Studies of the Association of Hepatitis C Virus and Host Lipoproteins *In Vivo* and in Cell Culture

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Declaration

This thesis contains no material which has been accepted for any other degree in any university. All work presented was performed by me at Newcastle University under the supervision of Prof. Geoffrey Toms and Dr. Soren Nielsen.

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Abbreviations used in this thesis

A	Adenine
aa	Amino acid
Ab	Antibody
ALT	Alanine transaminase
AMV-RT	Avian Myeloblastosis Virus – Reverse Transcriptase
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoC	Apolipoprotein C
ApoE	Apolipoprotein E
APS	Ammonium peroxodisulphate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
C	Cytosine
°C	Degrees celcius
$CaCl_2$	Calcium chloride
CAPS	3-cyclohexylamino-1-propanesulfonic acid
cDNA	Complementary DNA
CLDN1	Claudin-1
cm	Centimetre
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
CTP	Cytidine triphosphate
$CuCl_2$	Copper (II) chloride
CVID	Common variable immunodeficiency
D	Aspartic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DHA	Docosahexaenoic acid
dH_2O	Distilled water
DiI	Dioctadecylindocarbo-cyanine
DMEM	Dulbeccos modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsRNA	Double stranded RNA
DTT	Dithiothreitol (Dimercapto butanediol)
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene-diamine-tetra-acetic acid
EGTA	Ethylene-glycol-bis (2-aminoethylether)-N,N,N',N'-

	tetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagles minimum essential medium
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FAM	Carboxyfluorescein
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL	Fluorescence
FSC	Forward scatter
g	Grams, gravity
G	Guanine, Glycine
GAG	Glycosaminoglycan
gp	Glycoprotein
GTP	Guanosine triphosphate
H	Histidine
HBSS	Hank's Buffered Salt Solution
HCIG	Hepatitis C immune globulin
HCL	Hydrochloric acid
HCV	Hepatitis C virus
HCVcc	HCV cell culture
HCV-LP	Hepatitis C virus – like particle
HCVpp	HCV pseudoviral particle
HDL	High density lipoprotein
HEPES	N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid
HIV	Human immunodeficiency virus
hr	hour
HS	Heparan sulphate
HVR	Hypervariable region
IDL	Intermediate density lipoprotein
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRES	Internal ribosome entry site
ISG	IFN-stimulated genes
IU	International units
IV	Intravenous
JFH1	Japanese fulminant hepatitis 1
kb	Kilobase
kbp	Kilobase pair
KBr	Potassium bromide

NP-40	Nonidet P-40 (octyl phenoxylpolyethoxylethanol)
NRIg	Normal rabbit immunoglobulin
NS	Non-structural proteins
NTR	Non-translated regions
OA	Oleic acid
OCLN	occludin
OD	Optical density
25-OH	hydroxycholesterol
ORF	Open reading frame
oxLDL	Oxidised LDL
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
Pen/Strep	Penicillin/Streptomycin
PES	polyethersulfone
PMSF	Phenylmethylsulphonyl-fluoride
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene di-fluoride membrane
RdRp	RNA dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RIG-I	Retinoic acid inducible gene-I
RNA	Ribonucleic acid
RNP	ribonucleoprotein
rpm	Rotation per minute
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase – polymerase chain reaction
SAM-FITC	Sheep anti-mouse – fluorescein isothiocyanate
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
sec	second
S_f	Svedberg flotation units
shRNA	Short interfereing RNA
siRNA	Small interfereing RNA
SR-B1	Scavenger receptor class B 1
SSC	Side scatter
T	Thymidine
TAMRA	Carboxytetramethyl-rhodamine
TE	Tris EDTA
TEMED	N,N,N,N-tetramethyletylenediamine

TLR3	Toll-like receptor-3
Tm	Melting temperature
Tris HCl	Tris hydrochloride
U	Uracil
UTP	Uridine triphosphate
UV	ultraviolet
V	Voltage
VLDL	Very low density lipoprotein
VLP	Virus-like particle
WHO	World Health Organisation
w/v	Weight/volume

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Abstract

The purpose of this project has been to investigate the mechanism of hepatitis C virus/lipoprotein complexes or lipo-viro-particles (LVP) derived from liver (LLVP) binding to human hepatocytes and hepatoma cell lines. HCV LLVP was derived from transplanted liver macerates of an HCV infected immunodeficient patient (S6b) and density-fractionated from an iodixanol gradient.

Previous studies have indicated that serum derived HCV may bind to hepatocytes via the LDL-receptor (LDLr) or the scavenger receptor SR-B1. The role of these receptors in the uptake of LLVP in HepG2 cells was investigated by comparing LLVP uptake with that of dioctadecylindocarbocyanine-labeled-low density lipoprotein (DiI-LDL) and similarly labeled oxidized LDL (DiI-oxLDL), known to be taken up predominantly via the LDLr and SR-B1 respectively. DiI-LDL and DiI-oxLDL binding resembled that of LLVP in being significantly increased by insulin and LPDS treatment. DiI-LDL but not DiI-oxLDL binding was also decreased by hydroxycholesterol. These results suggest that all three lipoprotein particles may be taken up via the LDLr which binds predominantly via apolipoprotein B100. To confirm this, inhibition of binding studies were conducted. Whilst binding of DiI-LDL was reduced by 98% by pre-incubation with anti-apoB100 antibodies, binding of both LLVP and DiI-oxLDL was enhanced. Using confocal microscopy and FACS analysis, we compared the role of glycosaminoglycans (GAGs) in the binding of the three particles types by washing cells with suramine, previously shown to remove GAG bound-LDL. Like LLVP, DiI-LDL bound at 0°C was washed off with suramine but became resistant at 37°C. In contrast, washing of DiI-oxLDL with suramine at both 0°C and 37°C had no significant effect. The binding patterns of LLVP therefore differ from those of DiI-LDL and DiI-oxLDL. To investigate whether such differences might be due to viral glycoprotein E2, the effect of polyclonal antibody to E2 and a monoclonal antibody to the E2-hypervariable region (HVR1) on binding of LLVP to HepG2 cells was assessed. Neither anti-E2 nor anti-HVR1 blocked binding.

As the biological relevance of virus binding studies is questionable, we investigated ways of establishing an infection model in which to investigate LVP uptake. Huh7.5 hepatoma cells and primary hepatocytes were inoculated with LLVP and monitored for infection. No increase in HCV RNA was detected suggesting that this virus is unable to replicate in cell culture. We then set out to reproduce the cell culture model J6/JFH1 HCV (HCVcc) replication system and create 'infectious LVP' capable of producing productive infection in Huh7.5 cells. Upon transfection of the wild-type J6/JFH1 transcripts, there was an increase in HCV RNA output to $7X10^6$IU/ml detectable from day 7. No increase was seen in the transfection of the control mutant J6/JFH1-GND. Culture supernatant from J6/JFH-infected cells inoculated onto naïve Huh7.5 culture at 1IU/cell multiplicity of infection resulted in increasing titres of HCV genome in the infected cells peaking at approximately $9X10^6$IU/ml 21 days post-infection. It was postulated that the production of LVP would require HCV replication in hepatocytes actively secreting very low density lipoproteins (VLDL). To stimulate VLDL secretion, Huh7.5 cells were treated with oleic acid in a serum free medium leading to the release of apoB100 and apoE-containing lipoproteins in the density fraction of <1.006g/ml cell culture supernatants detectable by Western blotting and confirmed by mass spectrometry. Upon further fractionation, apoB100 and apoE were found to be in the VLDL-3 fraction. Oleic acid treatment of the J6/JFH1 HCVcc infected Huh7.5 cells led to an increase in HCVcc production and a decrease in the HCVcc density from 1.146g/ml to 1.135g/ml. Despite the decrease in the density, the particle size as analysed by gel filtration was found to be similar to that of non-oleic treated cells, that is in a VLDL-1 to VLDL-2 size range.

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Chapter 1

1 Introduction

1.1 Hepatitis C Virus – An overview

Hepatitis C virus (HCV) is a small enveloped virus containing a positive strand RNA which is approximately 9.6kb in size. HCV is classified under the genus *Hepacivirus* in the family *Flaviridae*. There are two other genera in the family *Flaviridae*: the flaviviruses and the pestiviruses (Francki, 1991) and both share a similar genome organization as hepaciviruses (Thomson, 2000). The sequencing of the genomes of HCV recovered from throughout the world has revealed that there are 6 major genotypes, denoted genotype 1 through 6. There is also considerable variation with each genotype. A HCV genome is highly variable due to the lack of proofreading mechanism of the viral polymerases which is a common phenomenon to all RNA viruses.

Until very recently culture of the virus in the laboratory was unsuccessful and characterization of the virus was difficult. The close relationship with the flaviviruses suggested that the particle would be enveloped and this was confirmed as the infectivity of the HCV from the various human samples was inactivated when exposed to chloroform (Thomson, 2000). In another study, the particles of HCV genotype 1b as seen under electron microscope were found in cytoplasmic vacuole possessing lipid bilayer envelopes (Thomson, 2000). The size of the virus in clinical material was estimated to be 30-60nm based on filtration studies (He et al., 1987). Studies of the buoyant density of virus in the blood of chimpanzees showed that the HCV buoyant density was heterogenous with a peak infectivity of 1.10g/ml. The heterogeneity and low density is thought to be because of the association of HCV

particles with serum components such as immunoglobulins and lipoproteins (Lindenbach, 2001, Bartenschlager et al., 2004).

Isolation of the virus genome from clinical material initially and subsequently by RT-PCR allowed determination of the genome organization. The function of the polypeptide encoded by the open reading frame (ORF) was initially inferred from that of similar ORFs in the flaviviridae and subsequently by studies in prokaryotic and eukaryotic expression systems, replicon systems and ultimately permissive infection in cell culture. Figure 1 below shows a diagram of the HCV genome organization, the translated protein products and also the known protein functions.

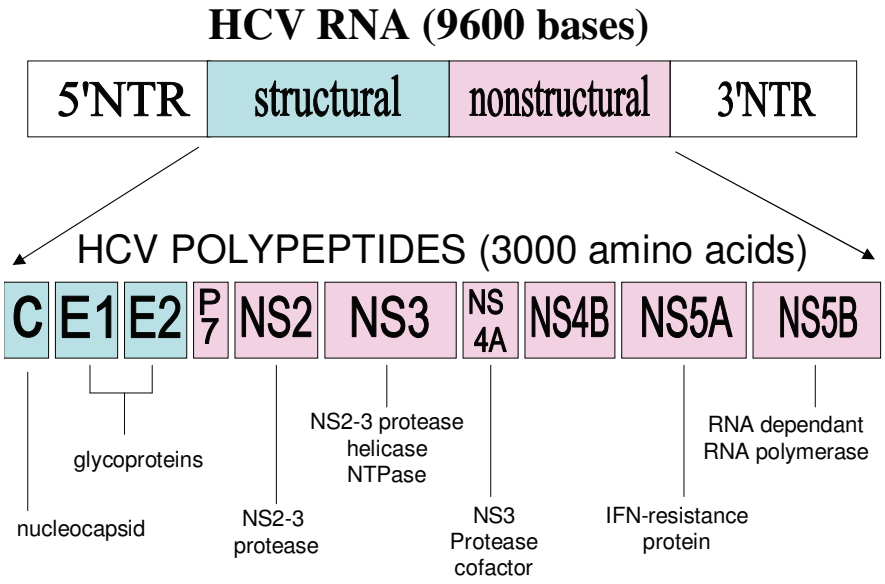


Figure 1: A diagram of an HCV RNA organization and the translated polypeptides.
Adapted from (Anzola, 2003).

The genome comprises a single ORF flanked by a 5' and 3' non-translated regions (NTRs) which are highly conserved. The 5'NTR is about 340 nucleotides in size and is the most highly conserved region of the HCV genome (Major, 1997). The 5'NTR acts as the internal ribosome entry site (IRES) for the initiation of protein translation

whereas the 3'NTR is important for viral replication and translation (Hoofnagle, 2002). The 5'NTR is comprised of residues 1 to 341 (Major, 1997) and the IRES is composed of residues 40-344 (Beales, 2001). Translation starts at the third or fourth AUG start codon which is found within the stem-loop of the 5'NTR. The 3'NTR is basically made up of 3 regions: a variable sequence after the stop codon of the ORF, a poly(U) tract and a 98 nucleotides sequence important for replication (Yanagi, 1999, Blight, 2000).

The ORF translates into one precursor polyprotein of about 3,000 amino acids in length (Major, 1997). The polyprotein is cleaved into ten different proteins: three structural proteins and seven non-structural proteins. The structural proteins include the nucleocapsid core protein (C) and two envelope glycoproteins (E1 and E2). The non-structural proteins include p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The translated polyprotein precursor is cleaved into its constituent proteins by host cellular signal peptidases and viral proteases during and after the translation process (Anzola, 2003).

1.2 Life cycle of HCV

The HCV life cycle is entirely cytoplasmic (Wieland and Chisari, 2005). The HCV particle first gains entry into a host cell via receptor mediated endocytosis using most probably CD81 as a secondary receptor and possibly other primary receptors such as SR-B1 and LDLr which will bind to the virus particle's E1/E2 glycoprotein. This is further discussed in section 1.11. HCV replication takes place using a minus-strand intermediate in a membranous compartment in the cytoplasm forming a double-stranded RNA intermediate (Moradpour et al., 2004, Wieland and Chisari, 2005).

Once in the cell, HCV releases its single positive stranded genome which acts as mRNA to direct viral protein synthesis in an IRES dependent manner from the 5' end of the sequence (Tsukiyama-Kohara et al., 1992, Wang et al., 1993). As mentioned in section 1.1, the ORF translates into one precursor polyprotein of about 3,000 amino acids in length (Major, 1997). The non-structural HCV proteins together with host factors form a ribonucleoprotein (RNP) viral replication complex in the rough endoplasmic reticulum which facilitates HCV genome replication (Moradpour et al., 2003). The characterization, processing and functions of HCV proteins are discussed in section 1.4. The genome also acts as a template for the synthesis of the complimentary negative strand which will then act as a template to produce more copies of the positive strands that eventually will be packaged as genomes into new viral protein capsids. Mature viruses will be secreted out of the cell together with a virus modified, host-derived lipid bilayer, and the virus envelope.

1.3 The genome and genotypes of HCV

The genetic diversity of HCV originated from the defective repair mechanism of the RNA dependent RNA polymerase of the virus and compounded by selective pressure. Comparisons made on major sequences of complete genomes obtained in chimpanzee and human studies gave an estimation of the mean frequency of nucleotide mutations ranging from $1.4x10^{3}$ to $1.9x10^{3}$ substitutions per nucleotide per year (Le Guillou-Guillemette et al., 2007, Ogata et al., 1991, Okamoto et al., 1992).

1.3.1 Genotypes

There are six HCV genotypes, genotype 1 to 6 and each of these genotypes are further divided into more than 50 subtypes for example subtype 1a, 1b, 2a, 2b, *etc*

(Hoofnagle, 2002). The 6 genotypes show 31-33% nucleotide sequence variation with each other whereas the subtypes show 20-25% nucleotide sequence variation (Simmonds et al., 2005). The common subtypes found in the Western countries are 1a, 1b, 2a, 2b, 2c, and 3a whereas genotype 2 is predominant in Western Africa, genotypes 1 and 4 are predominant in Central Africa, genotypes 4 and 5 primarily in Middle East and genotypes 3 and 6 are predominant in South and South Eastern Asia (Simmonds et al., 2005, Gottwein and Bukh, 2008). HCV genotype 7a was discovered in patients who may have been infected in Central Africa (Murphy, 2007).

Genotype identification is especially important clinically for drug therapy for example, genotypes 2 and 3 are more sensitive than genotypes 1 and 4 to pegylated interferon-α and ribavirin combination (Hnatyszyn, 2005).

1.3.2 Quasispecies variation

Quasispecies variation is commonly found in HCV-infected patients where a cluster of variant but related viruses has evolved due to mutations over time as a result of the error prone RNA dependent RNA polymerase. HCV quasispecies variability can be defined by two separate components; complexity and diversity within a quasispecies sample. Complexity is the average number of unique quasispecies variants and diversity is the average genetic distance (Martell et al., 1992).

In a study made by Shuhart (Shuhart et al., 2006), it was found that HCV quasispecies variability is decreased with HIV co-infection but is increased with highly active antiretroviral therapy (HAART). It has been suggested that the HCV variation

confers an advantage on the virus by allowing it to evade the immune response and to persist in an infected patient (Weiner et al., 1992).

1.4 HCV proteins

1.4.1 Structural proteins

The structural proteins of HCV include core protein (C) and envelope glycoproteins (E1 and E2). The structural proteins make up the first section encoded at the 5' end of the genome and at the N terminus of the polyprotein and are important in the architecture of the virion morphology as well as for the attachment and binding of the virus particle.

1.4.1.1 Core protein

The C or core protein is a viral capsid protein located at amino acids 1 to 191 of the polyprotein and it is cleaved from its polyprotein precursor by the host's cellular signal peptidases (Major, 1997). McLauchlan (2002) reported that the size of an immature form of the C protein containing the signal peptide and cleaved by signal peptidase at residue 191 is 21kDa whereas a 19kDa protein is produced when cleaved at the signal peptide by signal peptide peptidase at residue 179. The core protein is highly conserved and it contains some linear B-cell epitopes in the N-terminus and thus, it is immunogenic (Nasoff, 1991). The C-terminus of the C protein of the 21kDa form is hydrophobic but overall it is highly basic in nature (Major, 1997). The hydrophobic C-terminus of C protein acts as a signal for the translocation of protein E1 to the endoplasmic reticulum. The hydrophobic C-terminus is also important for the membrane-dependent processing of the core protein. The N-terminus contains three hydrophilic regions: residues 2-23, 39-74, and 101-121 that are conserved in 52 HCV isolates (Bukh, 1994). It also contains two nuclear localization signals, a DNA-

binding motif (Bukh, 1994) and an RNA binding activity. Hope (2000) reported that the core proteins are found mainly in the cytoplasm bound to the ER or to lipid droplets via the C-terminal hydrophobic region at residues 121-179.

Core protein has an RNA binding activity at the N-terminus; aa 1-75 (Santolini et al., 1994) presumably involved in the encapsidation of the RNA genome by the HCV particle (Major, 1997) forming a nucleocapsid which is the main component of the virion. The core protein is closely associated with lipid droplets as demonstrated in tissue culture by McLauchlan *et al.* (McLauchlan, 2002). In hepatocytes of HCV-infected chimpanzees, HCV core protein is also associated with cellular lipid droplets (Barba et al., 1997, Moradpour et al., 1996, Hope and McLauchlan, 2000). The association of core proteins with lipid droplets is thought to be important in virus assembly (McLauchlan, 2002). Core protein is also thought to be involved in cellular processes such as apoptosis and cell transformation as well as disrupting host immune response (McLauchlan, 2000, Lai and Ware, 2000). In cells infected with JFH1 HCV, core protein is shown to coat lipid droplets in a time-dependent manner and this coincides with increase rate of virus production (Boulant et al., 2007). Shavinskaya *et al* (Shavinskaya et al., 2007) demonstrated that the strength of the binding of core in domain D2 for lipid droplets is critical for determining the efficiency of virus assembly. Accumulation of mature core coincides with its localization to lipid droplets and an increase in infectious HCV titre. Mutations in core-E1 signal peptide delay the appearance of signal peptide peptidase-processed core thus affecting the maturation of core (Targett-Adams et al., 2008).

1.4.1.2 Envelope proteins

The E1 (gp31) and E2 (gp70) glycoproteins are viral envelope proteins. The C, E1, and E2 proteins are cleaved by the signal peptidases of the endoplasmic reticulum, and after maturation at this site, the proteins are assembled as part of the progeny virions in the internal membrane compartment (Tellinghuisen and Rice, 2002). The putative envelope proteins contain 190 amino acids from residues 190-380 (Takeuchi, 1990) and are N-glycosylated, E1 at 5 or 6 sites and E2 at 11 sites (Miyamura, 1993). Major (1997) suggested that the hydrophobic C-terminus of the envelope proteins may be important for membrane anchoring of the glycoproteins.

Inefficient cleavage at the p7 site produced two kinds of E2, E2 and E2-p7 with different C-terminal structures (Mizushima, 1994). E2 may inhibit the formation of p7 which functions as an ion channel when bound as an E2-p7 (Lavie et al., 2007). The existence of E2-p7 precursor was postulated to be a means of delaying p7 from being active of its ion-channel function during the maturation of glycoprotein and particle assembly or is a mechanism to prevent the premature fusion of E2 during the budding off of the virus (Carrere-Kremer et al., 2004). There is a suggestion that E2p7 is a component of the HCV virion (Isherwood and Patel, 2005) but this has not been proven. It has been suggested that E1 serves as a fusion protein and E2 for the binding to host-cell receptors (Tellinghuisen and Rice, 2002). It was found using mutational analysis of HCV E1 in HCV pseudoparticle (HCVpp) and HCV cell culture (HCVcc) that an internal hydrophobic domain within EI (aa262-290) of HCV acts as a fusion peptide (Russell et al., 2009).

E1 and E2 are retained in the ER membrane of the infected cells during replication. Many studies have reported that E1 and E2 interact to form a complex of

heterodimers that are noncovalently bonded (Major, 1997). The N-terminal ectodomain of E1, jutting into the lumen of ER, is 160aa long and the ectodomain for E2 is 334 aa long. The short transmembrane domains have an ER retention signals (Cocquerel et al., 1998, Cocquerel et al., 1999), have signal sequence function, and are involved in the noncovalent heterodimerization of E1 and E2 (Op De Beeck et al., 2000). The transmembrane domains also acts as anchors (Cocquerel et al., 2001). The correct folding of E1 during heterodimerization process is aided by E2 which itself is assisted by chaperone molecules for example calnexin (Dubuisson and Rice, 1996, Choukhi et al., 1998, Merola et al., 2001).

In the absence of HCV culture system, Semliki Forest virus replicon expressing genes encoding HCV structural proteins that assemble into HCV-like particles (HCV-LP) was used to study HCV morphogenesis. In this system, (Blanchard et al., 2003) showed that the HCV core protein constitutes the budding apparatus of the virus and that for budding to occur, the core must be targeted to the endoplasmic reticulum by means of the signal sequence of E1 protein. In the JFH1 HCVcc system, Miyanari, et al (Miyanari et al., 2007) suggested that infectious HCV particles are produced from LD-associated membranous environment as one of the results showed that spherical virus-like particles reacting with core- and E2-specific antibodies was found around the lipid droplets. A study using E1E2-HIV-SIN lentiviral vector to express HCV E1 and E2 proteins showed that HCV glycoproteins were secreted in association with triglyceride rich lipoprotein and apoB from the human liver cell line HepG2 but not by Huh-7 and Huh-7.5 hepatoma cells (Icard et al., 2009).

Glycoprotein variations may play an important role in HCV evasion of the host immune response (Flint and McKeating, 2000). The N-terminus of the E2 protein displays a high degree of amino acid variation (Mizushima, 1994). This hypervariable region, located at residue 1-27 of E2 or residue 384-410 of the polyprotein (Farci et al., 1996), is known as hypervariable region 1 or HVR1 and it is displayed on the virion surface forming a neutralizing epitope (Weiner et al., 1991). Forns *et al* demonstrated that antibodies to HVR1 change throughout the course of an infection suggesting that HCV is subjected to immune pressure (Forns et al., 1999). The second hypervariable region, HVR2, of E2 is found at amino acids 474-482 in the HCV genotype 1 (Yagnik et al., 2000). It has been implicated with CD81 receptor binding (Yagnik et al., 2000, Roccasecca et al., 2003). A third hypervariable region HVR3, aa 431-466, is probably involved in binding and viral entry (Troesch et al., 2006).

1.4.1.3 p7 protein

p7 is a small hydrophobic protein 63 amino acids in size and contains 2 trans-membrane alpha helices which are separated by a conserved basic loop (Lin et al., 1994a, Mizushima et al., 1994, Carrere-Kremer et al., 2002). Jones *et al* (Jones et al., 2007) showed that p7 was not needed for RNA replication but played an important role in the early stage of virion formation before the assembly of the infectious virus. p7, however, is not required for HCV entry into new host (Steinmann et al., 2007). Deleting the p7 did not result in any significant difference in RNA replication at various time points post-transfection but when the supernatant of cultures infected with the deletion mutant was used to infect naïve cells, no detectable infectious virus was obtained. However, other studies by Russell (Russell et al., 2008) showed that p7 mutations results in an increase of virus production.

Gonzalez and Carrasco (Gonzalez and Carrasco, 2003) showed that p7 belongs to a viral protein group called viroporins which includes the influenza A virus' M2 ion channel. p7, which is probably responsible for the flow of calcium ions from the ER into the cytoplasm as has been observed for other viroporins (Tian et al., 1995, van Kuppeveld et al., 1997), was demonstrated as an ion channel using black lipid membrane conductivity system where fusion protein GST-p7 caused a flow of current across an insulating membrane (Griffin et al., 2003). It is also unknown whether p7 is a structural component of HCV particles. Steinmann *et al* (Steinmann et al., 2007) showed that virus entry is not dependent on p7 because the specific infectivity of virions with a defective p7 released was not affected. Yet, a study by Griffin et al (Griffin et al., 2008) showed that cells cultured in infectious supernatants together with cation channel inhibitors partially inhibited infection implicating p7 as a structural component of the viral particle.

1.4.2 Non-structural proteins

The non-structural proteins of HCV include NS2, NS3, NS4A, NS4B, NS5A, and NS5B which make up the remaining portion of the polyprotein. These proteins play important roles as proteases and polymerase.

1.4.2.1 NS2 protein

The NS2 protein (21kDa) is comprised of amino acids 810 to 1026 of the polyprotein (Major, 1997). The NS2 protein together with part of the NS3 serine protease domain form the NS2-3 protease which allows autoproteolytic cleavage to occur at the junction between NS2 and NS3 (Grakoui, 1993). The crystal structure of the catalytic domain of the NS2-3 protease of NS2 has revealed that it functions as a dimer

(Lorenz et al., 2006). This virus-encoded protease requires zinc to function (Hijikata, 1993a). Santolini (1995) showed that NS2 is a transmembrane protein and Dubuisson (1994) showed that cleavage between E2 and NS2 occurs only after the completion of E2 folding. The E2 and NS2 cleavage is mediated by a cellular signal peptidase.

NS2 is not required for genome replication (Lohmann, 1999) although a study using J6/JFH chimeric viruses where NS2 has been deleted completely or partially showed a decrease in virus production (Jones et al., 2007). A study by Yi et al (Yi et al., 2009) showed that substitution of Ser-168 with Ala or Gly impaired the production of infectious virus particles in cells transfected with HJ3-5 chimera RNA and JFH1 RNA. The association of core or NS5A proteins with lipid droplets of host cell and the prevention of the assembly of core into particles with sedimentation and buoyant density properties similar to infectious virus were not changed with S168A mutation showing that NS2 acts subsequent to the core, NS5A, and NS3 involvement in particle assembly (Yi et al., 2009). NS2 is also found to be important for virus assembly probably by bringing HCV glycoproteins and nascent particles together to form infectious HCV virions (Jones and McLauchlan, 2010).

1.4.2.2 NS3 protein

NS3 protein is 70kDa in size and is hydrophilic in nature (Reed, 2000) and it encompasses amino acids 1027 to 1657 (Major, 1997). As mentioned in the paragraph above, part of NS3 and NS2 form the NS2-3 protease that cleaves the NS2 and NS3 junction. NS3 was initially predicted to be a serine protease, nucleotide triphosphatase (NTPase) and RNA helicase based on sequence motifs. Chambers (1990) provided the evidence that the N-terminal domain of NS3 is a serine protease in a flavivirus homologue. The domains between amino acids 1027 until 1207 of NS3

(Lin, 1994) protein are responsible for cleavages at the junctions 3/4A, 4A/4B, 4B/5A, and 5A/5B but these cleavages occur only after the release of the remaining downstream NS protein after the NS 2/3 cleavage (Major, 1997). The other two functions of NS3, NTPase and RNA helicase, are involved in the RNA replication of the virus unwinding the double stranded RNAs resulting from the synthesis of the complementary strand. These activities have been shown for the C-terminal two-thirds of NS3 protein when using synthetic substrates or full-length NS3 *in vitro* (Reed, 2000) and references therein). The functional domain of the NTPase/helicase resides between amino acids 1209 and 1608 of the NS3 protein (Kim, 1997). NS3 with NS4A function as a second viral protease responsible for cleaving four sites downstream within the HCV polyprotein as well as host proteins TRIF and Cardif. TRIF and Cardif play a role in double stranded RNA signaling to induce intracellular antiviral response (Li et al., 2005b, Meylan et al., 2005).

NS3 was shown to play a role in the assembly stage before the assembly of core-containing particles. In the absence of Q221L mutation, a mutation within subdomain 1 of the hepatitis C virus (HCV) NS3 helicase, NS3 were recruited to core protein on the surface of lipid droplets, but there was no assembly of core into high-density, rapidly sedimenting particles (Ma et al., 2008).

1.4.2.3 NS4A and NS4B proteins

NS4 protein is divided into NS4A and NS4B. NS4A (6kDa) spans amino acids 1658 to 1711 whereas NS4B (27kDa) spans amino acids 1712 to 1972 (Major, 1997). NS4A has a hydrophobic N-terminus and hydrophilic C-terminus (Failla, 1994). NS4A stimulates cleavages *in cis* and *in trans* at the junctions of 3/4A, 4A/4B, 4B/5A, and 5A/5B which are mediated by the NS3 serine protease (Major, 1997) and

references therein). The hydrophobic N-terminus of NS4A helps to anchor NS3 and other replication complex members to the cellular membranes (Hijikata, 1993c, Steer, 1996). NS4B is a hydrophobic protein and has a modular domain organization with the amino- (N) and carboxy- (C) terminal ends being cytoplasmic and a central region inserted in the ER membrane. Carboxy-terminal domain of NS4B is associated to membranes and is important for replication (Liefhebber et al., 2009). NS4B alters the ER to generate a scaffold for the replication complex (Gosert et al., 2003). M6 mutant of JFH1, producing a single amino acid change in NS4B, has a higher viral titers than the wild type but the same mutation in J6-JFH1 did not result in increase virus titers compared to J6/JFH1 with no M6 mutation (Jones et al., 2009). It is then suggested that NS4B interacts with other viral factors to modulate assembly (Jones and McLauchlan, 2010).

1.4.2.4 NS5A and NS5B proteins

NS5A protein is 58kDa in size and it encompasses amino acids 1973 to 2420 (Major, 1997). Earlier studies suggested that NS5A may play a role in causing sensitivity to interferon (Enomoto, 1995, Enomoto, 1996). Otherwise, the function of NS5A is not really clear although Appel et al (Appel et al., 2005) has shown that it is essential for viral replication. Interaction of NS5A with lipid droplets are prevented when mutations are introduced in domain I, one of the three domains of NS5A (Miyanari et al., 2007). Appel *et al* (Appel et al., 2008) showed that core and NS5A colocalize on the lipid droplets which are proposed as an assembly site of HCV particle and deletion in domain III of NS5A disrupts the colocalization abolishing the formation of infectious particle which leads to accumulation of core proteins on the lipid droplets. The substitutions of alanine for a cluster of serine residues in domain III of NS5A disrupts the phosphorylation of NS5A, decreasing NS5A-core interaction, disrupting

the subcellular localization of NS5A and eventually decreasing virion production (Masaki et al., 2008).

NS5B protein (68kDa) is an RNA-dependent RNA polymerase that encompasses amino acids 2421 to 3011 (Major, 1997). RNA-dependent RNA polymerase activity *in vitro* has been shown in an NS5B protein expressed in baculovirus system (Behrens, 1996, Lohmann, 1997). There could also be a nucleotidyl transferase activity (Behrens, 1996) but inconsistencies in other reported studies may suggest that this activity is due to contamination by host enzymes (Reed, 2000).

1.5 Hepatitis C virus infection

Hepatitis C virus (HCV) produces an infection of the liver, which is mainly spread by direct contact with human blood. The major causes of HCV infection globally are unscreened blood in blood transfusion and re-utilization of inadequately sterilized needles and syringes (WHO, 1999a). HCV has a narrow host range comprising humans and chimpanzees as the susceptible species (Dubuisson and Rice, 1996). The disease developed in both species is similar.

In 1999, WHO estimated 170 million people worldwide were infected with HCV. In general, acute HCV infection gives rise to a mild, acute illness but 70-80% of acutely infected individuals become chronically infected. Cirrhosis, an end-stage liver disease characterized by an irreversible scarring of the liver, occurs in less than 20% of the hepatitis C infected patients. Individuals with hepatitis C cirrhosis are also at risk of developing hepatocellular carcinoma where 1-5% of patients with chronic infection for a period of 20-30 years develop liver cancer (WHO, 1999b). In Asia and

Africa, 15% of hepatocellular carcinoma cases originate from HCV infection (WHO, 1999a).

The major site of HCV replication is the liver but there are other possible replication sites as well such as epithelial cells in the gut (Deforges, 2004), peripheral blood lymphocytes (Lindenbach, 2001), and central nervous system (Forton et al., 2004). It has yet to be determined the percentage of infected cells in the liver or the amount of viral RNA and protein associated with infected cells (Wieland and Chisari, 2005) although a study made by Bigger *et al* (Bigger et al., 2001) estimated using viral RNA measurement that about 10% of chimpanzee hepatocytes support HCV replication in an acute infection.

Chronically infected patients have typical viral loads in the serum ranging 10^3-10^7 genomes/ml estimated by Lindenbach (Lindenbach, 2001) or $2x10^5$ – $4x10^7$ genomes/ml estimated by (Adinolfi L, 2001). In a study of mathematical modeling of viral dynamics during treatment with α-interferon, it was found that HCV virion turn over is rapid with a half-life of 3 hours and up to 10^{12} viruses are produced per day in an infected person (Neumann et al., 1998).

HCV, once known as non-A, non-B hepatitis (NANBH), was first definitively identified by screening cDNA expression libraries made from total nucleic acid extracted from a large pool of plasma containing high titer of NANBH agents derived from chimpanzees infected with serum from patients diagnosed clinically with NANBH (Alter et al., 1989a). There have subsequently been studies on gene expression and RNA replication but the mechanism of HCV pathogenicity remains

unclear because, not until 2005, has there been a permissive cell culture system in which to study viral attachment, penetration, uncoating, assembly, release and reinfection.

Limited studies on the replication of synthetic replicons and observations of virus recovered from clinical material suggest that certain stages of HCV replication are interconnected with the lipid metabolism processes of the host cell and also that the virus may be complexed in lipo-viro-particles (LVP), and in assembly, release, protection from humoral mediated clearance and reinfection of susceptible hepatocytes, gain from its association with host lipoprotein.

1.5.1 Acute hepatitis

Acute hepatitis C is a short-term illness occurring in the first 6 months period after initial exposure to hepatitis C virus. The incubation period of HCV infection from the initial exposure to the clinical symptoms ranges from 6-8 weeks (Alter et al., 1989a, Barrera et al., 1995). Between 60-70% of the infected individuals is asymptomatic including those who progress to chronic infection. For the minority of those who have acute phase symptoms, the complaints are generally mild and non-specific with fatigue and jaundice as the more common ones. Other symptoms include decreased appetite, abdominal pain, nausea and dark urine.

HCV RNA can be found in the blood plasma as early as a few days after infection and usually peaks 6-8 weeks later (Abe et al., 1992, Beach et al., 1992, Alter et al., 1995). Specific antibodies against HCV can be detected about 7-8 weeks after infection (Pawlotsky, 1999). Serum alanine transaminase (ALT) level, which is commonly

used to screen for liver problems, peaks at approximately 2-3 months after infection and this coincides with the decline of HCV RNA (Rehermann and Nascimbeni, 2005). ALT level returns to normal level after approximately 3 months. About 80% of the acutely infected individuals progress to the chronic stage.

In a study by Bukh et al (Bukh et al., 2001), infected chimpanzees have viraemia with peak virus titres of 10^5-10^7 g.e./ml three to four days after exposure. This is followed by an elevated ALT level indicating the onset of hepatic damage (Abe et al., 1992, Farci et al., 1992b, Shimizu et al., 1990).

1.5.2 Chronic hepatitis

Chronic hepatitis is defined as HCV infection persisting more than 6 months where in the early stage, the symptoms are non-existent or mild (similar to those described in acute hepatitis section). The progression of chronic hepatitis C varies for each infected individuals with variable rates of fibrosis (liver scarring). Cirrhosis develops in 20-30% of chronically infected individuals with raised ALT levels (Di Bisceglie et al., 1991, Takahashi et al., 1993, Yano et al., 1996). Factors that contribute to speed up the progression to cirrhosis include old age, HCV genetic heterogeneity, alcohol consumption, immunodeficiency, viral titre, and HIV co-infection (Takahashi et al., 1993, WHO, 1999a, Yano et al., 1996, Di Bisceglie et al., 1991, Graham et al., 2001). In a Japanese study, the incidence of hepatocellular carcinoma was 10.4% in chronic hepatitis C cases which was 2.7 times higher than in hepatitis B cases (Takano et al., 1995).

During the chronic phase, HCV RNA can still be detected in the blood (Alter et al., 1989a, Farci et al., 1991, Conry-Cantilena et al., 1996) where most patients have stable viral titres that are 2-3 logs lower than in the acute phase (Rehermann and Nascimbeni, 2005). In most patients with chronic infection, ALT levels continue to fluctuate after the initial peak during the acute phase (Rehermann and Nascimbeni, 2005). For most chronic patients who have normal ALT levels, the symptoms are mild or subclinical with little damage occurring to the liver tissue whereas for most who have elevated ALT, they show mild hepatitis (Shindo et al., 1995, Healey et al., 1995).

The end stage liver disease in humans includes fibrosis and cirrhosis. Unlike in humans, no liver fibrosis or cirrhosis are observed in chimpanzees but instead there are changes to the liver morphology and necroinflammatory hepatic lesions which are similar to those found in humans (Alter et al., 1978, Tabor et al., 1978, Shimizu et al., 1979).

1.5.3 HCV-related steatosis

Infection of HCV is mainly restricted to hepatocytes (Huang et al., 2007a) which are the major cells involved in cholesterol homeostasis. Steatosis is a common histological feature in patients with chronic HCV hepatitis (Giusti et al., 1993). Steatosis is a condition where liver becomes a lipid storage space rather than as in otherwise normal conditions, adipocytes. There is an accumulation, in the liver, of neutral lipids (triacylglycerol and cholesterol esters). Other factors such as obesity, high alcohol intake, diabetes type II and hyperlidaemia may also contribute to

steatosis in chronic hepatitis C patients. HCV genotype 3 is particularly associated with steatosis.

1.6 Epidemiology

1.6.1 Prevalence

In 1999, WHO estimated prevalence of HCV to be 3% of the world's population (WHO, 1999a). Most of the prevalence studies have used blood donors and may be underestimations as the blood donor population may not be representative of the community at large.

The regions with the highest prevalence rates include Africa and Eastern Mediterranean regions with 5.3% and 4.6% prevalence rate respectively whereas the lowest include Americas and Europe regions with prevalence rates of 1.7% and 1.03% respectively (WHO, 1999a). South-East Asia and Western Pacific regions have prevalence rates of 2.15% and 3.9% respectively (WHO, 1999a). Figure 2 below shows a world map on the global prevalence of Hepatitis C infection in 2002.

