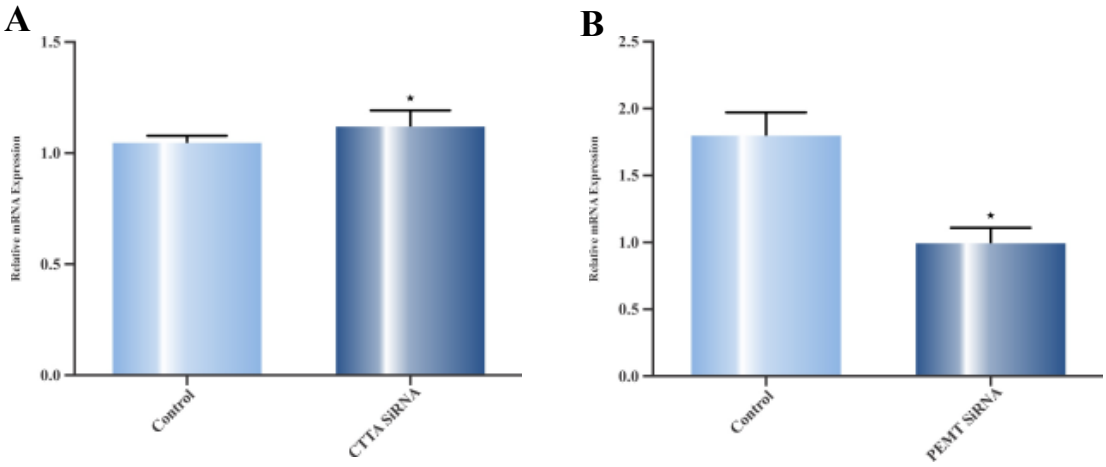


Figure 5.5 Effect of inhibiting PC synthesis on HCV infection.
HCV (JFH1) infected Huh-7 cells were transfected with target specific siRNA, or scrambled RNA control (20nM/ml). HCV RNA was measured 48 h after transfection. Cells transfected with PCYT1A (CTTA) siRNA did not show any effect in HCV RNA levels (A). PEMT knockdown cells showed significant reduction in HCV RNA levels, compared to mock treated cells (B). (*$p<0.05$).

To test whether reducing PEMT inhibited new HCV infection, infection assays were also performed PYCT1A or PEMT were knocked down using siRNA, as described above, with scrambled controls, then cells were infected with a JFH1-concentrated virus. After 48 hr, infected cells were labelled for virus protein using antibody against NS5A, and viral infectivity was determined through the use of a tissue culture infective dose (TCID50) calculation (Figure 5.6). Similar results were obtained and exhibited a significant reduction in infectivity of approximately 50% for cells that had been treated with PEMT siRNA, but not for those treated with PYCT1A siRNA. This suggests that PEMT is important for establishing HCV infection and ongoing RNA replication.

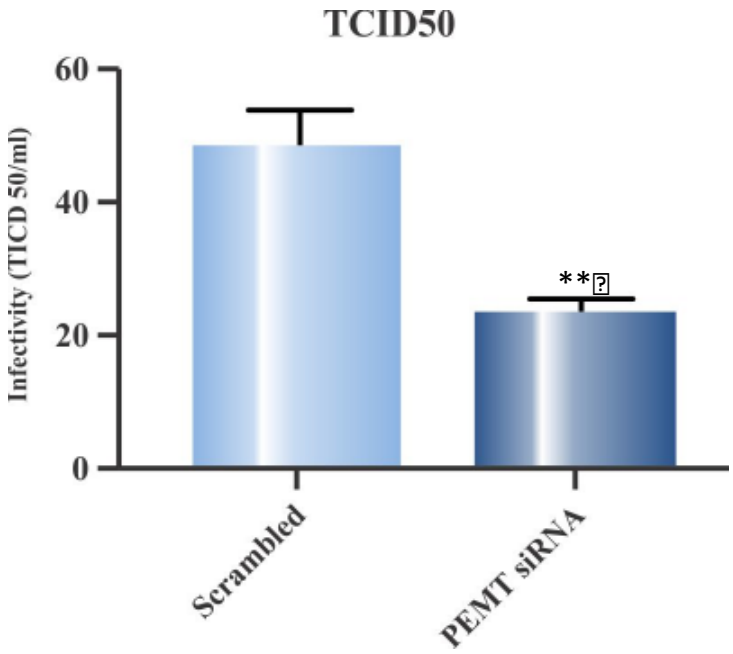


Figure 5.6 PEMT siRNA pre-treatment reduces HCV infection.
To determine the effect of PEMT knockdown on HCV infection, Huh-7 cells were treated with 20 nM/ml PEMT siRNA or scrambled siRNA for 48 h followed by JFH1 infection. TCID50 analyses demonstrate that PEMT siRNA pre-treatment significantly reduced JFH1 infection of Huh-7 cells (** $p<0.01$).

5.3 PEMT protein level is increased in JFH1 HCV-infected cells

Having confirmed that the expression of PEMT mRNA increases in HCV-infected cells; there was a requirement to measure the PEMT protein levels. Huh7 cells were infected with HCV (JFH1), the cells were then lysed and Western blotting was performed using anti-PEMT antibody, as described in Chapter 2 (Section 2.3.5). Western blot analysis of total cell lysates confirmed that PEMT protein levels were significantly elevated in JFH1-infected Huh-7 cells than they were in uninfected cells (Figure 5.7).

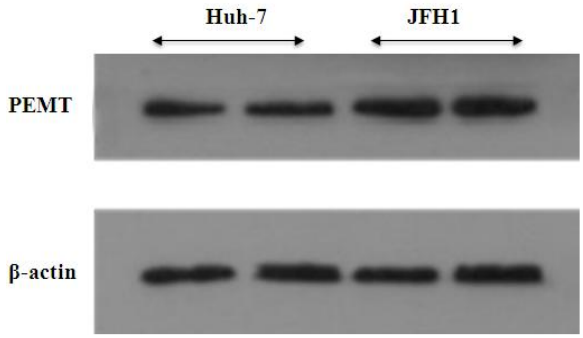


Figure 5.7 Western blot analysis of PEMT protein levels in Huh7 and JFH1 infected cells. Densitometry analysis demonstrated that JFH1 infection significantly increase in PEMT levels.

To further confirm the in vitro data obtained in cell lines, the expression of PEMT in human liver biopsies from patients infected with HCV genotype 1 and 3 was also investigated using qPCR. Due to the difficulties associated with obtaining normal liver biopsies as a control, liver biopsies from hepatitis B virus (HBV) infected patients were obtained as a control. Consistent with the in vitro findings, the HCV patientdemonstrated a significant increase in PEMT expression when compared with HBV patients (Figure 5.8), with the highest PEMT mRNA levels observed in HCV Genotype 3-infected patients, suggesting that HCV infection specifically induces PEMT expression.

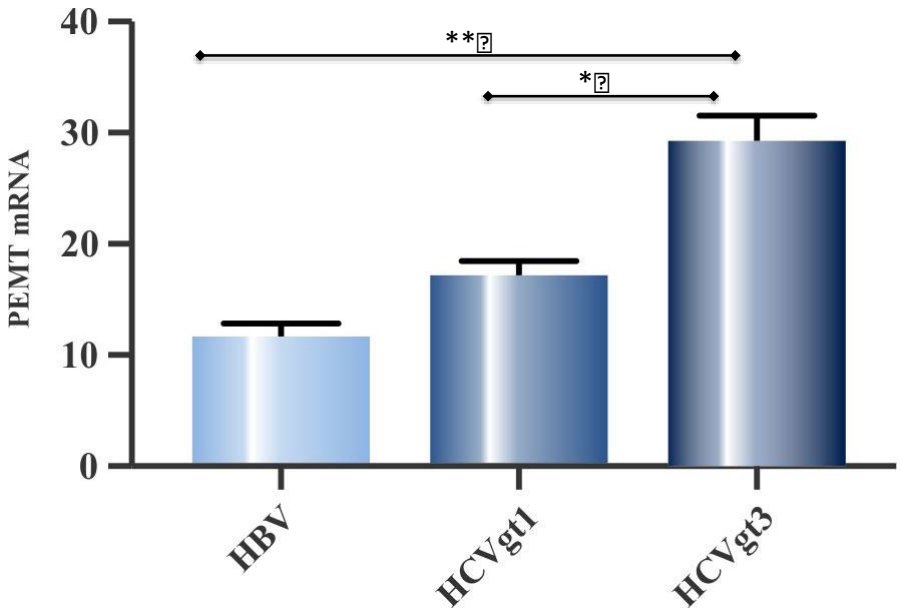


Figure 5.8 Increased PEMT expression is specific for HCV infected patients. Relative hepatic PEMT mRNA expression in 88 patients with Chronic Hepatitis C (genotype 1 and 3) and 12 Hepatitis B patients (normalized to 18s). PEMT was significantly increased in HCV patients compared to HBV patients, and higher for HCV genotype 3 than genotype 1. (*p<0.05, ** p<0.01).

5.3.1 PEMT pathway knockdown restores S-Adenosyl methionine (SAMe) levels to normal

SAMe is the major biological methyl donor in hepatocytes, where it is involved in a range of methylation reactions (Borchardt & Wu 1974), including the methylation of activated STAT1 (Figure 5.9) (Kerri A. Mowen et al. 2001).

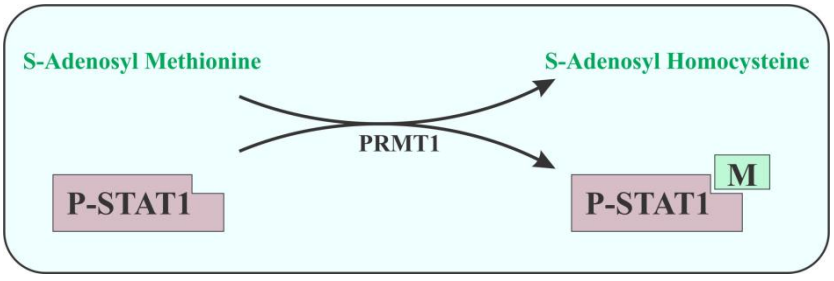


Figure 5.9 Methylation of activated STAT1 is catalysed by PRMT1 and requires SAMe.