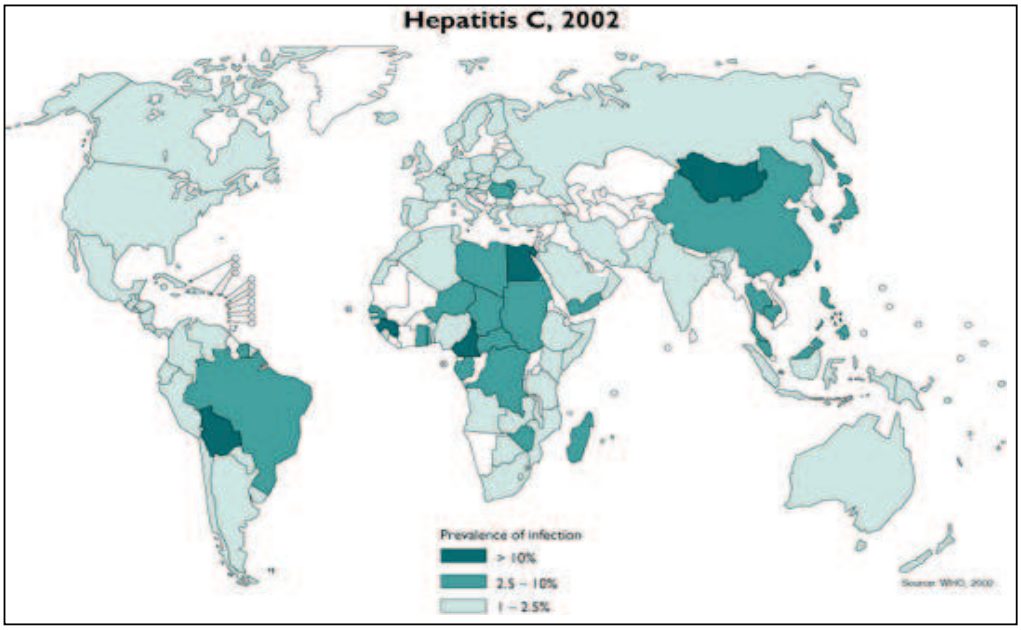


Figure 2: Global prevalence of Hepatitis C infection, 2002.
Source: World Health Organization (WHO)

Egypt was found to have probably the highest reported seroprevalence rate of 22% (Frank et al., 2000) and in another Egyptian study by (Saeed et al., 1991), the prevalence rate is 28%. The laboratory reports of hepatitis infection in England in the Annual Report of (Health Protection Agency, 2008), UK shows an increase of 12.2% to 7540 cases from the year 2006 to 2007.

1.6.2 Transmission

1.6.2.1 Intravenous drug users

Intravenous drug use is the main route of transmission for HCV infection in the developed world. In a study by (Judd et al., 2005) of 428 intravenous drug users with age below 30 in London, 44% had hepatitis C antibodies compared to 4% with HIV. Intravenous drug use has been the major mode of HCV transmission for more than 30 years accounting for 68% and 80% of current infections in the USA and Australia respectively (Alter, 2002, Dore et al., 2003). The prevalence rate in intravenous drug

users for HCV infection in seven European Union countries was about 80% (Touzet et al., 2000). In Chang Rai, Thailand 90% of the intravenous drug users were found to be positive for HCV (Apichartpiyakul et al., 1999).

1.6.2.2 Therapeutic injections

Unlike in the developed world, most countries in the developing world have limited supplies of sterile syringes in their health care settings. In the year 2000 Global Burden of Disease study by Hauri, *et al.*, it was found that contaminated injections in the health care settings caused about 2 million HCV infections of which most are found in the South-east Asian, Pacific, and Eastern Mediterranean regions (Hauri et al., 2004).

1.6.2.3 Blood Transfusions

In the past, blood transfusion has been the leading cause of HCV transmission in most developed countries but is now overtaken by intravenous drug use largely due to the improved screening method for hepatitis C. In England, cases of infected donations decreased from 1 in 520, 000 from 1993-1998 to 1 in 30 million in 1999 to 2001 when blood donations were tested for HCV RNA (Soldan et al., 2003).

In the developing world, the incidence of hepatitis C contaminated blood transfusion remains higher. WHO estimated that in the developing world, 43% of the donated blood is not adequately screened for transfusion-transmitted infections which include HCV (WHO, 2001). In a study of 147 Chilean chronic hepatitis C patients, the common risk factors include blood transfusion (54%), intravenous drug use (5%), and risky sexual practices (2%) (Soza et al., 2004).

1.6.2.4 Hemodialysis

A study based on data collected for hemodialysis patients from 136 European centres identifies the possible risk factors for virus transmission as failure to disinfect devices between patients, sharing of single-use vials for drug preparation or for infusions, inadequate sterilization technique or cleaning of machines, poor environmental cleanliness, and distance between chairs of less than a metre (Zampieron et al., 2004).

1.6.2.5 Sexual Activity

Non-percutaneous transmission such as sexual transmission of HCV is far less efficient compared to other sexually transmitted viruses. The efficiency of male to female sexual transmission of HCV is less than HIV (Eyster et al., 1991). In a study by Alter (Alter et al., 1989b), sex with infected partner or multiple partners was identified as risk factors for NANB virus transmission. In cases of acute NANB hepatitis intensive surveillance studies conducted by the USA Centers for Disease Control over a 7-year period, it was found that 6% of the patients were sexually exposed and this did not vary over time (Alter et al., 1990). However, the role of sexual activity in the transmission of HCV remains unclear and the increased likelihood of HCV transmissions could be due to high risk sexual practices (eg. promiscuity) or sexually transmitted coinfection (eg. HIV coinfection) or simply high risk activity in the high risk group (eg. intravenous drug use). In a 10-year prospective follow-up Italian study there is little or no risk of sexual transmission of HCV among heterosexual monogamous couples (Vandelli et al., 2004).

1.6.2.6 Vertical transmission

Mother to baby transmission of HCV, another example of non-percutaneous transmission, is uncommon. In a study by Reinus *et al* (1992), it was found that all the babies of anti-HCV positive mothers have anti-HCV antibody themselves but the

antibody disappeared or reduced with time. Vertical transmission is linked to the viremia level of the mother with higher than 10^6 copies/ml of HCV greatly increasing the probability of the babies being infected (Ohto et al., 1994, Lin et al., 1994b). A number of studies showed an increased likelihood of vertical HCV transmission in babies born to mothers with HCV/HIV coinfection (Ohto et al., 1994, Lin et al., 1994b, Lam et al., 1993, Zanetti et al., 1995).

1.7 Diagnosis

During the acute phase, diagnosis of HCV infection is not generally made as those infected are normally not ill enough to seek medical attention. Infected individuals may be diagnosed based on their medical history such as history of drug abuse, blood transfusion, *etc*. When suspected of HCV infection, infected individuals will normally have their blood tested for anti-HCV antibodies.

Enzyme immunoassay (EIA) is used in serological tests to detect anti-HCV antibodies using recombinant proteins or synthetic peptides of core, NS3, NS4, and NS5 proteins. The third generation enzyme-linked immunosorbent assay (ELISA3) has a sensitivity of 99% and specificity of 100% in patients with chronic liver disease (Colin et al., 2001). Acute hepatitis which may not be detected by the EIA technique can be detected using a more sensitive technique such as RT-PCR for the detection of HCV RNA.

Diagnostic genotyping assays using simultaneous PCR amplification and direct sequencing (CLIP sequencing) (Ross et al., 2000) and restriction fragment length polymorphism (RFLP) (Davidson, 1998) for HCV RNA based on sequence analysis

of the genome normally target the 5' untranslated region because although this region is highly conserved, there are well characterized polymorphisms that are used to predict HCV genotypes.

1.8 Treatment and vaccination

The development of an effective vaccine against HCV has been hampered because of the complexity of the immune response to the virus. Some studies have shown that HCV infection does not prevent reinfection (Farci et al., 1992a, Abe et al., 1992), others, however, indicate that prior immunity may be beneficial (Prince et al., 1992, Bassett et al., 2001, Major et al., 2002).

Passively administered anti-HCV antibodies do not provide protection to HCV infection as seen in the study of liver transplant patients made by Davis (Davis et al., 2005) where neither low dose (75 mg/kg) nor high dose (200 mg/kg) of human hepatitis C Immune Globulin (HCIG) (Civacir), a pooled HCV-positive plasma antibody, decrease serum HCV RNA. In a similar randomized, double-blind, dose escalation study by Schiano (Schiano et al., 2006) using a high-affinity, neutralizing, fully human, anti-E2 monoclonal antibody in liver transplant patients, there is a decrease of HCV RNA detected but the decrease is not sustained. In a study using chimpanzees, HCIG treatment only prolonged the incubation period of HCV infection but did not delay or prevent HCV infection (Krawczynski et al., 1996).

1.9 Immunological response to hepatitis C virus

HCV induces a strong innate immune response but is able to evade both innate and adaptive immune responses. Apart from liver cells, HCV RNA has been reported to

be found in B cells, dendritic cells, monocytes, and lymph node cells (Dustin and Rice, 2007) which are cells involved in the host immune response.

1.9.1 Innate immune respone

In humans, after infection, HCV begins to replicate immediately as viremia can be generally detected 1-2 weeks after exposure (Thimme et al., 2001). Interferon (IFN) production against HCV is rapidly induced by host's innate virus-sensing mechanisms as studies on HCV infected chimpanzee showed only up to 10% of hepatocytes support HCV replication at the peak of viraemia (Bigger et al., 2001) suggesting that an antiviral state, probably IFN mediated, is rapidly induced in most cells of the liver (Dustin and Rice, 2007). Following RNA helicase recognition of HCV dsRNA, RIG-I (retinoic acid inducible gene-I) is activated (Sumpter et al., 2005). TLR3 (Toll-like receptor 3) recognition of dsRNA also induced IFN production (Akira et al., 2006). Host response triggering of RIG-I and TLR3 results in IFN production and IFN-stimulated genes (ISG) expression which could result in resolution of the infection (15-25% of the cases) or attenuation of IFN production and ISG expression resulting in persistent infection (Li et al., 2005a).

In the liver of an acutely-infected chimpanzee, changes in the transcription of IFN-α occurs as early as 2 days after infection and these changes parallels the viral replication kinetics (Alter, 2002, Bigger et al., 2001). However in most cases IFN-α fails to inhibit replication of HCV (Alter, 2002). Treatment with exogenous IFN-α is however effective as it is currently in use for the treatment of chronically infected patients (Pawlotsky, 2003, Zeuzem, 2004).

The activation state of natural killer T (NKT) cells which are one of the many kinds of innate immune cells that can be found in the liver, may be altered during persistent HCV infection (Durante-Mangoni et al., 2004, Lucas et al., 2003).

1.9.2 Adaptive immune response

Among all persistent human viruses, HCV is among the most successful as it persists in nearly 70% of the infected individuals. In HCV infection, virus specific antibodies are usually detectable about 7-8 weeks after exposure (Pawlotsky, 1999). Patients with chronic HCV hepatitis have IgM persisting even after acute infection (Quiroga et al., 1991). In a study made by (Chen et al., 1999) where the humoral response to HCV was evaluated from 60 chronically and 12 acutely infected patients, it was found that except for HCV core antigens, the IgG response was highly restricted to IgG1 isotype, was of relatively low titer, and antibody appearance delayed. In both human and chimpanzee studies, the role of naturally acquired antibodies in HCV infection is limited because they do not prevent reinfection (Farci et al., 1992a, Lai et al., 1994). HCV specific antibodies are also not detected 10-20 years after recovery (Takaki et al., 2000). However, there ~~are~~ is evidence~~s~~ that HCV specific antibodies can influence the course of the infection. HCV clearance in acute self-limiting infections is associated with the production of anti-HVR1 (envelope) antibodies (Zibert et al., 1997). HCV-specific neutralizing antibodies have been found in the plasma of chronically infected patients by *in vitro* neutralization by the capacity of HCV-positive inocula to infect continuous T-cell lines (Shimizu, 1994) and chimpanzees (Farci, 1994). These antibodies can protect against infection by HCV strains previously present in the patients from which they are derived but fail to neutralize viral strains prevalent in patients at the time the antibodies are detected. The

neutralizing antibody response appears to be directed against epitopes located within the highly variable HCV envelope proteins (Chisari, 1997). In a study made by (Allander et al., 2000), antibodies against HCV E1 and E2 were able to modulate HCV RNA levels in vaccinated and rechallenged chimpanzees.

The risk of persistence upon re-exposure of HCV is greatly reduced after the resolution of an acute infection (Bowen and Walker, 2005). In both humans and chimpanzees, expansion of antiviral $CD4^+$ and $CD8^+$ T cells are important for the control of acute primary viral replication (Thimme et al., 2001, Thimme et al., 2002, Cooper et al., 1999). In the blood samples of recovered HCV-infected patients, specific multi-epitope $CD4^+$ and $CD8^+$ T cells are readily detected compared to chronic hepatitis C patients ~~which~~ who tend to have ~~a~~ much more focus T-cell responses (Lechner et al., 2000b, Wedemeyer et al., 2002, Diepolder et al., 1995). In the *in vivo* study using the chimpanzee model, depletion of either CD4+ or CD8+ T cells prevent the clearance of HCV and subsequently recovery from infection (Shoukry et al., 2003, Grakoui et al., 2003). In chronically infected individuals or in the individuals who had initially controlled infection but viraemia recurred, the CD4+ and CD8+ T cells response to the virus is not readily detectable (Gerlach et al., 1999, Thimme et al., 2001, Chang et al., 2001, Lechner et al., 2000a).

1.9.3 Immune escape variants

Immune escape quasispecies variants may arise from or is a result of selection pressure in CD8 cytotoxic T-lymphocyte (CTL) epitopes or antibody responses. These immune escape variants will then be able to replicate and predominate in the infected individuals particularly those who progress to the chronic stage.

Weiner (Weiner et al., 1992) suggested that E2 hypervariable HCV variants are a result of immune selection of escape mutants. In a study made by (van Doorn et al., 1995), the HVR1 was conserved for 6 years in a chronically HCV-infected chimpanzee and this coincides with no detectable anti-HVR1 but in the following year, anti-HVR1 IgG was produced and this coincides with changes in HVR1 suggesting HCV HVR1 evolution through immune selection. It was found in a 6-year-period study, common variable immunodeficiency (CVID) HCV-infected patients whose humoral immune response are compromised had 0.415 nucleotide substitutions a year compared to 6.954 in the control HCV-infected patients ($p < 0.02$) suggesting that the reduced humoral immune response pressure resulted in fewer immune escape variants emergence (Booth et al., 1998).

In an acute HCV infection, a strong T-cell immune response is associated with viral clearance but in a chronic HCV infection, a weak or absent T-cell response is ~~associated~~ found. Tsai (Tsai et al., 1998) showed that HVR1 HCV variants with altered peptide ligands with a more capable CTL activity emerged in 2 patients who developed chronic infection but no virus variants was found in 3 patients who recovered. Weiner (Weiner et al., 1995) demonstrated that immune escape variants arising from CTL response was aided by amino acid substitution (aspartic acid to glutamic acid) at position 1449 in all HCV genomes that were analysed.

1.10 HCV Model systems

1.10.1 HCV in animal model

The animal historically and still frequently used in HCV studies which can be infected with HCV is the chimpanzee (*Pan troglodytes*). In fact it was a large pool of chimpanzees blood plasma containing high titer of what was then known as NANBH agents that led to HCV being identified (Choo et al., 1989). HCV infection in chimpanzees has a similar course as that of humans making it a good animal model for HCV infection giving researchers better understanding on the nature of the innate and adaptive immune responses to HCV (Bukh et al., 2001, Bukh, 2004). HCV infection in chimpanzees is, however, milder because they clear HCV better than humans and persistent infection shows a milder form of the disease (Dustin and Rice, 2007). The disadvantages of using chimpanzees in the study of HCV infection include avaibility of chimpanzee, high maintenance costs and ethical problems regarding their usage.

Other animal like mice, Tamarins (*Saguinis* sp) and tree shrews have also been used in HCV studies. Transgenic mice that could express HCV genes in the liver have been developed but they have limitations as mice do not support HCV infection (Guha et al., 2005). SCID mice have been used to transplant human hepatocyte xenografts which can then be infected with HCV (Bartenschlager et al., 2004, Guha et al., 2005) but for the immunological study using these mice to be useful, they must have human immune cells operating with the same HLA type as the hepatocytes (Dustin and Rice, 2007). Tamarins infected with GB virus B (GBV-B) have been proposed as a surrogate model for HCV infection in humans. GBV-B is closely related to HCV phylogenetically and it has a tropism for hepatocytes (Ohba et al., 1996).

1.10.2 HCV in tissue culture

An early attempt of producing HCV *in vitro* includes the recombinant baculovirus expressing HCV glycoproteins of HCV virus-like particles (VLPs) generated in insect cells which have been used to study the characteristics of mammalian cell-derived soluble truncated E2 proteins (Clayton et al., 2002). It still remains unknown why most clinically isolated HCV is difficult to replicate in tissue culture (Lindenbach et al., 2005) making it difficult to study HCV replication. In an attempt to study the pathogenesis of HCV infection, several infection systems of the cultured cells had been developed. One of these is the development of the replicon system. Cloned virus genome is preferred over HCV-containing patient material because it can be easily manipulated and allows detailed genetic analysis of viral functions. Unlike other positive-strand RNA viruses, the production of HCV from cells transfected with RNA from an *in vitro* transcription of a cDNA copy of the viral genome has been difficult (Bartenschlager, 2000).

Lohmann (1999, Bartenschlager) developed selectable subgenomic HCV RNA molecules that were able to replicate after transfection into the human hepatoma cell line Huh7. The subgenomic HCV RNA contained the HCV 5'NTR, a small segment of the core gene, a neomycin phosphotransferase (*neo)* gene, the encephalomyocarditis virus IRES (E-1), HCV NS2-5B or NS3-5B and the 3'NTR. Upon transfection into Huh7 cells, only cells that support the replication of the HCV RNA would amplified the *neo* gene and developed resistance to G418 drug. Hence, only these cells survived whereas, the cells that did not support replication were eradicated. However, the original replicons replicated at low level.

Other successful attempts were also made to produce subgenomic replicons that were adapted for efficient replication in Huh7 cell line and other cultured cells (Blight, 2000, Kato, 2003, Bukh, 2004). In every case, successful replicons were shown to possess a number of adaptive mutations essential for high level replication of the replicon. However, full length genomes containing cell culture-adaptive mutations did not produce infectious virus particles in culture (Blight, 2002, Pietschmann, 2002).

The year 2005 had seen an important breakthrough for the study on HCV. Since HCV was first definitively identified by (Alter et al., 1989a), it proved to be difficult to study the mechanism of HCV pathogenicity because of the lack of a permissive cell culture system. In year 2005 three groups; Wakita, Chisari and Rice, have concurrently produced cell culture systems for the modelling of HCV infection and subsequently the production of infectious virus particles (Wakita et al., 2005, Zhong, 2005, Lindenbach et al., 2005).

Infectivity of HCV *in vitro* requires a permissive and robust cell culture system. A subgenomic replicon, JFH1 genotype 2a strain, transfected into a human hepatoma cell line (Huh7) successfully replicated and subsequently viral particles were secreted (Wakita et al., 2005). The strain was initially isolated from a patient with a severe and sudden onset of HCV infection. The replicon constructed was able to replicate efficiently in cell culture and did not require the adaptive mutation which had characterized previous successful replicons.

Meanwhile, Rice's group developed a new cell line, Huh7.5, derived from the Huh7 human hepatoma cell line with enhanced ability to support the replication of HCV replicons (Zhong, 2005). Introduction of the JFH1 replicon into Huh7.5 cells resulted in high levels of replication and the release of HCV virions capable of reinfection of naïve cells.

Lindenbach (2005) developed a chimeric genome consisting of JFH1 nonstructural genes with structural genes from J6, another genotype 2a strain which by itself is not infectious in cell culture, resulting in a production of infectious HCV cell culture (HCVcc) capable of more efficient replication than the original JFH strain. This is important for the study on the identification of genetic determinants of pathogenesis.

HCV pseudoviral particles (HCVpp) has provided us a lot of insights on the biology of HCV particularly on the binding and entry of HCV into cells but HCVpp has some shortcomings such as HCVpp are normally made in nonhepatic cells like 293T kidney cells which do not synthesize lipoproteins (Meunier et al., 2008). Most of the components are also non-HCV components for example the HCV proteins may not be folded in the correct way. HCVcc on the other hand, are made in the hepatic cells which are natural hosts of HCV and hence, the particles produced are more like its *in vivo* counterpart. The mechanism of its production would probably also follow~~s~~ the natural pathway (Meunier et al., 2008).

1.11 Cellular receptors involved in HCV entry into cells

There are four systems that have been used for investigating HCV receptors; *in vivo* derived HCV, recombinant viral glycoprotein, HCVpp, and HCVcc. Four putative

receptors have been implicated in the entry of HCV into cells, CD81, SR-B1 receptor, and tight junction molecules Claudin-1(CLDN1) and occludin (OCLN). Glycosaminoglycans (such as heparan sulphate) and LDL-receptor have also been implicated as initial attachment factors. Figure 3 shows the possible receptors / molecules involve in aiding the HCV entry into a cell.

In vivo or serum derived HCV had been used to study HCV binding, association, and internalization of purified materials. By quantifying HCV RNA to represent the amount of bound or internalized virus, Andre et al (Andre et al., 2002) found that there is maximum association with purified LVP when comparing serum, whole-density fraction corresponding to VLDL, the same fraction with depleted LVP, and purified LVP of the serum. There is, however, limitations to using serum-derived HCV because of low level of HCV replication in infected cells, difficulties in differentiating the input and output HCV RNA, the absence/low levels of HCV particles produced, and the heterogeneity of the virus in the serum (Burlone and Budkowska, 2009).

Pseudovirusparticles expressing E1 and E2 glycoproteins have been used in a number of HCV viral entry study (Bartenschlager et al., 2004, Bartosch et al., 2003a, Hsu et al., 2003).

CD81, a 26kDa cell surface protein, is found on a number of cells in human tissue and has many different kinds of functions being involved in cell adhesion, motility, metastasis, and signal transduction (Levy, 1998). It contains four transmembrane domains, two small extracellular loops (SEL), one large extracellular loop (LEL), and

N- and C- terminal intracellular domains. (Pileri et al., 1998) Non-hepatic human cells expressing CD81 were also found to be poorly or none permissive to infection (Bartosch et al., 2003a). Non-permissive hepatic cell lines such as HepG2 and HH29 which normally do not express CD81 are not susceptible to HCVcc and HCVpp infection but will become susceptible once they are transduced with and expressed CD81 (Bartosch et al., 2003a, Cormier, 2004, Lavillette et al., 2005, Zhang, 2004). Anti-CD81 monoclonal antibodies prevent the entry of HCV entry into cells (Cormier, 2004). These studies support the importance of CD81 for the entry of HCV into cells. However, there are studies that contradict these findings. Petracca (2000) demonstrate that CD81 has poor capacity to allow viral entry.

Scavenger receptor class B type I (SR-B1/Cla1), 509 aa cell membrane proteins (Acton et al., 1994), are alternative receptors to the LDL receptor for the uptake of cholesterol and lipids. They can be found in many mammalian cells but mostly expressed in liver and steroidogenic tissues. They bind chemically modified lipoproteins such as acetylated and oxidized LDL (Goldstein, 1979). SR-B1, which is 82kDa in size together with its non-protein components (Babitt, 1997), is a high affinity receptor for HDL and it allows the selective uptake of esterified cholesterol from HDL (Rigotti, 1995, Acton, 1996, Liadaki, 2000). SR-B1 can bind both HDL and LDL (Krieger, 2001) and it is found abundantly on hepatocytes (Calvo, 1997). Recombinant HCV E2 has been shown to bind specifically to SR-B1 and the HCV E2 recognition was inhibited by an HVR1-E2 monoclonal antibody (Scarselli et al., 2002). SR-B1 together with CD81 facilitates the entry of HCV. Some study showed that HDL, which is a ligand for SR-B1, enhanced HCVcc infectivity only when CD81 was expressed (Zeisel et al., 2007).

Claudin-1 (211 aa) is found in all epithelial tissues but mainly in the liver. In non-hepatic cell lines such as 293T and SW13, it was found that Claudin-1 expression, allows the infection of HCVpp in these cells to occur (Evans et al., 2007). Functional studies showed that binding to Claudin-1 occurs after CD81 and SR-B1 binding (Evans et al., 2007). Recently, it was shown that there is a correlation in the Claudin-1 distribution to the susceptibility to HCV infection in cells (Liu et al., 2009, Yang et al., 2008). Reducing the expression of Claudin-1 and occludin by siRNA and shRNA interference inhibited the entry of HCVpp and HCVcc (Liu et al., 2009). Studies made by (Liu et al., 2009, Benedicto et al., 2008) demonstrate that occludin interacts with E2 allowing the entry of the virus through the tight junctions of the hepatocytes.

Glycosaminoglycans (GAGS) are linear polysaccharides found on the cell surface. Heparan sulphate (HS) is an example of the many types of GAGS which act as binding sites for several viruses. Attachment of serum derived HCV to GAGS have been reported (Thomssen et al., 1992). Barth et al (Barth et al., 2006) demonstrated that by using HCVpp, HCV binding and entry are inhibited by highly sulphated HS and antiviral antibodies derived from HCV infected individuals.

LDL receptor, also known as apoB/E receptor, is a 160kDa cell surface glycoprotein important in the regulation of cholesterol homeostasis. It is an endocytic receptor that transports lipoproteins, especially LDL into cells (Nykjaer, 2002). LDL is taken up into a cell with LDL receptor first recognizing the LDL followed by the internalization of LDL via clathrin-coated pits (Steer, 1996, Cooper, 1996). Studies done by Monazahian (1999) and Agnello (1999) suggested that serum derived HCV

entry into cells is via LDL receptor. In another study, it was reported that the binding of low density HCV particles correlated with the number of LDL receptor on the cell surface (Wunschmann et al., 2000). A study by Owen *et al.* (Owen et al., 2009) using Huh7.5 cells stably expressing LDL receptor, constructed by using cytomegalovirus immediate-early promoter plasmid, showed that knockdown of the LDL receptor by treatment with 25-hydroxycholesterol or siRNA destroyed ligand uptake and reduced HCV infection of cells.

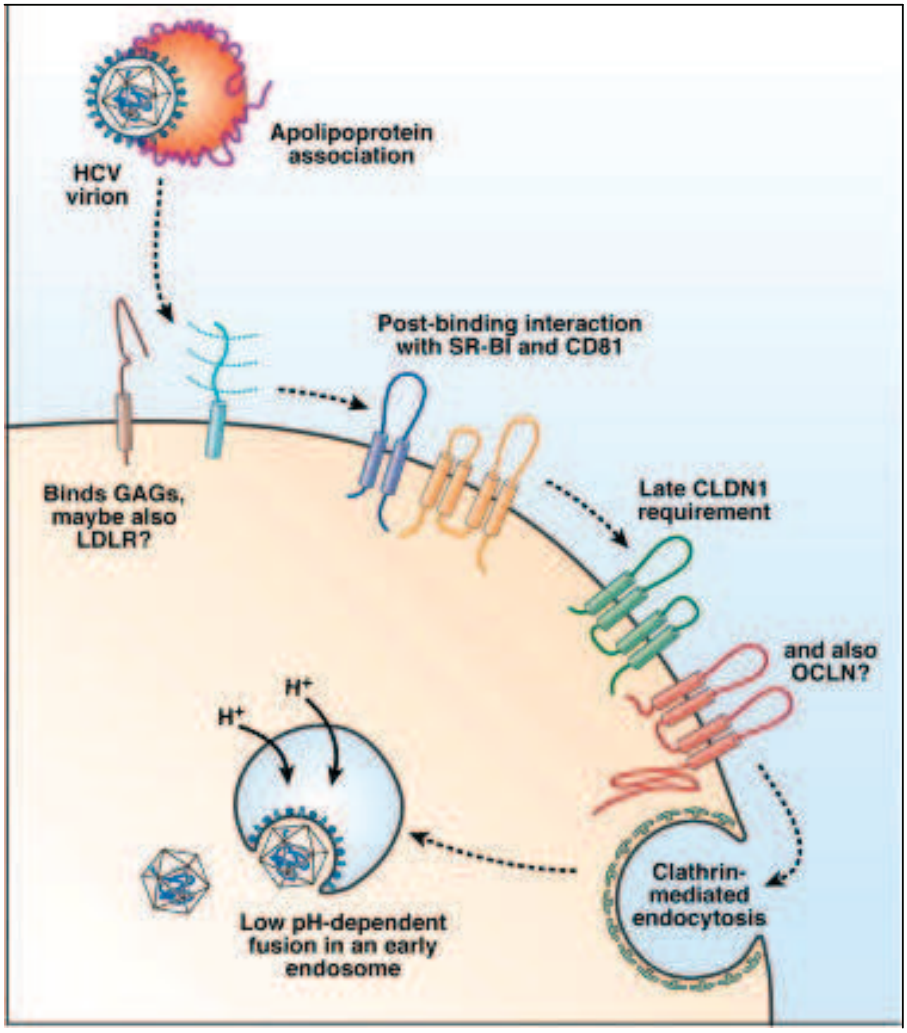


Figure 3: Overview of HCV cell entry.
The HCV entry process appears to require numerous interactions with host factors, both soluble and on the cell surface. The incoming virion, which appears to associate with apolipoprotein complexes, may

first bind a host cell by interacting with the low-density lipoprotein receptor (LDL-R) and glycosaminoglycans (GAGs) on the cell surface. Many HCV entry factors are required after virion binding to the host cell. These include an early requirement for CD81 and scavenger receptor class B type 1 (SR-B1), followed by a later utilization of the tight junction protein CLDN1. It is yet to be determined when the latest HCV entry factor OCLN, also a tight junction protein, is required. Such interactions result in endocytosis of the virion, where it fuses with an endosomal membrane upon acidification to release the viral nucleocapsid. Figure and legend adapted from (Lanford et al., 2009).

1.12 Binding and entry of HCV into cells

HCV found in the blood is heterogeneous in density and is often associated with lipoproteins and antibody. Andre (2002) demonstrated that lipo-viro-particles (LVP), purified by sequential ultracentrifugation followed by immunopurification with protein A-coated magnetic beads, bind and enter hepatocyte cell lines efficiently, whereas virus found in immunofractionated serum or whole-density fractions do not. He further confirmed the existence of LVP with electron microscopy. He also demonstrated that the binding of LVP is competed out by low concentration of VLDL and LDL and the binding of purified LVP to the cell line is blocked by anti-apolipoprotein B and E monoclonal antibodies. The internalization of the LVP was enhanced by the upregulation of the LDL receptor, whereas LDL receptor deficient fibroblasts had reduced ability to take up purified LVP. The authors concluded that LVP binding depends on host apolipoproteins and their receptors.

Favre (Favre, 2001) shows that cell-bound lipoproteins had to be removed by dextran sulphate before adding the virus to the hepatocyte cell lines in order to infect the cells. Maillard et al (Maillard et al., 2006) reported that binding of HCV (serum-derived) and SR-B1 is not affected by anti-E2 or anti-HVR1 antibodies but is inhibited by anti-betalipoprotein antibodies. The binding is also competed out by apoB-containing lipoproteins particularly VLDL.

Meunier, et al (Meunier et al., 2005) found that apoC1 (an apolipoprotein that is normally found in HDL), alone or as part of HDL, enhanced HCVpp cell entry when supplied exogeneously. Studies done by Chang et al (Chang et al., 2007) and Huang et al (Huang et al., 2007b) show that HCV production is reduced by apoE siRNA and microsomal triglyceride transfer protein inhibitor and apoB siRNA (agents that block VLDL production) respectively.

1.13 HCV-'LVP' from S6b liver macerate

Martin *et al.* (Martin, 2005) prepared liver derived HCV-'LVP' (henceforth termed as LLVP) from the low density fractions of liver macerate in an iodixanol gradient preparation. The liver macerate was from a female patient S6 who had common variable immunodeficiency (CVID) and was infected with HCV genotype 1a from a contaminated batch of Gammagaard intravenous immunoglobulin (Nielsen et al., 2004). The patient had a liver transplantation when her liver (liver S6a) failed. Liver S6a contained $5x10^6$ IU per gram of liver. A second liver transplantation was done 6 weeks later due to rejection, a leak from the biliary anastomosis, subtotal occlusion of the hepatic artery and re-infection by HCV. The transplant liver (S6b) containing $5x10^9$ IU per gram of liver was again removed and was kept for research purposes. All of the HCV RNA in the serum and 73% in the liver macerate were associated with low density lipoprotein and LLVP were recovered from the macerate by collection of fractions with a density below 1.08g/ml following iodixanol density gradient centrifugation.

A comparison was made on the binding of S6b LLVP, monitored by real time PCR for HCV RNA and low density lipoprotein labelled with the fluorescent dye 1,1'-

dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI), measured by FACS analysis. Binding was measured after washing the cells in suramine, which removes cell surface bound LDL leaving only internalized LDL associated with the cells. LLVP from S6b binding was greater to insulin treated cells than to hydroxyl-cholesterol treated cells and this is similar for DiI-LDL binding suggesting that LLVP may be binding to LDL receptor. However, although DiI-LDL binding was blocked by anti-apoB100 antibody, LLVP binding was blocked by neither anti-apoB100 nor anti-apoE antibody. This result suggests that LDL receptor may not be an important receptor for LLVP binding after all.

1.14 Host serum lipoproteins

Host serum lipoproteins are made up of macromolecular complexes of lipid and apolipoprotein. Lipoprotein particles are spherical in shape with the proteins and the polar lipids comprised of phospholipids and free (unesterified) cholesterol found on the outer layer and the hydrophobic lipids, esterified cholesterol and triglycerides found in the inner core (Mackness, 1992). The five different kinds of host serum lipoproteins namely; chylomicrons, VLDL, IDL, LDL and HDL, have different density, size, lipid and apolipoprotein contents and they can be separated by ultracentrifugation. Table 1 shows some aspects of the physical properties of lipoproteins.

	density (g/ml)	diameter (nm)	Apolipoproteins
Chylomicrons	<0.95	75-1200	A-I, A-II, A-IV, A-V, B48, C-1, C-II, C-III, E
VLDL	0.95-1.006	30-80	A-V, B100, C-1, C-II, C-III, E
IDL	1.006-1.019	25-35	B100, C-1, C-II, C-III, E
LDL	1.019-1.063	18-25	B100
HDL	1.063-1.21	5-12	A-I, A-II, A-IV, A-V, C-1, C-II, C-III,

D, E

Table 1: Physical properties of lipoproteins.
(Brown et al., 1986, Ginsberg, 1998, Obrien, 2005, Davis et al., 2005)

Serum lipoproteins act as carriers of lipids through the vascular and extravascular body fluids (Mackness, 1992). Lipids from the intestine are incorporated into chylomicrons in the endoplasmic reticulum of intestinal epithelial cells. The chylomicrons then enter the bloodstream where lipoprotein lipase acts on them to release free fatty acids and the chylomicron remnants, containing less triacylglycerol, then go to the liver. In the liver, VLDL are manufactured and released and are transported to muscle and adipose tissue via blood where they release free fatty acids from the VLDL triacylglycerol. The VLDL remnants (IDL) are once again taken up by the liver or continue to circulate when further removal of triacylglycerol produces LDL. LDL then carries cholesterol to the extrahepatic tissue or goes to the liver. Excess cholesterol found in the extrahepatic tissues is brought back to the liver as HDL (Davis et al., 2005).

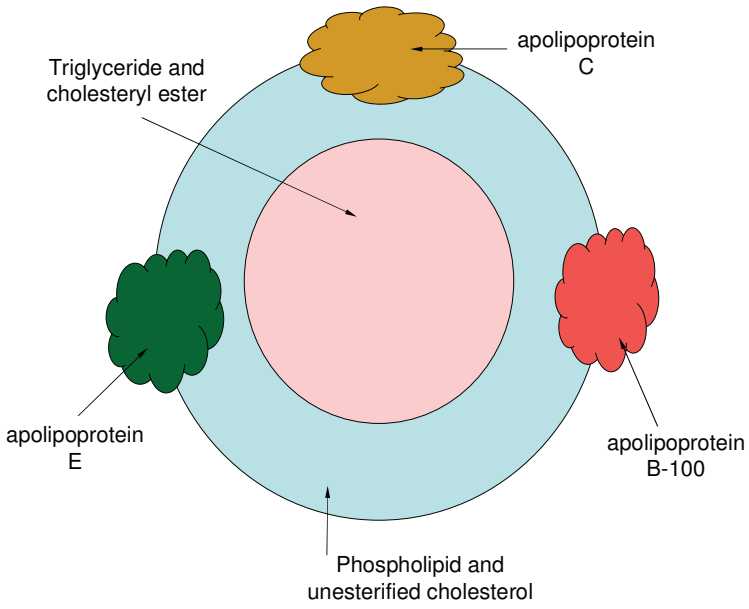


Figure 4: Structure of VLDL molecule.
Described by Brunzell and Failor, 2005 adapted from
http://www.acpmedicine.com/sample/ch0902s.htm

There are a minimum of nine different kinds of apolipoproteins that can be found in human blood plasma (Davis et al., 2005). They are usually grouped by function and divided into five classes A, B, C, D and E. The functions include a structural role, metabolic regulation, an immunological and homeostatic role unrelated with lipid transport, an enzymatic role (Mackness, 1992) and they also act as signals targeting lipoproteins to specific tissues (Davis et al., 2005).

Human apoA-I, encoded by exon 3 and 4 of apoA-I gene, is made up of 243 amino acids comprised of two domains: a globular N-terminal domain comprised of 43 amino acids (residues 1-43) and a C-terminal domain of the remaining amino acids (residues 44-243) (Bolanos-Garcia, 2003). The C-terminal domain possesses ten α-helices that form a horseshoe-shape structure. Reschly (2002) reported that helices 7 and 8 are important for the binding of lipid and that they contribute to apoA-I

association with different HDL subclasses. ApoA-I seems to be important in the regulation of HDL particle size distribution (Reschly, 2002). It is also a strong activator of lecithin acyl transferase (LCAT) which is an enzyme that catalyzes the formation of cholestryl esters in HDL (Fielding, 1972). ApoA-I interacts with other proteins such as SR-B1 (Liu, 2002) and the ATP-binding transporter (ABC) A1 (Arakawa, 2002).

Human ApoA-II is made up of 77 amino acids and it forms a homodimer which is linked with a disulfide bond (Vadiveloo, 1993) although there is a report suggesting that ApoA-II is formed from a heterodimer of apoE and apoH (Sprecher, 1984). Human apoA-II is produced in the liver and it is a main component of HDL particles (Bolanos-Garcia, 2003). The assembly of helices in both apoA-II and apoA-I structures is similar although the helices in apoA-I are longer. The two different forms of apoA-II structures, the lipid-free apoA-II crystal structure and apoA-II/β-octyl-glucopiranoside complex, show that the α-helix content increases upon association with lipids (Clement-Collin, 1999, Kawooya, 1986).

Apolipoprotein B (ApoB), a large amphiphatic protein, is generated primarily in the liver and in the small intestine. In the liver, apoB100 (4536 amino acids, 540kDa) is secreted in the form of VLDL particles whereas in the small intestine, a truncated version of apoB known as apoB48 (the N-terminal 2152 amino acids, 259kDa) is formed as a result of mRNA editing. The apoB48 is secreted in the form of chylomicron particles (Innerarity, 1996). ApoB100 has an LDL receptor binding site that is involved in the entry of plasma IDL and LDL and perhaps some VLDL into the cells. ApoB makes up 30-40% of the protein content of VLDL and more than 95% of

the protein content of LDL in the plasma (Dixon, 1993). Lipoproteins that contain apoB are assembled in the endoplasmic reticulum and matured at the Golgi apparatus before being secreted out of hepatocytes (Olofsson, 1987).

Apolipoproteins C-I, C-II and C-III, which are mainly expressed in the liver and can inhibit or stimulate a wide range of receptors and enzymes involved in lipid metabolism (Bolanos-Garcia, 2003). They mainly associate with HDL, VLDL and chylomicrons (Davis et al., 2005). The human apoC locus is located on chromosome 19 downstream from the apoE gene (Davison, 1986). ApoC-III is the main C apolipoprotein found in human plasma and it forms 60% of the total protein mass of HDL particles. One of its functions is inhibition of lipoprotein lipase thus, preventing lipolysis of the triglyceride-rich lipoproteins (Schaefer, 1985). ApoC-II, on the other hand, activates lipoprotein lipase (Davis et al., 2005).

Human apoE is made up of 299 amino acids that form an N-terminal domain of residues 1-191 and a C-terminal domain of residues 216-299 (Bolanos-Garcia, 2003). The N-terminal domain helps in the binding of apoE to the LDL receptor whereas the C-terminal domain make up the major lipid-binding elements. Chylomicron remnants and half of VLDL remnants, which are derived from lipolytic processing of intestinal chylomicrons and hepatic VLDL, are rapidly and efficiently cleared from the plasma by the liver through a mechanism mediated mainly by apolipoprotein E (Mahley, 1999).

1.15 Assembly of VLDL

VLDLs are lipoproteins ranging in size from 30nm to 80nm. They are made up of hydrophobic, neutral lipid (triacyglycerol and cholesterol esters) core which is surrounded by amphiphatic phospholipid and cholesterol monolayer. The major protein component is apolipoprotein B-100 which is found ~~on the~~ surface of VLDL and play a main role in VLDL assembly. ApoB-100 is also important for the structural integrity and the function of VLDL (Ginsberg et al., 2005). Other proteins found on the on the surface of VLDL are apoE and apoC which are exchangeable lipoproteins. ApoE and apoC proteins are structurally similar to the a-helical domains of apoB-100 and because of this, they can change between different lipoproteins (Segrest et al., 2001).

VLDL assembly starts with the translation of the apoB-100 protein which will reach 540kDa in size. The nascent apoB-100 protein fuses with lipid droplets which are rich in triacylglycerol and cholesterol esters in the lumen of endoplasmic reticulum and this fusion is fascilitated by microsomal triglyceride transfer protein (MTP) (Hussain et al., 2003). Hepatocytes and other cells that produce lipoprotein containing apoB have lipids in the lumen of the endoplasmic reticulum whereas most cells just contain cytosolic lipid droplets (Murphy and Vance, 1999). Secretion of apoB-100 will be halted as the protein will be highly unstable and degraded by proteosome if there is no MTP-mediated lipid transfer (Avramoglu and Adeli, 2004, Fisher and Ginsberg, 2002). As the apoB emulsion collects more and more neutral lipids, it forms what is known as VLDL-2 (Olofsson and Boren, 2005). Eventually, as more lipids are loaded and the particle matures through the Golgi apparatus, the now VLDL-1 will be the size of a secreted VLDL. Although some apoCI, apoCII, apoCIII, and apoE are present on the developing VLDL as they are secreted from the

hepatocyte, most of these apolipoproteins are probably added in the blood plasma (Ginsberg et al., 2005). The final VLDL are composed of hydrophobic core of triacyglycerol and cholesterol esters, phospholipid coat, free cholesterol, 2 main lipoproteins; apoB-100 and apoE which will ~~the~~ be secreted out by exocytosis (Gibbons et al., 2004).

1.16 Assembly of HCV

Scientists believe that HCV replication and assembly occur within the membranous structures derived from the endoplasmic reticulum in the cytosol of infected cells. The HCV RNA is thought to be assembled together with core, E1, E2, and lipid envelope as a particle without the non-structural proteins although the latter can be found in the infected cells. The infectious form of the virus *in vivo* has not been fully elucidated but is thought to be associated with lipids or lipoproteins.

1.17 HCV interaction with exosomes

Masciopinto (2004) has shown that the HCV envelope proteins were associated with exosomes. Exosomes are small membranous vesicles secreted by the cells upon fusion of multivesicular endosomes with the cell surface membrane (Stahl, 2002). It was shown that in the presence of CD81, HCV envelope proteins were transported through the Golgi apparatus, attained complex sugars and were eventually found associated with exoxomes extracellularly. However, in the absence of CD81, the envelope proteins were retained in the endoplasmic reticulum. It was then suggested that CD81 actually acts as an exit receptor for HCV (Masciopinto, 2004).

1.17.1 Association of HCV proteins with lipid metabolism

There have been reports showing that viral proteins interact with host cell proteins involved in lipid metabolism intracellularly. There are several studies that demonstrate association of HCV core protein with cytoplasmic lipid droplets. In a transfection study, HCV core protein expression causes a build up of lipid droplets in the cytoplasm (Barba et al., 1997, Hope and McLauchlan, 2000, Pietschmann, 2002, Clayton et al., 2002). In an in vivo study, transgenic mice expressing HCV core protein develop steatosis (Moriya, 1997). HCV core protein was shown to localize on the surface of the lipid droplets in a study done using an electron microscopy with immunolabeling (Barba et al., 1997). It has also been shown that there are similarities in the residues 125 until 144 of the HCV core with oleosin proteins that line the surface of lipid bodies in plant cells (Hope, 2002).

In a study using core-apoAII double transgenic mice, the effect of decreasing secretion of liver VLDL-triglycerides and apoB due to HCV core protein expression is abolished (Andre, 2005). ApoAII has been shown to co-localize with core protein in the HepG2 cytoplasm and also in the liver of infected patients (Perlemuter, 2002). In another study, when a region of the HCV core protein is deleted, there is a change in the localization of core protein and the ability to interact with apoAII is lost (Sabile, 1999). HCV core protein inhibits microsomal triglyceride transfer protein (MTP), a protein that stabilizes apoB by lipidation which is an important step in the VLDL production (Andre, 2005).

1.17.2 Association of HCV replication with lipid metabolism

Kapadia and Chisari (Huang et al., 2007a) demonstrated that HCV RNA expression of full length SfiI replicon (genotype 1b) in Huh-7 cells increased levels of ATP citrate

lyase and acetyl CoA synthetase genes, both of which are important in cholesterol and fatty acid biosynthesis. They confirmed that cholesterol-biosynthetic pathway regulates HCV RNA replication by controlling the cellular levels of geranylgeranyl pyrophosphate. They showed that saturated (lauric, myristic and palmitic) and monounsaturated (oleic) fatty acids stimulated HCV replication whereas polyunsaturated (arachidonic, eicosapentaenoic, and docosahexaenoic fatty acids (PUFA) inhibited HCV replication by their ability to downregulate lipogenic gene expression.

Several studies have shown that mevalonate pathway, one of the two ways hepatocytes acquire cholesterol, is involved in HCV RNA replication. The other way of acquiring cholesterol is through SR-BI – mediated uptake from HDL but this not as significant in humans as it is in mice (Abe et al., 2007). Ye (Dore et al., 2003) showed that HCV RNA replication is disrupted when host's protein geranylgeranylation is inhibited.

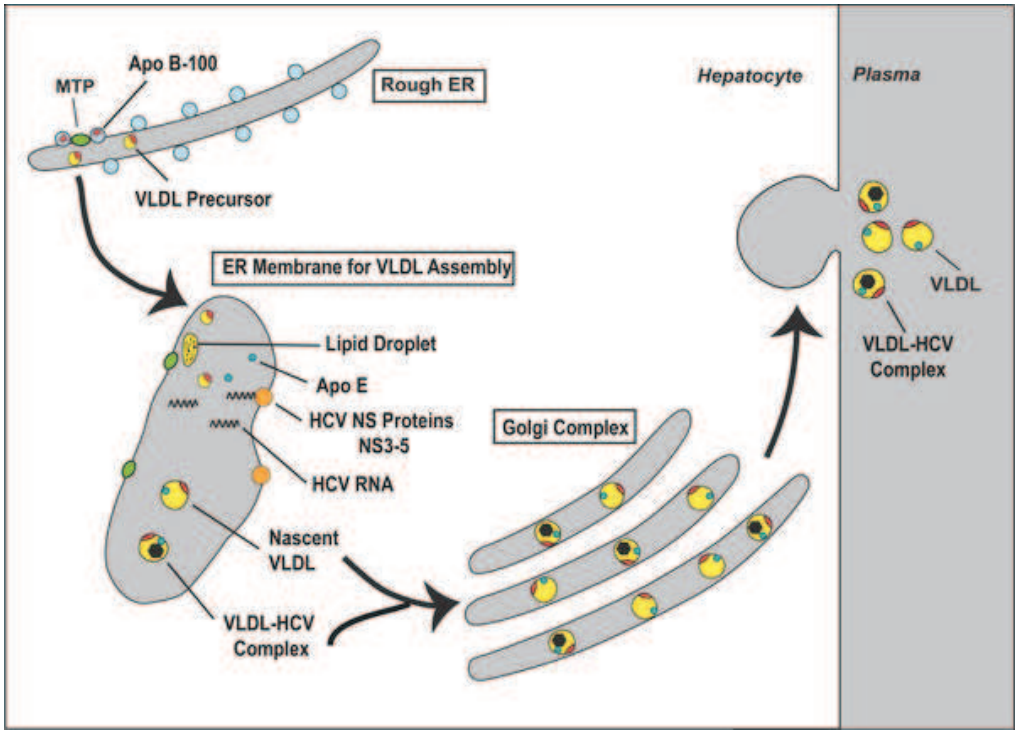


Figure 5: HCV replicates on ER membranes involved in the assembly of VLDL and is secreted together with VLDL.
The assembly of VLDL begins with the synthesis of apoB in the rough ER, resulting in the formation of a VLDL precursor that contains only a small amount of lipid. In the lumen of the ER this precursor is fused with lipid droplets (enriched in triglyceride and cholesterol) to generate the nascent VLDL. This reaction is mediated by MTP. The nascent VLDL particles, which contain both apoB and apoE, are secreted into plasma through exocytosis. The ER membranes involved in the assembly of VLDL are also enriched in HCV NS proteins and RNA. Replication of HCV on these membranes might allow the virus to attach or become incorporated into VLDL so that HCV is secreted together with VLDL. Figure and legend adapted from (Abe et al., 2007).

1.17.3 Association of HCV with low density or very low density lipoproteins

In chronically infected patients, HCV circulates as low-density lipoprotein virus particles (Andre et al., 2002, Nielsen et al., 2006) and ~~that~~ the density profile of the HCV from the infected patients varies depending on the stage of the infection when the samples were obtained (Carabaich et al., 2005, Pumeechockchai et al., 2002). Densities of HCV RNA-containing particles from serum are very heterogenous, ranging from less than 1.06g/mL to 1.30g/mL (Miyamoto et al., 1992, Kanto et al., 1994, Carrick et al., 1992). In a study by Carrick (Carrick et al., 1992), HCV RNA can be found in two fractions <1.03g/cm^3 and 1.18-1.21g/cm^3 when HCV-infected

plasma fractions were harvested from a sucrose and cesium chloride density gradients. The heterogeneity of the densities are attributed to factors associated with the viral structures, the viral interaction with different kinds of hosts lipoproteins (Thomssen et al., 1993) and the association of the different kinds of anti-HCV antibodies bound to the viral particles (Kanto et al., 1994, Choo, 1995, Hijikata, 1993a, Andre et al., 2002). (Thomssen et al., 1992) revealed following sucrose gradient density centrifugation on viral hepatitis C patients' sera, that the heterogeneity of HCV RNA density could be a result of association of the virus with serum lipoprotein as HCV-RNA banding at density 1.03-1.08g/cm^3 could be completely precipitated with anti-β lipoprotein whereas that found in higher density of 1.12-1.20g/cm^3 could only be partially precipitated or not at all. Thomssen et al (Thomssen et al., 1993) implicated both lipoprotein and immunoglobulin in the density heterogeneity of the HCV-RNA-carrying material from serum by immunoprecipitation studies. In another study, low density (1.03-1.07 g/ml) and not intermediate density (1.12-1.18g/ml) HCV particles bound to LDL receptors of MOLT-4 cells and fibroblasts (Wunschmann et al., 2000). A study by Lindenbach have shown that the J6/JFH HCVcc RNA was found widely distributed throughout the top of the iodixanol density gradient peaking at 1.13g/ml to1.14g/ml but not found beyond 1.18g/ml and the most infectious material containing J6/JFH HCVcc is at the buoyant density of 1.09 to 1.10g/ml whereas RNA containing material at a buoyant density of 1.14g/ml had a low specific infectivity (Lindenbach et al., 2005). In another study by Lindenbach, using the HCVcc system, there is a shift to lower buoyant density associated with increased specific infectivity of HCV grown *in vivo* (Lindenbach et al., 2006).

Andre et al showed that LDL-virus particles from plasma have abundant triglycerides and also contain HCV RNA, core protein, and apolipoproteins B and E (Andre et al., 2002). It has also been shown by Nielsen *et al* that HCV RNA from the serum can be immunoprecipitated with anti-apoB and anti-apoE antibodies and these apolipoproteins are components of VLDL which implicates that these host apolipoproteins are thus part of the circulating HCV particles (Nielsen et al., 2006). Using JFH1 HCVcc and shRNAs specific for apoB, Gastaminza et al demonstrated that reduced apoB expression would inhibit HCV assembly and secretion (Gastaminza et al., 2008).

Nahmias *et al* (Nahmias et al., 2008) demonstrated that silencing apoB messenger RNA in Huh7.5.1 cells infected with JFH1 causes a reduction in the secretion of both apoB-100 and HCV. They also demonstrated that the grapefruit flavonoid naringenin, previously shown to inhibit VLDL secretion, inhibits the MTP activity in infected cells and stimulation with naringenin reduces HCV secretion in infected cells by 80%.

(Andre, 2005) has proposed a model of the association of HCV particle with lipoprotein. The following figure is adapted from his model with some modifications:

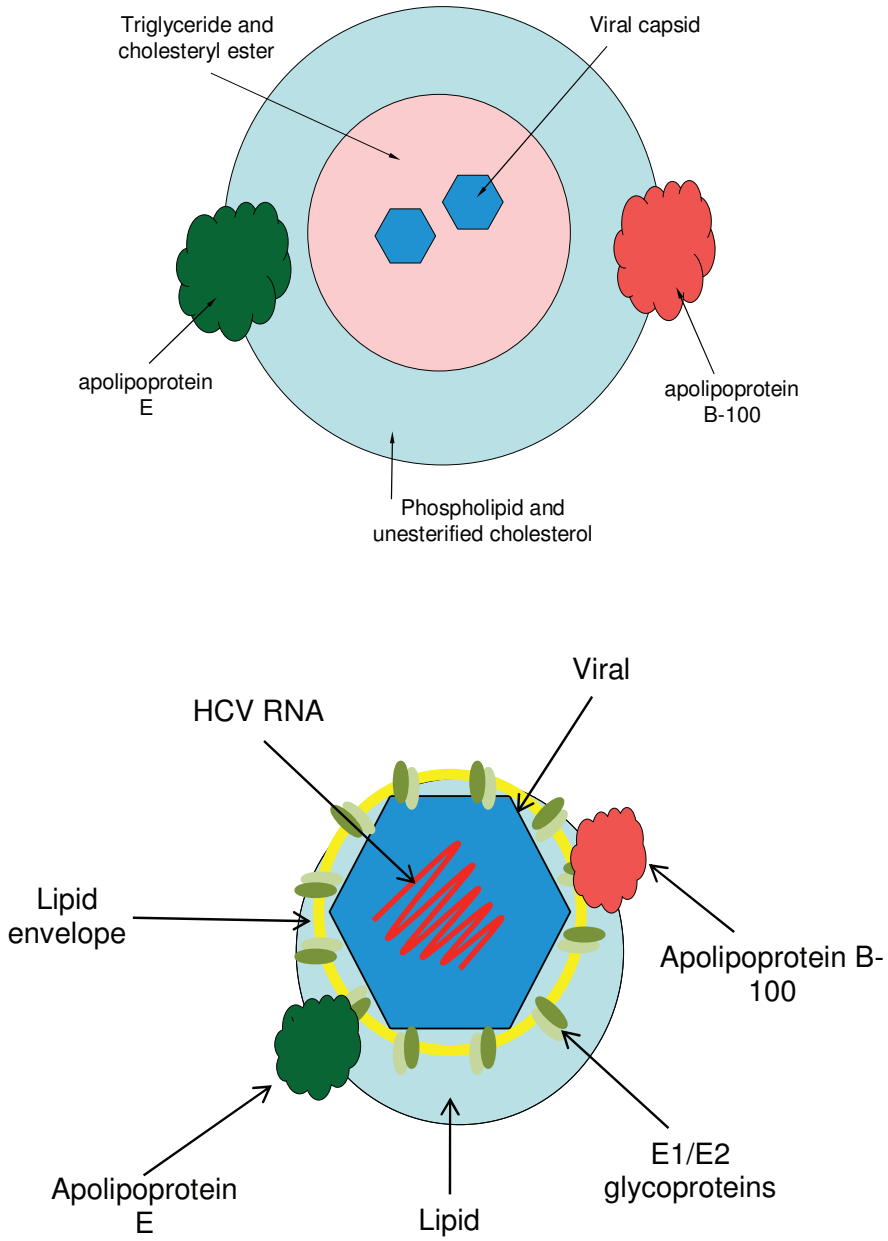


Figure 6: Model of LVP molecule.
Adapted from (Andre, 2005).

LVP appear as large spherical particles with a diameter of more than 100nm and with internal structures and consist of triglycerides, apolipoprotein B and E, HCV RNA

and core protein (Andre 2002). Delipidation of these particles results in capsid-like structures recognized by anti-HCV core protein antibody (Andre, 2005). Using iodixanol density gradients and gel filtration with Toyopearl, Nielsen *et al* have shown that serum HCV RNA is associated with particles of VLDL density and size and also by using mass spectrometry and Western blotting, the particles were found to contain apoB, apoE, and apoA1 (Nielsen et al., 2008). In an immunoprecipitation experiment, Nielsen et al (Nielsen et al., 2006) found that apoB lipoprotein but not apoE remained associated from the HCV after being stripped with deoxycholic acid or NP-40 and that 25% of the particles bind to anti-E2. Despite the heterogeneous density distribution of the HCV RNA in immunocompetent individuals, Nielsen et al suggested that the particles are coated with antibody (Nielsen et al., 2006). The amount of antibody in the VLDL fraction is too great to be accounted for by binding to HCV LVP alone and so either it is an autoantibody to VLDL or if it is anti-HCV, HCV proteins must be present as HCV RNA negative VLDL particles. Another study by (Owen et al., 2009) showed that in the presence of increasing concentrations of HDL, LDL, and β-VLDL, β -VLDL reduced 60% of JFH1 HCVcc infection of Huh7.5 cells. This is followed by moderate reduction by LDL and no reduction by HDL. They also showed that anti-apoE antibody blocks HCV entry and in the analyses of gradient-fractionated HCV, they demonstrate that apoE is associated with HCV virions exhibiting peak infectivity and dependence upon the LDL-R for cell entry.

The genetic diversity of LVP HCV genome population is less compared to the total HCV plasma population (Deforges, 2004). This may indicate that LVP is produced from a site other than the liver. D. Felmlee (unpublished) from our laboratory has

shown that HCV RNA is associated with both apoB48 and apoB100 and that post-prandially, there is a surge of apoB48 associated HCV RNA material in the blood before an increase in apoB100 associated HCV RNA.

Taken together, these data indicate that HCV assembly in vivo and cell culture requires apoB and apoE and that the LVP appears to be a hybrid of HCV particle with VLDL/chylomicrons. To examine this further, cell culture where VLDL synthesis is imperfect, does not seem to be an appropriate system to study it.

1.17.4 Association of HCV immune escape variants with lipoproteins

Viral escape from antibody response has been shown to be linked with HCV association with lipoproteins namely LDL and VLDL or the involvement of SR-B1 and HDL. Epitopes targeted by neutralizing antibody may be masked by through the association of HCV particles with lipoproteins allowing escape of antibody-mediated virus neutralization.

HCV has been shown to exist in heterogenous forms in density studies because of its association with VLDL, LDL, and HDL. Agnello (Agnello et al., 1999) showed that HCV particle complexes with VLDL or LDL but not HDL when it is endocytosed via the LDL receptor. HCV was immunoprecipitated from low infectivity samples found in the high density fractions but not from high infectivity samples found in low density fraction showing that HCV is complexed with anti-HCV antibodies in low density HCV complexes (Hijikata et al., 1993b). This study provides an indirect link that anti-HCV antibodies block HCV particles from associating with low density materials or the low density materials is protecting the virus particles from the anti-

HCV antibodies. Andre (Andre et al., 2002) found that binding of HCV lipo-viro-particles (LVP) was competed out by VLDL and LDL, blocked by anti-apolipoproteins B and E antibodies, and its endocytosis increased with the increase of the LDL receptors suggesting that the endogenous lipoproteins mediates the LVP infectivity providing an escape mechanism from the antibody-mediated immune response. An immunoprecipitation study by Nielsen (Nielsen et al., 2006) on the VLDL and LDL fractions of an iodixanol gradient from an HCV-infected CVID patient sample showed 25.0%, 91.8%, and 95.0% of the HCV sample being precipitated by anti-E2, anti-apoB, and and anti-apoE antibodies respectively.

Bartosch (Bartosch et al., 2005) on the contrary showed that HCVpp's interaction with HDL or human serum but not LDL allows for the protection of HCVpp from neutralizing antibodies (monoclonal antibodies and antibodies from the patients' sera) and together with HVR1's role facilitate the enhancement of cell entry and provide protection to the virus particles from the neutralizing antibodies. The mechanism of protection lies in the HDLs' ability to reduced HCVpp neutralization by hastening HCVpp entry via the lipid-transfer property of SR-B1 (Voisset et al., 2006). A study made by Grove *et al* (Grove et al., 2008) using JFH1 G451R cell culture-adapted mutants demonstrated that low density HCV particles are more resistant to antibody neutralization implicating that lipoproteins allows evasion of the virus particles from the host immune responses.

1.18 Aims of the study

There is evidence that *in vivo* LVP may enter hepatocytes by a viral glycoprotein independent route following binding of apoB100 to the LDLr. Investigations of this

phenomenon have been limited because of limitations to the supply of serum LVP and the effect of antibodies on these is unknown. S6b liver derived LVP (LLVP) is antibody-free and plentiful. Martin (Martin, 2005) had shown that treatment to raise LDLr increases binding of LLVP to HepG2 cells and that binding could be blocked with excess LDL. This suggests that LLVP binds in a similar way to LDL or VLDL via apoB or apoE binding to LDLr. To further test this hypothesis, I sought to confirm these results and extend them by testing the effect of anti-apolipoprotein antibodies on binding.

Studies in cell culture systems indicate that HCV may also bind via HCV E2 / SR-B1 interactions. To investigate the contribution of this interaction to LLVP binding, I sought to measure levels of SR-B1 expression in insulin treated cell and to assess the ability of anti-glycoprotein antibodies to block binding. These studies revealed the deficiencies of studying binding in a non-infectious system. I therefore sought to establish a source of infectious LVP. Initially, the possibility of establishing replication of the S6 virus in primary human hepatocytes was explored. Subsequently, the J6/JFH1 / Huh7.5 system was established. As VLDL production in Huh7.5 cells in normal medium is minimal, it is expected that LVP production will also be restricted. The possibility of increasing VLDL and LVP production by adding fatty acids to the medium was explored and the resulting virus was compared by density and size with serum-derived HCV LVP. Unfortunately, time limitations precluded binding studies with the putative LVP produced in cell culture.

Chapter 2

2 Materials and Methods

2.1 Chemicals

Unless otherwise stated, chemicals were purchased from BDH Ltd. (Poole, Dorset)

2.2 Cell culture

2.2.1 Growth medium for HepG2 and Vero cell lines

Eagles Minimum Essential Medium (EMEM) (Gibco BRL, Paisley, UK)
1% non-essential amino acids (NEAA) (Lonza, Verviers, Belgium)
1% 200mM glutamine (Lonza, Verviers, Belgium)
1% 10,000 units/ml penicillin and 10,000 units/ml streptomycin solution (PAA Laboratories GmbH, Haidmannweg, Pasching, Austria)
5% gassed 4.4% Sodium bicarbonate + 0.4% phenol red (Gibco BRL, Paisley, UK)
10% fetal calf serum (PAA Laboratories GmbH, Haidmannweg, Pasching, Austria)

2.2.2 Growth medium for Huh7 and Huh7.5 cell lines

Dulbeccos Modified Eagle Medium (DMEM) (PAA Laboratories GmbH, Haidmannweg, Pasching, Austria)
1% 100mM sodium pyruvate (Lonza, Verviers, Belgium)
1% non-essential amino acids (NEAA) (Lonza, Verviers, Belgium)
1% 200mM glutamine (Lonza, Verviers, Belgium)
1% 10,000 units/ml penicillin and 10,000 units/ml streptomycin solution (PAA Laboratories GmbH, Haidmannweg, Pasching, Austria)
10% fetal calf serum (PAA Laboratories GmbH, Haidmannweg, Pasching, Austria)

2.2.3 PBS solution

PBS (one tablet (Oxoid, Basingstoke, Hampshire)
100mls distilled water
Autoclaved at 15 lbs for 15 minutes

2.2.4 EDTA/Trypsin

PBS solution
0.86mM ethylene-diamine-tetra-acetic acid (EDTA) (0.25g/L)
Autoclaved at 15 lbs for 15 minutes
0.025% trypsin (Gibco BRL, Paisley, UK)

2.2.5 Cell lines

The HepG2 cell line was purchased from ATCC, Manassas, USA.

The Vero cell line was obtained from Clinical Virology Unit, Royal Victoria Infirmary, Newcastle upon Tyne, Tyne and Wear.

Huh7 cell line was obtained from Institut National de la Sante et de la Recherche Medicale, Lyon, France.

Huh7.5 cell line has been kindly supplied by Prof. J. McKeating from the University of Birmingham, U.K.

Cells were cultured in plastic tissue culture flasks (Corning, Schipol-Rijk, Netherlands) in 5% CO_2 at 37ºC. For sub-culture the cells were washed with 5ml PBS followed by 5ml of EDTA/Trypsin incubated at 37^{o}C for 5-10 minutes. The flask was shaken vigorously to detach the cells which were resuspended in 5ml of the appropriate growth medium and pipetted vigorously a few times to break up the clumps. 1ml of the cell suspension was seeded into 9ml fresh growth medium in a new 25cm^3 flask.

2.3 Cryopreservation of cells

For storage of cells in liquid nitrogen, cells grown to confluence from a T_{75} flask (Corning, Schipol-Rijk, Netherlands) were stripped with EDTA and trypsin and resuspended in 10ml medium. The cell suspension was centrifuged at 180g for 10 minutes after which the supernatant was discarded. The cells were suspended in 4ml foetal calf serum containing 10% dimethyl sulfoxide (DMSO) (Sigma, Gillingham, Dorset). The cell suspension was heat-sealed in a 1ml cryotube (Nalgene, Rochester, USA) and frozen down in a cryo l^{o}C freezing container (Nalgene, Rochester, U.S.A) at -80^{o}C overnight before transfer into liquid nitrogen.

2.4 Preparation and culture of primary hepatocytes

Primary hepatocytes were kindly supplied by Clare Selden from UCL Centre for Hepatology, Royal Free and University College Medical School, London. These were prepared according to the method originally described by Berry and Friend (Berry and Friend, 1969), modification by Hewes *et al* (Hewes et al., 2006) and carried out by (Selden et al., 2007).

A piece of human liver was cut out from tumor-free region from a secondary liver tumors that had been surgically removed from a patient with fully informed consent and local research ethics approval. The flushing was done with University of Wisconsin solution through available veins via cannulae insertion. The liver was then perfused with 0.5mM EGTA/PBS/20mM HEPES buffer at 37^{o}C for 30 minutes and 20mM HEPES- buffered PBS, pH 7.4 for a further 20 minutes. The liver was digested with 150mg collegenase type IV and 30mg DNAse I, 300ml digestion buffer (HBSS-Ca^{+} containing 0.5% albumin, 50μg/ml ascorbic acid, 100IU insulin followed by 20-30 minutes perfusion with 20mM HEPES. The liver capsule was disrupted with scalpel and shaken gently to release the hepatocytes in an ice-cold dispersal buffer (Williams medium containing 10% FBS, 50mg DNAse I. The hepatocyte suspension was centrifuged at 400rpm (240g) for 6min at 4^{o}C and resuspended in dispersal buffer. The washes were repeated three times and the pellet was eventually resuspended in serum-free Williams E medium.

Cells were counted using haemocytometer and viability checked after staining the cells with trypan blue. The cells were plated at 2 x 10^{5} cells/cm^{2} on 6-well-collagen I coated-plates in 4ml Williams E medium supplemented with 10% FCS, Pen/Strep,

Fungizone, insulin 10^{-8} M and incubated at 37°C in a humidified incubator at 5% CO_2.

2.5 Determination of HCV RNA quantity upon inoculation of HCV 'LLVP' from liver macerate to HepG2 and Huh7.5 cell lines and primary hepatocytes

For HepG2 cells, 0.7 X 10^6 HepG2 cells per well of a six-well plate were seeded in 4ml EMEM growth medium and incubated at 37ºC in 5% CO_2 for 2 days. The cells were washed twice with 4ml warm PBS and cultured in 8.5% LPDS + 0.1% insulin EMEM medium for another 3 days. The cells were washed twice with 4ml warm PBS and inoculated with 1.1 X 10^7 IU S6b LLVP in 800µl labelling medium (8.5% LPDS and serum free medium) and incubated for 3 hours at 37°C shaking every half an hour. The cells were washed twice with 4ml warm PBS, grown in 8.5% LPDS + 0.1% insulin EMEM medium and the medium was harvested from the well for RNA extraction at 2/3 day intervals for a 7-day period. This was repeated two more times on day 5 and day 7 post-inoculation.

For Huh7.5 cells, 1 X 10^5 Huh7.5 cells per well of a six-well plate were seeded in 4ml DMEM growth medium and incubated at 37ºC in 5% CO_2 for 2 days. The cells were washed twice with 4ml warm PBS and cultured in 8.5% LPDS + 0.1% insulin EMEM medium for another 3 days. The cells were washed twice with 4ml warm PBS and inoculated with 1.1 X 10^7 IU S6b LLVP in 800µl labelling medium (8.5% LPDS and serum free medium) and incubated for 3 hours at 37°C shaking every half an hour. The cells were washed twice with 4ml warm PBS, grown in DMEM growth medium with 10% FCS, 8.5% LPDS + 0.1% insulin EMEM medium or Williams E (Sigma, St. Louis, USA) growth medium with 10% FCS and the medium was harvested from

the well for RNA extraction at 2 day intervals for a 6-day period. This was repeated two more times on day 4 and day 6 post-inoculation.

For primary hepatocytes, 2 x 10^5 cells/cm^2 of primary hepatocytes were seeded on 6-well-collagen I coated-plates in 4ml Williams E medium (Sigma, St. Louis, USA) supplemented with 10% FCS, Pen/Strep, Fungizone, insulin 10^{-8} M and incubated at 37°C in 5% CO_2 overnight. The cells were washed once with 4ml warm PBS and cultured in 8.5% LPDS + 0.1% insulin MEM (Gibco, Paisley, UK) medium for another day. The cells were washed twice with 4ml warm PBS and inoculated with 3 X 10^6 IU S6b LLVP in 800µl labelling medium (8.5% LPDS and serum free medium) and incubated for 3 hours at 37°C shaking every half an hour. The primary hepatocytes were washed twice with 4ml warm PBS, grown in two types of medium – Williams E growth medium with 10% FCS or 8.5% LPDS + 0.1% insulin MEM medium with or without 10µg/ml recombinant human growth factor (Chemicon International, Temecula, USA) added to each medium and the medium was harvested from the well for RNA extraction at 2 day intervals for a 6-day period. This was repeated two more times on day 4 and day 6 post-inoculation.

Samples were extracted for HCV RNA using QIAamp viral RNA mini kit (Qiagen, UK) following the manufacturer's instructions. The viral RNA was stored at -20°C or -70°C. On a different day the RNA was titrated by quantitative RT-PCR as described in sections 2.28 and 2.29.

2.6 Preparation of lipoprotein deficient serum

Lipoprotein deficient serum (LPDS) was prepared following the method of Bierman and Albers (1977). 109g of inactivated foetal calf serum (Invitrogen, Paisley) was thawed out and centrifuged at 1610g for 10min to remove debris. 400µl of 0.5M EDTA, 200µl of 20% NaN_3, 400µl of 30mg/ml phenylmethylsulphonyl-flouride [PMSF] (Sigma, Gillingham, Dorset) and 41.8g of KBr were added to the foetal calf serum to achieve a density of 1.24g/ml.

The mixture was dissolved on a Sucroagitator (Boots-Celltech Diagnostics Limited, Slough) and 10ml was aliquoted into twelve tubes. The solution was then centrifuged at 50,000rpm for 24hrs at 4^{o}C in a Ti50 rotor, acceleration 8, deceleration 0 in a Beckman L8-70 ultracentrifuge. The yellowish, top layer containing the lipoproteins was removed while the bottom layer containing the LPDS was pooled into a 12-14 kDa MWCO dialysis tubing (Medicell International Ltd, Liverpool) and dialysed three times in 4L dialysis buffer pH 7.2 containing 80ml 0.5M phosphate buffer (0.28M Na_2HPO_4 and 0.21M $NaH_2PO_4.H_2O$ and 36g NaCl at 4^{o}C. The LPDS was heat inactivated at 56^{o}C for 30minutes and filter sterilized with a 0.22µm polyethersulfone (PES) 500ml bottle top filter (Corning) and stored at -20^{o}C.

2.7 Preparation of low density lipoprotein by sequential floatation ultracentrifugation from blood plasma

Low density lipoprotein (LDL) was prepared by a modification of the method of Stephan (1993).

480ul of 0.5M EDTA, which acts as anticoagulant, was added to 30ml of human blood and these were well mixed. The mixture was incubated at 4^{o}C for 1 hour and

centrifuged at 2862g, 30min, 4°C to separate red blood cells and buffy coat from plasma. 45 ul of 0.5M EDTA, 23ul of 20% NaN_3 and 45ul of 30mg/ml PMSF (Sigma) stock were added to 10ml of the plasma. The plasma was centrifuged at 50,000rpm 18-24hrs at 10°C in a thick-wall polycarbonate tube in a Ti50 rotor in a Beckman L8-70 ultracentrifuge. The whitish, opaque, top layer consisting of chylomicrons and very low density lipoproteins (VLDL) with a density of less than 1.006g/ml together with one-third of the plasma was removed. The pellet was resuspended in the bottom 2/3 plasma layer and the suspension density (ρ) adjusted with 1.132g/ml solution of potassium bromide (KBr) to reach a density of 1.019g/ml confirmed by measuring both weight and volume. The mixture was distributed between Ti50 tubes and centrifuged under the same condition as the above. One-third of the mixture's top layer consisting of an intermediate density lipoprotein (IDL) with a density of greater than 1.006g/ml but less than 1.019g/ml was removed. The remaining volume was adjusted to a density of 1.063g/ml with 1.218g/ml KBr solution. The solution was again distributed between Ti50 centrifuge tubes and spun again under the condition described above. The yellowish top layer consisting of low density lipoproteins (LDL) with a density of greater than 1.019g/ml but less than 1.063g/ml was removed and pooled. The LDL was dialysed three times at 4°C for 4 hours in dialysis buffer consisting of PBS + 1mM EDTA. The protein concentration was determined using a Biorad protein assay kit. The LDL was preserved by adding sucrose to a final concentration of 10% sucrose using a 50% sucrose stock solution. The LDL and sucrose solution was filter sterilized using a 0.45μm filter (Scientific Laboratory Supplies, North Shields, Tyne and Wear) and stored at -80°C.

2.8 Preparation of oxidized low density lipoprotein (Low Aggregation Procedure)

Oxidized LDL (oxLDL) was prepared by the method of (Lopes-Virella et al., 2000) and modified to reduce protein aggregation Hoff (1993). 10mg LDL in a dialysis membrane was dialysed three times in 2L of dialysis buffer, pH 7.4 containing 12.5mM tris(hydroxymethyl)aminomethane (Tris) and 140mM NaCl at 4^{o}C for 4 hours (each dialysis) in an aluminium foil-wrapped container for protection against light. The LDL was then dialysed for the fourth time in 3L dialysis buffer (12.5mM Tris, 140mM NaCl and 23.8mM sodium bicarbonate ($NaHCO_3$)) oxygenated by bubbling 95% oxygen gas for 10 minutes at 4^{o}C for 4 hours .

A stock solution for oxidation was prepared by adding 25µl of 10mM $CuCl_2$ to 20ml LDL solution making a final concentration of LDL to 0.5mg/ml in a dialysis membrane and placed in 3L oxygenated dialysis buffer containing 0.0125mM $CuCl_2$ in a 21^{o}C waterbath. 10ml of the dialysis buffer containing the $CuCl_2$ was set aside to be used as a reference solution.

500µl of the stock solution was transferred to a 15ml Falcon tube containing 4.5ml reference solution, and referred to as sample oxidation. The sample oxidation was used for OD_{234} and fluorescence measurements and it will be incubated under the same condition as the remaining stock solution to be oxidized. The remaining stock solution and sample oxidation were placed in a 21^{o}C waterbath and were protected from light during the course of the preparation. OD_{234} and fluorescence (excitation 360nm, emission 430nm) readings of the sample oxidation were taken at 0hr, 0.5hrs, 1hr, 2hrs and 24hrs to assess the degree of Cu^{2+}-induced oxidation.

After the oxidation process, LDL stock solution was dialysed in the dark three times against PBS containing 1mM EDTA to remove Cu^{2+} as further oxidation was no longer needed. The volume of oxLDL solution was measured after dialysis to determine the LDL concentration. The oxLDL was then filter sterilized using a 0.45µm filter (Scientific Laboratory Supplies, North Shields, Tyne and Wear) and warmed to 37 ^{o}C for labelling.

2.9 DiI labelling of oxidized LDL

LDL was labelled with dioctadecylindocarbocyanine (DiI) by the method of Arnold *et al.* (Arnold, 1992).