STAT1 is a member of the signal transducers and activators of transcription (STAT) family of transcription factors. It is involved in mediating up-regulation of genes in response to a signal from either Type I, Type II or Type III interferons. STAT1 is activated by phosphorylation of a single residue, Tyr-701, following binding of a number of cytokines to their cognate receptors, including interferons (IFNs), interleukin 6 (IL-6), and epidermal growth factor. Phosphorylation on the Tyr-701 residue of STAT1 is required for its nuclear translocation, dimerization, DNA binding and gene activation (Shuai et al. 1994; Shuai et al. 1993). IFN-α-induced gene transcription can be inhibited by the protein inhibitor of activated STAT1 (PIAS1), which adds a SUMO group to a conserved lysine residue of STAT1, inhibiting its ability to bind to STAT1 promoter elements (Liu et al. 1998). The binding of PIAS1 to STAT1 is affected by methylation of STAT1, which is regulated by protein arginine methyltransferase 1 (PRMT1) (Figure 5.9). Arginine methylation of STAT1 inhibits binding of PIAS, whereas demethylation of STAT1 enhances its association with PIAS1 (Duong et al. 2006; Kerri A. Mowen et al. 2001). Duong et al. have shown that HCV induces hypomethylation of STAT1, resulting in increased binding of PIAS1 to STAT1, increased methylation of STAT1, decreased binding of STAT1 to promoter elements and ultimately down-regulation of ISG expression (Duong et al. 2004).

Based on the findings of the current research and those outlined in published data, it can be hypothesized that:

1. HCV activation of the PEMT pathway in HCV-infected hepatocytes results in a high consumption of SAMe.
2. Depletion of the level of SAMe can lead to decreased methylation of STAT1.
3. Decreased STAT1 methylation results in decreased STAT1-DNA binding and a reduction in the induction of ISGs.

To investigate this hypothesis, SAMe levels were measured in JFH-infected Huh-7 cells 48 hours post transfection with PEMT siRNA (20 nM/ml) or scrambled siRNA, and compared to uninfected Huh-7 cells as a negative control. As expected, PEMT knockdown in JFH1-infected cells showed partial, but significant, restoration of SAMe towards the normal levels of the control cells (Figure 5.10). This suggests that activation of the PEMT pathway in JFH1-infected cells resulted in the increased methylation reactions of PE and the depletion of SAMe levels.

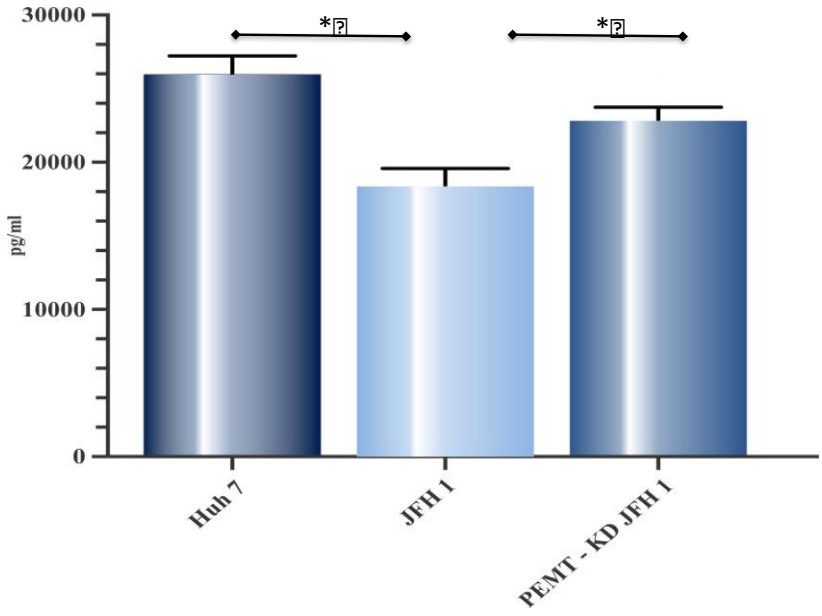


Figure 5.10 PEMT knockdown restores S-adenosylmethionine (SAMe) levels to the normal.
Total protein was extracted from JFH1 infected Huh-7 treated or mock treated with 20 nM/ml PEMT siRNA for 48 hr, then SAMe levels was quantified by commercial ELISA kit and compared to uninfected Huh7 cells as a control. (*$p<0.05$).

5.3.2 PEMT pathway knockdown modulates interferon-stimulated gene expression

Having confirmed that PEMT knockdown restored the SAMe levels in JFH1-infected cells to nearly normal levels, there was a requirement to explore the impact that this had on ISG induction. As outlined in the introduction, IFN-α signals via the JAK-STAT pathway to induce the expression of hundreds of genes, known as interferon-stimulated genes (ISGs). The methylation status of STAT1 is an important determinant of ISG expression. The manipulation of STAT1 methylation had consequences on DNA binding of STAT1 and the induction of IFNα target genes (ISGs) (Duong et al. 2006; K A Mowen et al. 2001). A series of experiments was therefore designed to investigate whether PEMT knockdown enhances the expression of antiviral ISGs following treatment with IFN-α.

For the purpose of these experiments, JFH1-infected Huh-7 cells were pre-treated with PEMT siRNA for 48 hr, or scrambled RNA control, then treated with 100 U/ml IFN-α. The expression of key ISGs was then measured using qPCR. Selected well-characterised ISGs were chosen to determine whether PEMT knockdown affected the regulation of the antiviral response. The expression of ISGs over a 24 h period was examined at key time points (0 hr, 2 hr, 6 hr and 24 hr after IFN-α treatment). The results revealed that the PEMT knockdown significantly increased the IFN-induced expression of Viperin, 2'5' oligoadenylate synthetase 3 (OAS3), and MX1, but had no significant effect on ISG15 (Figure 5.11).

These findings suggest that PEMT functions beyond its recognized role as a compensatory pathway for PC biosynthesis and that, in contrast, PEMT activity is involved in many physiologic processes including the flux of lipid between liver and plasma and the delivery of essential fatty acids to blood and peripheral tissues via the liver-derived lipoproteins. This is consistent with existing clinical data that shows that HCV G3a infection directly induces hepatic steatosis in humans.

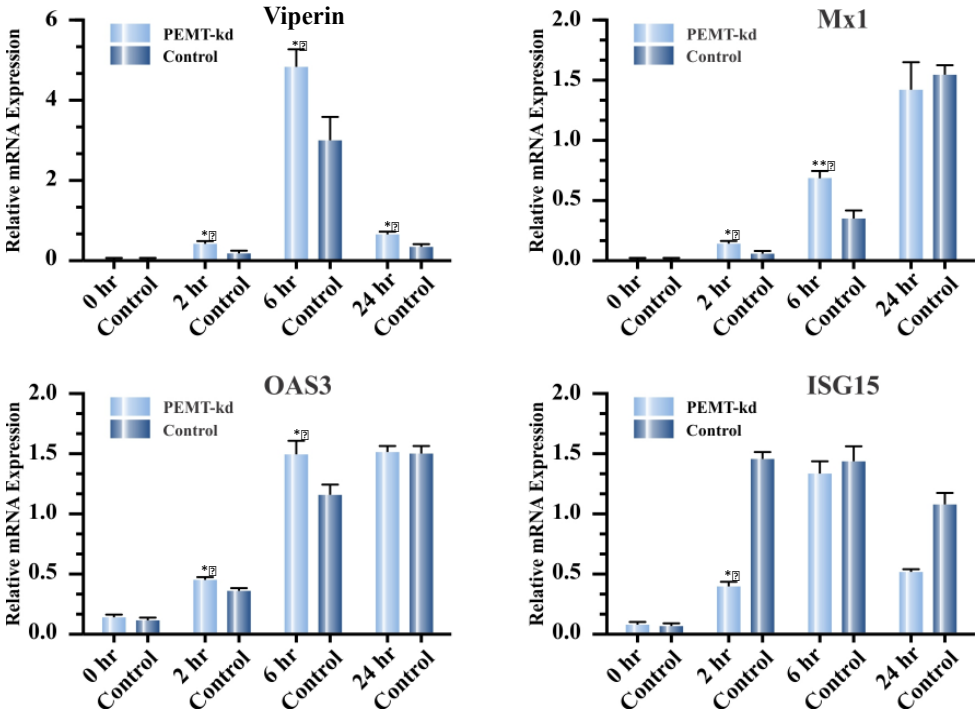


Figure 5.11 PEMT knockdown pre-treatment variably effects interferon stimulated genes expression following interferon alpha treatment.
JFH1 infected Huh-7 cells were pre-treated with PEMT siRNA for 48 hr, or scrambled RNA control, then treated with 100 U/ml IFN-α, and the expression of key ISGs measured by qPCR after 0, 2, 6, and 24 hr post INF-α. Significant up-regulation at early time points was observed in Viperin, OAS3, and MX1 in PEMT knockdown pre-treated cells, whereas ISG15 expression was not effected (*p<0.05, ** p<0.01).

To explore the mechanism of this effect, a Western blot analysis was performed to examine the effect of IFN-α and PEMT siRNA pre-treatment on STAT1 phosphorylation. JFH1-infected cells (>90% infection) were treated with PEMT siRNA (20 nM), or mock treated as a control. After 48 hr, the cells were treated with 100 U/ml IFN-α and were then lysed after 30 min and 2 hours, protein was extracted, and STAT1 phosphorylation was detected using Western blot. STAT1 phosphorylation was evident at 30 and 120 min post-IFN-α treatment in both PEMT-kd and control cells, but was more prominent for PEMT-kd cells(Figure 5.12A). Baseline STAT1 was also modestly elevated in PEMT-kd. Densitometry analysis confirmed that IFN-induced STAT1 phosphorylation was stronger in PEMT siRNA-treated cells than it was in the control cells (Figure 5.12B).