10µl of 30µg/µl DiI (Sigma, Gillingham, Dorset) stock solution in DMSO (Sigma, Gillingham, Dorset) was added to 1mg of oxLDL. The mixture is incubated at 37^{o}C for 24 hours on a DynaI sample mixer (Invitrogen) and made up to 27ml with PBS containing 1mM EDTA. 2.25g KBr was added to the DiI-oxLDL mixture to adjust the density of the solution to 1.063g/ml which was then distributed into three thick-walled Ti50 tubes and centrifuged at 4^{o}C for 18-24hrs in the Ti50 rotor at 50,000rpm. The pink, top layer of oxidized LDL labelled with DiI was harvested and dialysed three times in the dark against 2L of dialysis buffer (PBS containing 1mM EDTA) at 4^{o}C. The protein concentration was determined by the method of Bradford (Bradford, 1976) with a protein assay kit (Biorad, Hertfordshire). The volume was adjusted so that the protein concentration was 0.5µg/µl. The DiI-oxLDL was then filter sterilized with a 0.45µm filter and a ¼ volume of 50% sucrose was added to make a final concentration of 10% sucrose and a final protein concentration of 0.4µg/µl. The DiI-

oxLDL was stored in small aliquots at -80°C. When the DiI-oxLDL is used, it is first thawed and then centrifuged at 10,000g for 5min to reduce aggregation.

2.10 Iodixanol gradient preparation of HCV liver derived lipoviroparticles (LLVP)

Liver macerate was prepared from liver S6b by Nielsen (Nielsen et al., 2004) and stored in -80°C. 2.5ml of thawed liver macerate was layered onto each of the seven Ti50 tubes containing 4.2ml 60% iodixanol (Axis-Shield, Bridport, Dorset), 100µl of 0.5M EDTA (BDH) pH 8.0, and 3.3ml of 0.25M sucrose (BDH) on ice. All work dealing with the liver macerate was done in a category III laboratory. The samples were centrifuged at 50 000rpm for 24hrs at 4°C with no brake in a Ti50 rotor of Beckman L8-70 ultracentrifuge. After centrifugation, 500µl of the gradient fractions was harvested sequentially starting from the top. The density in the form of sucrose percentage of each fraction was measured in a refractometer (Atago, Tokyo, Japan). The sucrose percentage was converted to g/ml and fractions with a density of less than 1.067g/ml were pooled, the volume measured and concentrated using a 20ml Vivaspin ultrafiltration spin column (Vivascience, Hannover, Germany) with a molecular weight cut off (MWCO) 1,000,000. These were centrifuged at 2146g at room temperature until 1.4ml was left. Aliquots (100µl) of the concentrated samples were made and were stored at -80°C.

2.11 LLVP uptake assay

Labelling medium

1.275ml Heat-inactivated LPDS
13.725ml Serum free medium

Serum free medium (HepG2)
90ml Sterile dH_2O
10ml 10X Eagles Minimum Essential Medium (GIBCO)
1ml Penicillin/Streptomycin (GIBCO)
1ml 200mM L-Glutamine (GIBCO)
1ml Non-essential amino acid (NEAA)
5ml Gassed 4.4% Sodium bicarbonate + 0.4% phenol red

LPDS + Insulin medium
54.9ml Serum free medium (Growth medium with no FCS)
5.1ml LPDS (lipoprotein deficient serum)
60µl Insulin (Sigma, Gillingham, Dorset)

On day 0, HepG2 cells were seeded at a density of $0.7X10^6$ cells per well in a 6-well-plate (Corning, Schipol-Rijk, Netherlands) in 4ml per well EMEM medium and incubated at 37°C in a 5% CO_2 incubator. On day 2, the attached cells were washed twice with 4ml warm sterile PBS before addition of 4ml warm growth medium, LPDS (8.5%) + insulin (0.1%) medium (to up-regulate LDL receptor) and incubated at 37°C in a 5% CO_2 incubator. On day 5, the cells were washed twice with 5ml warm PBS before addition of 800µl/well pH equilibrated labelling medium, made up of serum free medium containing 8.5% LPDS and the appropriate amount of 3µl HCV LLVP with or without 20µg/ml normal rabbit IgG or 20µg/ml polyclonal rabbit anti-apoB100, a combination of 20µg/ml anti-apoB-100 and 50µg/ml anti-apoE antibodies, or a combination of 20µg/ml anti-apoB-100 and 100µg/ml anti-apoE antibodies, and pre-incubated for 30 minutes in 5% CO_2 incubator. HCV LLVP was thawed and centrifuged at 10,000g for 12 seconds before use. The plates were incubated for 3 hours at 37°C in CO_2 incubator and shaken at every 30 minutes interval. After 3 hours the cells were prepared for RNA extraction for real-time PCR.

2.12 Aggregation test for LLVP

800µl of LPDS labelling medium incubated at 37°C overnight in CO_2 incubator was mixed with 3µl of LLVP and 20µg/ml of apoB100 antibody (Dako) or normal rabbit IgG and incubated for 3 hours in 37°C CO_2 incubator. The samples were centrifuged at 3000g for 40min. The supernatant was removed from each Eppendorf tube leaving behind 30µl, transferred to a new tube and stored at 4°C. 600µl of ice-cold, sterile PBS was added to the 30µl pellet fraction and the tube inverted a few times to mix before centrifuging at 16000g for 5 min and removing the supernatant and leaving about 30µl at the bottom of the tube. The wash step was repeated before proceeding to the RNA extraction step using QIAamp kit (Qiagen).

140µl from each of the two tubes with supernatant was transferred to a new Eppendorf tube, 560µl of AVL lysis solution (QIAamp kit, Qiagen) was added, and the remaining RNA extraction steps were according to the manufacturer's instructions.

560µl of AVL lysis solution from the Qiaamp kit (Qiagen) was added to each of the two 30µl pellet fractions, 110µl DEPC water was added to each sample, and vortexed. The remaining RNA extraction steps were according to the manufacturer's instructions.

All RNA samples were eluted in 100µl volume. The samples were diluted 1 in 15 in DEPC water with carrier RNA for real-time PCR. The supernatant value from the real time PCR was corrected for the amount of HCV RNA found in the total supernatant (800µl) initially.

2.13 DiI-LDL/DiI-oxLDL uptake assay

LPDS + Insulin medium
54.9ml Serum free medium (Growth medium with no FCS)
5.1ml LPDS (lipoprotein deficient serum)
60µl Insulin (Sigma, Gillingham, Dorset)

2mg/ml Hydroxycholesterol solution
5mg Hydroxycholesterol
2.5ml100% ethanol

1mg/ml Hydroxycholesterol solution
1ml 2mg/ml Hydroxycholesterol solution
1ml 100% ethanol
Stored in -80^{o}C

2.5 µg/ml Hydroxycholesterol medium
25µl 1mg/ml Hydroxycholesterol solution
10ml HepG2 growth medium
Mixed very well to ensure hydroxycholesterol is in solution and incubated in HepG2 growth medium in 37^{o}C CO_2 incubator one day before use.

On day 0, HepG2 cells were seeded at a density of $0.7X10^6$ cells per well in a 6-well-plate (Corning, Schipol-Rijk, Netherlands) in 4ml per well EMEM medium and incubated at 37^{o}C in a 5% CO_2 incubator. On day 2 (day 3 for hydroxycholesterol [25-OH] treatment) , the attached cells were washed twice with 4ml warm sterile PBS before addition of 4ml warm growth medium, LPDS (8.5%) + insulin (0.1%) medium (to up-regulate LDL receptor) or growth medium + hydroxycholesterol [25-OH] (to down-regulate LDL receptor) and incubated at 37^{o}C in 5% CO_2 incubator. On day 5, the cells were washed twice with 5ml warm PBS before addition of 1ml/well pH equilibrated labelling medium, made up of serum free medium containing 8.5% LPDS and 25ul DiI-LDL (10µg/well) or 35ul DiI-oxLDL (14µg/well). The plates were wrapped in aluminium foil and incubated for 3 hours at

37°C in CO_2 incubator. The plates were shaken at every 30 minutes interval and after 3 hours the cells were prepared for FACS analysis.

2.14 DiI-LDL/DiI-oxLDL and/or LLVP uptake assay

LPDS + Insulin medium
54.9ml Serum free medium (Growth medium with no FCS)
5.1ml LPDS (lipoprotein deficient serum)
60µl Insulin (Sigma, Gillingham, Dorset)

Labelling medium
1.275ml Heat-inactivated LPDS
13.725ml Serum free medium

Serum free medium (HepG2)
90ml Sterile dH_2O
10ml 10X Eagles Minimum Essential Medium (GIBCO)
1ml Penicillin/Streptomycin (GIBCO)
1ml 200mM L-Glutamine (GIBCO)
1ml Non-essential amino acid (NEAA)
5ml Gassed 4.4% Sodium bicarbonate + 0.4% phenol red

2mg/ml Hydroxycholesterol solution
5mg Hydroxycholesterol
2.5ml100% ethanol

1mg/ml Hydroxycholesterol solution
1ml 2mg/ml Hydroxycholesterol solution
1ml 100% ethanol
Stored in -80°C

2.5 µg/ml Hydroxycholesterol medium
25µl 1mg/ml Hydroxycholesterol solution
10ml HepG2 growth medium
Mixed very well to ensure hydroxycholesterol is in solution and incubated in HepG2 growth medium in 37°C CO_2 incubator one day before use.

On day 0, HepG2 cells were seeded at a density of $0.7X10^6$ cells per well in a 6-well-plate (Corning, Schipol-Rijk, Netherlands) in 4ml per well EMEM medium and incubated at 37°C in a 5% CO_2 incubator. On day 2 (day 3 for hydroxycholesterol [25-OH] treatment), the attached cells were washed twice with 4ml warm sterile PBS

before addition of 4ml warm growth medium, LPDS (8.5%) + insulin (0.1%) medium (to up-regulate LDL receptor) or growth medium + hydroxycholesterol [25-OH] (to down-regulate LDL receptor) and incubated at 37°C in 5% CO_2 incubator. On day 5, the cells were washed twice with 5ml warm PBS before addition of 1ml/well (800µl/well for LLVP) pH equilibrated labelling medium, made up of serum free medium containing 8.5% LPDS and the appropriate amount of DiI-LDL/DiI-oxLDL [25ul DiI-LDL (10µg/well) or 35ul DiI-oxLDL (14µg/well)] alone or HCV LLVP with or without 20µg/ml normal rabbit IgG or 20µg/ml polyclonal rabbit anti-apoB100 antibodies, and pre-incubated for 30 minutes in 5% CO_2 incubator. HCV LLVP was thawed and centrifuged at 10,000g for 12 seconds before use. The plates were wrapped in aluminium foil (for DiI-LDL/DiI-oxLDL assay) and incubated for 3 hours at 37°C in CO_2 incubator. The plates were shaken at every 30 minutes interval and after 3 hours the cells were prepared for FACS analysis or RNA extraction for real-time PCR.

2.15 Preparation of cells for fluorescence activated cells sorting (FACS) analysis

PBS + 0.5% BSA / Wash solution
200ml PBS
1g BSA (BDH, Poole, Dorset)

Trypsin + EDTA solution
58.5ml PBS
642µl 100mM EDTA
1200µl 2.5% Trypsin (Gibco BRL, Paisley, UK)

FACS rinse buffer
200ml PBS
2g BSA

BSA was added on the day of FACS

The plate of cells inoculated with DiI labelled lipoproteins described in section 2.13 were placed on ice and washed once with 5ml PBS containing 0.5% BSA per well. 1ml of 10mg/ml suramine (Sigma, Gillingham, Dorset) was then added to each well and the incubated at 4^{o}C for 30minutes to remove surface bound lipoprotein. The cells were washed once with 5ml PBS + 0.5% BSA on ice and then removed from the ice before adding 5ml of warm (37^{o}C) Trypsin + EDTA solution containing PBS, 100mM EDTA and 2.5% Trypsin to dissociate the cells from the well. Approximately 4ml of the solution was removed immediately and the plates were incubated at 37^{o}C for 10 minute. The action of trypsin was stopped by adding 2ml of EMEM growth medium to each well. The cells were pipetted vigorously using a Pasteur pipette to break them up into single cell suspension and then topped up with 3ml of EMEM growth medium before filtering them through 11um filter (Millipore, Watford) into a 14ml Falcon tube. The cell suspension was centrifuged at 1500rpm for 10min and brake set at 1 in MSE 1000 centrifuge at room temperature. After removing the supernatant, the cell pellet was resuspended in 1500ul freshly prepared FACS rinse buffer, transferred into a 12x75mm, 5ml polystyrene, round bottom FACS tube (Becton Dickinson, Devon), and stored on ice, ready for FACS analysis. In each sample, 10,000 cells were analysed on the FACScan, (Becton Dickinson, Devon) using detectors FSC, voltage E-1 and FL-2, voltage 474 and Cell Quest Pro software where cells are sorted according to the light scattering and fluorescent characteristics of each cell, in this case the cells labelled with DiI, and translated into mean fluorescent intensity. The figure 7 below shows an example of the FACS analysis. From the dot plot (figure 7A) a single cell population (gated) was selected and the fluorescence intensity distribution, expressed in logarithmic scale, of this cell population is shown in figure 7B.

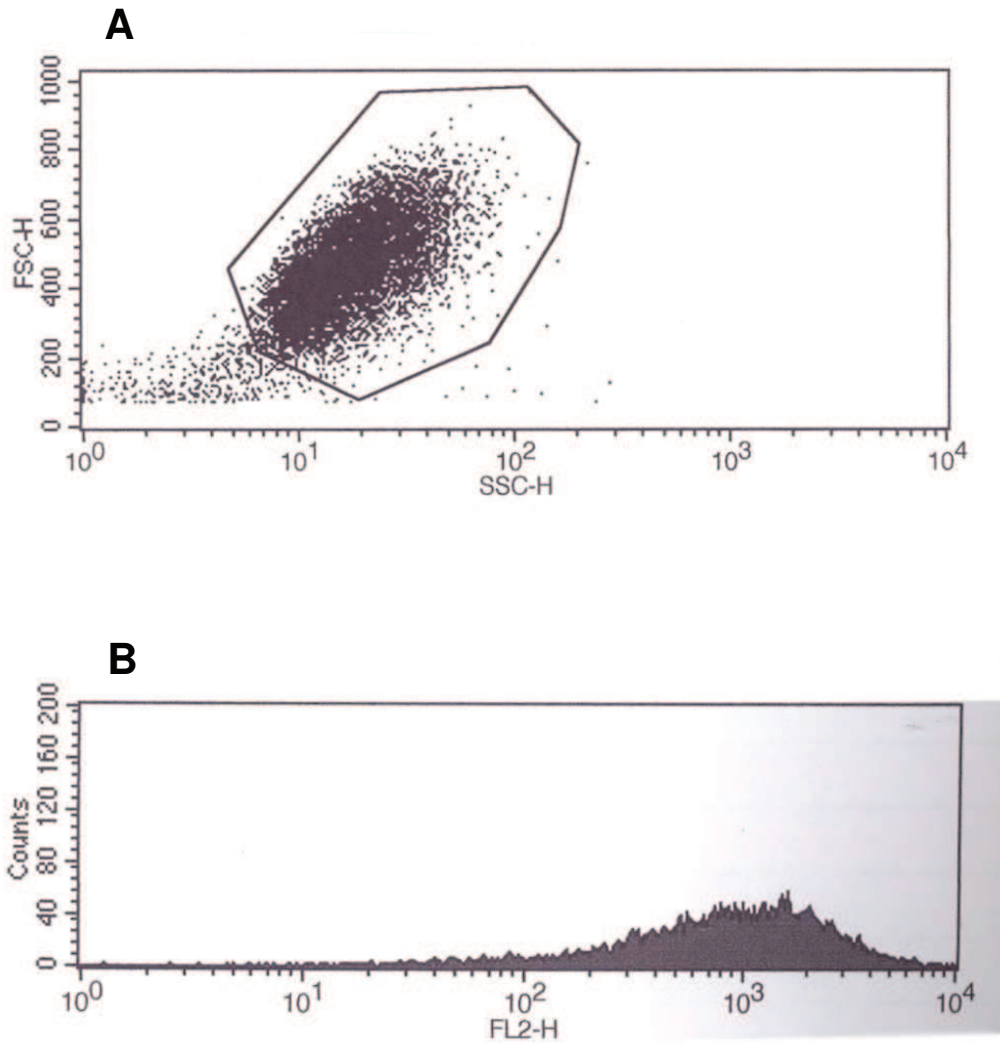


Figure 7: A. Dot plot and B. histogram of the population of HepG2 cells used to analyse entry of DiI-LDL.
FSC-H shows the size and SSC-H shows the granularity of 10,000 cells out of the sample. FL2-H shows the fluorescence intensity distribution across the sample of gated cells.

2.16 Propidium iodide staining for FACS analysis

1mg/ml propidium iodide solution

1mg Propidium iodide (Sigma, Gillingham, Dorset)
1ml distilled water

To determine the percentage cell death for cells treated with oleic acid, 30µl of 1mg/ml propidium iodide (Sigma, Dorset) was added to the cell suspension immediately before FACS analysis. In each sample, 10,000 cells were analysed on the FACScan, (Becton Dickinson, Devon) using detectors FSC, E-1 and FL-2, voltage 474 and Cell Quest Pro software where cells are sorted according to the light scattering and fluorescent characteristics of each cell, in this case the cells taking up propidium iodide, and expressed in percentage of dead cells. The figure 8 below shows an example of the FACS analysis. From the dot plot (figure 8A) a single cell population (gated) was selected and the fluorescence intensity distribution, expressed in logarithmic scale, of this cell population is shown in figure 8B.

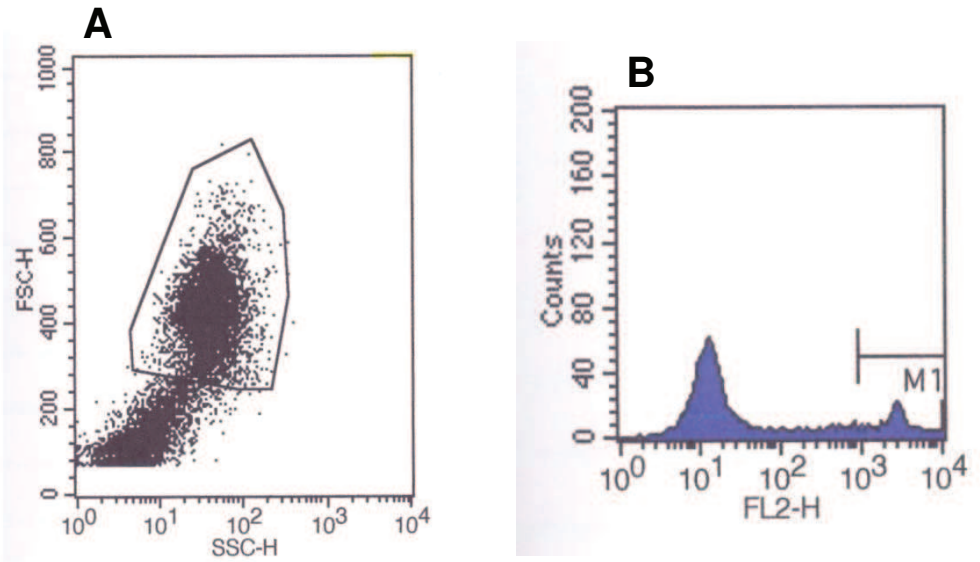


Figure 8: A. Dot plot and B. histogram of the population of HepG2 cells used to analyse cells taking up propidium iodide.
FSC-H shows the size and SSC-H shows the granularity of 10,000 cells out of the sample. FL2-H shows the fluorescence intensity distribution across the sample of cells. M1 shows fluorescence intensity distribution of the gated cell population taking up the propidium iodide.

2.17 Antibodies

Antibodies were used in various experimental procedures in this project. The antibodies used are summarised in Table 2.

Antibody	Isotype	Source	Other information
Anti-SR-B1 NB 400-104	n/a	Novus Biologicals, Inc.	-purified rabbit polyclonal antibody against the human and mouse SR-B1 receptor - recognizes an epitope in the C-terminal intracellular domain of the SR-B1 receptor
Anti-LDLr C7	IgG2b	Santa Cruz Biotechnology, California, U.S.A	-mouse monoclonal antibody -binds to the N-terminus of the LDL-R, an extracellular domain
Anti-LDLr 61099	IgG	Progen Biotechnik, Germany (UK distributor: Insight Biotechnology)	-affinity purified rabbit polyclonal, monospecific - specific for the human LDLr extracellular domain
Anti-apoB-100	n/a	DAKO, Ely, UK	-polyclonal rabbit antibody against human apoB-100
Anti-apoE	n/a	DAKO, Ely, UK	-purified rabbit immunoglobulin against human apoE
anti-E2 HCV B65581G	n/a	Biodesign, Maine	-goat antibody against HCV E2
anti-HVR-1 3C7-C3	IgG1	M.U. Mondelli, Pavia, Italy (Cerino et al., 2001)	-HCV E2 mouse monoclonal antibody
Anti-HCV E2 AP33	n/a	A. Patel, Glasgow, UK (Clayton et al., 2002)	-HCV E2 mouse monoclonal antibody
Anti-HCV core ϕ126	n/a	Biogenesis, Poole, UK	-HCV core protein mouse monoclonal antibody
Normal Rabbit Immunoglobulin	IgG	Dako	-purified rabbit polyclonal antibody
Normal Goat Immunoglobulin	n/a	Sigma, Poole, UK	-goat antibody
Anti-RSV-P 2G 12_2	IgG1	E. Routledge, UK (Routledge et al., 1985)	-mouse monoclonal antibody against phosphoprotein
Swine anti rabbit IgG-FITC	n/a	Dako	-swine antibody
Sheep anti mouse IgG-FITC	n/a	Chemicon	-sheep antibody
rabbit anti-mouse IgG-FITC	n/a	Dako	-rabbit antibody

Table 2: Antibodies used throughout the project

2.18 Confocal microscopy

0.6 x 10^6 cells were seeded in a 60mm x 15mm Petri dish (Corning, Schipol-Rijk, Netherlands) in 6ml EMEM growth medium and incubated at 37°C in 5% CO_2 incubator for two days. The cells were washed with 5ml PBS twice, 6ml LPDS + insulin medium per dish was added before further incubation of 3 more days. The labelling medium was equilibrated in 5% CO_2 incubator a day prior to the experiment.

For 0ºC treatment, the Petri dishes were incubated for 30 minutes on ice in a refrigerator to cool the cells. The Petri dishes were washed twice with 5 ml of ice-cold PBS containing 0.5 % BSA per dish. The PBS containing 0.5 % BSA was removed and 2.5ml of ice-cold labelling medium containing either 10μg/ml of DiI-LDL or 2.5ml of ice-cold labelling medium containing 14μg/ml of DiI-oxLDL or 2.5ml of ice-cold labelling medium without DiI was added. The Petri dishes were shaken to distribute the medium and wrapped in aluminium foil. Then they were incubated in refrigerator sitting on ice for 3 hours. The dishes were shaken every half an hour interval.

After 3 hours incubation, the medium was removed and washed with 5ml of ice-cold PBS containing 0.5 % BSA. If the cells were to be washed with suramine, 2ml of 10mg/ml suramine solution per dish was added and incubated for 30 minutes in the refrigerator. The wash step was repeated using 5ml ice-cold PBS containing 0.5 % BSA. 4ml ice-cold EMEM growth medium with 10 % FCS was then added. These dishes were stored in the refrigerator sitting on ice inside a lunchbox wrapped in aluminium foil until they could be viewed by confocal microscopy.

For 37°C treatment, the medium was removed and the Petri dishes were washed twice with 5 ml of warm (37°C) PBS containing 0.5 % BSA per dish. The wash buffer was removed and 2.5ml of warm (37ºC) labelling medium containing either 10µg/ml of DiI-LDL or 2.5ml of warm labelling medium containing 14µg/ml of DiI-oxLDL or 2.5ml of warm labelling medium without DiI control was added. The Petri dishes were shaken to distribute the medium and wrapped in aluminium foil. Then they were incubated in 37ºC, 5% CO_2 incubator for 3 hours. The dishes were shaken every half an hour interval. After 3 hours incubation, the steps involved were the same as for the 0°C treatment of the cells.

The cells were analysed on a Leica TCS SP2 UV CLSM microscope (Leica Microsystems, Heidelberg, Germany) using excitation wavelength of 488nm and emission wavelength of 550-600nm. Leica Confocal Software Lite was used to analyse the images.

2.19 FITC-Staining of HepG2 cells for FACS

Versene
59.358ml PBS
642µl 100mM EDTA

FACS rinse buffer
1 g Bovine Serum Albumin (BDH)
100 ml PBS
0.5 ml 20 % NaN_3
Filtered through 0.22 µm filter.
Stored at 4 ºC for up to 1 week.

0.1% Saponin in PBS
100 mg Saponin (Sigma, Gillingham, Dorset)
100 ml PBS
Dissolved by magnetic stirring; prepared day before experiment.

FACS diluent with sheep serum
95 ml FACS rinse buffer
5 ml normal sheep serum (Sigma, St. Louis, Missouri)
Serum was heat inactivated at 56 ºC for 45 min.
Filtered through 0.22 μm filter
Stored at 4 ºC for up to 1 week

0.1% Saponin in FACS diluent containing sheep serum
50 mg Saponin (Sigma, Gillingham, Dorset)
50 ml FACS diluent with sheep serum (0.22 μm filtered)
Dissolved by magnetic stirring; prepared day before experiment.

FACS diluent with swine serum
95 ml FACS rinse buffer
5 ml normal swine serum (Sigma, St. Louis, Missouri)
Serum was heat inactivated at 56 ºC for 45 min.
Filtered through 0.22 μm filter.
Stored at 4 ºC for up to 1 week.

0.1% Saponin in FACS diluent containing swine serum
50 mg Saponin (Sigma, Gillingham, Dorset)
50 ml FACS diluent with swine serum (0.22 μm filtered)
Dissolved by magnetic stirring; prepared day before experiment.

On day 0, $0.7X10^6$ HepG2 cells/well were plated into each well of a 6-well-plate (Corning, Schipol-Rijk, Netherlands) in 4ml EMEM growth medium and incubated at 37^oC in a 5% CO_2 incubator for two days. On day 2 (day 3 for 25-OH), the cells were washed with 4ml PBS twice, 4ml 8.5% LPDS + (0.1%) insulin or growth medium + 25-OH per dish was added before further incubation of 2/3 more days. On day 5, the cells were washed once with PBS, washed once with 5ml versene before adding 4ml of fresh warm versene. The plate was incubated at 37ºC in an incubator for 20 minutes before pipetting off the versene. HepG2 growth medium (2ml) was added to each well, cells were pipetted vigorously using Pasteur pipette until a single cell suspension was obtained, 2ml FACS rinse buffer was added to each well, and the cell suspension was pooled into a beaker.

The pooled cell suspension was filtered through an 11 μm filter (Millipore, Watford) into a 50 ml Falcon tube, centrifuged at 402g (1500rpm) for 5 min at +4 ºC in a swing out rotor (cat. 34121-613) Chilspin 2 centrifuge (M.S.E., UK), the supernatant removed, and cells resuspended in 4ml PBS. The cells were counted and the volume of cell suspension needed for the staining experiment was calculated. The number of cells needed per well was 2 x 10^5 cells/well of a 96-well-plate. The cell suspension was adjusted to 2 x 10^6 cells/ml by adding PBS, added with an equal volume of 4% paraformaldehyde (Sigma) (room temperature), incubated for 30min at room temperature, centrifuged at 1500 rpm for 5 min at 4 ºC, washed twice in 20ml PBS, split into 2 Falcon tubes equally, and centrifuged at 1500rpm for 5 min at 4 ºC in an tabletop centrifuge before removing the supernatant. The cell pellet was added with PBS containing 0.1% Saponin (Sigma) in the same volume calculated earlier, incubated for 20 minutes at room temperature, centrifuged at 1500 rpm for 5 min at 4ºC, and supernatant removed.

The cells were resuspended in either FACS diluent with sheep serum or FACS diluent with swine serum to give 2x10^6cells/ml cell suspension and incubated on ice until required. Diluted antibody (25 μl) to be tested, which had been diluted in either 0.1% Saponin in FACS diluent containing sheep serum (for LDL receptor antibody) or 0.1% Saponin in FACS diluent containing swine serum (for SR-B1 receptor antibody), was added into the appropriate wells of a 96-well round bottom (3910 Falcon plates, non-tissue culture treated) or Sero-wel (Sterilin, Hounslow, UK). The appropriately diluted cell suspension (25 μl) was added to the appropriate well making sure the cells were in suspension. The plate was covered with parafilm, agitated for 30 second on the Easiashaker (Medgenix Diagnostics, Brussels, Belgium) at setting 10, and incubated at 4 ºC for 1 hour wrapped in aluminium foil. The cells

were rinsed by adding 200µl of cold FACS diluent with either sheep serum (for LDL receptor wells) or swine serum (for SR-B1 receptor wells), spinning the plate (with parafilm plus lid) for 5 min at 982g in the plate tabletop centrifuge Mistral 2000 (M.S.E, UK), the supernatant removed, and the plate shaken on the Easiashaker to break up the cell pellets. The cells were rinsed twice with 200µl FACS rinse buffer, and 50µl of 1/50 of rabbit anti-mouse IgG FITC (Dako) or 1/50 of swine anti-rabbit IgG FITC (Dako) was added to each well, the plate covered and shaken as before and incubated at 4 ºC for 1 hour wrapped in aluminium foil. The cells were rinsed twice with 200µl FACS rinse buffer, resuspended in 100µl FACS rinse buffer, shook for 60 seconds on the plate shaker, topped up with 100µl FACS rinse buffer, transferred into a 12x75mm, 5ml polystyrene, round bottom FACS tube (Becton Dickinson, Devon), and stored on ice, ready for FACS analysis. In each sample, 5,000 cells were analysed on the FACScan, (Becton Dickinson, Devon) using detectors FSC, E-1 and FL-1, voltage 474 and Cell Quest Pro software where cells are sorted according to the light scattering and fluorescent characteristics of each cell, in this case the cells labelled with FITC, and translated into mean fluorescent intensity. The figure below shows an example of the FACS analysis. From the dot plot (figure 9A) a single cell population (gated) was selected and the fluorescence intensity distribution, expressed in logarithmic scale, of this cell population is shown in figure 9B.

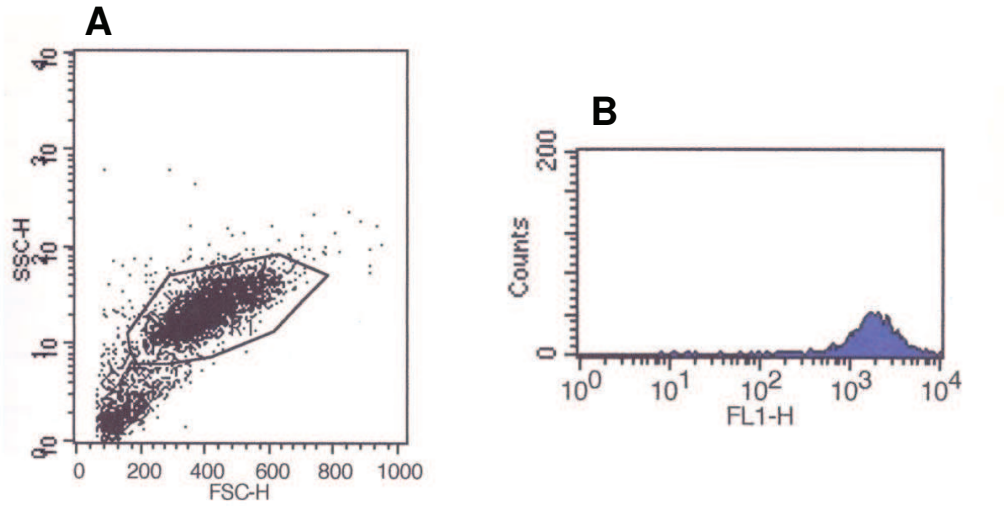


Figure 9: A. Dot plot and B. histogram of the population of HepG2 cells used to analyse FITC-conjugated anti-SR-B1 for staining of cells with SR-B1.
FSC-H shows the size and SSC-H shows the granularity of 5000 cells out of the sample. FL1-H shows the fluorescence intensity distribution across the sample of gated cells.

2.20 Quantitation of HCV RNA

2.21 RNA extraction from cell cultures

HepG2, Huh7.5, and primary hepatocytes cells were washed once with cold 5ml PBS containing 0.5% BSA per well and 1ml of 10mg/ml suramine added per well. The cells were incubated at 4°C for 30min. The suramine was removed and the cells were washed again with 5ml PBS containing 0.5% BSA.

1ml of solution D (4M guanidium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1M β-mercaptoethanol) was added to denature the protein. A cell scraper (Corning, Schipol-Rijk, Netherlands) was used to scrape the cells from the bottom of the well and the well content was transferred into a sterile eppendorf tubes. The well was rinsed with 300µl of solution D and this was pooled with the 1ml initial cell mixture. The cell mixture was shaken for 10 minutes at full speed using an Eppendorf shaker of 24-well capacity. The Eppendorf tubes were centrifuged at

10000g for 30 seconds. The cell homogenate was either frozen in liquid nitrogen and stored in -80^{o}C freezer or extracted for RNA immediately.

To extract the RNA, the cell homogenate was layered onto 4ml of 5.7M cesium chloride (Sigma, Gillingham, Dorset) in a DEPC treated Ti50 tube (Beckman Coulter, Buckinghamshire). Tubes were centrifuged at 47,000rpm without a brake at 18^{o}C for 18-22 hours in a Ti50 rotor Beckman L7 ultracentrifuge. After centrifugation, the supernatant was removed and the tubes were centrifuged upside down at 500rpm for 2 minutes in MSE 1000 centrifuge. The RNA pellet, now visible, was further purified using RNAEasy mini kit, (Qiagen) according to the manufacturer's instructions. In the final stage of the RNAEasy mini kit procedure, the RNA can be eluted in 30-50ul once or twice depending on the RNA yield. One time elution of 30µl volume and two times elution of 100µl total volume were chosen for comparison purposes.

RNA was extracted from the cell homogenate and the supernatant of the cell culture using QIAamp viral RNA mini kit (Qiagen, UK) following the manufacturer's instructions. The viral RNA was stored at -20^{o}C or -70^{o}C.

2.22 Determination of RNA quantity

The optical density (OD) measurement on the RNeasy extracted RNA was done to determine the purity of RNA. The extracted RNA was diluted 1 in 45 for a 30µl extraction volume and 1 in 13.5 for a 100µl extraction volume in 10mM Tris-HCl. The OD at wavelengths of 260 and 280 was measured using the GeneQuant II spectrophotometer (Pharmacia Biotech, Little Chalfont, Buckinghamshire). An OD_{260}/OD_{280} ratio between 1.8 to 2.1 was of acceptable purity.

2.23 Real time polymerase chain reaction

To minimize the possibility of contamination of PCR-reactions with amplicons from previous experiments, RT-PCR was carried out at a series of stations in separate rooms with dedicated equipment and protective clothing. These were clean area for preparation of mastermixes, an RNA station for RNA extraction and adding RNA to mastermix, a DNA station for handling cDNA and an analysis area for reading and analysis of amplicons.

2.24 HCV RNA standard samples for RT-PCR Curve

In order to determine the amount of HCV RNA in the samples, a standard curve ranging from 700,000 IU to 3.6 IU was used. 1 IU is equal to 3-8 genomes equivalent as defined by the WHO HCV working group (Saldanha, 1996). HCV RNA kindly supplied by the National Institute for Biological Standards and Control (NIBSC, Potters Bar, Hertfordshire) code 96/790 was used to make the standard curve ranging from 300 IU to 3.6 IU. The standard curve ranging from 700,000 IU to 960 IU was prepared from HCV RNA purified from liver PF2 and stored at -80^{o}C and titered against the NIBSC by Pumeechockchai (Pumeechockchai, 2001). All of the HCV RNA standards were prepared in a three fold dilution series and stored at -20^{o}C (Martin in 2005).

2.25 HCV RNA standard curve from qRT-PCR

An HCV RNA standard curve is needed to quantitate HCV RNA in samples of subsequent experiments using quantitative Real-Time PCR technique. A standard curve ranging from 700,000 IU to 3.6 IU was used with 1 IU being equivalent to 3-8

genomes (Saldanha, 1996) as defined by the WHO group working on HCV. The higher ends of HCV RNA standards were from 1:3 dilutions of high titer PF2 liver macerates (Pumeechockchai in 2001) and the lower ends were from 1:3 dilutions of NIBSC standards. Figure 10 below is an example of the amplification plot of the HCV RNA standards from the qRT-PCR.

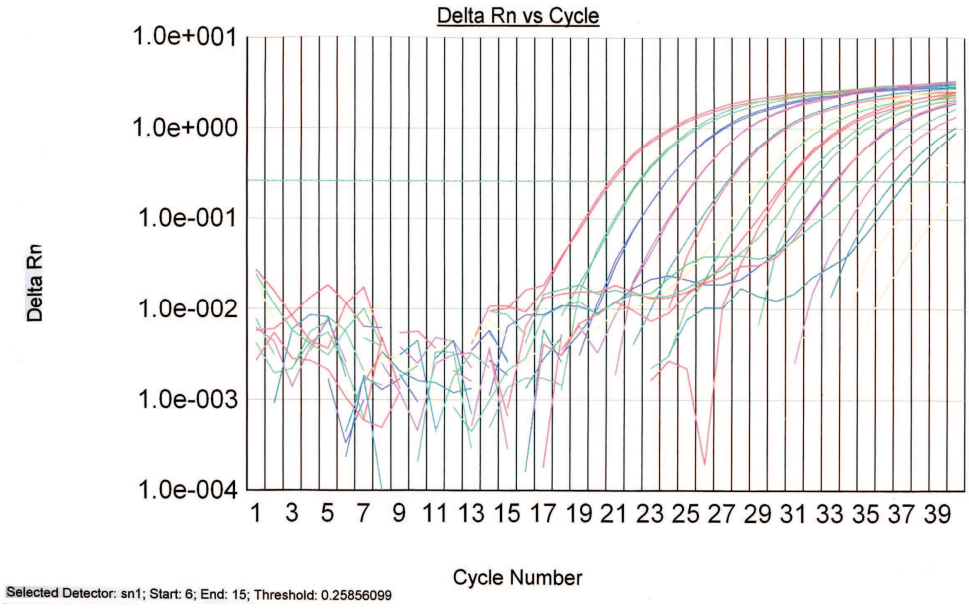


Figure 10: Amplification plot of the HCV RNA standards from the qRT-PCR.

The following figure 11 shows an example of the HCV RNA standard curve derived from the amplification plot of a Real-Time PCR.

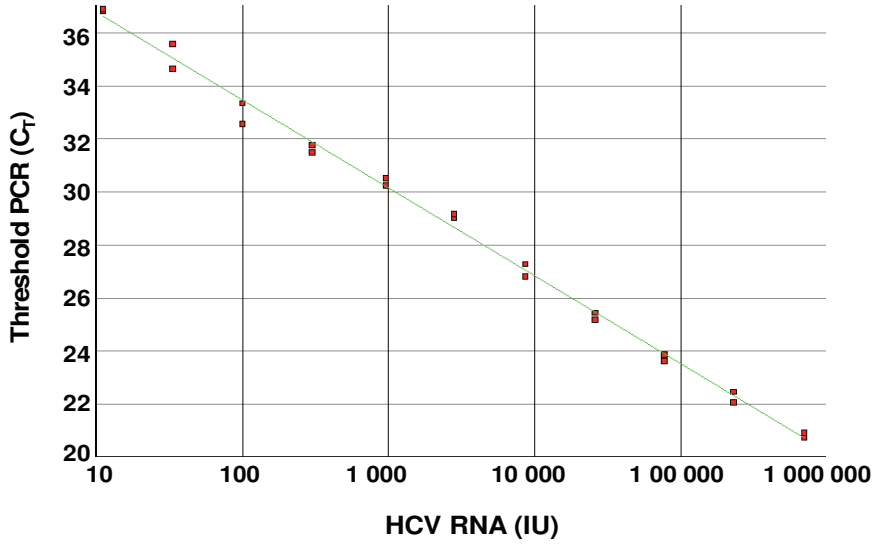


Figure 11: Real-time PCR standard curve for HCV RNA.

2.26 Primers and probes

The primers, NCR-3 and NCR-5 and probe SN1 used in Real-Time PCR during the course of the projects are shown in table 3 below.

Name	Base sequence	Base pairs *	Reference	Source
NCR-3 Reverse Primer	5' ACCACAAGGCCTTTCGCGACCCAAC 3'	263 - 287	(Mercier, 1999); modified by Nielsen)	Invitrogen, Paisley
NCR-5 Forward Primer	5' CCCCCCCTCCCGGGAGAGCCAT 3'	120 - 141	(Mercier, 1999); modified by Nielsen)	Invitrogen, Paisley
SN1 Probe	5' FAM-ATTCCGGTGTACTCACCGGTTCCGCAGA -TAMRA 3'	147 - 174	(Mercier, 1999); modified by Nielsen	Applied Biosystems, Warrington

Table 3: The sequences of NCR-3 Reverse Primer, NCR-5 Forward Primer, and SN1 Probe.
NCR refers to non-coding region of the HCV genome, FAM is a reporter dye and TAMRA is a quencher dye.
* Base pair numbers refer to the sequence described by (Kolykhalov, 1997).

2.27 Dilutions of RNA for RT-PCR

Extracted RNA was diluted at 1 in 15 for 100µl extraction volume and 1 in 50 for 30µl extraction volume. The diluent used consisted of DEPC treated water with 8µg/ml carrier RNA from the QIAAMP kit (Qiagen) and was stored at -20^{o}C. 5µl HCV RNA was added to 70µl diluent, vortexed briefly and centrifuged at 13 000 rpm briefly before reverse transcription.

2.28 Reverse Transcription with Primer NCR-3

12.5µl reaction mastermix per tube was prepared consisting of 2.5µl of each of 10x RT buffer (Promega, Southampton), 25mM $MgCl_2$ (Promega), dNTPs (10mM each of dATP, dTTP, dCTP and dGTP in Diethyl pyrocarbonate (DEPC) water from Invitrogen, Paisley) and 25µM NCR-3 primer (in DEPC water, Invitrogen), 0.625µl RNasin Ribonuclease Inhibitor (in 20mM HEPES-KOH, 50mM KCl, 8mM DTT and 50% glycerol, Promega) and 0.938µl of each of 10U/µl Avian Myeloblastosis Virus-Reverse Transcripase (AMV-RT in 200mM potassium phosphate, 0.2% Triton X-100, 2mM DTT and 50% glycerol, (Promega) and DEPC treated water. The mastermix was thoroughly mixed and briefly centrifuged before aliquoting 12.5µl into each of the required number of wells on a reaction plate, on ice. This was done in a PCR clean room. The plate was transferred to the RNA area. 12.5µl of the 1 in 15 or 1 in 50 dilution of RNA was added into the appropriate wells of a 48-well plate (Applied Biosystems, Warrington, Cheshire) and covered with Microseal 'A' film (M.J.Research, Waltham, U.S.A). The plate was centrifuged briefly and placed in a DNA Engine PTC-200 Peltier Thermal Cycler (M.J. Research) at 37ºC for 20

minutes, 42ºC for 40 minutes, 48ºC for 20 minutes, 95ºC for 5 minutes and then 4ºC indefinitely. The plate was stored at -20°C freezer until the next step.

2.29 RT-PCR with primers NCR-3 + NCR-5 and probe SN1

A 24µl reaction mastermix per tube consisting of 15µl Taqman Universal PCR mastermix (Applied Biosystems, Warrington, Cheshire), 1.08µl of each of the primers NCR-3 and NCR-5 (25µM each in DEPC water, Invitogen, Paisley), 0.9µl probe SN1 (in DEPC water, Applied Biosystems) and 5.94µl DEPC water was prepared.The mastermix was thoroughly mixed and briefly centrifuged before aliquoting 24µl into each of the required number of wells on a 96-well PCR plate (Applied Biosystems), on ice. This was done in the clean room. The plate was transferred to the cDNA area. 6µl of cDNA from the previous step was added into the appropriate wells. The plate was covered with a clear plate seal and centrifuged briefly. The cDNA was amplified in an ABI-Prism 7000 Real-Time PCR machine, at 95°C for 10 minutes then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. After amplification, the Real-Time PCR machine analysis was done by ABI Prism 7000 SDS software.

2.30 Optimization of RNA dilution to minimize the effect of RNA inhibitors

Martin (Martin, 2005) showed that there were inhibitors present in the liver macerate which affected the Real-Time PCR values. The effect of RNA inhibitors from HepG2 cells derived during the RNA extraction process on real-time PCR was tested using two different methods.

In the first method (Method 1), the HepG2 cells were inoculated with LLVP and incubated for 3 hours and the RNA from the inoculated cells was extracted for real-time PCR quantitation. On day 0, HepG2 cells were seeded at a density of $0.7X10^6$ cells per well in a 6-well-plate (Corning, Schipol-Rijk, Netherlands) in 4ml per well EMEM medium and incubated at 37°C in a 5% CO_2 incubator. On day 2, the attached cells were washed twice with 4ml warm sterile PBS before addition of 4ml warm growth medium, LPDS (8.5%) + insulin (0.1%) medium (to up-regulate LDL receptor) and incubated at 37°C in a 5% CO_2 incubator. On day 5, the cells were washed twice with 5ml warm PBS before addition of 800µl/well pH equilibrated labelling medium, made up of serum free medium containing 8.5% LPDS and 6µl (4 X 10^5IU) HCV LLVP, incubated for 3 hours at 37°C in CO_2 incubator and shaken at every 30 minutes interval. After 3 hours, the RNA from the cells was extracted using RNA extraction for cell culture method described in section 2.21 before proceeding to real-time PCR.

In the second method (Method 2), RNA from uninfected HepG2 cells was extracted using RNA extraction for cell culture method described in section 2.21 and then approximately 1.3 X 10^4IU of HCV RNA standard was added to each dilution of HepG2 RNA before proceeding to real-time PCR.

In both methods, two elution volumes: 30µl and 100µl, were used because in the final stage of the RNAEasy mini kit procedure, the RNA can be eluted in 30-50ul once or twice depending on the RNA yield. One time elution of 30µl volume and two times elution of 50µl (100µl total volume) were chosen for comparison purposes. Dilution

factors of 100, 500, 1000, 2000, and 5000 (refer to table 4) of extracted RNA were also made to test if the inhibitor effect could be avoided by dilution.

100µl RNeasy elution volume		30µl RNeasy elution volume	
Dilution	**Dilution factor**	**Dilution**	**Dilution factor**
1:3	100	1:10	100
1:15	500	1:50	500
1:30	1000	1:100	1000
1:60	2000	1:200	2000
1:150	5000	1:500	5000

Table 4: Corresponding dilution factor from the RNA dilutions of the RNA extracted from the LLVP inoculated HepG2 cells and HepG2 cells alone.

The results for the first method are presented in figure 12. The optimal dilution factor for real-time PCR was 500 for the 100µl elution and 500 for the 30µl elution in the final stage of RNA extraction using RNeasy kit. For both elution volumes, the sample with a final dilution factor of 100 gave a reduced yield compared to higher dilutions indicating that inhibitors are present. The yield derived from the OD_{260} spectrophotometer readings (data not shown) shows that RNeasy elution of 100ul gave 1.29 times higher yield than 30µl RNeasy elution.

The results of the second method are presented in figure 13. Inhibitors were evident as judged by a reduced yield at final dilution factors of 100 and 500 but not at 1000.

Both methods showed that inhibitor effect could be minimized by dilution. It is not clear where the inhibitors originated from; either the inoculum or the HepG2 lysates. It is clear though that there is a reduction of inhibitor effect when dilution of RNA is made prior to carrying out real-time PCR.

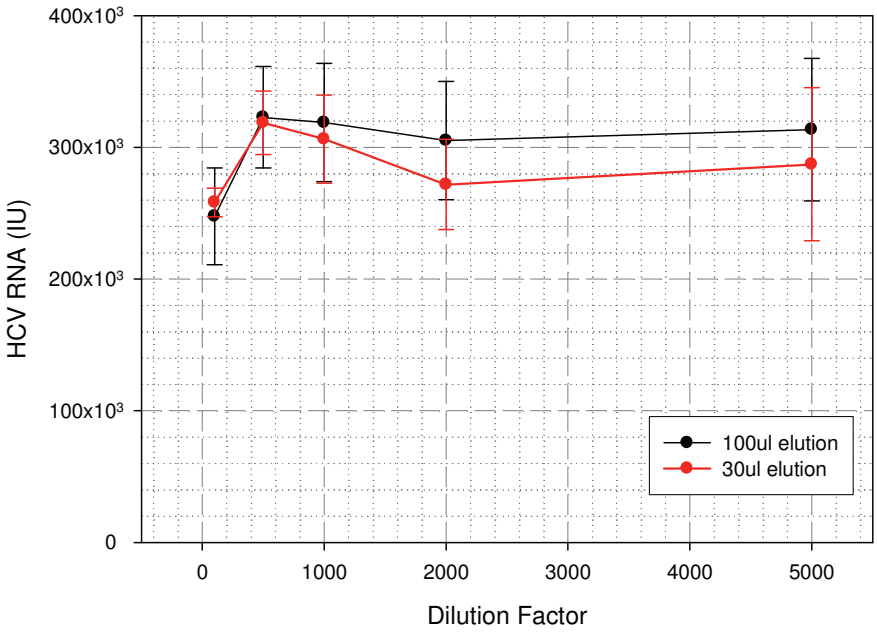


Figure 12: RT-PCR quantification of HCV RNA from HepG2 cells that were infected with LLVP.
The HCV RNA at different dilution factors was quantitated.

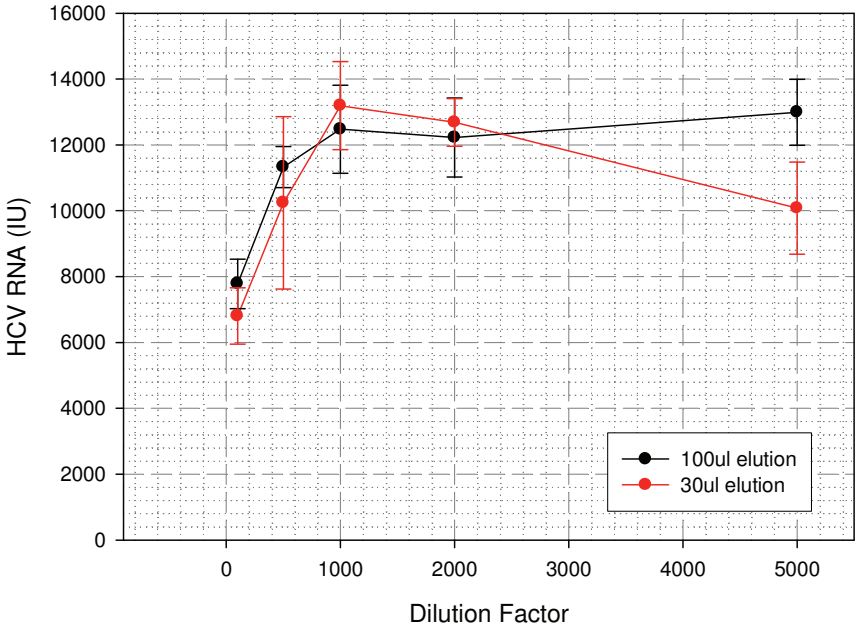


Figure 13: RT-PCR quantification of HCV RNA from HCV RNA standards that were added to RNA of uninfected HepG2 cells.
The HCV RNA standards at different dilution factors was quantitated.

2.31 The J6/JFH1 HCV replication system

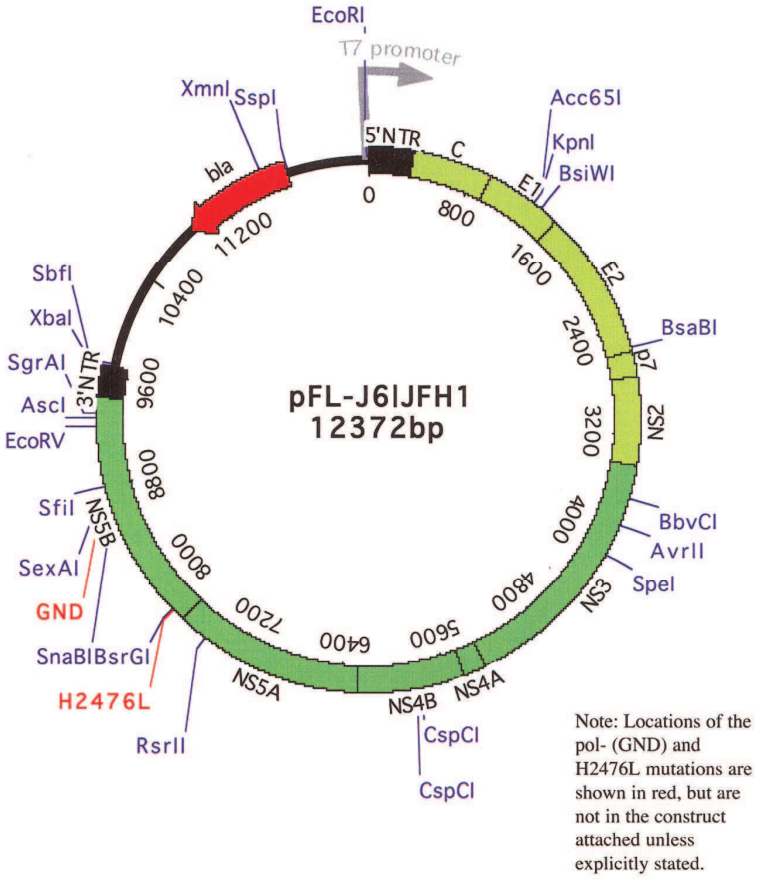


Figure 14: A map of pFL-J6/JFH1 DNA.
The map shows size, HCV proteins coding regions, T7 promoter site, restriction enzyme sites, as well as, locations of the pol-(GND) and H2476L mutations.

2.32 Extraction of pFL-J6/JFH1 and pFL-J6/JFH1-GND plasmids from a filter

Wild-type pFL-J6/JFH1 DNA, and also mutants pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND DNA adsorbed to filter paper were kindly provided by Prof. Charlie Rice from Rockefeller University, were cut out from the filter paper. Each filter paper containing 3µg of pDNA was placed in eppendorf tube and 50µl of TE buffer pH 7.4

was added to elute out the DNA. The filter paper was incubated for 30min at room temperature, vortexed every 10 minutes for a few seconds and finally centrifuged briefly. The supernatant was transferred to a new Eppendorf tube and stored at -80°C.

LB medium

8g Bactotryptone (Oxoid)
4g Yeast extract (Oxoid)
8g NaCl
240µl 5M NaOH

Dissolved in 800ml distilled water, autoclaved and stored at 4°C
Before use, 100µl of 50mg/ml Ampicillin (Sigma) was added per 100ml.

LB agar plates

4g Bactotryptone (Oxoid)
2g Yeast extract (Oxoid)
4g NaCl
120µl 5M NaOH
6g Bacto™ Agar (Benton Dickinson)

Dissolved in 400ml deionised water, autoclaved and stored at 4°C.
The agar was melted in boiling waterbath on a different day, cooled to 50°C in waterbath, ampicillin added to 50µg/ml and stored at 4°C.

SOB medium

8g Bactotryptone (Oxoid)
2g BActo Yeast (Oxoid)
234mg NaCl
75mg KCl

Dissolved in 400ml distilled, autoclaved and stored at 4°C. 4ml from 1M $MgCl_2$ and 4ml from 1M $MgSO_4$ added on the day of use.

Transformation buffer

200ml 2-(*N*-morpholino)ethanesulphonic acid (MES) [Mr 195.2]
490mg 2-(*N*-morpholino)ethanesulphonic acid (MES) dissolved in 200ml Millipore water and adjusted to pH 6.3.
1.85g KCl
2.22g Manganese II chloride tetrahydrate
0.367g Calcium chloride
200mg Hexamine Cobalt III chloride

The volume make up to 250mL with distilled water and mixed with stirrer.

0.2µM filter was rinsed with sterile 2 X 20ml Millipore water before filter sterilising the transformation buffer into 5 X 50ml tubes and the buffer was stored at -20°C.

2.33 Competent bacteria preparation

Competent bacteria were prepared by growing a seed culture of *E. coli* TGI the day before carrying out the transformation. Two universals containing 5ml SOB each were inoculated with *E. coli* TG1 using a sterile toothpick and incubated overnight in an orbital shaker at 37°C. The following day, 100µl of the *E. coli* TG1 culture was inoculated into a pre-incubated (37°C) 60ml SOB medium in a conical flask plugged with cotton wool. The *E. coli* TG1 were incubated for 2 hours in the orbital shaker at 37°C. After 2 hours, the OD at 550nm was measured using SOB medium as a reference. Incubation was continued until an OD of 0.45 to 0.55 was reached. 50ml of *E. coli* TG1 culture was then transferred into a 50ml Falcon tube and incubated on ice for 15 minutes. The culture was centrifuged at 2862g for 10min at 4°C. The supernatant was discarded and the bacterial pellet resuspended first in 5ml followed by another 15ml of thawed ice-cold transformation buffer. The bacterial suspension was then incubated on ice for 10min and centrifuged at 2862g for 10min at 4°C. The supernatant was discarded and the bacterial pellet resuspended in 4ml ice-cold transformation buffer. Dimethyl sulfoxide (DMSO) (140µl) was added and mixed. The bacterial suspension was incubated on ice for 10min and 140µl 1M DTT (dithiothreitol) was added and mixed. The bacterial suspension was again incubated on ice for another 10min and finally another 140µl DMSO (dimethyl sulfoxide) was added and swirled gently to mix.

2.34 Transformation of E. coli TG1 with J6/JFH1 and J6/JFH1-GND pDNA

Ten four fold dilutions of pDNA were made in sterile distilled water. 20µl of each dilution was pipetted into a 14ml Falcon tube for each ligation reaction and kept on ice. 210µl competent bacteria preparation was aliquoted into each of the tube and incubated on ice for 40 minutes. The bacteria were heat shocked by placing the tubes in a 42^{o}C waterbath for 90seconds and incubated on ice for 2 minutes. 800µl of the complete SOB medium was then pipetted to each tube and the tubes were incubated for 30 minutes in a 37^{o}C waterbath. In a Class II cabinet, 200µl of the bacterial solution from each tube was plated onto an LB + Ampicillin agar plate using a plate spreader. The plates were left open for approximately 30 minutes at room temperature until the surface was dry. The plates were then inverted and incubated at 37^{o}C overnight.

2.35 Cloning the transformed E. coli TG1

Isolated colonies of transformed bacteria were picked and restreaked on LB + Ampicillin agar plate using a sterile disposable plastic loop. The plates were inverted and incubated at 37^{o}C overnight. Cloning was repeated twice with positive clones.

2.36 Mini prep for plasmid purification of transformed E. coli TG1

Solution I
50mM Glucose (Mw 180g/mol)
25mM Tris base (Mw 121g/mol)
10mM EDTA

Dissolved in 250ml distilled water, adjusted pH to 8.0 and autoclaved.

Small scale plasmid purification was carried out using a QIAprep^{R} miniprep kit (Qiagen, UK) following the manufacturers instructions except that after harvesting,

the cell pellet was washed with solution I by resuspending the pellet in 1.5ml sterile solution I, vortexing, centrifuging at 16,000g for 5min, and discarding the supernatant. Also, since the plasmid J6/JFH1 is ~12kb (>10kb) big, Buffer EB (10mM Tris.Cl, pH 8.5) was pre-heated to 70^{o}C prior to eluting DNA from the QIAprep membrane. The purified plasmid was stored at -20^{o}C.

2.37 Maxi prep for plasmid purification of transformed E. coli TG1

STE (TEN)
0.1M NaCl (MW 58.44g/mol)
10mM Tris (MW 121.14g/mol)
1mM EDTA.2H_2O (MW 372.24g/mol)

The volume was made up to 1L with distilled water. The pH was adjusted to 8.0 with concentrated HCl.

10mM Sodium acetate (pH 5.2)
1.36g Sodium acetate.3H_2O (MW 136.08g/mol)

The volume was made up to 800mL with distilled water, pH adjusted to 5.2 with glacial acetic acid, volume adjusted to 1000mL with distilled water, and the solution was autoclaved.

1M Tris (pH 7.4)
121.1g Tris base (MW 121.14g/mol)

pH was adjusted to 7.4 by adding concentrated HCl, the solution was allowed to cool to room temperature before making final pH adjustment. The volume adjusted to 1000mL with distilled water and and the solution was autoclaved.

DNAase free 10mg/ml RNAase
10mg/ml RNAase A (pancreatic)
2ml 0.01M Sodium acetate (pH 5.2)
0.1 volume 1M Tris.Cl (pH 7.4)

RNAase A was dissolved in 2ml 10mM sodium acetate, the solution heated to 100^{o}C for 15 minutes in a boiling waterbath to deactivate DNAase, allowed to cool slowly to room temperature, pH adjusted by adding (0.1 volume) 200µl of 1M Tris.Cl (pH 7.4), and filter sterilized with 0.2µm filter.

P1
6.06g Tris base (MW 121.14g/mol)
3.72g $Na_2EDTA.2H_20$ (MW 372.24g/mol)

pH was adjusted to 8.0 with HCl, volume was adjusted to 1L, and the solution autoclaved before RNAase was added. 100mg RNAase A was added per liter of P1. Stored at 4°C once RNAase was added.

P2
8g NaOH (MW 40.0g/mol)
50ml 20% SDS (w/v)

The volume was adjusted to 1000mL with distilled water.

P3
294.5g Potassium acetate (MW 98.15g/mol)
~110ml Glacial acetic acid

pH was adjusted to 5.5 with glacial acetic acid. The volume was adjusted to 1000mL with distilled water, and the solution was autoclaved.

10mMTris or (pH 8.0)
1.21g Tris base (MW 121.1g/mol)

pH was adjusted to 8.0 by adding concentrated HCl. The solution was allowed to cool to room temperature before making final pH adjustment. The volume was adjusted to 1000mL with distilled water, and the solution was autoclaved.

10mg/ml lysozyme
200mg Lysozyme (Sigma)
20mL 10mM Tris.Cl (pH 8.0)

Stirred, filter sterilized with 0.2µm filter, aliquoted in sterile Eppendorf tube, and stored at -20°C.

Large scale plasmid purification was done using QIAfilter Plasmid Maxi Kit (Qiagen, UK) following manufacturers instructions with some modifications. The modifications include 12h-16h incubation of starter culture, the starter culture was diluted 1/100 into 500ml selective (ampicillin) LB medium, the bacterial pellet was washed in 200ml sterile cold (4°C) STE buffer before adding 40ml buffer P1 containing 100ug/ml RNase A, 2ml of 10mg/ml lysozyme (in 10mM Tris.Cl pH 8.0)

was added to buffer P1/bacterial mixture and gently swirled, 40ml each of P2 and P3 were used, the lysate was centrifuged at 5000rpm for 15min at 4^{o}C without brake in a JLA10.5 rotor in a Beckman Coulter Avanti J-25 centrifuge (Beckman Coulter UK Ltd) get rid most of the debris before the lysate was poured into the barrel of the QIAfilter cartridge, the DNA was eluted with warm (60^{o}C) 15ml Buffer QF provided, and the pellet was air-dried for 30min.

The DNA was dissolved in 120-240µl TE buffer, pH 8.0 or EB buffer from the QIAprep^{R} miniprep kit (Qiagen, UK). The DNA solution was next transferred to a sterile screw-capped tube and 3µl of the DNA was aliquoted for yield determination. The rest was stored at -20^{o}C.

2.38 DNA yield Determination

The concentration of DNA was determined by diluting the sample 1:100 in TE buffer and reading the OD in a spectrophotometer at 260nm. The concentration of DNA was calculated using the formula below:

Concentration = OD_{260} reading x 100 x 50µg/ml

NanoDrop ND-1000 Spectrophotometer (Isogen-Lifesciences) was also used to measure the concentration of DNA where 1µl of the sample DNA was pipetted directly onto the lower measurement pedestal before closing the sampling arm and the spectral measurement initiated and analyzed using the Nanodrop software V3.0.0.

2.39 Restriction Enzyme digestions of pFL-J6/JFH1 and pFL-J6/JFH1-GND plasmids

2.39.1 Material 1

No **digest**
8 µl DEPC distilled water
10 µl Plasmid
2 µl React Buffer 3 (Invitrogen)

EcoR I **digest**
7 µl DEPC distilled water
10 µl plasmid
1 µl EcoR I enzyme (Invitrogen)
2 µl React Buffer 3 (Invitrogen)

EcoR I + Xba I **digest**
6 µl DEPC distilled water
10 µl plasmid
1 µl EcoR I enzyme (Invitrogen)
1 µl Xba I enzyme (Invitrogen)
2 µl React Buffer 6 (Invitrogen)

Kpn I + Xba I **digest**
6 µl DEPC distilled water
10 µl plasmid
1 µl Kpn I enzyme (Invitrogen)
1 µl Xba I enzyme (Invitrogen)
2 µl React Buffer 1 (Invitrogen)

2.39.2 Material 2

Xba I digest
62.2µl DEPC distilled water
10.3µl (30µg) pFL-J6/JFH1 plasmid
7.5µl XbaI (NEB)
10µl 10X NEB Buffer 2
10µl 1 mg/ml BSA (NEB)

2.39.3 Material 3

No digest
14.5µl DEPC distilled water
1.5µl (2µg) pFL-J6/JFH1-GND plasmid
2µl 10X NEB Buffer 2
2µl 1 mg/ml BSA (NEB)

EcoR I digest
15.5µl DEPC distilled water
1.5µl (2µg) pFL-J6/JFH1-GND plasmid
1µl EcoR I enzyme (Invitrogen)
2µl React Buffer 3 (Invitrogen)

XbaI digest
14µl DEPC distilled water
1.5µl (2µg) pFL-J6/JFH1-GND plasmid
0.5µl XbaI (NEB)
2µl 10X NEB Buffer 2
2µl 1 mg/ml BSA (NEB)

SspI digest
14.5µl DEPC distilled water
1.5µl (2µg) pFL-J6/JFH1-GND plasmid
2µl SspI (NEB)
2µl10X NEB Buffer SspI

XmnI digest
14µl DEPC distilled water
1.5µl (2µg) pFL-J6/JFH1-GND plasmid
0.5µl XmnI (NEB)
2µl10X NEB Buffer 2
2µl 1 mg/ml BSA (NEB)

XmnI + XbaI digest
13.5µl DEPC distilled water
1.5µl (2µg) pFL-J6/JFH1-GND plasmid
0.5µl XmnI (NEB)
0.5µl XbaI (NEB)
2µl 10X NEB Buffer 2
2µl 1 mg/ml BSA (NEB)

SspI + XbaI digest
12µl DEPC distilled water
1.5µl (2µg) pFL-J6/JFH1-GND plasmid
2µl SspI (NEB)
0.5µl XbaI (NEB)
2µl 10X NEB Buffer 2
2µl 1 mg/ml BSA (NEB)

Note: XbaI (NEB) is 20 unit/µl; Buffer 2, BSA
XmnI (NEB) is 20 unit/µl; Buffer 2, BSA
SspI (NEB) is 5 unit/µl; SspI buffer, Buffer 2

The samples were incubated for 4 hours in a 37°C waterbath, 5µl of 6X sample loading buffer (Promega, Southampton, UK) was added to each sample, vortexed, centrifuged briefly at 16,000g at room temperature and run on 0.7% TBE agarose gel (see below).

2.40 Large scale restriction Enzyme digestions of pFL-J6/JFH1, pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND plasmids

XbaI digest
124.4µl DEPC distilled water
20.6µl (60µg) pFL-J6/JFH1 plasmid
15µl (300U) XbaI (NEB)
20µl 10X NEB Buffer 2
20µl 1 mg/ml BSA (NEB)

XbaI digest
121.9µl DEPC distilled water
23.1µl (60µg) pFL-J6/JFH1-H2476L plasmid
15µl (300U) XbaI (NEB)
20µl 10X NEB Buffer 2
20µl 1 mg/ml BSA (NEB)

XmnI + XbaI digest
42.4µl DEPC distilled water
22.6µl (30µg) pFL-J6/JFH1-GND plasmid
7.5µl (150U) XmnI (NEB)
7.5µl (150U) XbaI (NEB)
10µl 10X NEB Buffer 2
10µl 1 mg/ml BSA (NEB)

The samples were incubated for 4 hours in a 37°C waterbath. 5µl of 6X sample loading buffer (Promega, Southampton, UK) was added to 2µl of each sample (diluted in 18ml in distilled water), vortexed, centrifuged briefly at 16,000g at room temperature and run on 0.7% TBE agarose gel to check for complete digestion.

2.41 Mung bean nuclease digestions of pFL-J6/JFH1, pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND plasmids

Mung bean digest (pFL-J6/JFH1 or pFL-J6/JFH1-H2476L)
~200µl XbaI digested pFL-J6/JFH1 or pFL-J6/JFH1-H2476L DNA
12µl (120U) Mung bean nuclease (NEB)

Mung bean digest (pFL-J6/JFH1-GND)
~100µl XbaI digested pFL-J6/JFH1-GND DNA
6µl (60U)Mung bean nuclease (NEB)

The digested DNA samples from section above were re-digested with Mung bean nuclease enzyme. The samples were incubated for 30min in a 30°C waterbath.

2.42 Proteinase K and SDS treatment of pFL-J6/JFH1, pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND plasmids

Proteinase K and SDS treatment (pFL-J6/JFH1 or pFL-J6/JFH1-H2476L)
15µl 10.3mg/ml Proteinase K
10µl 10% SDS
~212µl Digested pFL-J6/JFH1 or pFL-J6/JFH1-H2476L DNA

Proteinase K and SDS treatment (pFL-J6/JFH1-GND)
15µl 10.3mg/ml Proteinase K
10µl 10% SDS
~106µl Digested pFL-J6/JFH1-GND DNA

The digested DNA samples from section above were treated with Proteinase K and SDS. The samples were incubated for 1hour in a 50°C waterbath. The samples were ready for double phenol-chloroform purification and could be stored at -20°C.

2.43 DNA electrophoresis on agarose gel

1X TBE buffer (pH 8.2-8.4)
43.2g Tris Base (MW 121g/mol)
22g Boric acid
3.7g EDTA
pH adjusted to 8.2 - 8.4, volume adjusted to 4L with distilled water, and the solution stored at room temperature.

Ethidium Bromide (1mg/ml)
10mg Ethidium Bromide (MW 394.31g/mol) (Northumbria Biologicals Ltd., Cramlington, UK)
10ml distilled water
Stored at 4°C.

The Bio-Rad Mini-Sub Cell GT horizontal gel electrophoresis apparatus (Bio-Rad, USA) was first assembled and securely sealed at the ends of the gel trough with autoclave tape. Agarose (1.7%) was added to 70ml 1X TBE (pH 8.2) per gel. The agarose mixture was boiled and swirled occasionally until completely dissolved. Ethidium bromide was added at 1µg/ml and the hot agarose solution was poured into the assembled gel electrophoresis apparatus and allowed to set for approximately 60min. Once the gel was set, the autoclave tape and comb were removed and the gel was placed in the gel tank containing 250ml running buffer (1X TBE) with 0.5µg/ml ethidium bromide. The samples containing 6X sample loading buffer (see above) and GeneRuler 1kb DNA ladder (Fermentas) were then loaded into the wells and run at 70 volts for 2-3 hours using a Northumbria Biologicals powerpack (Northumbria Biologicals Ltd., Cramlington, UK). Results were analyzed and recorded with a Bio-Rad Fluor-S MAX2 MultiImager gel scanner (Bio-Rad, California) and Bio-Rad Quantity One Quantitation software V4.2.3 (Bio-Rad).

2.44 Partial sequencing of pFL-J6/JFH1, pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND plasmids

Primers for pFL-J6/JFH1-GND mutant
SI-1 (forward plus strand)
5'- CCA CTA GCA TGG GTA ACA CC -3'

- 20bp length, 11 C+G, 9 A+T
- Location on J6-JFH1 sequence: 8523-8542
- Melting temperature (Tm): DNAstar – 49.4°C, PCgene – 53.8°C

SI-2 (reverse complementary strand)
5'- ATT AGC TCC AGG TCA TAT TCC -3'

- 21bp length, 9 C+G, 12 A+T
- Location on J6-JFH1 sequence (complementary): 8734-8754
- Melting temperature (Tm): DNAstar – 47.0^{o}C, PCgene – 50.2^{o}C

Primers for pFL-J6/JFH1-H2476L mutant

SI-3 (forward plus strand)
5'- TGT GCT GCT CCA TGT CAT AC -3'

- 20bp length, 10 C+G, 10 A+T
- Location on J6-JFH1 sequence: 7659-7678
- Melting temperature (Tm): DNAstar – 48.9^{o}C, PCgene – 51.7^{o}C

SI-4 (reverse complementary strand)
5'- GAA GCC GCT AGC TTG ATG TC -3'

- 20bp length, 11 C+G, 9 A+T
- Location on J6-JFH1 sequence (complementary): 7874-7893
- Melting temperature (Tm): DNAstar – 51.8^{o}C, PCgene – 53.8^{o}C

The following primers were designed to locate the T7 promoter region on pFL-J6/JFH1:

Primers for T7 promoter region

SI-5 (reverse complementary strand)
5'- AGT GGT TCA TGG CGG AGT G -3'

- 19bp length, 11 C+G, 8 A+T
- Location on J6-JFH1 sequence (complementary): 22-40
- Melting temperature (Tm): 53^{o}C

SI-6 (reverse complementary strand)
5'- GGC TGT ACG ACA CTC ATA C -3'

- 19bp length, 10 C+G, 9 A+T
- Location on J6-JFH1 sequence (complementary): 93-111
- Melting temperature (Tm): 51^{o}C

Tm calculated using http://www.promega.com/biomath/calc11.htm

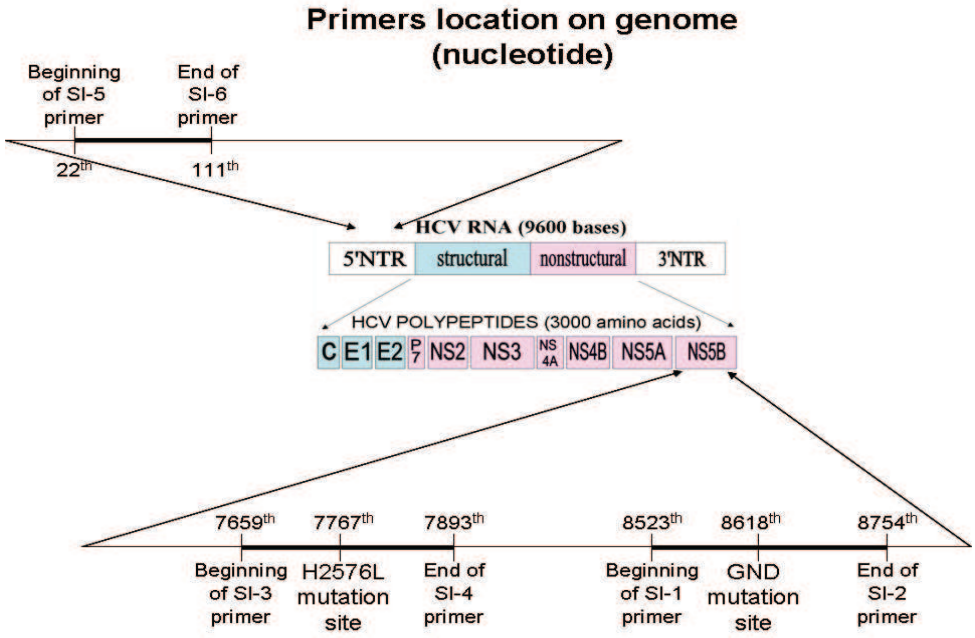


Figure 15: A diagram of the locations of SI-1, SI-2, SI-3, SI-4, SI-5, and SI-6 primers on the HCV genome.

HCV-J6 AND HCV-JFH1 sequences were obtained from the following websites using these accession numbers.

HCV-J6 sequence
Hepatitis C virus genomic RNA for polyprotein, complete cds
http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=D00944
ACCESSION D00944

HCV JFH-1 sequence
Hepatitis C virus (isolate JFH-1) genomic RNA, complete genome
http://getentry.ddbj.nig.ac.jp/getstart-e.html
ACCESSION AB047639

HCV-J6 AND HCV-JFH1 sequences obtained were aligned together over part of the NS2-NS3 regions to make the complete HCV J6/JFH1 sequence.

2µg of maxiprep pFL-J6/JFH1, pFL-J6/JFH1-H2476L or pFL-J6/JFH1-GND DNA in a 10µl volume each together with 2µM primer pair (SI-1 and SI-2, SI-3 and SI-4 or SI-5 and SI-6 primer pair) in a 5µl volume/primer were aliquoted and sent to Pinnacle in Newcastle University for the DNA to be sequenced. The sequencing result was then sent back for analysis. Analysis was done using the megalign (clustal V and W methods) of dnastar software.

2.45 Purification 1-Phenol-chloroform extraction/ethanol precipitation

Phenol:chloroform:isoamyl alcohol
25 ml phenol
24 ml chloroform
1 ml isoamyl alcohol

Chloroform:isoamyl alcohol
24 ml chloroform
1 ml isoamyl alcohol

75% ethanol
75 ml 100% ethanol

25 ml DEPC dH_2O
Mixture was well-mixed, kept on ice when in use and stored at 4°C.

In a fume hood, an equal volume (500µl) of neutral phenol:chloroform:isoamyl alcohol was added to a volume of 500µl DNA sample. The mixture was vortexed for 1min, incubated for 4min, and vortexed again for 1min before centrifuging for 16,000g for 2 minutes. The top aqueous layer was pipetted out into a new RNAase-free microfuge tube (Fisher, UK). An equal volume of chloroform/isoamyl alcohol was added to the aqueous layer. The mixture was vortexed for 1min, incubated for 4min, and vortexed again for 1min before centrifuging for 16,000g for 2 minutes. The top aqueous layer was pipetted out into a new RNAase-free microfuge tube (Fisher, UK). One-tenth volume of 10M NH_4OAc and 2 volumes of 100% ethanol were added to the sample and the mixture was incubated in -20°C overnight. The mixture was centrifuged at 16,000g for 15 minutes at 4°C, the supernatant was discarded, 500µl of ice-cold 75% ethanol was added to the pellet before centrifuging again at 16,000g for 2 minutes at 4°C and removing the supernatant. The pellet was left to air-dry for 20min and resuspended by vortexing in 20µl DEPC distilled water. Finally OD_{260} and OD_{280} measurement was made using a NanoDrop ND-1000 Spectrophotometer (Isogen-Lifesciences) and the DNA sample was stored at -80°C.