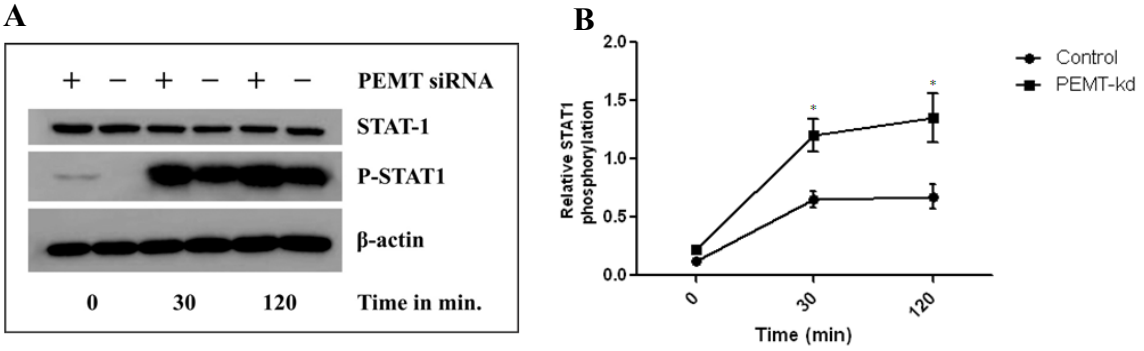


Figure 5.12 PEMT knockdown modulates STAT1 phosphorylation post interferon treatment.
JFH1 infected Huh-7 cells were mock treated or treated with 20 nM/ml PEMT siRNA for 48 hr followed by 100 U/ml IFN-α treatment. (A) STAT1 and pSTAT1 Western blots were performed over 2 h post- IFN-α to examine cellular response. (B) PEMT knockdown significantly increased STAT1 phosphorylation following IFN-α treatment (* $p<0.05$).

5.3.3 PEMT knockdown enhances the anti-HCV activity of interferon-alpha

To examine the effects of PEMT knockdown on IFN-α antiviral activity, JFH1 HCV-infected Huh-7 cells were cells were transfected with PEMT siRNA, or scrambled RNA control; then, cells were treated with 100 U/ml IFN-α. HCV replication was determined at 0 hr, 2 hr, 6 hr and 24 hr by qPCR. PEMT knockdown significantly decreased viral replication compared to scrambled RNA control cells (Figure 5.13A). To examine the average reduction in viral replication, each treatment was normalized to baseline (0 hr) expression and compared. PEMT siRNA treated cells were the most sensitive to IFN-α treatment, exhibiting over 40% more sensitivity than scrambled RNA-treated cells (Figure 5.13B). This suggests that PEMT sensitised cells to IFNon ISG expression.

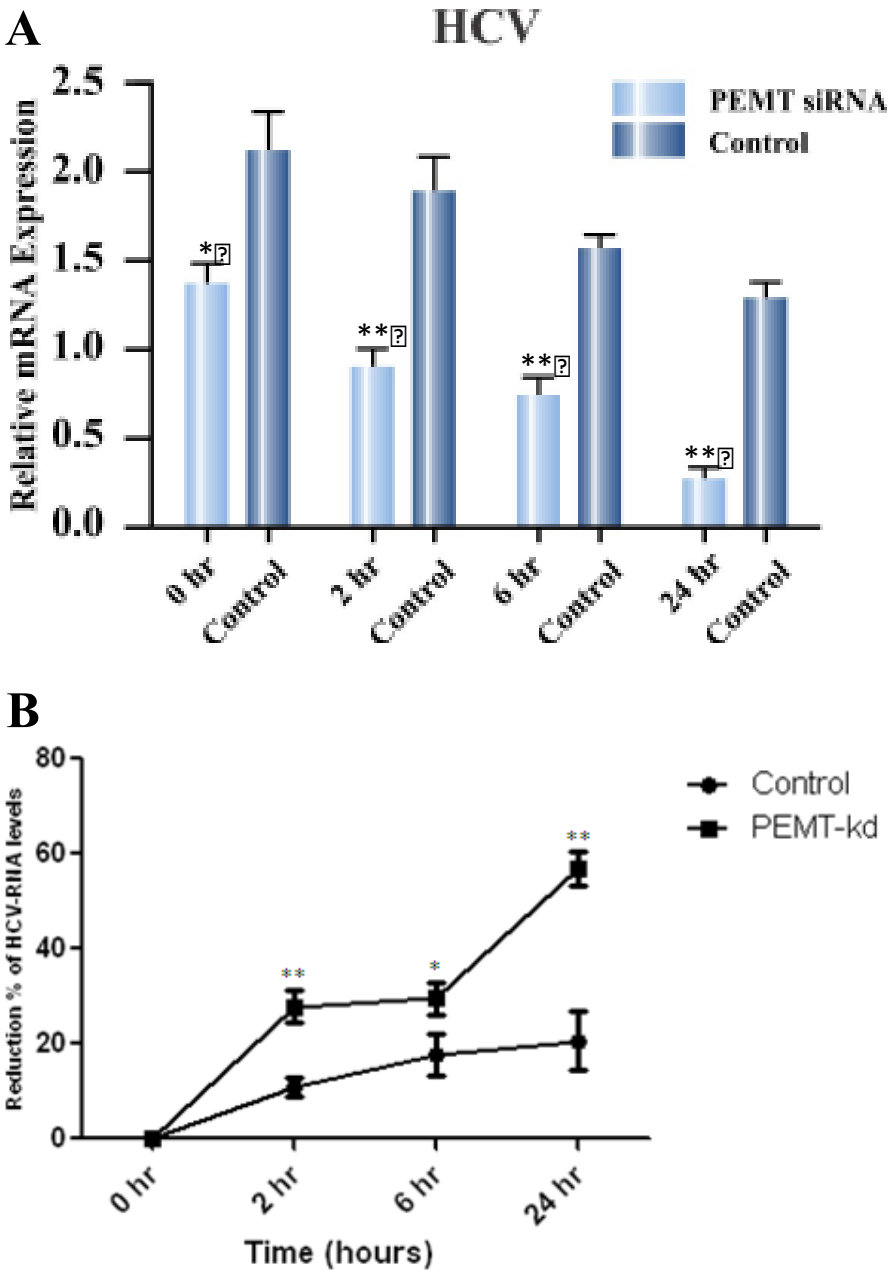


Figure 5.13 PEMT knockdown act synergistically with interferon alpha to reduce HCV replication.
JFH1 (HCV) infected Huh-7 cells were transfected with PEMT siRNA, or scrambled RNA control, for 24 h then cells were treated with 100 U/ml IFN-α. (A) HCV replication was determined at 0 hr, 2 hr, 6 hr and 24 hr by qPCR. In all time points, PEMT knockdown significantly decreased viral replication compared to control. (B) The average reduction in viral replication, each treatment was normalized to baseline (0 hr) expression and compared (* $p<0.05$, ** $p<0.01$).

5.4 The PEMT enzyme controls hepatic intracellular lipids homeostasis and turnover

SAMe is involved in mediating hepatic TG synthesis via the PEMT pathway (Figure 5.14). Currently, there is evidence that decreased content of SAMe is linked with the development of NAFLD in different experimental models of steatosis in rodents and humans (Lu & Mato 2012). This research has shown that chronic HCV infection is associated with increased cellular triglyceride and cholesterol content (Hourioux et al. 2007; Negro & Sanyal 2009). As such, the hypothesis that PEMT knockdown with SAMe at normal levels will be reflected in decreased cellular triglyceride content in the JFH1 HCV-infected Huh7 cells was developed. To test this hypothesis, the cellular distribution of triglyceride and cholesterol following PEMT knockdown was examined using immunofluorescence labelling. PEMT expression was knocked down in JFH1-infected Huh-7 cells using 20 nM PEMT siRNA and, 48 h later, cells were immune-labelled with NS5A antibodies and secondary 594 anti-Sheep IgG conjugated antibodies. BODIPY® 493/503 was used to label the neutral lipids and DAPI was used to label the nucleus. PEMT knockdown cells were compared with both JFH1 cells that were treated with scrambled RNA and non-infected Huh7 cells.

As expected, HCV-infected cells showed a significant increase in neutral lipid compared to uninfected cells (Fig 5.15, $p<0.01$). Consistent with the hypothesis, the PEMT knockdown led to a significant decrease in the accumulation of BODIPY®-stained neutral lipid in HCV-infected cells, but did not impact uninfected controls. These results suggest that PEMT and its target genes are intimately involved in the lipid metabolism of hepatocytes, especially in LD formation and the persistent infection of HCV.

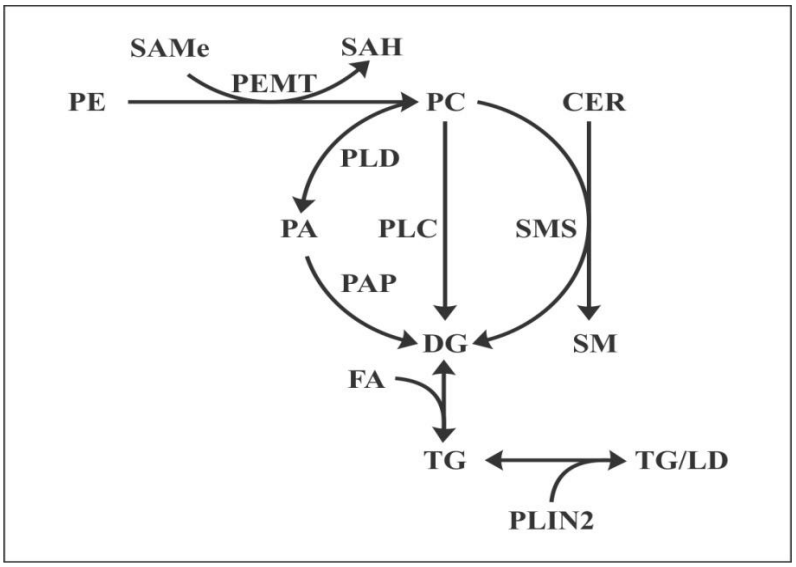


Figure 5.14 Schematic representation of the role of SAMe in mediating TG synthesis via PEMT. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; CER, ceramide; SM, sphingomyelin; DG, diglycerides; FA, fatty acids; TG, triglycerides; LD, lipid droplets; PEMT, PE N-methyltransferase; PLD, phospholipase D; PAP, PA phosphatase; PLC, phospholipase C; SMS, sphingomyelin synthase; PLIN2, perilipin2. (Martínez-Uña et al. 2013).