2.46 Purification 2-Double phenol purification of linearized plasmid DNA

Phenol
50 ml phenol

Phenol:chloroform:isoamyl alcohol
25 ml phenol
24 ml chloroform
1 ml isoamyl alcohol

Chloroform:isoamyl alcohol

24 ml chloroform
1 ml isoamyl alcohol

Ether

25 ml Ether
25 ml DEPC dH_2O
The mixture was well shaken to get a-DEPC water-saturated ether. The mixture was stored at room temperature.

75% ethanol

75 ml 100% ethanol
25 ml DEPC dH_2O
Mixture was well-mixed, kept on ice when in use and stored at 4^oC.

3M Sodium acetate pH 5.6

40.83g Sodium acetate.$3H_2O$
70ml DEPC dH_2O
pH adjusted to 5.6 with glacial acetic acid in the fume hood, volume adjusted to 100ml with distilled water, 100µl DEPC distilled water was added, the mixture was mixed well, incubated overnight at 37^oC, and autoclaved.

In a fume hood, an equal volume (500µl) of neutral phenol was added to a volume of 500µl DNA sample. The mixture was vortexed for 1 min, incubated for 2 min, and vortexed again for 1 min before centrifuging for 16,000g for 2 minutes. The top aqueous layer (A) was pipetted out into a new Eppendorf DNA lobind tube (Fisher, UK) leaving behind the phenol phase (A1), 100µl fresh DEPC distilled water was added to the phenol phase A1, and the previous steps were repeated. The top aqueous layer of A1 was pipetted out and pooled with the previous aqueous layer A.

An equal volume of phenol/chloroform/isoamyl alcohol was added to the pooled aqueous layer (A+A1). The mixture was vortexed for 1 min, incubated for 2 min, and vortexed again for 1 min before centrifuging for 16,000g for 2 minutes. The top aqueous layer (B) was again pipetted out into a new DNA lobind tube leaving behind the phenol/chloroform/isoamyl alcohol phase (B1), 100µl DEPC distilled water was

added to the phenol/chloroform/isoamyl alcohol phase B1, and the previous steps were again repeated. The top aqueous layer of B1 was pipetted out and pooled with the previous aqueous layer B.

An equal volume of chloroform/isoamyl alcohol was added to the pooled aqueous layer (B+B1). The mixture was vortexed for 1 min, incubated for 2 min, and vortexed again for 1 min before centrifuging for 16,000g for 2 minutes. The top aqueous layer (C) was again pipetted out into a new DNA lobind tube leaving behind the chloroform/isoamyl alcohol phase (C1), 100µl DEPC distilled water was added to the chloroform/isoamyl alcohol phase C1, and the previous steps were again repeated. The top aqueous layer of C1 was pipetted out and pooled with the previous aqueous layer C.

DEPC water-saturated ether (1 ml) was added to the pooled aqueous layer (C+C1). The mixture was vortexed for 1 min and the top layer of ether was then removed. The microfuge tube was left opened on a 50^{o}C heatblock for 10 minutes to evaporate any remaining ether.

The DNA sample was split into two 250µl aliquots and placed on ice. One-tenth volume of 3M sodium acetate pH 5.6 was added to each and mixed by inversion followed by 2.5 volumes (625µl) cold absolute (100%) ethanol. The mixture was mixed, incubated at -70^{o}C overnight and centrifuged at 16,000g for 15 minutes at 4^{o}C. The supernatant was discarded, 500µl of ice-cold 75% ethanol was added to the pellet, and the sample centrifuged immediately at 16,000g for 15 minutes at 4^{o}C. The supernatant was discarded and the sample centrifuged again at 4^{o}C for 2min to

remove last drop of supernatant. The pellet was air-dried for twenty minutes before resuspending it in 20µl DEPC distilled water or more (depending on pellet size). The pellet was left to soak for 30-60min at 42°C in the heat block and vortexed to facilitate dissolving. Finally OD_{260} and OD_{280} measurement was made using a NanoDrop ND-1000 Spectrophotometer (Isogen-Lifesciences) and the DNA sample was stored at -80°C.

2.47 In vitro transcription

Plasmids were linearized as described in section 2.40 and purified as described in section 2.46 before carrying out *in vitro* transcription below.

Ribonucleotide mix solutions
7.5 µl ATP
7.5 µl CTP
7.5 µl GTP
7.5 µl UTP
The ribonucleotide solutions mixed and kept on ice.

In vitro transcription reaction mix
8 µl ribonucleotide mix solutions
2 µl 10X Reaction buffer
2 or 1 µg Linear template DNA
2 µl Enzyme mix (**T7**)
2 hrs 1st incubation time
2 µl Enzyme mix (**T7**)
4 hrs 2nd incubation time
Volume was made up to 20µl with Nuclease free water (Ambion)

In vitro transcription of linearized, purified DNA was carried out with an AMBION MEGAscript T7 kit (Austin, Texas, US) as the T7 promoter is present in plasmid pFL-J6/JFH1 and also in the H2476L and GND mutants. The transcription reaction was performed at room temperature. The reaction mixture was mixed thoroughly by gently flicking the tube or pipetting the mixture up and down gently. The tube was centrifuged briefly and incubated at 37°C for 2 hours (first incubation). The second T7 enzyme mix was then added and mixed thoroughly by gently flicking the tube or

pipetting the mixture up and down gently. The tube was centrifuged briefly and incubated at 37°C for another 4 hours (second incubation). The RNA was stored at -20°C until the next step.

2.48 RNA purification and precipitation

The RNA (20µl) was first thawed out, 1µl TURBO DNase from MEGAscript T7 kit was added and mixed well and then incubated at 37°C for 15 minutes. The reaction was stopped by adding 15µl stop solution (5M Ammonium Acetate) from the Megascript kit. The RNA was purified using Sigma Tri reagent LS (St. Louis, Missouri, US) following the method of Kato *et al* (Kato et al., 2006) with some modifications. Nuclease-free water (115µl) was added and the mixture pipetted gently and thoroughly before adding another 100µl nuclease-free water making the total volume to approximately 250µl. A 3:1 ratio (750µl) of Trizol LS was added and pipetted gently and thoroughly before incubating at room temperature for 5min. Pure chloroform (200µl) was then added, the mixture vortexed vigorously for exactly 15sec and this was followed by incubation at room temperature for 3min. The mixture was centrifuged at 16,000g for 25min at 4°C. The upper layer was transferred in a new RNAase-free tube, 600µl isopropanol was added and the mixture was incubated at room temperature for 10min. This was then centrifuged at 16,000g for 20min at 4°C. The supernatant was discarded and 1ml of 75% ethanol was added to the pellet. This was then centrifuged at 16,000g for 10min at 4°C and the supernatant discarded. After another round of centrifugation at 16,000g for 2min at room temperature, the RNA pellet was air-dried for 5-10 min. The pellet was resuspended in 11ul (varied according to pellet size) nuclease-free water. The RNA concentration

was read at OD_{260} and RNA quality checked on an RNA agarose gel (see below). The RNA was stored at -80°C and frequent freeze-thawing of RNA was avoided by aliquoting the RNA samples.

2.49 Glyoxal RNA agarose gel electrophoresis

DEPC water
1L distilled water
1mL Diethylpyrocarbonate (DEPC)
The mixture was vigorously shaken, incubated at 37°C overnight, and autoclaved.

1% RNA agarose gel
1% Agarose (Life Technologies)
70ml 10mM sodium phosphate (pH 7.0)
10mM sodium iodoacetate (M.W.-207.93, Sigma)

The Bio-Rad Mini-Sub Cell GT horizontal gel electrophoresis apparatus (Bio-Rad, USA) was made RNAase-free by soaking in 3% hydrogen peroxide for 10mins and rinsing with DEPC distilled water. Glassware was baked at 180°C overnight.

Agarose was dissolved by boiling in boiling waterbath and the solution cooled to 70°C in waterbath set to 70°C. Solid sodium iodoacetate was added and the mixture swirled. The solution was cooled to 50°C in a waterbath set to 50°C. Finally, the gel was poured and allowed to set for at least 1 hour before the RNA samples were loaded.

RNA samples (30µg maximum per sample) were mixed with an equal volume of Glyoxal Denaturing Load Dye (Ambion, Austin, USA). The samples were incubated for 1 hour at 50ºC in a dry heat block. After incubation, the samples were placed on ice-water immediately for 10 minutes. The samples were then centrifuged briefly before being loaded into the wells. The samples were run in the gel submerged in

10mM sodium phosphate buffer at 3-4 V/cm (measured between the electrodes) for 2 hours. The buffer was pipetted from one side of the gel chamber to the other every 15-20 minutes to prevent H^+ build-up. The gel was incubated for 15min in 0.5M ammonium acetate on an Easiashaker (Medgenix Diagnostics, Brussels, Belgium) and photographed on a Bio-Rad Fluor-S MAX2 MultiImager gel scanner (Bio-Rad, California) immediately.

2.50 Electroporation optimization

The protocol was adapted from Kato *et al* (Kato et al., 2006) with some modifications. Huh7.5 cells were cultured in T_{225} flasks (Corning, Schipol-Rijk, Netherlands) in complete DMEM growth medium seeded the day before with 60-70% confluence on the day of transfection. The cells were washed with 40ml PBS and then trypsinized with 5ml of warm (37^{o}C) Trypsin-EDTA per flask. The cells were suspended in 40ml of cold Opti-MEM I medium (Gibco, Paisley, UK) and centrifuged at 1000rpm for 5min at 4^{o}C. The supernatant was discarded and the cells resuspended in 40ml of cold Opti-MEM medium. The cells were counted on a haemacytometer and then centrifuged at 1000rpm for 5min at 4^{o}C. The supernatant was discarded and the cells resuspended in cold Opti-MEM I medium to make 7.5 x 10^6 cells/ml, 2 x 10^6 cells/ml, or 5 x 10^5 cells/ml suspension as specified. The cell suspension (1ml) was transferred into a sterile, new Eppendorf tubes placed on ice.

The incomplete Cytomix buffer (120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4/KH_2PO_4 pH 7.6, 2mM EGTA pH 8.0, 5mM $MgCl_2$, 25mM HEPES pH 7-7.6) was completed by adding 8µl of 100mM ATP (Melford, Ipswich, UK) pH 7.6

and 8µl of 250mM Glutathione (Melford, Ipswich, UK) to 400µl Cytomix buffer, vortexed, and placed on ice.

The cells were pelleted by centrifuging at 5000rpm at 4°C for 2min in a microcentrifuge and the supernatant discarded. The cells were resuspended in an Eppendorf tube with 400µl cold complete Cytomix buffer. This was mixed well, transferred to a 0.2cm/0.4cm electroporation cuvette and pulsed immediately at 220V/260V/300V as specified, 960µF using a Gene pulser. The cells were incubated at room temperature for 15min or on ice for 10min as specified before being pipetted into a plastic universal containing 24ml warm, complete DMEM growth medium. The cell suspension was aliquoted 4ml per well in a 6-well-plate and incubated at 37°C, in 5% CO_2 for 24 hours. The culture medium was removed, the electroporated cells washed 3X with warm PBS, and 4ml fresh, warm complete medium was finally added to each well.

The cells were 1) allowed to grow for another 3 days before the cells were stripped with EDTA and trypsin and counted with a haemacytometer or 2) replaced with 4ml fresh, warm complete DMEM growth medium and allowed to grow for another 3 days before being counted again or 3) sub-cultured/passaged on day 4 and allowed to grow for another 3 days before being counted.

2.51 RNA transfection by electroporation

The transfection protocol was adapted from Kato *et al* (Kato et al., 2006) with some modifications. Huh7.5 cells were cultured in T_{225} flasks (Corning, Schipol-Rijk,

Netherlands) in complete DMEM growth medium seeded the day before with 60-70% confluence on the day of transfection. The cells were washed with 40ml PBS and then trypsinized with 5ml of warm (37^{o}C) Trypsin-EDTA per flask. The cells were suspended in 40ml of cold Opti-MEM I medium (Gibco, Paisley, UK) and centrifuged at 1000rpm for 5min at 4^{o}C. The supernatant was discarded and the cells resuspended in 40ml of cold Opti-MEM medium. The cells were counted on a haemacytometer and then centrifuged at 1000rpm for 5min at 4^{o}C. The supernatant was discarded and the cells resuspended in cold Opti-MEM I medium to make 7.5 x 10^{6} cells/ml suspension. The cell suspension (1ml) was transferred into a sterile, new Eppendorf tubes placed on ice.

The incomplete Cytomix buffer (120mM KCl, 0.15mM $CaCl_{2,}$ 10mM K_2HPO_4/KH_2PO_4 pH 7.6, 2mM EGTA pH 8.0, 5mM $MgCl_{2,}$ 25mM HEPES pH 7-7.6) was completed by adding 8µl of 100mM ATP (Melford, Ipswich, UK) pH 7.6 and 8µl of 250mM Glutathione (Melford, Ipswich, UK) to 400µl Cytomix buffer, vortexed, and placed on ice.

The cells were pelleted by centrifuging at 5000rpm at 4^{o}C for 2min in a microcentrifuge and the supernatant discarded. The cells were resuspended in an Eppendorf tube with 400µl cold complete Cytomix buffer and 10µg of RNA was added to the cell suspension. This was mixed well, transferred to a 0.2cm electroporation cuvette and pulsed immediately at 260V, at 960µF using a Gene pulser. The cells were incubated on ice for 10min before being pipetted into a plastic universal containing 20ml warm, complete DMEM growth medium. The cell suspension was aliquoted 1ml per well in a 24-well-plate and incubated at 37^{o}C, in

5% CO_2 for 24 hours. The culture medium was removed, the transfected cells washed 3X with warm PBS, and 1ml fresh, warm complete medium was finally added to each well. The supernatant of each well was harvested every day consecutively, frozen in liquid nitrogen and stored at -80°C. RNA was then extracted from these supernatant using QIAamp kit (Qiagen) followed by reverse transcription and quantative real time PCR.

2.52 Infection, propagation and harvest of HCVcc

2 x 10^4 cells/ml per well (4 x 10^5 cells/10ml T_{25} flask) of naïve Huh7.5 cells was seeded in a 24-well-plate (or T_{25} flask) the day before infection in 10% DMEM growth medium and incubated at 37°C, in 5% CO_2. The supernatant from the naïve cells was discarded. The supernatant from the transfection or infectivity experiment was thawed and centrifuged at 1500 x g for 10 min to avoid carryover of viable cells and 200µl per well of this was inoculated on to each well of the naïve Huh7.5 cells. In a T_{25} flask, the 200µl inoculum was topped up with 800ml medium to cover a bigger surface area. The plate/flask was incubated at 37°C, 5% CO_2 for 1 hour rocking the plate every 15 minutes. The well was topped up to 1ml with 3% FCS DMEM growth medium (10ml for T_{25} flask) and incubated at 37°C, 5% CO_2. The cells were incubated until day 16 post-infection and the supernatant harvested and replaced with fresh medium as required. The supernatant was snap-frozen in liquid nitrogen and stored at -80°C.

2.53 Indirect immunofluorescence assay

The method was adapted from Gardner and McQuillin (Gardner, 1980).

2.53.1 Slide preparation

The medium from the infected cell cultures were removed leaving behind residual volume. A small area of cells was scraped into the medium using a plastic Pasteur pipette. 15µl of the cell suspension was pipetted onto a spot (one well/spot) on the multi-well PTFE-coated glass slide (Hendley-Essex Ltd., UK). The spots were air-dried in the hood before fixing the spots in ice-cold acetone for 10min. The slides were air-dried again and stored at -20^{o}C until needed or proceeded to indirect immunofluorescence staining immediately.

2.53.2 Indirect immunofluorescence staining for HCVcc

Slides stored at -20^{o}C were defrosted and air-dried. Each spot was covered with 15µl primary antibody diluted in PBS and incubated for 30min at 37^{o}C in a moist box. The spots were washed gently with PBS using a Pasteur pipette and the slides soaked in PBS for 5mins. The slides were dried by first blotting excess liquid with tissue and then exposing them to a hair-dryer on cold setting. Each spot was covered with 15 µl of sheep anti-mouse FITC conjugated antiserum diluted 1:50 in Evans blue (0.5% w/v in PBS containing 0.1% sodium azide diluted 1:1000in PBS) and incubated as described above. The slides were washed as before and soaked in distilled water for 1min before being dried as described previously. The staining was viewed under Type FF Cargille immersion oil (R.P. Cargille Laboratories Inc, USA) using a darkfield immunofluorescence microscope (Labphot 2, Nikon).

2.54 Effect of oleic acid concentration on Huh7.5 cell growth

4mM 10X oleic acid stock solution (Wolfbauer's)
9.5ml DMEM serum free medium
1.5g BSA
11.3mg Oleic acid (Sigma, St. Louis, USA)
Dissolved BSA in medium at 37°C, oleic acid added, stirred at 37°C until the solution becomes clear, pH adjusted to 7.0 with 1N NaOH, filter sterilized with 0.2µm filter, aliquoted in sterile screw-cap tubes, snap-frozen in liquid nitrogen and stored at -80°C.

2mM 10X oleic acid stock solution (Wolfbauer's)
9.5ml DMEM serum free medium
1.5g BSA
5.65mg Oleic acid (Sigma, St. Louis, USA)
Prepared as above.

0.5mM 10X oleic acid stock solution (Wolfbauer's)
9.5ml DMEM serum free medium
1.5g BSA
1.41mg Oleic acid (Sigma, St. Louis, USA)
Prepared as above.

BSA stock solution (no oleic acid)
9.5ml DMEM serum free medium
1.5g BSA
Dissolved BSA in medium at 37°C, pH adjusted to 7.0 with 1N NaOH, filter sterilized with 0.2µm filter, aliquoted in sterile screw-cap tubes, snap-frozen in liquid nitrogen and stored at -80°C.

PBS + 0.5% BSA / Wash solution
200ml PBS
1g BSA (BDH, Poole, Dorset)

Trypsin + EDTA solution
58.5ml PBS
642µl 100mM EDTA
1200µl 2.5% Trypsin (Gibco BRL, Paisley, UK)

FACS rinse buffer
200ml PBS
2g BSA
BSA was added on the day of FACS

Huh7.5 cells ($1X10^5$ cells/well) were plated in each well of a 6-well-plate and were grown in DMEM growth medium containing 10% FCS with a final volume of 4ml/well in a 37°C, 5% CO_2 incubator. On day 2, the medium was replaced with 4ml 5% FCS DMEM growth medium. 450µl of 4mM, 2mM, 0.5mM of 10X oleic acid stock solution, and BSA stock solution only was added to each well and incubated in a 37°C, 5% CO_2 incubator for another 2 days. These were repeated on day 4 and day 6.

On day 8, the supernatant was discarded and cells were washed twice with 5ml PBS + 0.5% BSA (wash solution). The cells were detached by adding 5ml of warm 37°C Trypsin + EDTA solution of which 4ml was removed and the cells were incubated at 37°C for 5 minute. 2ml of Huh7.5 growth medium was added to each well and a single cell suspension was made by pipetting the medium vigorously using a Pasteur pipette. 3ml of Huh7.5 growth medium was added and the cells were filtered through an 11µm filter (Millipore, Watford) into a 14ml Falcon tube. The cells were centrifuged at 180g for 10min, supernatant was discarded, and cell pellet resuspended in 1500ul FACS rinse buffer. The cell suspension was transferred into a 12x75mm, 5ml polystyrene, round bottom FACS tube (Becton Dickinson, Devon), stored on ice and covered with aluminium foil.

Propidium iodide staining was done immediately just before FACS analysis and the detail method is described in section Propidium iodide staining for FACS analysis. FACS analysis was recorded at time 0, 5, 10, and 20 minutes.

2.55 Treatment of cells with oleic acids

HepG2 cells, Huh7 cells, or Huh7.5 cells ($0.7X10^6$ cells/well) were plated in each well of a 6-well-plate. HepG2 cells were grown in EMEM and Huh7.5 cells were grown in DMEM growth medium containing 10% FCS with a final volume of 4ml/well in a 37°C, 5% CO_2 incubator. The respective growth medium was replaced on day 2 or day 3. On day 5, the cells in each well were washed twice with warm (37°C) PBS and once with warm (37°C) EMEM or DMEM serum free medium as appropriate. Serum free medium (4ml) and 450µl BSA with or without oleic acid was added to each well and then incubated in 37°C, 5% CO_2 incubator. Supernatants (conditioned media) were harvested in sterile 15ml conical tubes on day 7 and centrifuged at 1500rpm for 5min in MSE 2000 centrifuge to pellet any cells. The supernatants were then transferred into fresh tubes.

2.56 Separation of VLDL and LDL by Potassium Bromide sequential ultracentrifugation from cell culture medium

Initially, 8ml (4ml x 2 wells of 6-well plates) each of HepG2 and Huh7.5 cell culture medium were harvested from cell culture as described in section Treatment of cells with oleic acids.

KBr (0.57g) was added to 4ml conditioned media per tube to obtain a density of 1.10g/ml. The solution was carefully pipetted into the bottom of an ultra-clear 14 X 95mm centrifuge tubes (Beckman, Palo Alto, USA). The medium was overlaid with 3ml each of 1.063g/ml and 1.019g/ml KBr solutions and 2ml of 1.006g/ml NaCl solution sequentially and ultracentrifuged at 40 000rpm, 15°C for 21 hours. The top layer (1ml) VLDL with density < 1.006g/ml followed by a 4.5ml of IDL/LDL with density of 1.02g/ml-1.063g/ml were collected from two tubes and pooled. The samples were concentrated to 140µl each using a Vivaspin 2ml concentrator with

100,000 MWCO (Fischer, Loughborough, Leicestershire) by centrifuging at 4000g at 4°C for 30min or more.

2.57 Delipidation / Protein Precipitation

All solvents / solutions were prechilled on ice and method performed on ice. Four (560ul) volumes of chilled methanol were added to one volume (140µl) of sample protein and vortexed. Two (280µl) volumes of chilled chloroform were then added and vortexed. This was then followed with 3 (420µl) volumes of chilled water and again vortexed. The samples were then centrifuged at 16,000g for 2.5minutes at 4^{o}C and using a drawn-out pipette, most of the upper phase was removed leaving the inter- and lower phases behind. Three (420µl) volumes of chilled methanol were then added and samples vortexed before being incubated on ice for 3 minutes. Next, the sample was centrifuged at 14 000rpm for 2.5minutes at 4^{o}C, supernatant removed and the pellet air-dried at room temperature.

2.58 Preparation of VLDL 1, 2, and 3 by flotation ultracentrifugation with Sodium Chloride gradient from cell culture medium

VLDL was fractionated into VLDL 1, 2, and 3 using the method described by Lindgren and Jensen (Lindgren, 1972).

Initially, 48ml (4ml x 12 wells of 6-well plates) each of Huh7 and Huh7.5 cell culture medium harvested from cell culture as described in section Treatment of cells with oleic acids was concentrated using Vivaspin 6ml concentrators with 100,000 MWCO

(Fischer, Loughborough, Leicestershire) by centrifuging at 4000g at 4°C for 30min or until a 9ml-volume was achieved.

A 10ml NaCl gradient was prepared, containing layers of different NaCl densities in an ultra-clear 14 X 95mm centrifuge tubes (Beckman, Palo Alto, USA), on top of the sample layer. The bottom-most layer in the tube contained 3ml of the concentrated cell culture medium containing NaCl to achieve a density of 1.1g/ml. This was overlaid by 1ml of 1.0464g/ml, 1ml of 1.0336g/ml, 2ml of 1.0271g/ml, 2ml of 1.0197g/l, 2ml of 1.0117g/ml and 2ml of 1.0064g/ml NaCl. The gradient was centrifuged at 35000rpm in a SW40 swing-out rotor in a Beckman L8-70 ultracentrifuge at 23ºC for 144 minutes. The top 1ml of VLDL1 (S_f >100) was harvested and the remaining material was topped up with fresh 1.0064g/ml NaCl. The gradient was centrifuged for a further 108 minutes, 1ml of top VLDL2 (S_f >60) layer was collected, and the gradient topped up with more 1.0064g/ml NaCl before centrifuging the gradient again for 18 hours 38 minutes. The top 0.5ml layer of VLDL3 (S_f >20) was collected. VLDL 1, VLDL 2 and VLDL 3 fractions were concentrated to about 240µl each using a Vivaspin 6ml concentrator with 100,000 MWCO (Fischer, Loughborough, Leicestershire) by centrifuging at 4000g at 4°C for ~10min or more depending on the flow rate using a Jouan centrifuge CR422 (Henderson Biomedical Ltd, Kent, UK). The samples were transferred into microfuge tube, topped up to 280µl each, and stored at -20^{o}C.

2.59 3%-18% gradient SDS-PAGE

Low concentration acrylamide stock
25ml 40% acrylamide (AMS Biotechnology, Oxon)
500 mg methylene-bis-acrylamide (Sigma, Gillingham, Dorset)
75ml of distilled water

High concentration acrylamide stock

80ml 40% acrylamide (AMS Biotechnology, Oxon)
960mg methylene-bis-acrylamide (Sigma, Gillingham, Dorset)

3% acrylamide solution

9ml "low concentration acrylamide stock solution"
15ml resolving gel buffer pH 8.8
6ml distilled water
200µl 10% ammonium peroxodisulphate (APS) (Sigma, Gillingham, Dorset)
11µl N,N,N, N-tetramethyletylenediamine (TEMED) (BDH, Poole, Dorset)
The solution was degassed with a vacuum pump before adding freshly prepared 10% APS and TEMED.

18% acrylamide

11.7ml "high concentration acrylamide stock solution"
13ml resolving gel buffer pH 8.8
3.9g sucrose (BDH, Poole, Dorset)
180µl of fresh 10% APS (Sigma, Gillingham, Dorset)
9µl TEMED (BDH, Poole, Dorset)
The solution was degassed with a vacuum pump before adding freshly prepared 10% APS and TEMED.

2x resolving buffer pH 8.8

36.3g Tris (BDH, Poole, Dorset)
0.8g SDS (BDH, Poole, Dorset)
350ml distilled water
The pH was adjusted to 8.8 and made up to 400ml with distilled water

8x stacking gel buffer

6.05g Tris (BDH, Poole, Dorset)
40mls distilled water
The pH was adjusted to 6.8 and made up to 50ml with water

10% APS

500mg APS (BDH, Poole, Dorset)
4.5ml distilled water
This was used fresh.

6x sample buffer

15ml 8x stacking gel buffer
30g glycerol (BDH, Poole, Dorset)
4.8g SDS (BDH)
6mg bromophenol blue (BDH)
The volume was adjusted to 40ml with distilled water
6ml β-mercaptoethanol (BDH) was added before use

Running buffer for acrylamide gels
12.1g Tris HCl (BDH, Poole, Dorset)
4.0g SDS (BDH)
57.6g glycine (Sigma, Gillingham, Dorset)
4 litres distilled water

18%-3% gradient gels were prepared in a Protein II xi cell (BioRad, Hemel Hempstead, Hertfordshire) using a two chamber gradient maker (Jencons, Forest Row, East Sussex). The gel was left to set, with a 15-well comb, for 2 hours. 100µl of sample were mixed with 25µl of 6x sample buffer, boiled for 3 minutes and spun before application to the gel. Molecular weight markers (MWM) (Broad Range Mol.Wt Standard Mixture from NEB) containing 212kDa Myosin, 158kDa MBP-β-galactosidase, 116kDa β-Galactosidase, 97kDa Phosphorylase b, 66kDa Serum albumin, 56kDa Glutamic dehydrogenase, 43kDa Maltose-binding protein2 and 36kDa Thioredoxin reductase, 27kDa Triosephosphate isomerase and 20kDa Trypsin inhibitor, 14kDa Lysozyme, and 6kDa Aprotinin were added to each of two wells. Once the samples were added, the gel was connected up to a current at 24-26 mA on the Power Supply Model EPS 600 (Pharmacia Biotech, Little Chalfont, Buckinghamshire), and run overnight, in running buffer.

2.60 Western blotting

This protocol was taken from Harlow and Cane in 1988.

Blocking solutions
360ml PBS
40ml FCS (Invitrogen, Paisley) (heat inactivated at 56ºC for 30 minutes)
20g Marvel milk powder (Sainsburys, South Shields, Tyne and Wear)
800µl 20% NaN_3 (BDH, Poole Dorset)
0.1% Tween (Sigma, Gillingham, Dorset) or 4g BSA

Blotting buffer with 10% methanol

500ml 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer (Sigma, Gillingham, Dorset)
4000ml distilled water
500ml methanol

CAPS buffer

11g CAPS (Sigma, Gillingham, Dorset)
500ml distilled water
The solution was stored at 4ºC. One day before use, the bottle was placed at room temperature and the pH adjusted to 11.1 with 5M NaOH

Ponceau S Red

500mg Ponceau S Red Powder (3-hydroxy-4-[2-sulfophenyllazo0 phenylazo]-2,7-napthalenedisulfonic acid)
25ml acetic acid
500ml distilled water

Following polyacrylamide gel electrophoresis, the gel was placed in three changes of blotting buffer and mixed on the Model Sucro Agitator (Boots-Celltech Diagnostics Limited, Slough) for 20 minutes each change. 2 pieces of Polyvinylidene di-flouride membrane PVDF (Amersham Biosciences, Little Chalfont, Buckinghamshire) and 8 pieces of 3MM chromatography paper (Whatman, Middlesex) were soaked in the blotting buffer and the gel was placed between the membranes. This assembly was placed between two pieces of blotting paper and clipped together in a plastic cassette, placed in a Bio-rad 'Trans –blot' cell and connected to the Power Supply Model EPS 600 (Pharmacia Biotech, Buckinghamshire) at 120 volts for 4 hours.

After transfer, the membrane was washed with 250ml of 5% acetic acid followed by 250ml 1% Ponceau S Red and 250ml distilled water to visualize the protein bands. The MWM were cut off, and the rest of the membrane was washed in distilled water and then PBS. The membrane was blocked with 'PBS with 10% FCS, Milk and BSA' overnight.

After the blocking stage, the solution was removed and the membranes were incubated for an hour in 'PBS-Tween and 10% FCS, Milk and BSA' and then washed twice for 5 minutes in PBS-Tween. The membrane was washed for 5 minutes in PBS-Tween containing 10% FCS and BSA.

The membrane was incubated for 90 minutes with the first antibody, at a dilution of 1:6,000. The membrane was washed three times for 20 minutes and once for 30 minutes in PBS-Tween. The membrane was incubated for 5 minutes in PBS-Tween containing 10% FCS and BSA before a 90 minute incubation with the second antibody, swine anti-rabbit conjugated to horse radish peroxidase (DAKO, Ely, Cambridgeshire) at a dilution of 1:6,000.

The stained membrane was given three 15 minute and one thirty minute wash with PBS-Tween, and then one 5 minute wash with PBS. It was developed using the Amersham ECL Plus Western Blotting detection system, following manufacturers instructions.

2.61 Protein visualization

Coomassie blue

500ml methanol
1g coomassie blue dye
100ml acetic acid
400ml distilled water

Destain solution

800ml distilled water
1000ml methanol
200ml of acetic acid

The gel was incubated with 250ml coomasie blue overnight and destained the following day by soaking in 250ml destain solution. The results were recorded using a Bio-Rad GS-800 calibrated densitometer and Quantitiy One software.

2.62 Stripping the membrane

Stripping solution
200ml distilled water
50ml 2x stacking gel buffer, pH 6.8
4g sodium dodecyl sulfate (SDS) (Sigma, Gillingham, Dorset)
1.6ml β mercaptoethanol

2x Stacking gel buffer
6.06g Tris
0.4g SDS (Sigma, Gillingham, Dorset)
150ml distilled water
The pH was adjusted to 6.8 and then made up to 200ml with distilled water

The protocol was used from 'Amersham Biosciences Manual for ECL Plus Western Blotting Detection Reagents', with extra wash steps using water. The membrane was soaked in 100ml acetonitrile for 10 minutes and 100ml of stripping buffer and incubated at 50ºC for 30 minutes. It was washed twice with PBS-0.1% Tween (Sigma, Gillingham, Dorset) for 10 minutes. The membrane was left in PBS with FCS, Milk and BSA overnight, before the membrane was stained with another antibody the following day.

2.63 Infection and culture of the HCVcc in Huh7.5 cells in the presence or absence of oleic acid – an optimization

2 x 10^4 cells/ml per well of naïve Huh7.5 cells was seeded in a 24-well-plate the day before infection in 1ml 10% DMEM growth medium and incubated at 37^{o}C, in 5% CO_2 as described in section 2.52 except that the cells were incubated until day 11 post-infection and the supernatant harvested for RNA samplings on day 1, 3, 5, 7, 9,

and 11 and replaced with fresh medium as required. The inoculum used was a passage 4 of J6/JFH1 RNA-transfected Huh7.5 cells or a pooled inoculum of varying passages of the alleged J6/JFH1 HCVcc with HCVcc RNA quantity of 1.8 X 10^5 IU per well from snap-frozen, thawed and centrifuged supernatant samples. The cell culture medium used until day 11 was 3% FCS DMEM growth medium. The harvested supernatant was snap-frozen in liquid nitrogen and stored at -80^{o}C.

On day 11, the cells were washed twice with warm PBS after saving the supernatant and 1ml of 1)3% FCS DMEM growth medium + 1.5% BSA, 2)3% FCS DMEM growth medium + 0.4mM oleic acid, 3)DMEM serum free medium + 1.5% BSA or 4) DMEM serum free medium + 0.4mM oleic acid was added to each well. The supernatant was harvested on alternate day until day 16 post-infection replacing the cells with respective fresh medium. The harvested supernatant samples were snap-frozen in liquid nitrogen and stored at -80^{o}C. The bulk of day 16 samples were not frozen but concentrated immediately for iodixanol gradient preparation and gel filtration column as described in the following sections 2.64 and 2.65 respectively.

2.64 Iodixanol gradient preparation of supernatant from J6/JFH1 HCVcc-infected Huh7.5

<u>6% Iodixanol solution</u>
1.7ml 60% Iodixanol
340µl 500mM Tris.HCl pH 8.0
340µl 100mM EDTA pH 8.0
14.62ml 0.25M sucrose in DEPC water
85µl 2M $MgSO_4$ pH 7.8
The solution was mixed well.

56% Iodixanol solution
16ml 60% Iodixanol
340µl 500mM Tris.HCl pH 8.0
340µl 100mM EDTA pH 8.0
320µl 0.25M sucrose in DEPC water
85µl 2M $MgSO_4$ pH 7.8
The solution was mixed well.

A 5ml pool of day 16 post-infection supernatant samples from section Infection and culture of the HCVcc in Huh7.5 cells in the presence or absence of oleic acid – an optimization was concentrated using Vivaspin 6ml concentrators with 100,000 MWCO (Fischer, Loughborough, Leicestershire) by centrifuging at 3000g at 4°C for 30min or until 0.5ml volume is achieved. The concentrated supernatants were snap-frozen in liquid nitrogen and stored at -80°C.

Iodixanol (Optiprep, Axis-Shield) density gradients (preformed) were prepared from 2 iodixanol buffered solutions (6% and 56%) above. The 6% iodixanol (13.24ml) was loaded to the right chamber and 10.76ml of the 56% iodixanol was loaded to the left chamber of a two-chamber gradient maker (Jencons, Leighton Buzzard, UK). The 6% and the 56% iodixanol solutions were pumped into an SW40 centrifuge tube containing 1.5ml of the 56% iodixanol at the bottom creating a density gradient.

The frozen concentrated supernatants (0.5ml each) from J6/JFH1 HCVcc-infected Huh7.5 treated with or without oleic acid were thawed in 37°C waterbath and applied onto the Iodixanol gradients. The samples were then centrifuged at 27,000rpm for 22hrs at 4°C with acceleration setting 7 and deceleration setting 0 in an SW40 rotor, Beckman L8-70 M ultracentrifuge. The gradient was harvested from the bottom by puncturing the tube with a heated Model 184 tube piercer (Isco, Lincoln, USA) and collected in 27 (0.5ml each) fractions. The density of each fraction was measured

using a digital refractometer (Atago, Japan). 300µl of each of the 27 fractions was used for RNA quantitation using real-time PCR as described in section RT-PCR with primers NCR-3 + NCR-5 and probe SN1.

2.65 Gel filtration preparation of supernatant from J6/JFH1 HCVcc-infected Huh7.5

Elution buffer
20mM Tris.HCl pH 8.0
0.25M sucrose
2mM EDTA
2mM $MgSO_4$
2mM $MgCl_2$
0.02% NaN_3

A 30ml pool of day 16 post-infection supernatant samples from section Infection and culture of the HCVcc in Huh7.5 cells in the presence or absence of oleic acid – an optimization was concentrated using Vivaspin 6ml concentrators with 100,000 MWCO (Fischer, Loughborough, Leicestershire) by centrifuging at 3000g at 4°C for 30min or until 4ml volume is achieved. The concentrated supernatants were snap-frozen in liquid nitrogen and stored at -80^{o}C.

Gel filtration column was prepared by packing an XK 26/100 (GE Healthcare) column with Toyopearl HW-75S (Tosoh Corporation) as described by Nielsen *et al.* (Nielsen et al., 2008). The column was heated to 37^{o}C and ran with a flow rate of 1ml/min. Calibration of the Toyopearl column was carried out using purified chylomicrons, VLDL-1, VLDL-2, IDL, and LDL (Nielsen et al., 2008, Okazaki et al., 2005).

The frozen concentrated supernatants (4ml each) from J6/JFH1 HCVcc-infected Huh7.5 treated with or without oleic acid were thawed in 37^{o}C waterbath and injected on to the gel filtration column. 48 fractions (4ml per fraction) were collected for analysis. For RNA analysis, two fractions (8ml) were pooled from the 48 fractions reducing the number of samples to 24. 600µl from each of the 24 fractions was used for RNA quantitation using real-time PCR as described in section RT-PCR with primers NCR-3 + NCR-5 and probe SN1.

Chapter 3

3 Results I

3.1 Studies on the binding of liver-derived HCV LVP and host lipoproteins to HepG2 cells

Martin (Martin, 2005) found that LLVP paralleled LDL in being taken up more efficiently in LPDS/insulin treated cells suggesting that LLVP bind and are taken up via LDLr. Agnello et al (Agnello et al., 1999) have shown that the binding and entry of blood derived particles containing HCV RNA shows an association with LDLr expression on cells and this can be completely inhibited by pre-incubating the cells with anti-LDLr antibody.

Martin (Martin, 2005) has demonstrated that LLVP binds better to LPDS/insulin treated cells than hydroxyl 25-OH treated cells and that this correlates with raised LDLr expression. Also binding can be blocked by pre-incubation with LDL. If HCV is binding via the interaction of the host lipoprotein moiety of the LLVP to LDLr, it should be inhibited by anti-apoB and anti-apoE antibodies. To test this hypothesis, the following experiment was done.

3.2 Anti-apoB-100 and anti-apoE antibodies do not block binding of LLVP

The purpose of this experiment was to see if polyclonal rabbit anti-apoB-100 antibody or a combination of anti-apoB-100 and anti-apoE antibodies prevented the uptake of LVP of the liver macerate into the HepG2 cells.