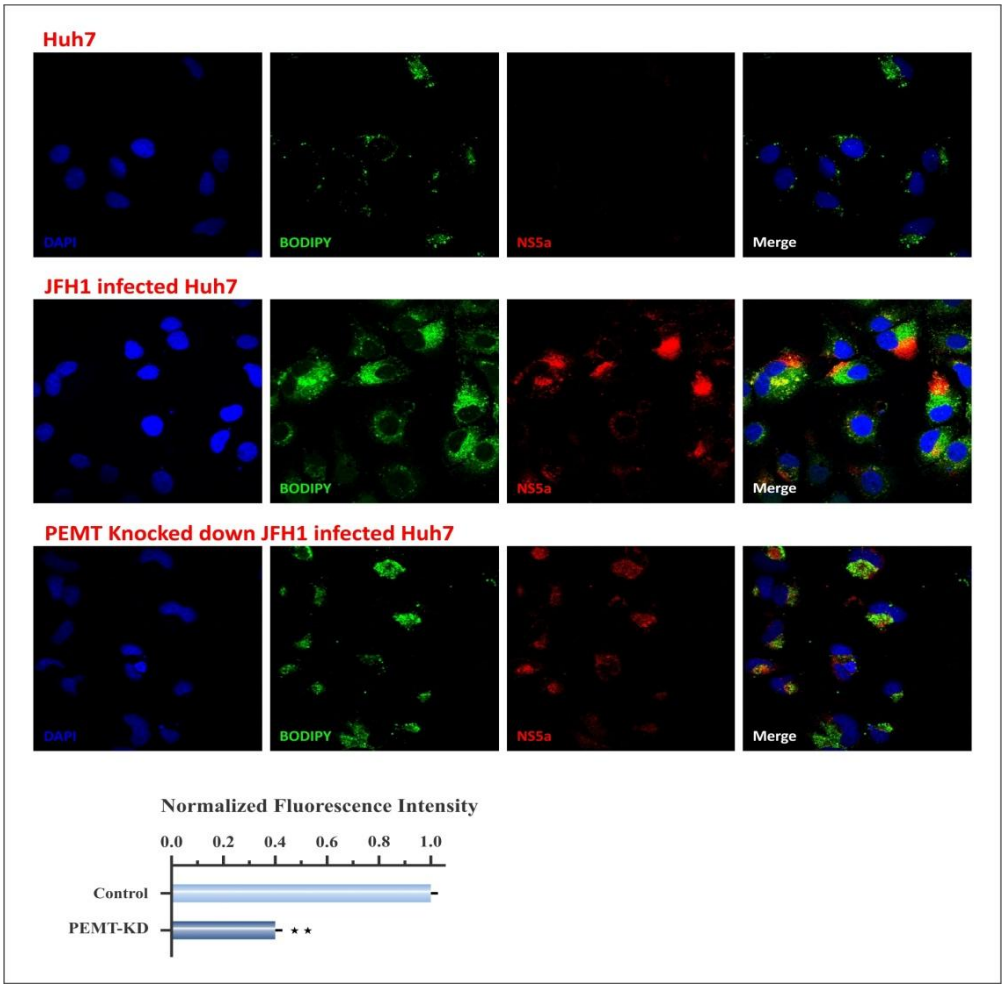


Figure 5.15 Decrease in lipids accumulation in PEMT knocked down JFH1 infected Huh-7 cells.
Huh7 cells were electroporated with 5µg of *in vitro* transcribed JFH-1 RNA. After 14 days JFH1 infected Huh7 cells were treated with PEMT siRNA or scrambled RNA. 48 hr post-transfection, cells were fixed in methanol and labelled with sheep polyclonal anti-NS5A antiserum (anti NS5a) and red 594 antiSheep IgG conjugated secondary antibody, neutral lipids and nucleus were stained with BODIPY 493/503 or DAPI respectively, then examined by fluorescence microscopy.PEMT knocked down JFH1 infected cells showed a decrease in the accumulation of neutral lipid. For quantification of lipid abundance, images were captured and analyzed using Imaris software. **Significant difference from JFHI infected Huh7 cells ($P < 0.01$).

5.4.1 The effect of PEMT-knockdown on intracellular lipid turnover

Various studies have shown that HCV hijacks the host lipid machinery to facilitate its own replication, inducing lipogenesis, while reducing fatty acid secretion (Syed et al. 2010). For example, HCV modulates lipid homeostasis by reducing oxidation and lipid export (Negro & Sanyal 2009b) and increasing lipogenesis via SREBP activation (Waris et

al. 2007b). Moreover, HCV downregulates very-low-density lipoprotein (VLDL) particle secretion by inhibiting the activity of microsomal triglyceride transfer protein (MTP) activity (Perlemuter et al. 2002) Therefore, the effect of PEMT knockdown on the expression of key lipid synthesis and trafficking genes was examined in order to develop an understanding of the mechanism of modulation of cell lipid content following PEMT knockdown in JFH1-infected cells. Huh-7 cells were infected with JFH1 then, after two weeks (when > 90 % of cells were infected), JFH1-infected and uninfected control cells were seeded in 6-well plates and incubated at 37 °C. 24 hours later, JFH1-infected cells were transfected with 20 nM/ml PEMT or scrambled RNA then, after 48 hr, cells were lysed, the total RNA was extracted and reverse transcribed to produce cDNA. The expression of key enzyme genes was measured using quantitative real-time PCR. Figure 5.16 shows the expression of entry factors LDL-R (A), cholesterol synthesis transcription factor SREBP1 (B), VLDL pathway components MTP (C) and cholesterol transporters ABCA1 (D).

SREBP-1c is the isoform of the sterol regulatory element-binding protein (SREBP) family that controls the biosynthesis of fatty acids and triglycerides in the liver (Brown & Goldstein 1997). The amount of (active) SREBP-1c protein in the nucleus is highly correlated with SREBP-1c mRNA levels. Therefore, mRNA expression of the SREBP-1c gene is the key regulatory step for its activity and ultimately determines hepatic lipogenesis. The expression of SREBP-1c was significantly up-regulated in HCV-infected cells (Waris et al. 2007b). As such, the expression of SERBP-1c mRNA in JHF1-infected cells transfected with 20 nM/ml PEMT siRNA, or mock transfected, was measured and compared to uninfected Huh-7 controls. SREBP-1c expression was increased in JFH1-infected cells, but there was a significant down-regulation of the normal levels in PEMT knockdown observed in the JFH1-infected cells (Fig 5.16A).

Next, the expression of the two genes (MTP and ABCA1) involved in lipid trafficking was assessed. MTP was significantly down regulated in JFH1-infected cells compared to mock-infected cells; importantly, the JFH1-infected cells that were treated with PEMT siRNA elevated MTP expression to normal levels (Figure 5.16B). MTP protein transfers lipids from various donor lipid sites to acceptor sites and plays an essential role in transferring triglycerides to the ER lumen and thus in the formation of very low density lipoprotein (VLDL) (Raabe et al. 1999). HCV core protein interferes with the assembly of VLDL by decreasing the expression of MTP protein (Perlemuter et al. 2002).

Importantly, an increase in ABCA1 expression following PEMT knockdown in JFH1-infected cells was also demonstrated. These data are consistent with ABCA1 positive regulation (Figure 5.16C). ABCA1 mediates the transport of phospholipids and cholesterol across cellular membranes and their export from cells by high-density lipoproteins (HDL) (Oram 2003). Interestingly, a recent study showed that the stimulation of ABCA1 expression inhibited HCV infection of both human primary hepatocytes and isolated human liver slices.

Lastly, PEMT knockdown had no effect on LDL-R expression in JFH1-infected cells (Figure 5.16D). Low density lipoprotein receptor (LDL-R) is a cell surface receptor that is involved in the binding and uptake of HCV lipoviral particles that circulate in the blood (Koutsoudakis et al. 2006; Zeisel et al. 2011). The inhibition of LDL-R in HCV-infected patients through the use of anti-LDLR has been shown to significantly reduce HCV endocytosis (Germi et al. 2002).

The inhibition of PEMT in HCV-infected cells led to decreased lipogenesis via SREB1-c activity inhibition and enhanced intracellular lipids transport and export by increasing ABCA1 and MTP activities respectively. Taken together, these data suggest

that the effect of PEMT knockdown on lipid metabolism genes reflects the accumulation of lipids within infected cells.