The method used followed that of section 2.11 (LLVP uptake assay). Confluent, duplicate (in a 6-well-plate) cultures of HepG2 cells were maintained in HepG2

growth medium for 2 days and treated with LPDS (8.5%) + insulin (0.1%) medium for 3 days. On day 5, the cells were washed and treated with 20µg/ml normal rabbit IgG, 20µg/ml polyclonal rabbit anti-apoB-100 antibody, a combination of 20µg/ml anti-apoB-100 and 50µg/ml anti-apoE antibodies, or a combination of 20µg/ml anti-apoB-100 and 100µg/ml anti-apoE antibodies added to 3µl of LLVP for 30 minutes prior to incubation with HepG2 cells in 800µl labelling medium.

The results are presented in figure 16. There was a significant increase ($p \leq 0.05$) of 59.2% HCV RNA uptake when LLVP was treated with rabbit anti-apoB100 antibody compared to normal rabbit IgG. There seemed to be an enhancing effect rather than a blocking effect when the LLVP was exposed to anti-apoB100 antibody. This experiment was repeated twice more with a similar result; an increase of 74.1% and 57.71% following treatment of LLVP with 20µg/ml polyclonal rabbit anti-apoB-100 antibody.

For the combination of 20µg/ml anti-apoB Ab + 50µg/ml or 100µg/ml anti-apoE antibodies treatment, the result showed an enhanced uptake of LLVP above that of LLVP uptake when treated with anti-apoB antibody treatment alone. The 20µg/ml anti-apoB Ab + 50µg/ml or 100µg/ml anti-apoE antibodies combination treatment showed a further increase of 35.7% ($p=0.04$) or 15.0% ($p=0.12$) respectively of the LLVP uptake in comparison to LLVP treated with 20µg/ml anti-apoB antibody treatment alone. In a second experiment, there was only a slight further increase of 4.1% ($p=0.87$) or 1.9% ($p=0.96$) increment respectively.

In a further experiment, 50µg/ml or 100µg/ml anti-apoE was used to see if anti-apoE antibody alone has a blocking effect on the LLVP binding. There was a slight increase of 7.2% or 11.9% respectively over that of normal rabbit IgG treatment and the results were not significantly different.

The results of this experiment showed that anti-apoB enhances the uptake of LLVP into HepG2 cells whereas anti-apoE does not.

Instead of anti-apoB100 and anti-apoE blocking the uptake of LLVP into the HepG2 cells, anti-apoE was without effect and anti-apoB100 actually enhanced the uptake of LLVP. This paralleled the increase in oxLDL uptake into HepG2 cells when it was exposed to anti-apoB100 antibody. A possible reason for the enhanced uptake could be aggregation of the LLVP mediated by the polyclonal antibodies. To test if the anti-apoB100 polyclonal antibodies did cause the aggregation of LLVP, an immunoprecipitation experiment in the next section was carried out.

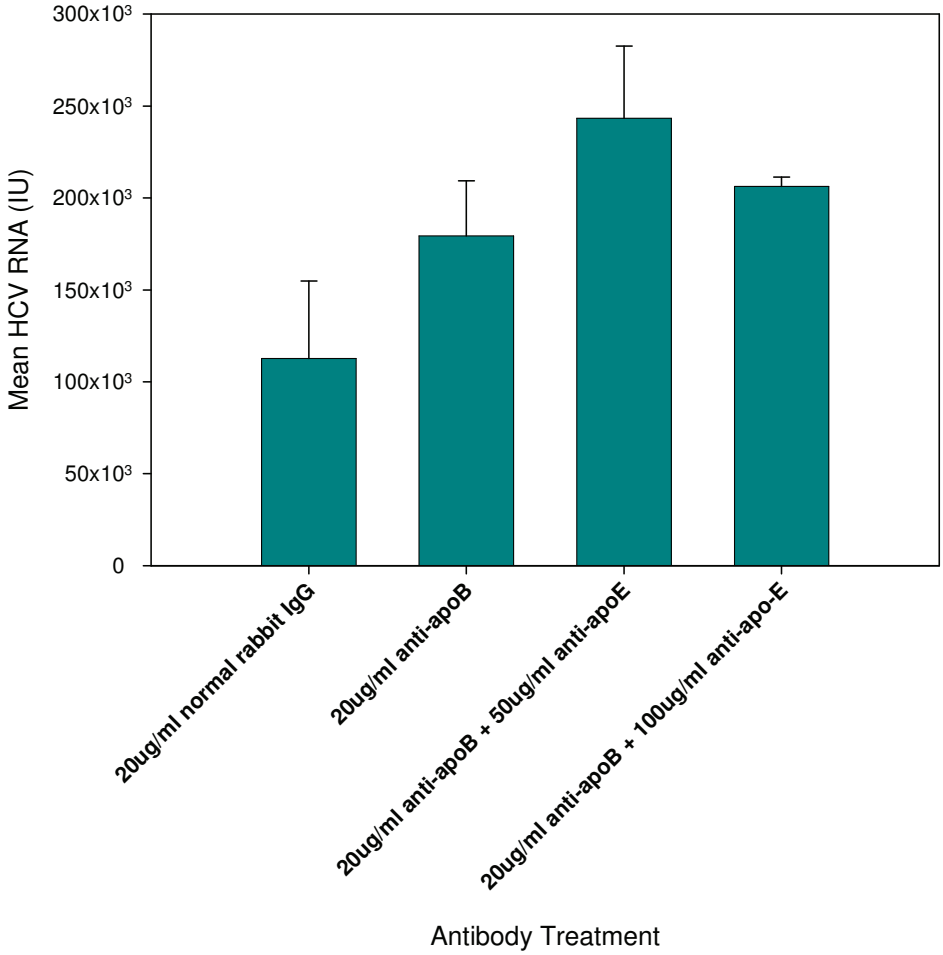


Figure 16: Mean HCV RNA from LLVP infected HepG2 cells.
The LLVP was incubated with anti-apoB100, combinations of anti-apoB100 and anti-apoE, or control antibody prior to infection.

3.3 Immunoprecipitation experiment to test for aggregation of LLVP

Following the result above, an experiment was done to see if anti-apoB100 antibody caused aggregation of the LLVP. If anti-apoB recognizes and binds the LLVP, it would aggregate the LLVP and the aggregation could be brought down by centrifugation at low speed (3000g) and hence, less LLVP would be found in the supernatant.

LLVP (1.2 X 10^6 IU HCV RNA in 3µl) was incubated with 20µg/ml (16µg) polyclonal rabbit anti-apoB100 or normal rabbit IgG in 800µl labelling medium (8.5% LPDS and serum free medium) for 3 hours in 37°C CO_2 incubator. After incubation, the tubes are centrifuged at 3 000g for 40 minutes. RNA from both pellets and supernatants were extracted using a QIAamp kit (Qiagen) according to the manufacturer instructions. The final RNA elution volume was 100µl. The HCV RNA was quantitated using RT-PCR as described in section 2.23.

The results as shown in table 5 and figure 17 confirmed that LLVP was aggregated by anti-apoB100 antibody because there is a higher mean HCV RNA reading of 4 162 583 IU (62.5%) in the pellet when compared to 2 495 327 IU (37.5%) in the supernatant. This aggregation effect is a common phenomenon for experiments involving polyclonal antibody. This effect gave an enhanced reading of LLVP uptake into HepG2 cells in the LLVP uptake or infectivity experiment as opposed to if there is no aggregation taking place and hence, less amount of LLVP will be taken up by the cells. The polyclonal antibodies mediated aggregation could promote Fc receptor-mediated uptake or the aggregation of LLVP would probably increase the surface area allowing easier access to the cellular receptors and hence increase uptake by the cells.

Antibody (20µg/ml)	Supernatant/pellet	mean HCV RNA (IU)	standard deviation	Percentage of total
Normal rabbit IgG	supernatant	3447684	469792.59	72.5%
Normal rabbit IgG	pellet	1310940	231450.19	27.5%
anti-apoB	supernatant	2495327	91047.423	37.5%
anti-apoB	pellet	4162583	359326.92	62.5%

Table 5: Percentage of LVP HCV RNA in supernatant and pellet in a test for LLVP aggregation by anti-apoB100 polyclonal antibody.

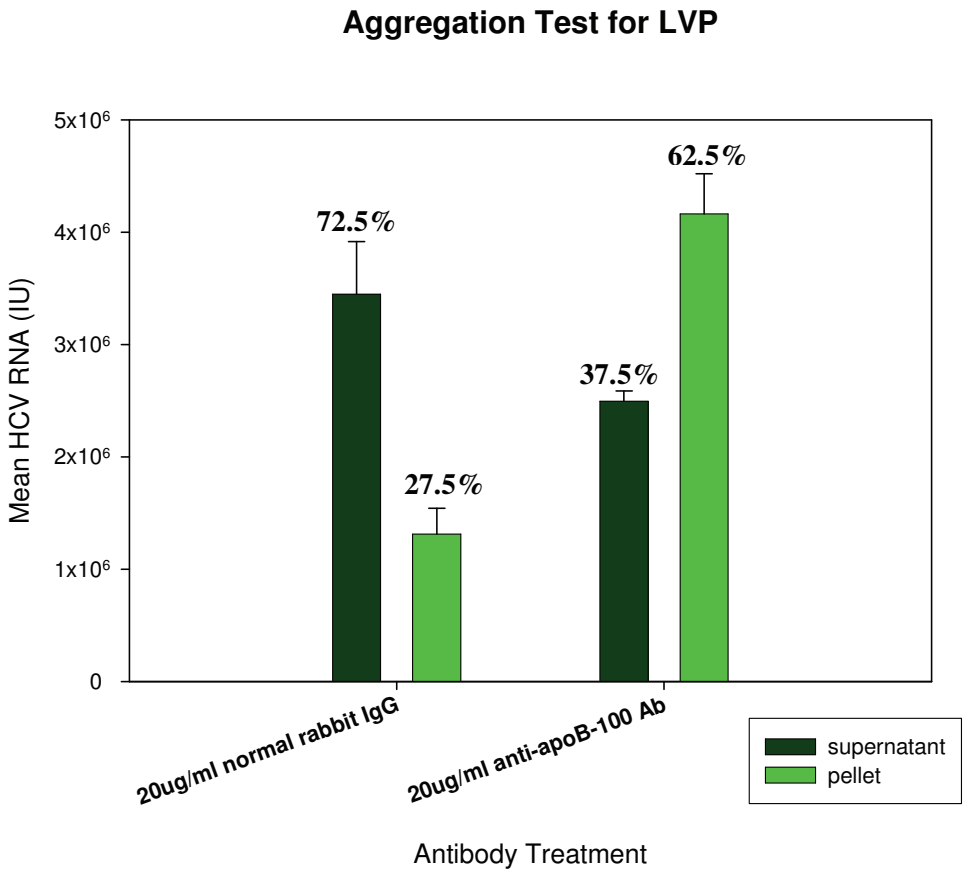


Figure 17: Aggregation test for LLVP.

3.4 DiI-oxLDL uptake by HepG2 cells under different drug treatments

LDL binds both to the LDL receptor (Hajjar et al., 1997) and scavenger receptor B1 (SR-B1) (Steinbrecher, 1999). Oxidized LDL binds to SR-B1 but not to the LDL receptor (Hajjar, 1997). LDL receptor expression can be reduced with hydroxycholesterol (25-OH) (Srivastava et al., 1995) and increased by removal of low density lipoproteins from the medium and addition of insulin (Agnello et al., 1999, Wade et al., 1989, Wade et al., 1988). The effect of these conditions on the expression of SR-B1 is not clear.

The result obtained in section 3.2 above ~~in~~ that anti-apoB100 antibody enhances the uptake of LLVP suggests that LLVP is not binding to LDLr. As Martin (Martin, 2005) has shown that LLVP binding can be blocked by pre-incubation to both LDL and oxLDL, LLVP may mimic binding of oxLDL, which binds to SR-B1 via oxidised ligands on the particle's surface. To test whether oxLDL binding is also affected by 25-OH and LPDS plus insulin treatment the following experiment was carried out.

The purpose of this experiment was to make comparisons of DiI-oxLDL uptake by HepG2 cells to that of DiI-LDL under different conditions of the LDL receptors expression.

Confluent, triplicate (in a 6-well-plate) cultures of HepG2 cells were maintained in HepG2 growth medium containing either 10% FCS for 3 days, 8.5% LPDS and 0.1% insulin for 3 days, or in growth medium containing 10% FCS and 0.00025% 25-OH for 2 days (25-OH is toxic to cells) as described in section 2.7. The cells were washed and incubated with 1ml labelling medium containing either 25ul DiI-LDL (10µg/well) or 35ul DiI-oxLDL (14µg/well) 3 hours in 37^{o}C CO2 incubator. The cells were harvested and prepared for FACS analysis as described in section 2.15.

The results are presented in figures 18 and 19, as well as, tables 6, 7, 8, and 9. There is a significant decrease of 28% mean intensity of DiI-LDL uptake by HepG2 cells when cells are pre-incubated in hydroxycholesterol, whereas, there is a significant 150% increase in mean intensity when cells are cultured in LPDS + insulin. However, there is little change in the DiI-oxLDL uptake by HepG2 treated with

hydroxycholesterol, whereas, there is an 88% significant increase in mean intensity when cells were treated with LPDS + insulin.

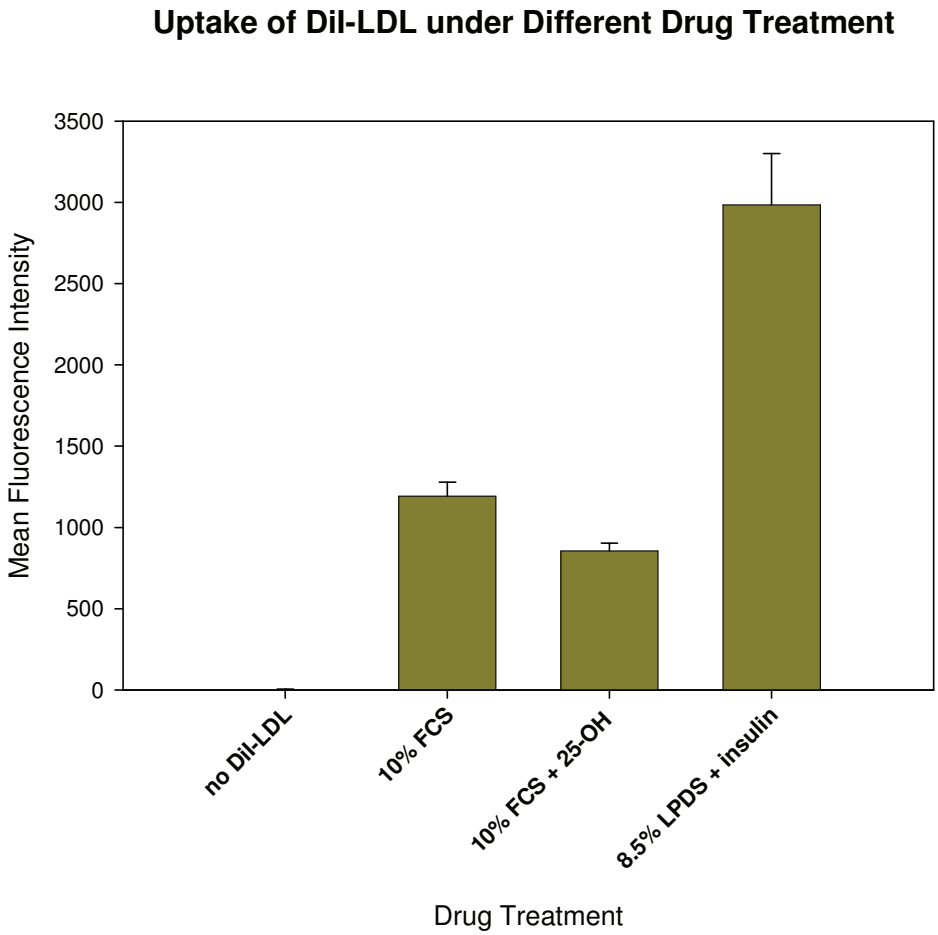


Figure 18: Mean fluorescence intensity of DiI-LDL uptake into HepG2 cells treated with different drugs.

Treatment		Mean Intensity	Standard Deviation
HepG2 Medium	**LDL**		
10% FCS, LPDS + insulin, FCS + 25-OH	No DiI-LDL	4	1
10% FCS	DiI-LDL	1 191	87
FCS + 25-OH	DiI-LDL	856	46
LPDS + insulin	DiI-LDL	2 984	317

Table 6: Mean intensity and standard deviation of DiI-LDL uptake into HepG2 cells treated with FCS + 25-OH and LPDS + insulin.

Samples compared	P value	Significant if $p \leq 0.05$	% change
10% FCS vs.			
FCS + 25-OH	0.0042	yes	28% decrease
10% FCS vs.			
LPDS + insulin	0.0006	yes	150%
			increase

Table 7: P value and percentage change of the sample comparisons of DiI-LDL uptake into HepG2 cells treated with FCS + 25-OH and LPDS + insulin.

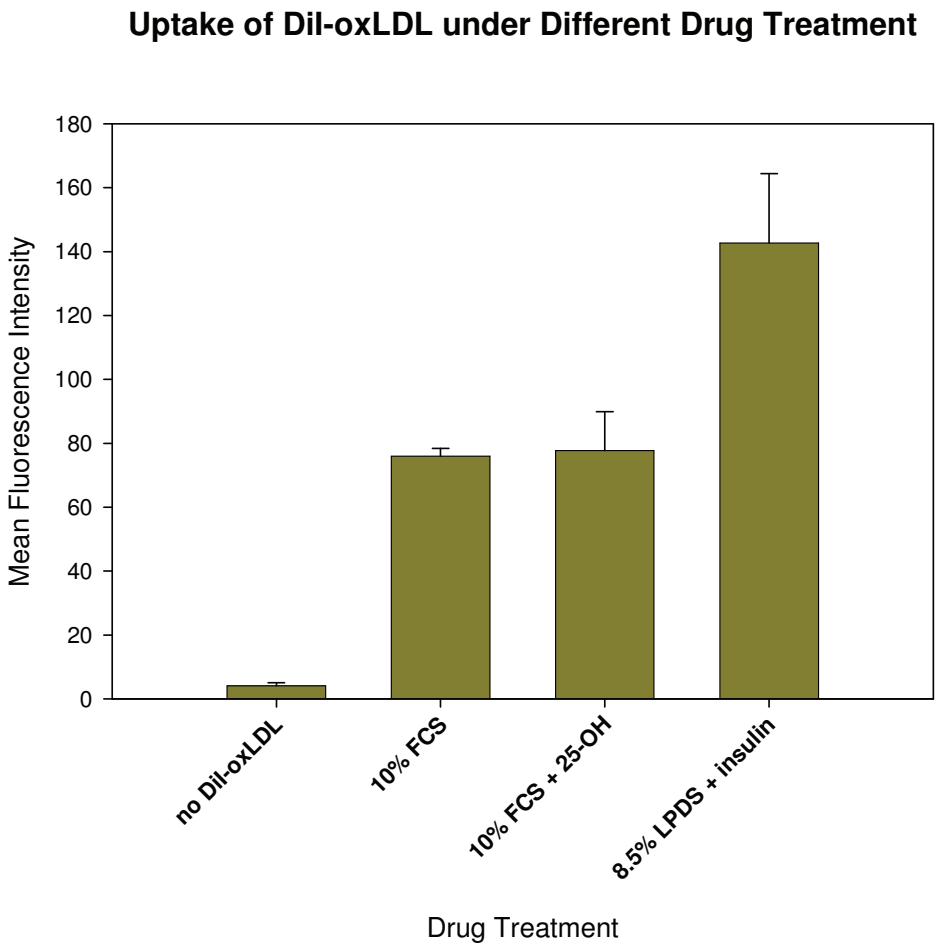


Figure 19: Mean fluorescence intensity of DiI-oxLDL uptake into HepG2 cells treated with different drugs.

Treatment		Average Intensity	Standard Deviation
HepG2 Medium	LDL		
10% FCS, LPDS + insulin, FCS + 25-OH	No DiI-oxLDL	4	1
10% FCS	DiI-oxLDL	76	2
FCS + 25-OH	DiI-oxLDL	78	12
LPDS + insulin	DiI-oxLDL	143	22

Table 8: Mean intensity and standard deviation of DiI-oxLDL uptake into HepG2 cells treated with FCS + 25-OH and LPDS + insulin.

Samples compared	P value	Significant if $p \leq 0.05$	% change
10% FCS vs.			
FCS + 25-OH	0.821	no	2.63% increase
10% FCS vs.			
LPDS + insulin	0.006	yes	88% increase

Table 9: P value and percentage change of the sample comparisons of DiI-oxLDL uptake into HepG2 cells treated with FCS + 25-OH and LPDS + insulin.

3.5 Effect of anti-apoB100 antibody on the binding of DiI-LDL and DiI-oxLDL

The increased binding of ox-LDL in cultures incubated in LPDS and insulin, designed to upregulate LDLr expression suggests that oxLDL may be taken up by residual binding of apoB100 to LDLr rather than by the SR-B1 receptor through recognition of oxidized ligands on the surface of oxLDL particle. If so, binding should be blocked with anti-apoB100 antibody. In this experiment therefore, we have tested the ability of anti-apoB100 antibody to block DiI-oxLDL uptake.

Duplicate cultures of HepG2 cells seeded at $0.7X10^6$ cells/well treated with LPDS + insulin (0.1%) medium as decribed in in section 2.14. Treated cells were incubated with either 25µl DiI-LDL or DiI-oxLDL preincubated with or without 20µg/ml normal rabbit IgG or 20µg/ml polyclonal rabbit anti-apoB100 antibody. The cells were harvested and prepared for FACS analysis as in section 2.15.

The results are presented in figures 20 and 21. Statistical analyses of the results are presented in Table 10. Whereas preincubation of DiI-LDL with anti-apoB100 antibody reduced binding by 98%, significant reduction when compared to pre-incubation with normal IgG, similar treatment of DiI-oxLDL produced an increase, not a decrease although this change did not achieve significance.

As seen previously, the binding of DiI-oxLDL was much lower (6.5%) than that of DiI-LDL. These results suggest that binding of oxLDL is not apoB100 dependent and therefore quite different from binding of LDL. The mechanism of increased binding of oxLDL mediated by LPDS + insulin thus remains unclear.

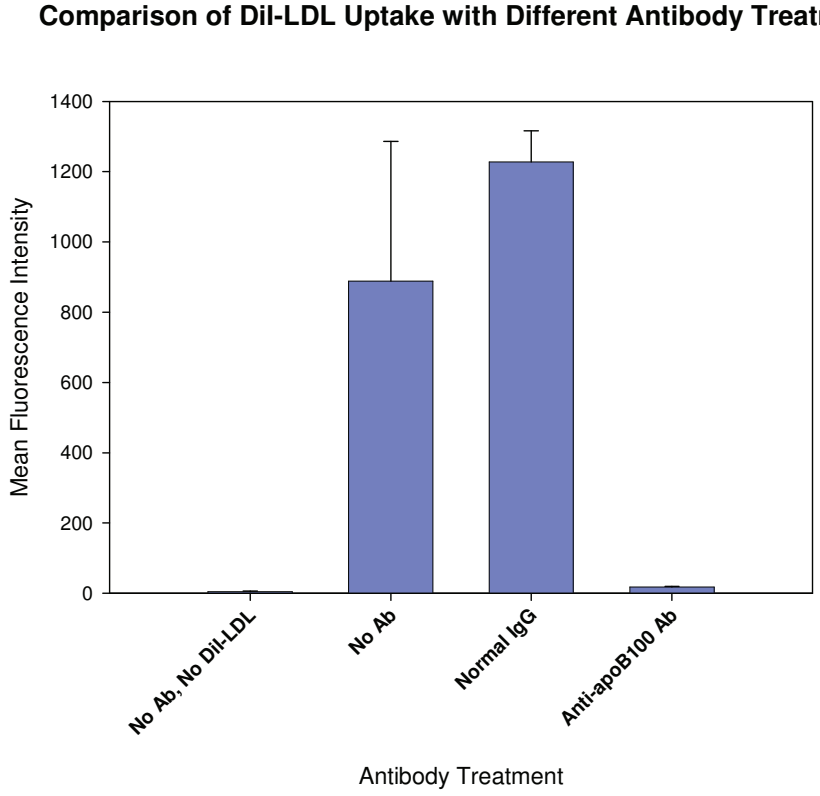


Figure 20: Mean fluorescence intensity of DiI-LDL uptake into HepG2 cells following different antibody treatment.

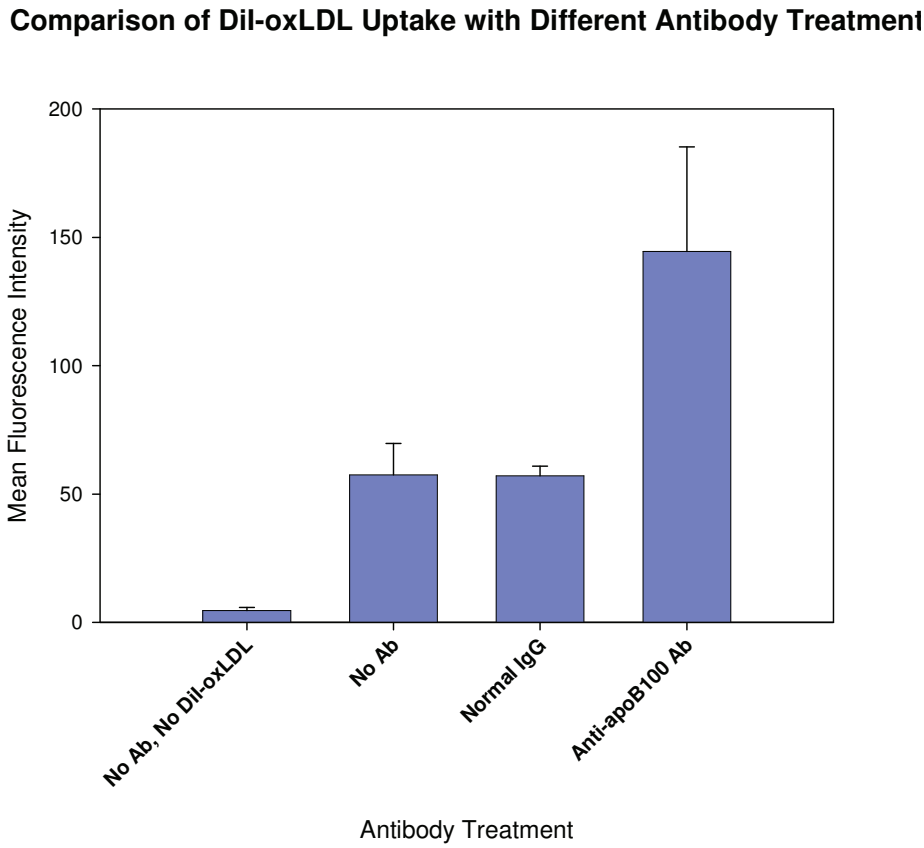


Figure 21: Mean fluorescence intensity of DiI-oxLDL uptake into HepG2 cells following different antibody treatment.

Antibody and LDL/oxLDL Treatment	P value	% change
no Ab + DiI-LDL vs anti-apoB Ab + DiI-LDL	0.090	- 98%
normal rabbit IgG + DiI-LDL vs anti-apoB Ab + DiI-LDL	0.002	- 98%
no Ab + DiI-oxLDL vs anti-apoB Ab + DiI-oxLDL	0.101	+ 151%
normal rabbit IgG + DiI-oxLDL vs anti-apoB Ab + DiI-oxLDL	0.094	+ 154%

Table 10: A comparison of percentage differences on the mean fluorescence intensity of DiI-LDL or DiI-oxLDL uptake into HepG2 cells following different antibody treatment.

3.6 Effect of suramine wash on DiI-ox-LDL bound to HepG2 cell surface

3.6.1 Assessed by confocal microscopy

In the following experiments, we set out to test the suramine sensitivity/resistance of oxLDL binding compared to that of LDL. Suramine is a polyanion that can remove LDL bound to glycosaminoglycans (GAG) on the cell surface (Brown and Goldstein, 1986). At 0^{o}C, cell membrane's fluidity decreases, blocking any uptake of bound material whereas at 37^{o}C, bound material on the cell's surface will be taken up by the cell (Jarvis, 2000).

Martin (Martin, 2005) demonstrated that when HepG2 cells were incubated at 4ºC with DiI-LDL, the dye bound onto the surface and was visible by confocal microscopy. After a suramine wash, DiI-LDL was removed (Martin, 2005). At 37^{o}C, the cells internalised the DiI-LDL and hence, the result before and after the suramine wash was similar with only a slight reduction post-suramine wash representing the proportion of DiI-LDL which remained on the cell surface. The effect of a suramine wash on LLVP binding was more complex. Although after 3h at 0^{o}C, the binding of HCV LLVP was found significantly suramine sensitive; with time, it became progressively resistant to suramine. At 37^{o}C, suramine had removed only a minority of bound material in a manner analogous to the reduction in DiI-LDL binding. The purpose of this experiment was to see how DiI-oxLDL behaves under the same conditions.

HepG2 cells cultured in 60mm x 15mm Petri dish, treated with 8.5% LPDS plus 0.1% insulin, were incubated with 10μg/ml of DiI-LDL or 14μg/ml of DiI-oxLDL at 0°C or 37°C for 3 hours. Cells were then washed with cold PBS containing 0.5 % BSA, incubated with or without 10mg/ml suramine solution for 30 minutes at 4°C. The wash step was repeated and 4ml ice-cold EMEM growth medium with 10 % FCS was then added. The cells were examined using confocal microscopy. The platform of the microscope was adjusted to move the point of focus through the cells allowing the mid-sections of the cells to be visualised. Detailed confocal microscopy method is described in section confocal microscopy.

The results of the confocal microscopy are presented in figures 22 and 23. As expected, low levels of DiI-LDL were bound to cells at 0°C and remained associated with the cell membrane. This was largely removed after a suramine wash. At 37°C, much higher levels of DiI-LDL binding were evident which was not removed by suramine washing and appeared to be intracellular. Although because of the low levels of binding it was difficult to assess the effect of suramine wash on the DiI-oxLDL either at 0°C or at 37°C, binding of DiI-oxLDL did not appear substantially increased at 37°C and any effect of suramine wash on the DiI-oxLDL was not discernible either at 0°C or at 37°C.

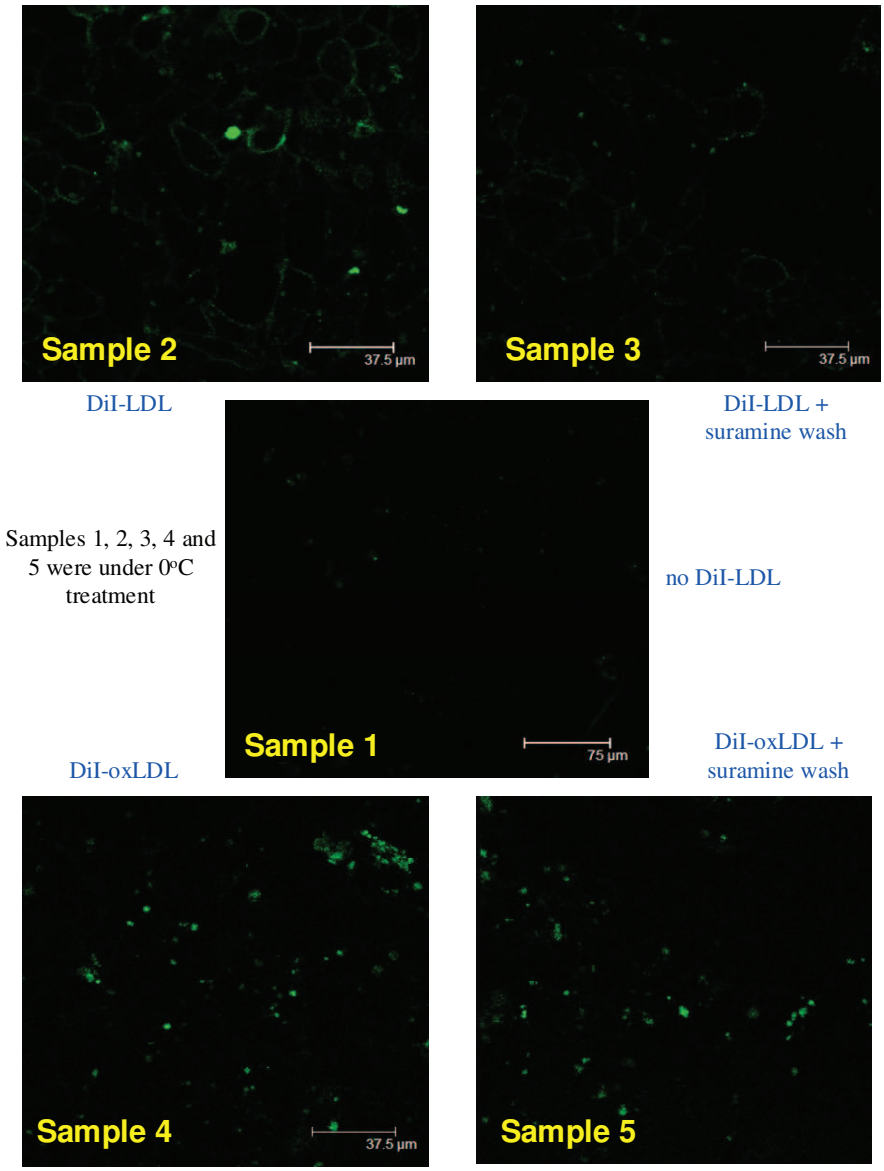


Figure 22: Confocal pictures of DiI-LDL or DiI-oxLDL uptake into HepG2 cells at 0°C with or without suramine wash.

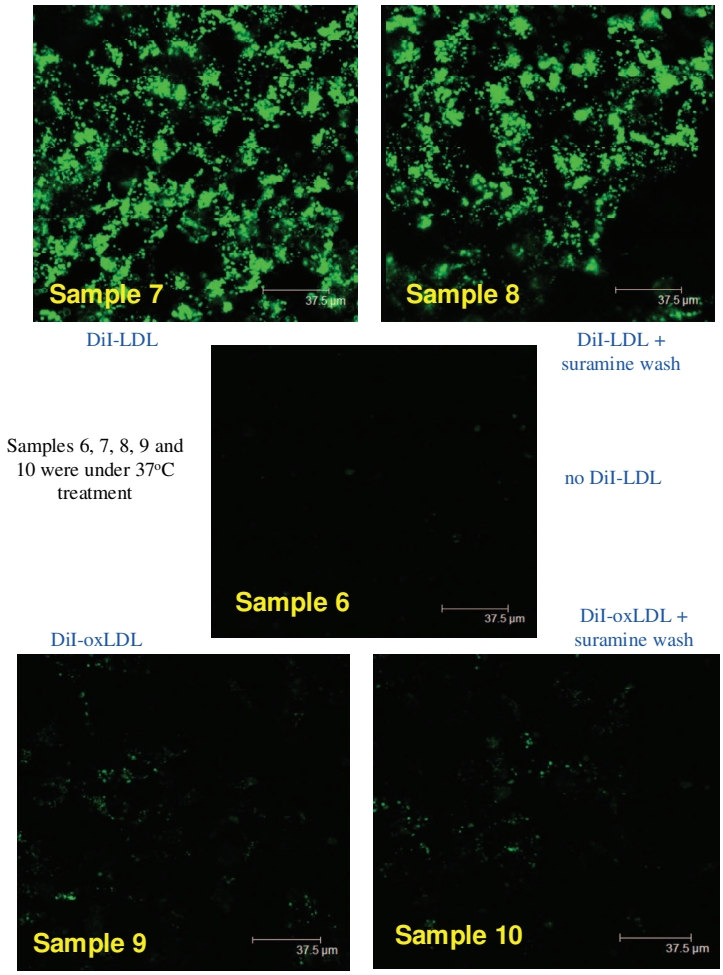


Figure 23: Confocal pictures of DiI-LDL or DiI-oxLDL uptake into HepG2 cells at 37°C with or without suramine wash.

3.6.2 Assessed by FACS analysis

In order to quantitate the results from the above analysis the experiment was repeated but DiI labelled lipoprotein binding was measured by FACS analysis rather than confocal microscopy.

Confluent, duplicate (seeded at 0.3 x 10^6 cells/well in a 6-well-plate) cultures of HepG2 cells, treated with 8.5% LPDS plus 0.1% insulin, were incubated with 10µg/ml of DiI-LDL or 14µg/ml of DiI-oxLDL at 0°C or 37°C for 3 hours. Cells

were then washed with cold PBS containing 0.5 % BSA, incubated with or without 10mg/ml suramine solution for 30 minutes at 4°C. The wash step was repeated and 4ml ice-cold EMEM growth medium with 10 % FCS was then added. Detailed method is described in section 2.15 except that cell cultures were maintained in a 6-well-plate and that the incubation with suramine step was eliminated since it had been done.

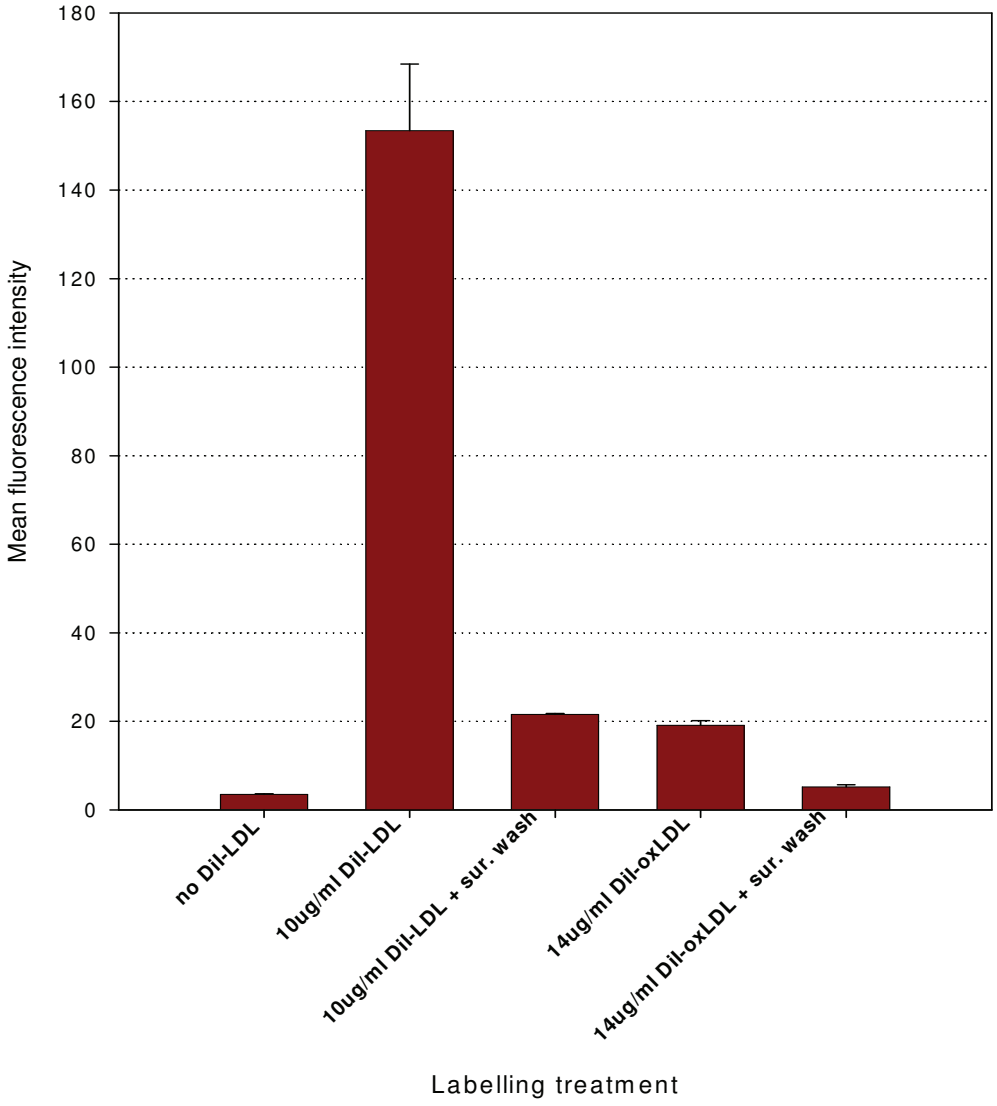


Figure 24: A graph showing the mean fluorescence intensity of DiI-LDL and DiI-oxLDL taken up by HepG2 cells after a 3-hour-exposure at 0°C without or after a suramine wash.