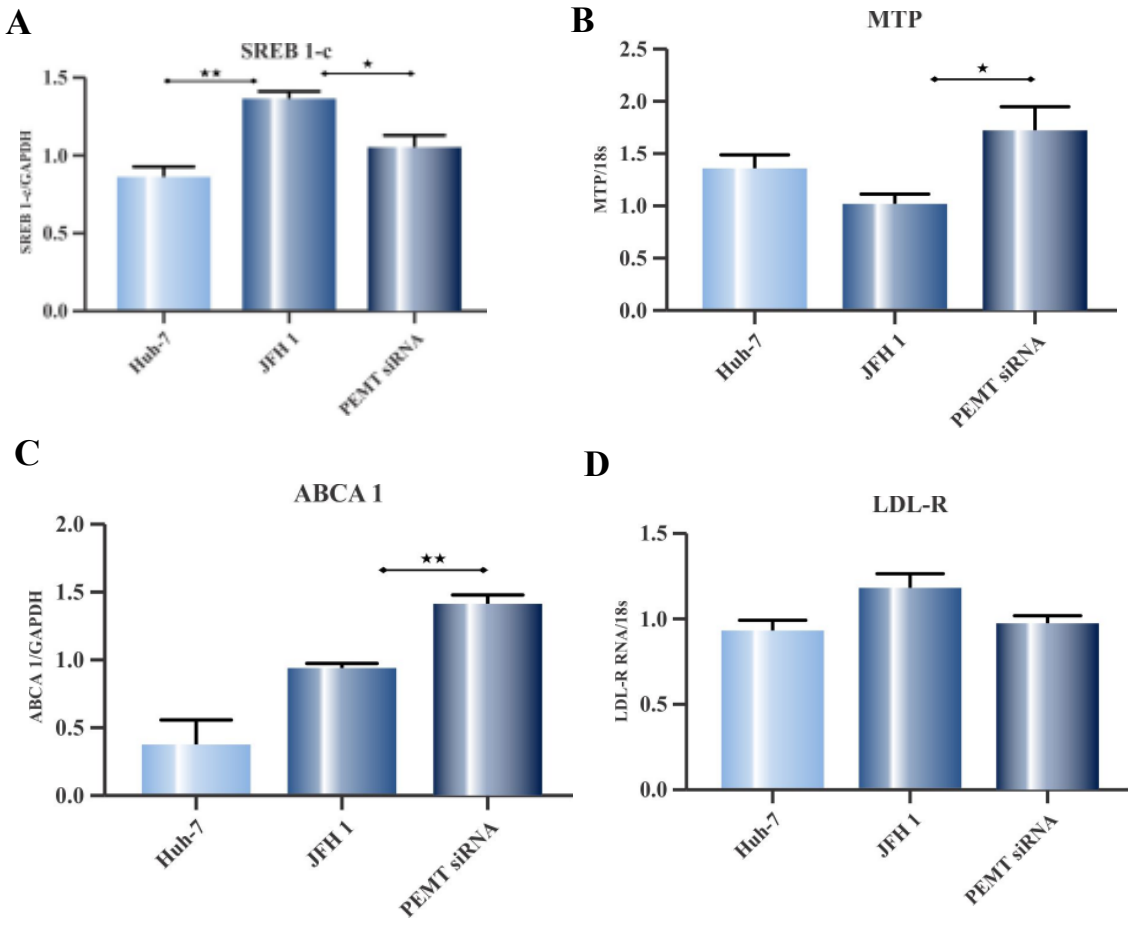


Figure 5.16 Representative sample (Three replicates) of gene expression responsible for lipids turnover.
HCV (JFH1) infected Huh-7 cells were treated or mock treated with PEMT siRNA for 48 h, RNA was extracted and SREBP-1c, MTP, ABCA1, and LDL-R mRNA were measured by qPCR. (A) Significant reduction in SREBP-1c expression with PEMT siRNA pre-treatment compared to mock treated cells. (B and C) MTP and ABCA1 were significantly increased in JFH1 infected cells pre-treated with PEMT siRNA. (D) PEMT knockdown had no effect in LDL-R expression in JFH1 infected cells. (* $p<0.05$, ** $p<0.01$).

5.4.2 PEMT pathway knockdown significantly reduced HCV replication through reduced DGAT1

The diacylglycerol acyltransferase-1 (DGAT1) enzymes catalyse the final step in triglyceride biosynthesis and are essential in LD biogenesis (Yen et al. 2008). Herker et al. identified DGAT1 as a key factor for HCV infection, as it binds the HCV core protein and localizes core protein to DGAT1-generated LDs (Figure 5.17). The inhibition of DGAT1 activity robustly inhibits HCV particle production, suggesting that assembly requires DGAT1-mediated LD formation (Herker et al. 2010). A subsequent study showed that DGAT1 is required for the development of hepatic steatosis in DGAT1 knockout mice (Harris et al. 2011). Recently, research has also indicated that, besides the core protein, NS5A protein is translocated from the ER onto LDs through the actions of DGAT1 (Camus et al. 2013).

To examine the impact of PEMT knockdown on DGAT1 expression, Huh-7 cells were electroporated with JFH1 RNA. Then, after two weeks, JFH1-infected cells and uninfected cells were seeded in 6-well plates. After 24 h incubation, JFH1-infected cells were transfected with PEMT siRNA or scrambled RNA for 48 h, the cells were lysed, the total RNA extracted, reverse transcribed to produce cDNA, and mRNA was quantified using qPCR. As expected, there was an up-regulation of DGAT1 expression in JFH1-infected cells, but PEMT knockdown restored DGAT1 expression to control levels (Figure 5.18). These results support the view that increased PEMT activity induces both TG synthesis and its accumulation into newly formed LDs.

Because phosphatidylcholine is required for the formation of LDs, there is a potential that the loss of PEMT activity interferes with triglyceride turnover and reduces cellular lipid droplet levels, thereby resulting in a reduction the production of infectious particles. Consistent with this hypothesis, the results of this study indicate that the flux

from PE to PC is stimulated in the HCV-infected cells via PEMT pathway, and that this produces a marked increase in DG and TG.

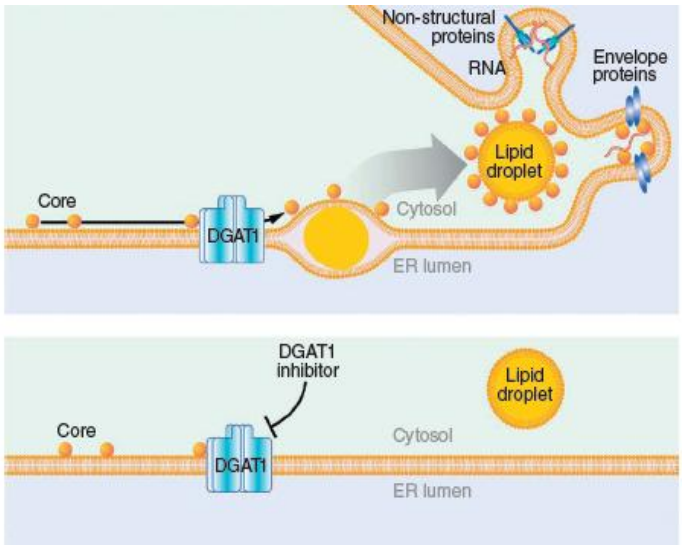


Figure 5. 17 DGAT1 is necessary for HCV particle assembly at lipid droplets. HCV core association with lipid droplets depends on DGAT1. Inhibition of DGAT1 prevents HCV core protein from accessing lipid droplets (Herker et al. 2010).

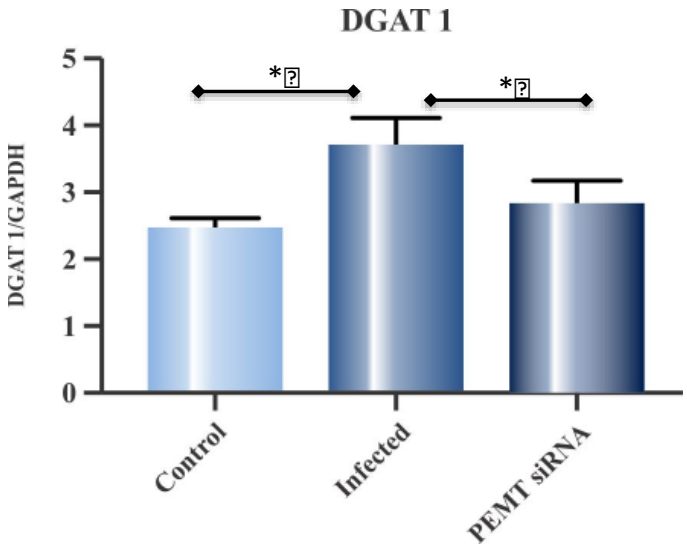


Figure 5.18 Real-time RT-PCR analysis of DGAT1 expression.
RNA isolated from Huh-7 cells or from of Huh-7 cells electroporated with JFH1 RNA and treated with PEMT inhibitor (20nM) or scrambled RNA. Results are expressed as DGAT1 RNA copy numbers per 1 µg total cellular RNA normalized to GAPDHRNA (intracellular) at day 2-post transfection (*p< 0.05).

5.5 Correlation of PEMT expression and steatosis in HCV-infected patients

It has been reported that the severity of steatosis is associated with the progression of hepatic fibrosis and hepatocellular carcinoma in chronic hepatitis C (CHC) (Kurosaki et al. 2010). Moreover, of the CHC patients who are infected with HCV genotype 2, those with hepatic steatosis are more resistant to interferon therapy (Kurosaki et al. 2010). The mechanism by which hepatocyte steatosis plays a role in IFN resistance is still unclear.

Previous sections of this chapter described the increased expression of the PEMT enzyme in HCV-infected hepatic cells and confirmed the impact that PEMT knockdown had on HCV replication, lipid accumulation and interferon signalling. Therefore, the last section of this chapter investigates the association between PEMT expression and the severity of steatosis in HCV infection. To investigate how HCV infection may influence PEMT expression in the liver, PEMT expression was measured in liver biopsies from three independent HCV cohorts, using three different techniques: Microarrays, RNA high throughput sequencing (RNA-Seq) and real-time qPCR.

All liver biopsies samples were collected from HCV genotype 1-infected patients. A total of 26 samples for RNA-Seq, 22 for microarray analysis and 37 for qPCR were used. Ethics approval was obtained from the Human Research Ethics Committees of Sydney West Area Health Service and the University of Sydney. All subjects provided written informed consent (HREC2002/12/ 4.9(1564) prior to the research.

PEMT expression was positively correlated with steatosis severity in HCV patients in all three cohorts (Figures 5.19 a, b, and c). Gene microarray, RNA-seq and qPCR analysis consistently exhibited higher PEMT levels in patients who suffered from moderate-to-severe steatosis than those with mild-to-moderate steatosis.

In a similar manner, the extent to which hepatic PEMT expression correlates with

plasma HCV RNA levels was assessed. As was the case with steatosis, there was a positive correlation between HCV RNA levels and PEMT expression in the three independent cohorts, using the three different techniques (Figure 5.20).

Following this, the correlation between liver fibrosis and PEMT expression in HCV-infected patients was assessed. Liver biopsy samples were divided into high (METAVIR or Scheuer fibrosis score ×2) or low fibrosis (METAVIR or Scheuer fibrosis score p1). In contrast to the positive correlation between steatosis and PEMT expression in the liver, the opposite effect was observed with fibrosis. As demonstrated in Figure 5.21, PEMT expression was negatively correlated with fibrosis severity in HCV-infected patients in all three cohorts.

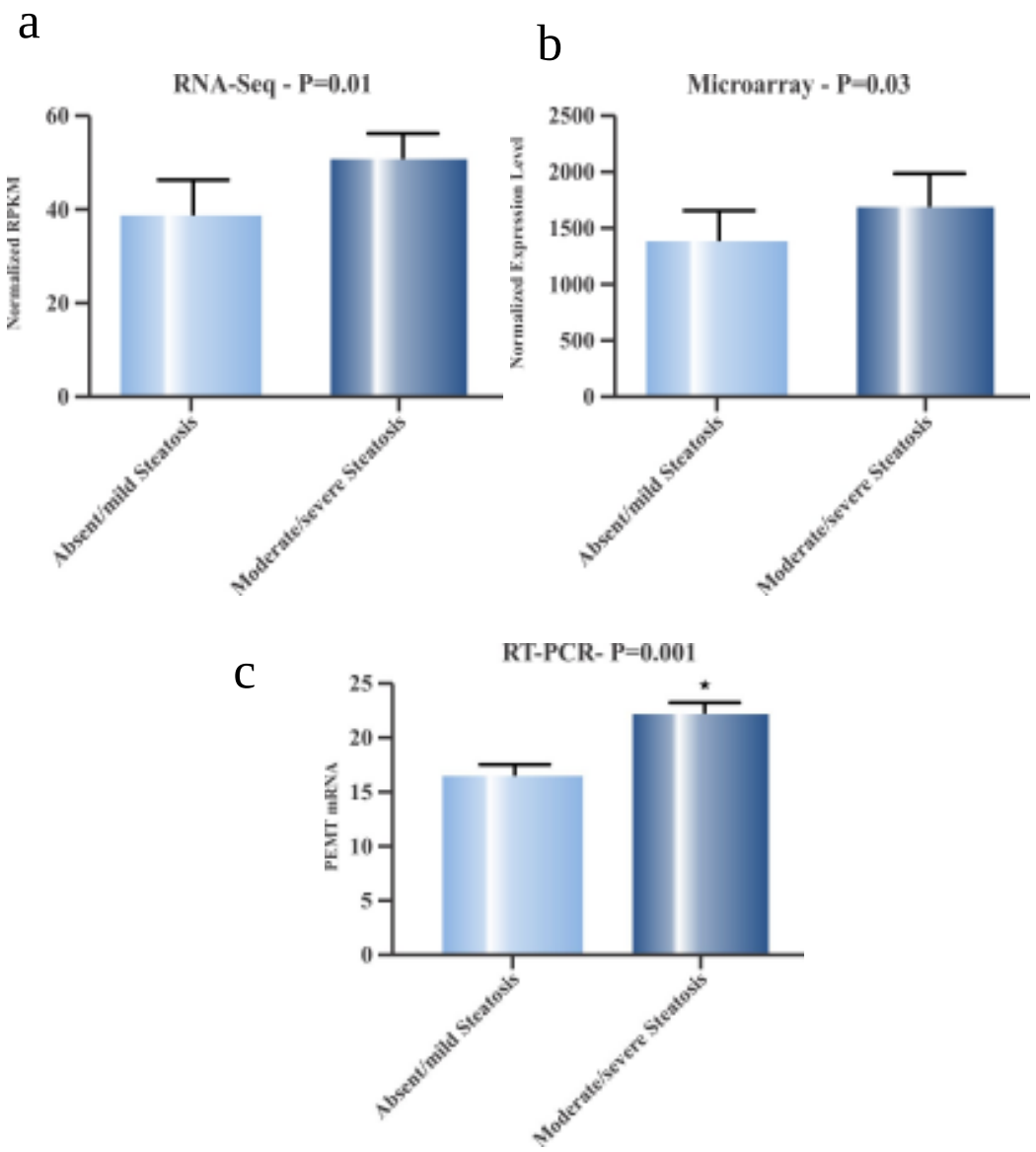


Fig. 5.19 Analysis of PEMT expression level in low versus high steatosis liver biopsy samples. Panel (a) performed by RNA-Seq (n 1⁄4 26), panel (b) by microarray (n 1⁄4 22) and panel (c) qPCR (n 1⁄4 37). High fibrosis samples exhibit higher MT expression by all three methods.

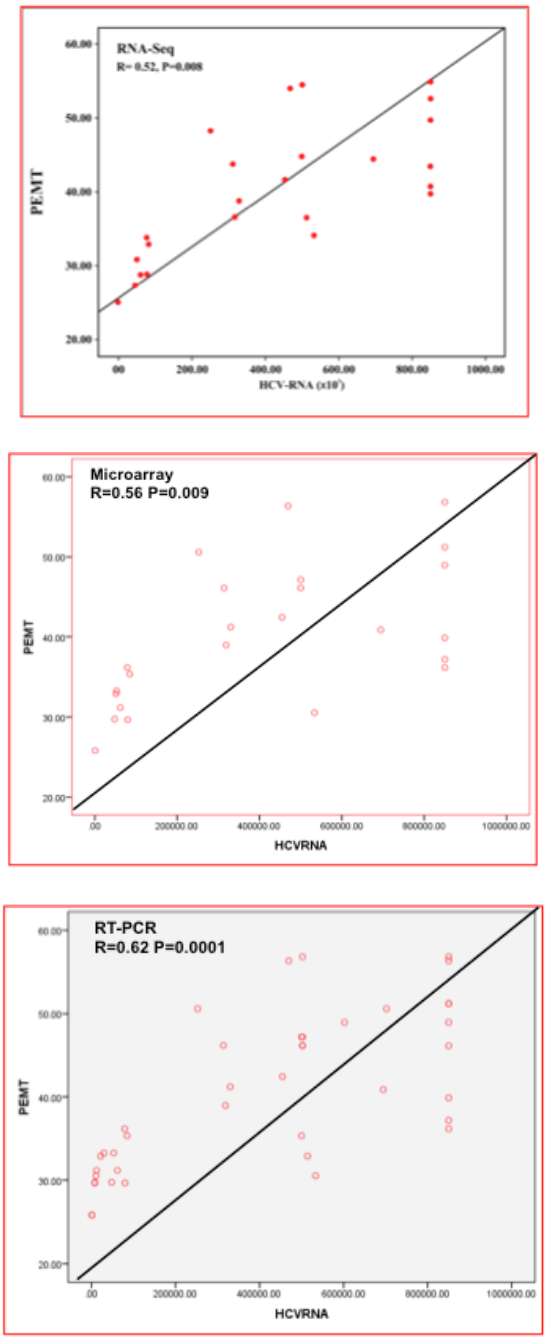


Figure 5.20 Correlation between HCV RNA levels and PEMT expression in hepatocytes. Panel (a) performed by RNA-Seq (n 1⁄4 26), panel (b) by microarray (n 1⁄4 22) and panel (c) qPCR (n 1⁄4 37). High fibrosis samples exhibit higher MT expression by all three methods.

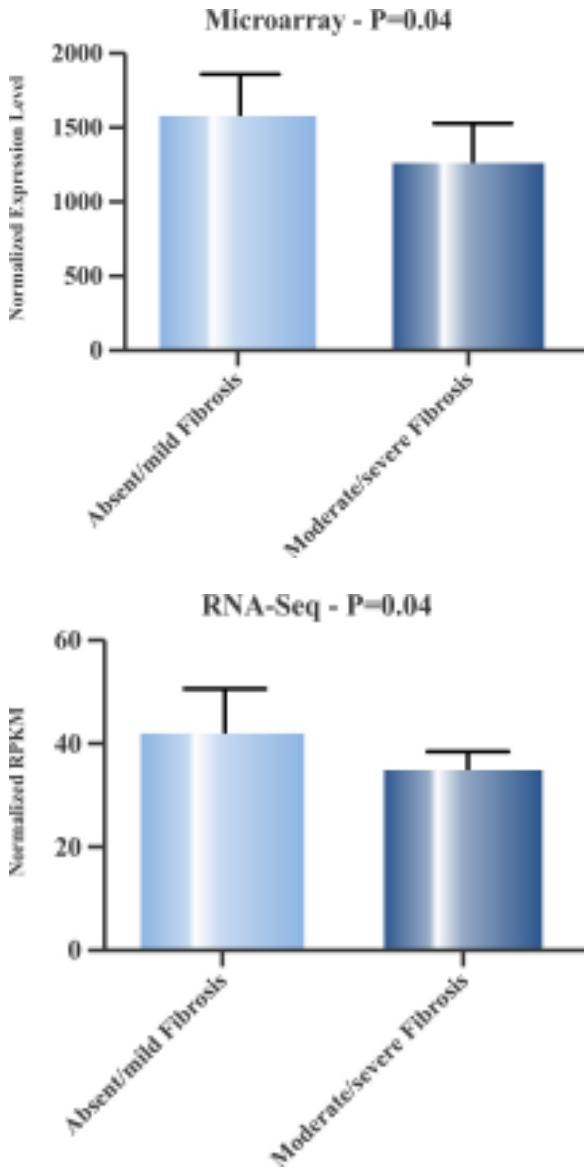


Fig. 5.21 Correlation between hepatic fibrosis in HCV infected patients and PEMT expression. Independent comparisons RNA-Seq (A) and microarray (B) of expression of PEMT in HCV-infected liver biopsies.

5.6 Summary

In summary, based on the in vitro and in vivo data gathered through this research, HCV infection increases the expression of PEMT in hepatocytes. Furthermore, inhibiting PEMT activity using PEMT siRNA led to two important observations:

1. Inhibiting PEMT reduced HCV infection in vitro, most likely by altering the synthesis and simultaneously enhancing the secretion of lipids. HCV-infected cells have increased expression of genes involved in lipogenesis (SREB1-c ABCA1 and LDL-R), and more intracellular lipid.

2. PEMT siRNA pre-treatment enhanced the sensitivity of HCV-infected cells to IFN-α treatment, as demonstrated by the increased STAT1 phosphorylation, the increased ISG induction and, most importantly, the reduction in HCV RNA.

The researchers hypothesize that this improved response to IFN-α may be due to increased intrahepatocellular concentrations of SAMe, which restore STAT1 methylation and improve IFNα signalling. This suggests that PEMT inhibitors may be useful as a result of their ability to improve treatment response in patients with chronic HCV infection who are refractory to treatment with IFN-α.

3. Finally, the research revealed that PEMT expression is elevated in patient's CHC and is coupled with elevated HCV RNA levels and more severe steatosis. The relationship between HCV viral load and PEMT suggests the possibility of a causative interplay between the virus and PC metabolism, which will need to be further examined.

6. Final Discussion

6.1 Study aims

The original aim of this project was to examine, in detail, hepatitis C virus-induced lipid changes in whole cells and sub-cellular organelles, and explore the implications for novel anti-host targeted HCV therapies. Upon finding that HCV induced the significant accumulation of several phosphatidylcholine (PC) species, the research sought to assess the effect of silencing the enzymes responsible for the activation of this pathway (PCYT1A and PEMT) on HCV replication and the antiviral effects of IFN. The results indicated that the PEMT pathway is involved in HCV replication and probably HCV-induced interferon resistance, in mechanisms mainly related to methylation and partially independent of changing PC levels. The lipid-modifying effects of PEMT knockdown were examined in more detail to further understand how the PEMT pathway may contribute to HCV-induced metabolic reprogramming.

6.2 Findings and significance

The results of this study demonstrated, for the first time, that PEMT enzyme knockdown can increase the sensitivity of Huh-7 cells to IFN-α treatment, resulting in reduced HCV replication in vitro and reduced HCV-associated lipid accumulation.

The research demonstrated that the use of two complementary approaches (high-resolution HPTLC and mass spectrometry) on the JFH1 HCV infection of Huh-7 hepatoma cells induces global changes in the cell lipidome, affecting both neutral lipids and phospholipids. The clinically derived JFH1 strain and its derivatives are the only HCV

strains that reproduce the entire virus life cyclein vitro, and only replicate efficiently in the Huh-7 cell line, which is why this model was selected for the in vitro studies.

Chapter 3 described HCV-induced global changes in the content of cell lipids. These findings provide useful insights into the metabolic reprogramming induced by chronic HCV infection, and how this benefits both the energetic and biosynthetic needs of the viral life cycle. The increased abundance of certain phospholipids (PC, PE, and PI) potentially reflects their role as crucial constituents in the various structural entities supporting viral replication, including the lipid droplet and membranous replicase compartments. In contrast, a major change in other lipid classes (triacylglycerol) was expected; however, only minor changes were observed. This probably reflects their consumption during incorporation into lipoprotein-associated viral particles, reflecting potentially important roles in infectivity.

However, the detail of these changes at the subcellular level was not known, so this was examined further. Of the various subcellular compartments, the endoplasmic reticulum (ER) is a prime candidate for HCV infection. The ER plays a central role in HCV replication: following infection, viral RNA is translated at the ER, where viral proteins induce membrane modifications, giving rise to the ‘membranous web’. This web acts as the platform for the synthesis of HCV RNA. Virus structural proteins are then packaged with RNA at the interface between ER and cytoplasmic LDs (Herker et al. 2010), assembled into ‘lipoviral’ particles in the ER and exported through the ER-Golgi pathway (Gastaminza et al. 2008). Thus, understanding of HCV/host lipid homeostasis and turnover in the ER should provide novel insights, over and above examining changes in the whole infected cell. This research incorporated the hypothesis that HCV-induced lipid changes would be more profound in the ER organelle than they would be in the whole cell. Consistent with this, dramatically greater changes in both neutral lipids and phospholipids

in the ER we observed in comparison to the whole cell. The most striking difference was the relatively greater HCV-induced increase in phosphatidylcholine (150% vs. 30%, $p < 0.001$) and free cholesterol (200% vs. 50 %, $p < 0.01$) in the ER, compared to whole cell extracts. This emphasizes the importance of the ER in the HCV life cycle. Further studies are required to explore this in depth both in terms of HCV and perhaps also for viruses that affect cellular lipid.

In the view of the findings, more detailed analysis of the effect of chronic HCV infection on ER lipid rafts was performed. Recent studies suggest that the HCV replication complexes are formed on lipid rafts, which are detergent-insoluble micro domains of intracellular vesicular membranes.

Lipid rafts are small platforms in the outer exoplasmic leaflet of the lipid bilayer, composed of sphingolipids and cholesterol, which are connected to phospholipids and cholesterol in the inner cytoplasmic leaflet. These assemblies are fluid, but more ordered and tightly packed than the surrounding lipid bilayer. Lipid rafts were first discovered as a detergent-insoluble fraction, and the concept of lipid rafts has changed our understanding of cell membrane organization. In mammalian cells, lipid rafts are first assembled in the Golgi complex (Simons & Ehehalt 2002), and are involved in cell signalling and intracellular trafficking (Helms & Zurzolo 2004). Some viruses utilize these sub-cellular membrane domains for cell entry, virion assembly or budding (Chazal & Gerlier 2003; Salaün et al. 2004). Viruses can also perturb cell signalling pathways by interfering with proteins in lipid rafts (Krautkramer et al. 2004; Avota et al. 2004). Moreover, several studies have shown that HCV replication is associated with ER lipid rafts (Shi et al. 2003; Aizaki et al. 2004). Therefore, lipidomic analysis of HCV-lipid raft interactions may help to explain the abnormalities of lipid metabolism that is associated with HCV infection.

Interestingly, after isolating the ER-lipid rafts and analysing each fraction individually, it was elucidated that the HCV-induced increase in free cholesterol levels in the ER was lipid raft specific. This was evidenced by the fact that the ER-lipid raft fraction in the HCV-infected cells exhibited the highest cholesterol band density when analysed using HPTLC and commercial standards to identify free cholesterol.

Next, it was important to perform further detailed lipid analyses of other sub-cellular compartments, particularly cytoplasmic LDs (LDs). LDs are organelles that are used for the storage of neutral lipids within hepatocytes. They dynamically move through the cytoplasm, interacting with other organelles, especially the endoplasmic reticulum (ER) (Martin & Parton 2006; Blanchette-Mackie et al. 1995; Vock et al. 1996). These interactions are thought to facilitate the transport of lipids and proteins to other organelles throughout the cell.

LDs have recently attracted considerable attention because of the link between the accumulation of LDs and human diseases such as obesity, atherosclerosis and HCV-associated liver malfunctions. Additionally, the size and neutral lipids content of LDs increases in Huh7 cells transfected with HCV genotype 3a (Piodi et al. 2008). However, the actual role of LDs in the complexity of the HCV life cycle, especially HCV assembly and release, is still poorly understood. This research sought to clarify this issue by investigating how the HCV virus particles interact with LDs, with the ultimate aim of identifying new therapeutic strategies that can protect against HCV.

Notably, the results indicated that triacylglycerol was the most prominent neutral lipid class in LDs and was significantly up-regulated in HCV-infected cells (Figure 4.13). This finding is consistent with data published in existing literature, which highlights the important role of triglycerides in the HCV life cycle (André et al. 2005; Negro 2010b).

Phospholipid was also higher in LDs extracted from HCV-infected cells than it was in those extracted from naive cells (Section 4.4.2).

To provide deeper insights into the effect that HCV have on LDs, the protein content of purified LD from JFH1 HCV-infected Huh-7 cells was compared with LDS from uninfected Huh-7s. Using protein gel electrophoresis and Western blot, an HCV-induced increase in DEAD-box RNA helicase (DDX3) in LDs was observed. This finding was consistent with that of previous studies (Ariumi et al. 2007; Oshiumi et al. 2010). However, work by Li et al. identified interactions between the HCV 3'UTR and DDX3, which contributed to lipogenesis by stimulating SREBP-1c (Li et al. 2013). This data suggests that the interaction between DDX3 and HCV core on LDs is potentially important in the replication of HCV. Understanding the viral strategies for manipulating or co-opting DDX3 could provide valuable insights that could benefit the development of strategies for preventing HCV by targeting DDX3. Interestingly, there is accumulating data in existing literature that indicates DDX3 is also important in the life cycle of several other viruses, including human immunodeficiency virus-1 (HIV-1) (Yedavalli et al. 2004) and brome mosaic virus (Noueiry et al. 2000).

This research also included the hypothesis that the interaction between DDX3 and HCV core is required for the process of shuttling HCV core protein between the cellular compartments (a vital step in the life cycle of HCV) and mediated by CRM-1/exportin. To test this hypothesis, the effect of leptomycin B (LMB, Sigma), a specific inhibitor of CRM-1/exportin, on DDX3 was investigated using Western blot. Notably, leptomycin B significantly reduced the accumulation of DDX3 in purified LDs from cells infected with HCV (JFH1).

Collectively, this data suggests that targeting the DDRX3/CRM-1/exportin pathway may result in a novel host-targeted agent that prevents HCV. Generally, host

targeted therapies for HCV have a higher genetic barrier to resistance than direct acting antivirals (DAA), which target variable virus proteins. This makes them attractive candidates when used as part of a combination, as oral antiviral therapies in the future.

Finally, the research sought to compare the relative HCV-induced increases in PC levels in whole cells, ER fraction and LD fraction, to determine whether the increase in PC is global, or more specific to some sub-cellular organelles. The results revealed that ER-specific fractions from HCV-infected cells contained a higher level of PC than whole cell extracts and LD fractions. On the other hand, there was no significant difference in PC increase between whole cell and LD fractions. Again, this emphasizes the vital role ER plays in HCV replication.

Another comparison was performed for the changes in PL and neutral lipid in LDs compared to the whole cells changes. After analysing the neutral lipid content of LD using HPTLC, TAG was the predominant lipid class detected, with up to 40% increase in LDs from JFH1-infected Huh-7 cells, compared to less than 20% increase in whole cell lysates. Similarly, during the PL analysis of LDs, PC was the only PL detected, with significant increase that was similar to that found in the whole cell. This reflects the importance of the LDs organelle in the HCV life cycle.

Based on these findings, a hypothesis that HCV infection induces wide metabolic reprogramming, resulting in the accumulation of PC species in the ER, through effects on the choline pathway (through CTP enzyme) and methylation pathway (PEMT enzyme) was developed. Effects on PEMT may be particularly significant as they could lead to higher rates of consumption of SAMe, the major biological methyl donor, which may ultimately induce interferon resistance and lipid accumulation. The roles of SAMe in steatosis and interferon signalling are well established (Duong et al. 2006; Lu & Mato 2012).

Chapter 5 described a series of experiments that were designed to investigate whether inhibition of PEMT in HCV-infected cells: (1) affected lipid accumulation; and (2) enhanced IFN-α-induced expression of antiviral ISGs.

In the cell culture model implemented, HCV infection increased the expression of PEMT in Huh-7 cells (Figure 5.3). Consistent with this, PEMT expression was elevated in patients who had chronic hepatitis C (CHH), particularly in those with genotype 3 infection. This is associated with elevated HCV RNA levels and more severe steatosis (Figure 5.8).

Inhibiting PEMT activity using PEMT siRNA led to two important observations:

1. Inhibiting PEMT reduced HCV infection in vitro, most likely by altering the synthesis and simultaneously enhancing the secretion of lipids. HCV-infected cells have increased expression of genes involved in lipogenesis (SREB1-c, ABCA1 and LDL-R), and more intracellular lipid (Figure 5.16). Consistent with this, in human liver biopsies from three independent cohorts using three different techniques (microarray, RNA-Seq and RT-PCR), the hepatic expression of PEMT mRNA correlated with the degree of hepatic steatosis (Figure 5.19). Furthermore, the hepatic expression of PEMT was higher in patients who were infected with genotype 3 than it was in those infected with genotype 1 (Figure 5.8). These findings are consistent with the well-described direct steatogenic effect of HCV genotype 3 (Adinolfi et al. 2001).

2. PEMT siRNA pre-treatment enhanced the extent to which HCV-infected cells were sensitive to IFN-α treatment, as demonstrated by increased STAT1 phosphorylation, increased ISG induction and, most importantly, the reduction in HCV RNA levels and infectivity (Figure 5.11).

This improved response to IFN-α may be due to the increased intracellular concentrations of SAMe, restoring STAT1 methylation and improving IFNα signalling. PEMT catalyses the synthesis of PC by the sequential addition of methyl groups from SAMe to the amino head group of phosphatidylethanolamine (PE). Methylation of activated STAT1 is catalysed by PRMT1 and requires SAMe (Figure 6.1A).

Thus, silencing the PEMT pathway should prevent the consumption of SAMe in the synthesis of PC, a process that is enhanced by HCV infection. This should lead to restored STAT1 methylation, decreased STAT1 association with the inhibitory protein PIAS1, activation of STAT1 binding to DNA and, consequently, increased IFN sensitivity and decreased HCV replication (Figure 6.1B), partially independent of decreased levels of PC, as the latter can be compensated by the PCYT1 pathway. In support of this hypothesis, silencing of PCYT1, the enzyme responsible for the majority of PC synthesis had no significant effect on HCV replication or infectivity (Figure 5.5). This suggests that the effect of PEMT silencing on HCV replication is mainly related to altered methylation STAT1 and is independent of PC levels.

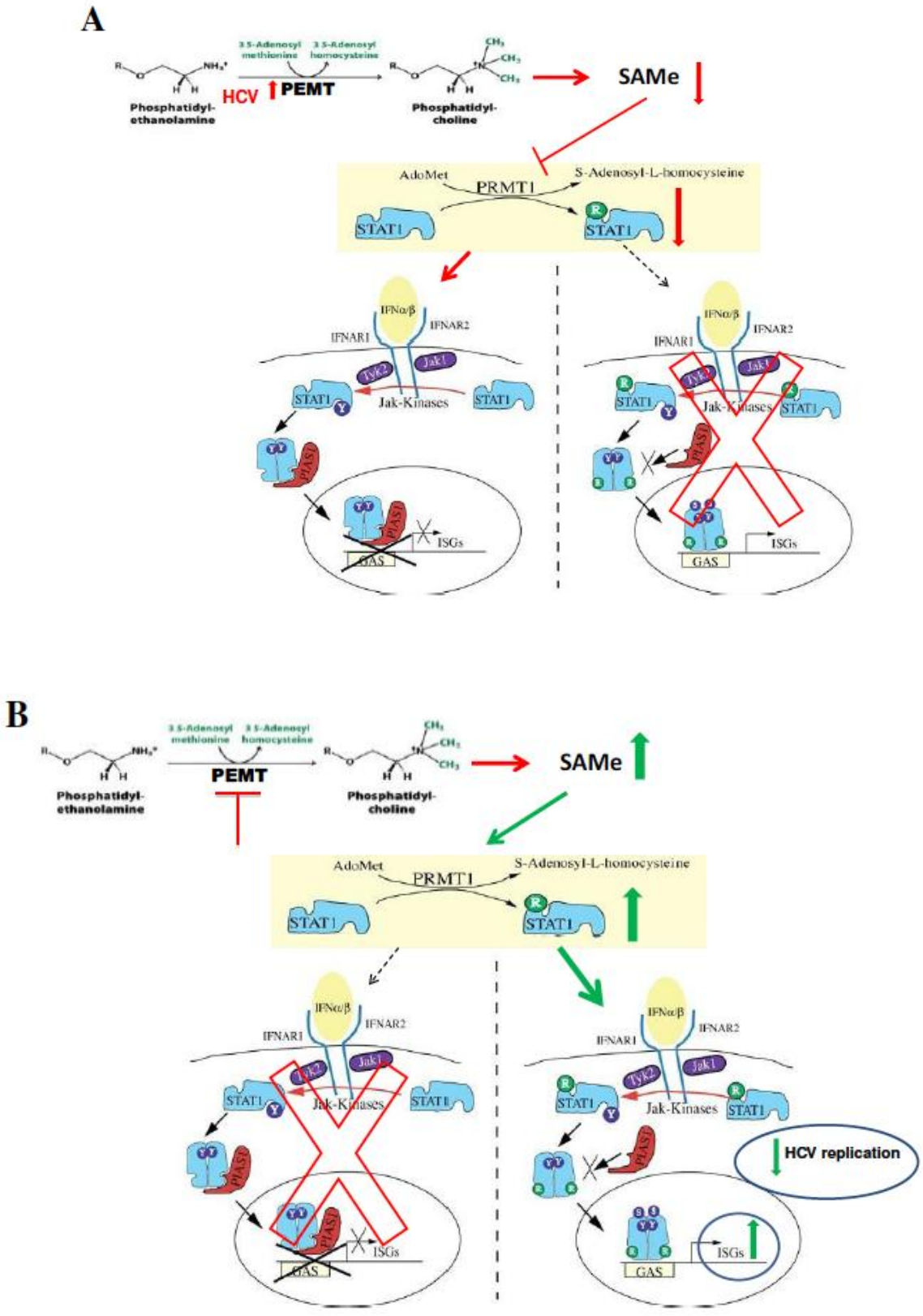


Figure 6.1 Schematic diagrams to explain the effect of PEMT inhibition on INF signaling in HCV infected cell.

HCV induces an upregulation PEMTleads to higher consumption of SAMeand decreased methylation of STAT1 byprotein arginine methyltransferase 1 (PRMT-1) enzyme inhibits

the enzymatic activity of protein arginine methyltransferase 1 (PRMT-1) resulting in hypomethylated STAT1 associates with the inhibitory proteinPIAS1 (protein inhibitor of activated STAT1) leading to inhibition of STAT1-DNA binding and,therefore, reduced transcriptional activation of IFN target genes activation (A).
Silencing the PEMT pathway should prevent the consumption of SAMe in the synthesis of PC, a process which is enhanced by HCV infection. This should lead to restored STAT1 methylation, decreased STAT1 association with the inhibitory protein PIAS1, activation of STAT1 binding to DNA and, consequently, increased IFN sensitivity and decreased HCV replication (B). form(Duong et al. 2006) with modification.

Taken in combination, the data presented in this research suggests that PEMT inhibitors may be useful in patients who are suffering from chronic HCV infection. They not only have the potential to improve the treatment response of those who are refractory to IFN-α, but may also improve many of the HCV-associated sequels, including hepatic steatosis. The possible importance of the PEMT pathway in other HCV-related complications (e.g., liver fibrosis and HCV-associated HCC) deserves further investigation.

6.3 Future directions

The results of this thesis suggest that reversing the metabolic reprogramming induced by HCV infection is an effective novel treatment strategy that deserves further investigation. Until now, there have been no robust therapies developed to target HCV-associated consequences such as steatosis and fibrosis. Understanding and correcting HCV-induced metabolic reprogramming and its deleterious consequences could open the door for future novel therapies.

The results associated with the use of HCV as a model are encouraging and could inform future studies involving other viruses that are known to be dependent on cell lipids, such as HIV. Furthermore, the results of this study may also have implications for other fatty liver diseases (e.g., NAFLD). Indeed, several candidate gene studies on mice have emphasised the role of the PEMT pathway in hepatic steatosis due to NAFLD (Lu & Mato 2012).

6.4 Conclusion

In conclusion, the use of an HCV cell culture model in this study provided a valuable example of the importance of dissecting the role of various host cell lipid species in the virus life cycle. The research demonstrated HCV-induced global changes in cell lipids and highlighted the fact that the effect was more profound in the ER fraction, particularly for PC and cholesterol. HCV increased hepatic PEMT expression in vitro and in vivo, increasing consumption of SAMe and resulting in hepatic lipid accumulation and interferon resistance. PEMT knockdown in the model reversed HCV-induced metabolic reprogramming, with reduced lipid accumulation, improved interferon sensitivity and decreased HCV replication. The observations made in this study open the door to several new and exciting avenues of investigation. These should further improve understanding of HCV-associated pathogenesis, and hopefully lead to the development of novel antiviral therapies, not only for HCV, but also for other viruses that interact with cell lipid pathway

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