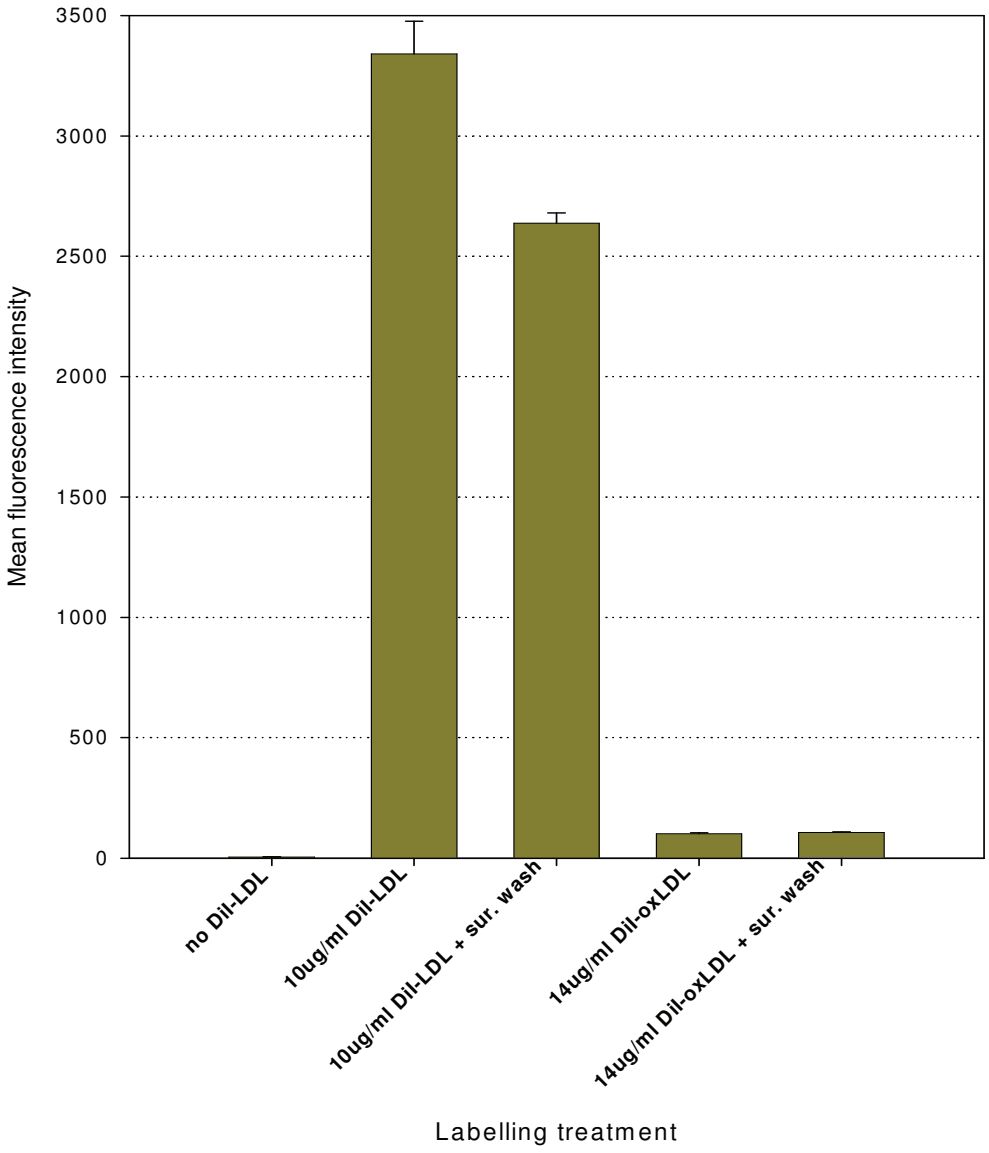


Figure 25: A graph showing the mean fluorescence intensity of DiI-LDL and DiI-oxLDL taken up by HepG2 cells after a 3-hour-exposure at 37°C without or after a suramine wash.

From the results in figures 24 and 25, it can be seen that there is a significant 6.10 fold ($P = 0.006$) reduction of DiI-LDL binding after the suramine wash at 0°C and a significant 0.27 fold ($P = 0.003$) reduction at 37°C.

Although oxLDL, appeared to be sensitive to suramine at 0°C as seen in figure 24, with a 2.67 fold reduction, the result is not significant (P = 0.06). At 37°C however, the intensity reading remains similar before and after the suramine wash. In fact, there is a slight increase in oxLDL uptake after the suramine wash but this result is not significant (P = 0.5).

At 0°C, the reduction in DiI-oxLDL binding by suramine to insulin treated HepG2 parallels that observed by Martin for LLVP 44.3% reduction (Martin et al., 2008). However at 37°C, whereas Martin reports a 1.5 fold reduction in LLVP binding, here, oxLDL binding is increased by suramine wash. Thus, the mechanism of uptake of oxLDL, although differing in degree from that of LDL, shows a similar pattern to that of LLVP – being suramine sensitive after 3 hours at 0°C.

3.7 Result summary of the LLVP uptake assay

I have shown that LLVP binding to HepG2 resembles binding of oxidised low density lipoprotein (oxLDL) more closely than that of low density lipoprotein (LDL). Thus, whereas anti-apoB100 antibodies blocked uptake of LDL by HepG2 cells, the same antibody actually enhanced the uptake of both oxLDL and LLVP. We subsequently hypothesized that LLVP like oxLDL, binds to SR-B1 to enter and infect cells.

This hypothesis predicts that the established properties of LLVP binding to HepG2, including increased uptake by insulin treated cells and removal by suramine will be shared by the oxLDL/SR-B1 system.

3.8 Anti-LDLr and anti-SR-B1 antibodies titration

In an attempt to compare the expression of SR-B1 and LDLr on HepG2 cells under different drug treatments, antibodies against these receptors were used to quantify receptor expression. The antibodies to be used were first titrated before the actual comparison experiment was carried out. Anti-SR-B1 antibody NB400-104 and anti-LDLr antibodies 61099 and C7 were titrated on HepG2 cells maintained for 3 days on medium containing 8.5% LPDS plus 0.1% insulin. Normal rabbit IgG was titrated in parallel as a control for NB400-104 and 61099 which are both polyclonal rabbit antisera. An irrelevant anti-RSV-2G122 was titrated as a control for the mouse Mab C7. The rest of the detailed method is described in section 2.19.

The results of the titrations of anti-SR-B1 and anti-LDLr (61099 and C7) antibodies are presented in figures 26, 27 and 28. NRIg at 13µg/ml gave only 13.5% of the binding seen with the anti-SR-B1 (NB400-104) antibody at 15µg/ml (figure 26). Higher binding of anti-SR-B1 antibodies occurred at higher concentrations but suitable control immunoglobulin was not available for comparison. At 10µg/ml NRIg gave a value which is only 14.3% of that for anti-LDLr (61099). Anti-RSV monoclonal antibody (2G 12-2) at 7.5µg/ml gave only 2.9% of the binding of anti-LDLr (C7) at 4µg/ml. However, C7 binding was low at this concentration and a more concentrated antibody was not available.

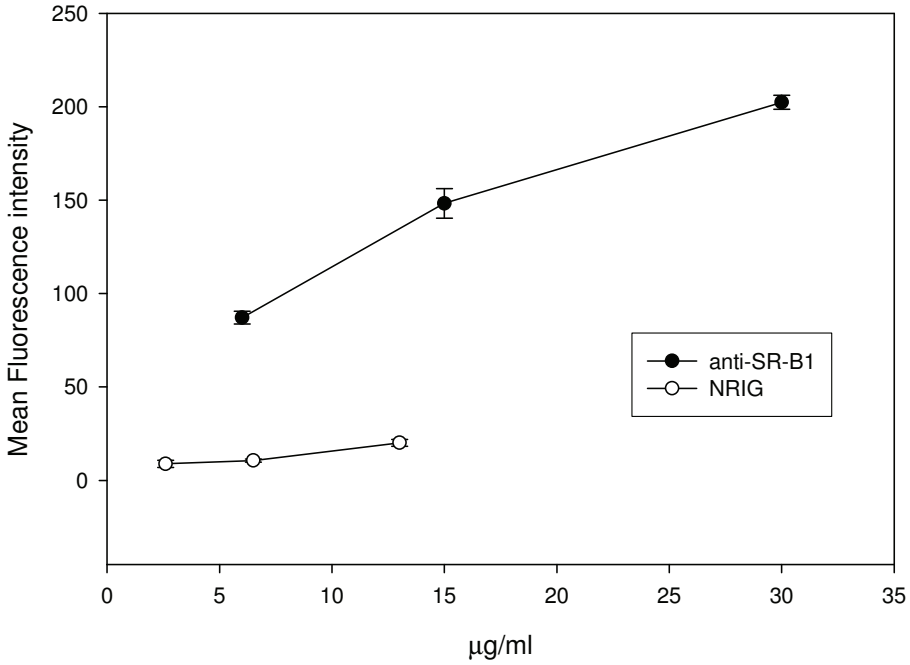


Figure 26: Optimization of anti-SR-B1 (NB 400-104) antibody dilutions.

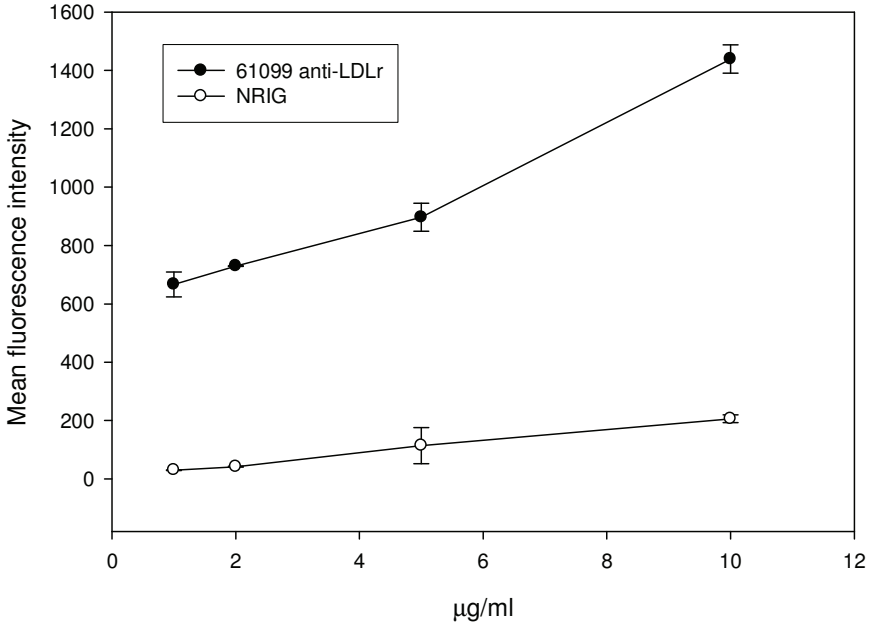


Figure 27: Optimization of anti-LDLr (61099) antibody dilutions.

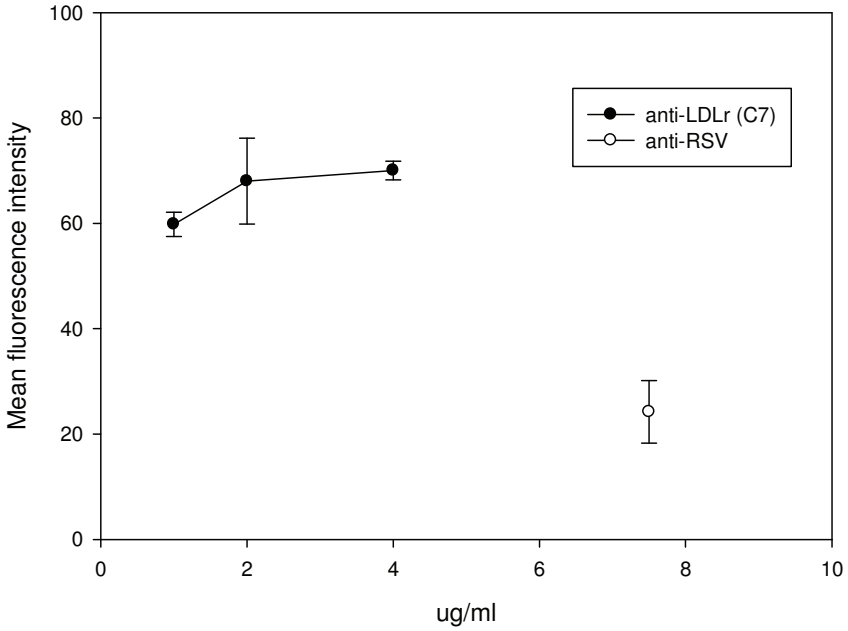


Figure 28: Optimization of anti-LDLr (C7) antibody dilutions.

3.9 Effect of LPDS plus insulin and 2,5 hydroxycholesterol on the expression of LDLr and SR-B1 on HepG2 cells

In order to compare the effect of insulin on SR-B1 expression with the previously determined effect on LDLr expression and LLVP binding, SR-B1 expression was quantitated by FACS analysis with 15µg/ml anti-SR-B1 (NB400-104) on normal untreated HepG2 cells grown in 10% Foetal Calf Serum, cells grown in LPDS plus insulin and cells grown in FCS plus hydroxylcholesterol as described in section 2.19.

Having found suitable titres for anti-SR-B1 NB 400-104 and anti-LDLr 61099 antibodies, the following experiment was carried out to determine the effect of LPDS plus insulin and 2,5 hydroxycholesterol on the expression of LDLr and SR-B1 on HepG2 cells.

HepG2 cells were seeded and treated with either EMEM growth medium with 10% FCS and 8.5% LPDS plus 0.1% insulin for 3 days or 25-OH for 2 days. The treated cells were trypsinized and pipetted vigorously until a single cell suspension was obtained. The cells were treated with 4% paraformaldehyde and 0.1% Saponin before being exposed to anti-SR-B1 (15µg/ml in 25 µl/well) or anti-LDLr (10µg/ml in 25 µl/well) antibodies and subsequently the bound antibody was detected with appropriate species specific secondary fluoresceinated anti-immunoglobulin and quantitated by FACS analysis as described in section 2.19.

The results are presented in figure 29 and 30. SR-B1 was down-regulated by LPDS plus insulin 1.2-fold (P = 0.002) and by hydroxycholesterol 1.4-fold (P = 0.002) compared to SR-B1 expressed in normal growth medium with 10% FCS.

In similar experiments the effect of insulin or hydroxycholesterol treatment on LDLr was determined using 10ug/ml anti-LDLr (61099). LDLr was up-regulated 2.2-fold (P = 0.0002) by LPDS plus insulin and down-regulated 1.3-fold (P = 0.001) by hydroxycholesterol when compared to LDLr expressed in normal growth medium with 10% FCS.

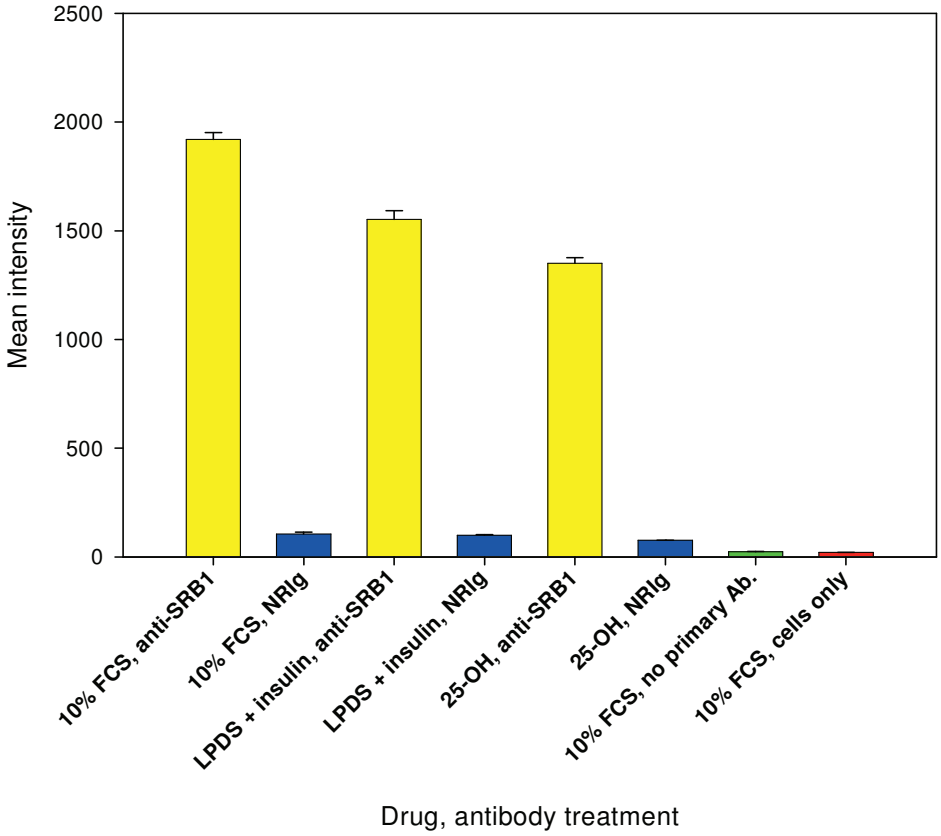


Figure 29: The effect on SR-B1 receptor expression of different drugs treatment in HepG2 cells. The cells were stained with anti-SR-B1 or normal rabbit Ig (NRIg), secondary antibody or no primary and secondary antibody.

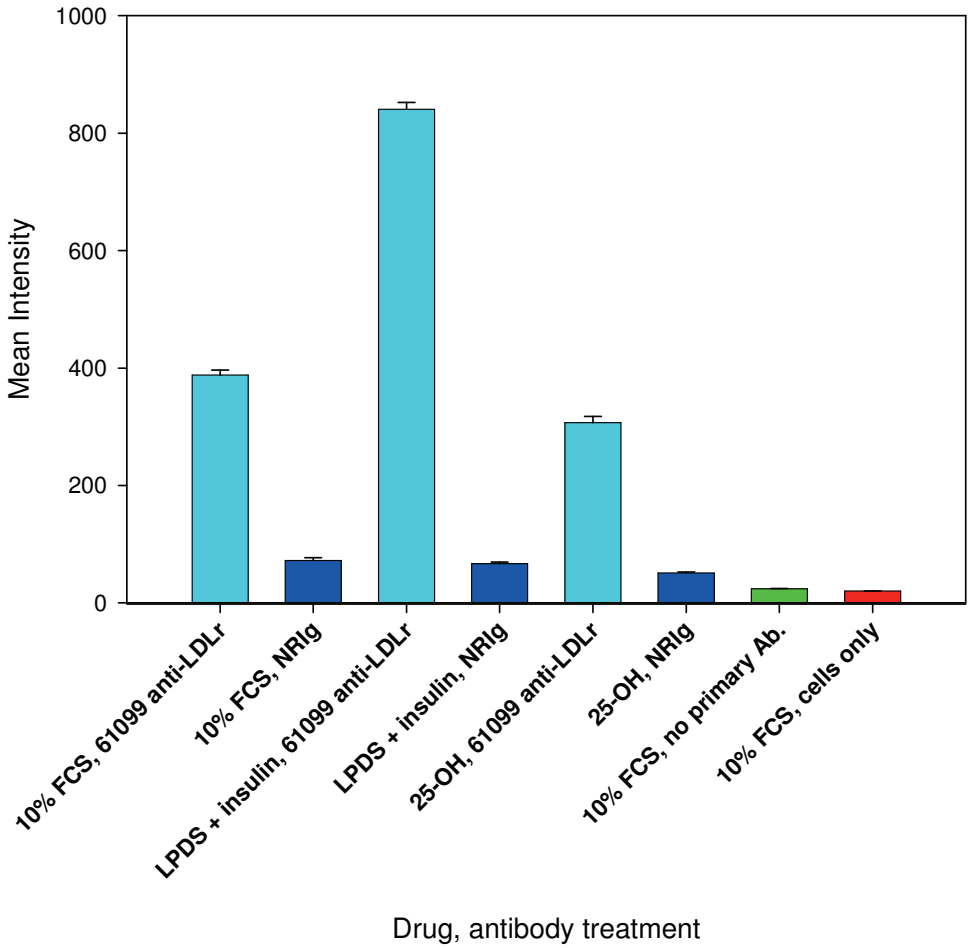


Figure 30: The effect on LDL receptor expression of different drugs treatment in HepG2 cells. The cells were stained with 61099 anti-LDLr, NRIg, secondary antibody or no primary and secondary antibody.

3.10 The effect of LPDS plus insulin on LDLr and SR-B1 in non-hepatic cells

To see if the same phenomenon occurs in non-hepatocyte derived cells, a similar kind of experiment was carried out using Vero cells (monkey kidney cells) to compare with HepG2 cells.

$0.7X10^6$ cells/well of either HepG2 or Vero cells were seeded and treated with either 8.5% LPDS plus 0.1% insulin or normal EMEM growth medium with 10% FCS for 3

days. The treated cells were trypsinized and pipetted vigorously until a single cell suspension was obtained. The cells were treated with 4% paraformaldehyde and 0.1% Saponin before being exposed to anti-SR-B1 (15µg/ml in 25 µl/well) or anti-LDLr (10µg/ml in 25 µl/well) antibodies and subsequently the bound antibody was detected with appropriate species specific secondary fluoresceinated anti-immunoglobulin and quantitated by FACS analysis as described in section 2.19.

The results are presented in figures 31 and 32. Figure 31 shows that the LDLr expression in HepG2 cells is higher by 1.6-fold (P = 0.001) and 2.6-fold (P = 0.0006) compared to the expression in Vero cells when treated with 10% FCS and LPDS plus insulin respectively. In Vero cells however, LPDS plus insulin down-regulate LDLr by 10%. Figure 32 show the SR-B1 expression in HepG2 is also higher by 1.9-fold (P = 0.002) and 2.4-fold (P = 0.0003) compared to the expression of SR-B1 in Vero treated with 10% FCS and LPDS plus insulin respectively. In Vero cells however, LPDS plus insulin down-regulates SR-B1 by 7.5%. Binding of LLVP to Vero cells was higher than that to HepG2 cells (Martin, 2005) and hence, does not correlate with SR-B1 and LDLr as shown in the experiments above.

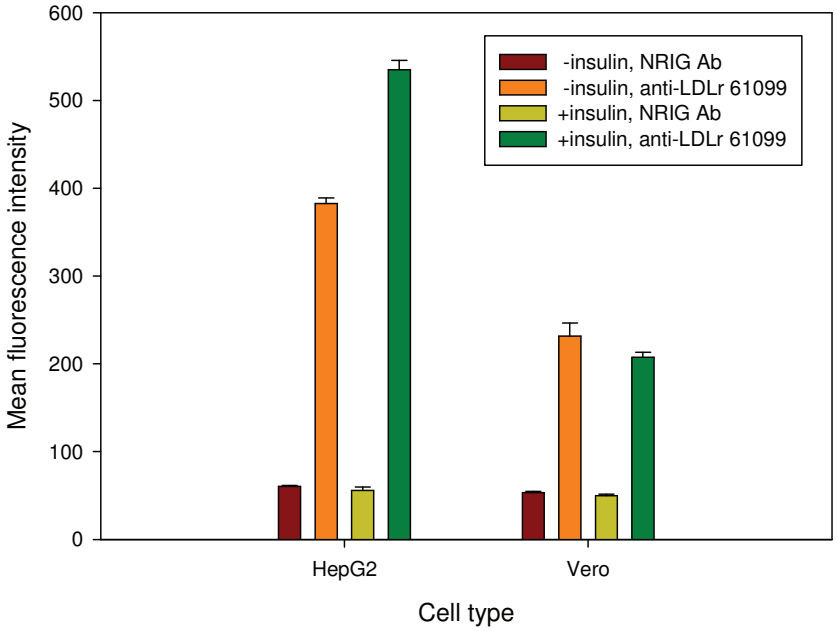


Figure 31: The effect of LPDS plus insulin on the expression of LDLr on HepG2 cells and Vero cells.

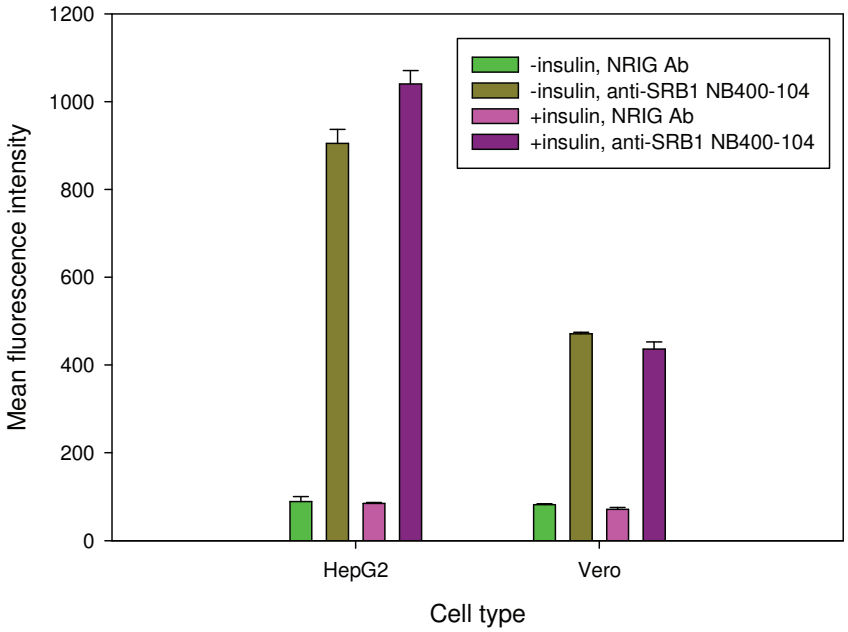


Figure 32: The effect of LPDS plus insulin on the expression of SR-B1 on HepG2 cells and Vero cells.

Summary

SR-B1 expression in HepG2 cells was slightly lowered by insulin treatment in the first experiment and slightly raised in the second suggesting that this receptor is not affected by insulin. These experiments, therefore, do not confirm the predictions for the hypothesis that LLVP and oxLDL, binds to insulin treated HepG2 cells via SR-B1. These results do not preclude the involvement of SR-B1 in binding but indicate that other insulin-dependent factors are as or more important.

Recent development in some of the experiments carried out by Dr. Nielsen indicate that LLVP semi-purified on iodixanol gradients may contain viral RNA containing microsomal membrane particles created during the maceration of the liver (Nielsen et al., 2008). In light of this, it is not clear whether the HCV RNA bound to HepG2 cells in the binding studies presented here represents the binding of LLVP or microsomal membrane fragments.

3.11 Effect of anti-E2 HCV antibody and anti-HVR-1 monoclonal antibody blocking binding of LLVP

Bartosch *et al* (Bartosch et al., 2003b) have suggested that binding of HCV pseudotype particles to SR-B1 is dependent upon the E2 glycoprotein. In order to assess the role of E2 in the binding of liver derived HCV LVP to HepG2 cells, polyclonal anti-E2 antibody and a monoclonal antibody to the E2 hypervariable region previously shown in an immunoprecipitation experiment to bind to S6 HCV (Nielsen et al., 2006) were tested for their ability to block LLVP binding.

The method used followed that of section 2.11 (LLVP uptake assay). Confluent, duplicate (in a 6-well-plate) cultures of HepG2 cells were maintained in HepG2

growth medium for 2 days and treated with LPDS (8.5%) + insulin (0.1%) medium for 3 days. On day 5, the cells were washed and treated with 20µg/ml normal goat antibody, 20µg/ml or 100µg/ml polyclonal goat anti-E2 HCV (B65581G) antibody, 20µg/ml anti-RSV-F (2G122) monoclonal antibody, or 20µg/ml or 100µg/ml anti-HVR-1 (3C7-C3) of HCV E2 monoclonal antibody added to 3ul of LLVP for 30 minutes prior to incubation with HepG2 cells in 800µl labelling medium.

The results are presented in figure 33. There was no significant difference between the binding of LLVP to HepG2 cells following treatment with normal goat IgG and either 20µg/ml or 100µg/ml goat anti-E2 HCV (B65581G) antibody treatment to the LLVP uptake assay. This experiment was done three times with similar results.

Similarly, treatment of LLVP with 20µg/ml or 100µg/ml HVR-1 (3C7-C3) of HCV E2 monoclonal antibody produced no significant change in the binding compared to 20µg/ml anti-RSV-F (2G122) monoclonal antibody.

From the results above, neither polyclonal antibody against E2 nor monoclonal antibody against the hypervariable region of E2 blocks binding of LLVP to HepG2 cells.

LLVP binding parallels ox-LDL binding rather than LDL binding - that is being increased by insulin, enhanced by apoB-100, and GAG dependent. Although this suggests that LLVP may be binding to SR-B1, insulin does not increase SR-B1 expression suggesting that insulin increases binding by a different mechanism. HCV

binding to SR-B1 has been shown to be via E2 but LLVP binding was not blocked by anti-E2 antibodies.

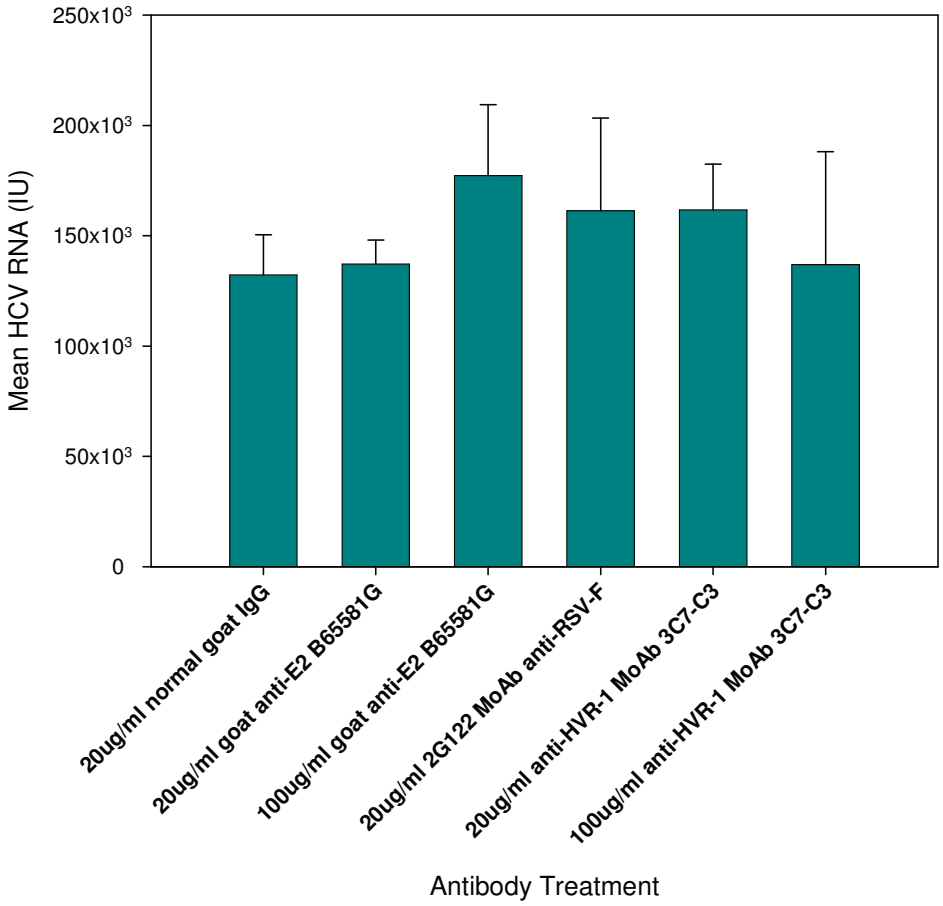


Figure 33: Mean HCV RNA from LLVP infected HepG2 cells.
The LLVP was incubated with different anti-E2 or control antibodies prior to infection.

Chapter 4

4 Results II

4.1 Determination of HCV RNA quantity upon inoculation of HCV LLVP from liver macerate to HepG2 and Huh7.5 cell lines and primary hepatocytes

Binding of HCV LLVP from liver macerate to HepG2 cells, which appears to be mediated via host cell receptor-ligand interactions, may not be biologically relevant and may not lead to infection in the cell. An infection system, in which the ability of adsorbed virus to penetrate and initiate infection in the cells can be monitored, is required to investigate whether binding of LLVP via host cell ligands can lead to infection. The purpose of this experiment therefore was to see if LLVP can infect HepG2 cells.

0.7 X 10^6 HepG2 cells per well of a six-well plate were seeded and treated with 8.5% LPDS + 0.1% insulin EMEM medium after 2 days as described in section 2.14. The cells were inoculated with 1.1 X 10^7 IU S6b LLVP and incubated for 3 hours at 37^{o}C. HepG2 cells were grown in 8.5% LPDS + 0.1% insulin EMEM medium post-inoculation and the medium were sampled at 2/3 day intervals for a 7-day period. Samples were extracted for HCV RNA and titrated by quantitative RT-PCR. The results are presented in figure 34. There is a general decrease of HCV RNA in the HepG2 cells that were inoculated with LLVP. There is an 87% decrease from day 0 to day 2, a 94% decrease from day 0 to day 5 and a 98% decrease from day 0 to day 7. These results indicate that HepG2 cells do not support the replication of HCV from the LLVP.

The experiment was repeated in Huh7.5 cells ~~with~~ which is a different human hepatoma cell line. The Huh7.5 cell line was used because it is known to support the replication of HCVcc J6/JFH1. 1 X 10^5 Huh7.5 cells per well of a six-well plate were seeded and treated with 8.5% LPDS + 0.1% insulin DMEM medium after 2 days as described in section 2.14. The cells were inoculated with 1.1 X 10^7 IU S6b LLVP and incubated for 3 hours at 37°C. Huh7.5 cells were grown in DMEM growth medium with 10% FCS, 8.5% LPDS + 0.1% insulin DMEM medium or Williams E medium post-inoculation and the media were sampled at 2 day intervals for a 6-day period. Samples were extracted for HCV RNA and titrated by quantitative RT-PCR. The results are presented in figure 35. There is a general decrease of HCV RNA in Huh7.5 cells that were infected with LLVP.

The experiment was again repeated in primary hepatocytes. 2 x 10^5 cells/cm^2 of primary hepatocytes were seeded on 6-well-collagen I coated-plates in Williams E medium supplemented with 10% FCS, Pen/Strep, Fungizone, insulin 10^{-8} M and incubated at 37°C in a humidified incubator at 5% CO_2 overnight as described in section 2.4. The primary hepatocytes were cultured for another day in two types of medium – Williams E growth medium with 10% FCS or 8.5% LPDS + 0.1% insulin MEM medium with or without growth factor added to the medium. Williams E medium and growth factors were used because primary hepatocytes grow best in them. However, hepatocytes grown in LPDS +insulin needed to be tested as well to upregulate LDLr which was important for binding of LLVP. The cells were inoculated with 3 X 10^6 IU S6b LLVP and incubated for 3 hours at 37°C and the media were sampled at 2 day intervals for a 6-day period. Samples were extracted for HCV RNA and titrated by quantitative RT-PCR. The results are presented in figure

36. There is a general decrease of HCV RNA in the primary hepatocytes that were infected with LLVP. However, the decline of HCV RNA titres was less rapid in Williams E medium whereas the decline was most rapid in LPDS plus insulin plus growth factor.

Figure 37 compares the LLVP inoculation of the three kinds of cells; HepG2, primary hepatocytes and Huh7.5 after LPDS plus insulin treatment. All three kinds of cells showed a similar trend of declining HCV titres over the six/seven days after initial exposure providing no evidence of virus replication over this period. Huh7.5 cells show the highest percentage (8.5%) of binding to LLVP at day 0 whereas primary hepatocytes and HepG2 cells show similar binding percentage of 3.8% and 4.6% respectively.

The results above fail to demonstrate any increase in HCV RNA post-inoculation of two hepatoma cell lines, HepG2 and Huh7.5 or primary hepatocytes with S6b liver LLVP.

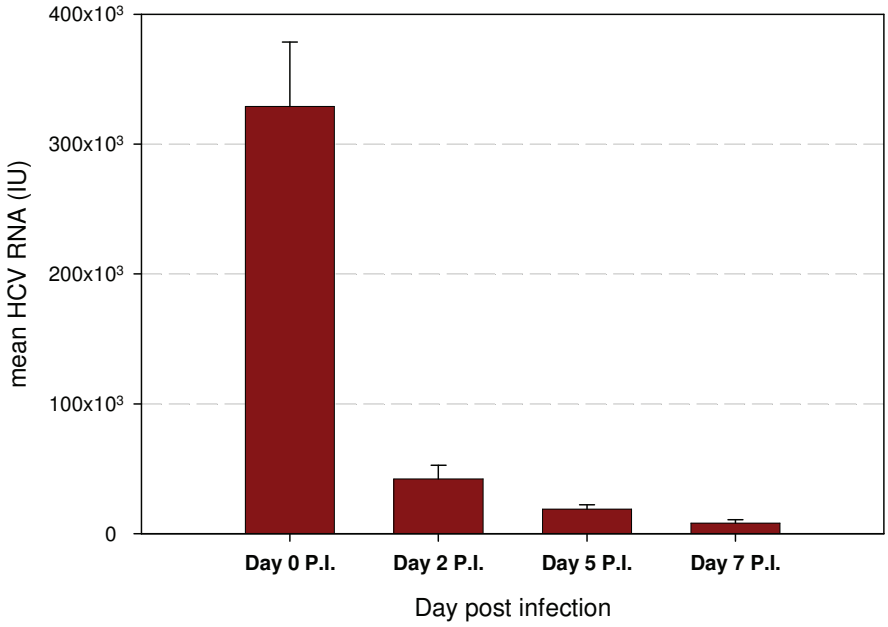


Figure 34: Mean HCV RNA from LLVP inoculated HepG2 cells harvested on day 0, 2, 5 and 7 post-infection.

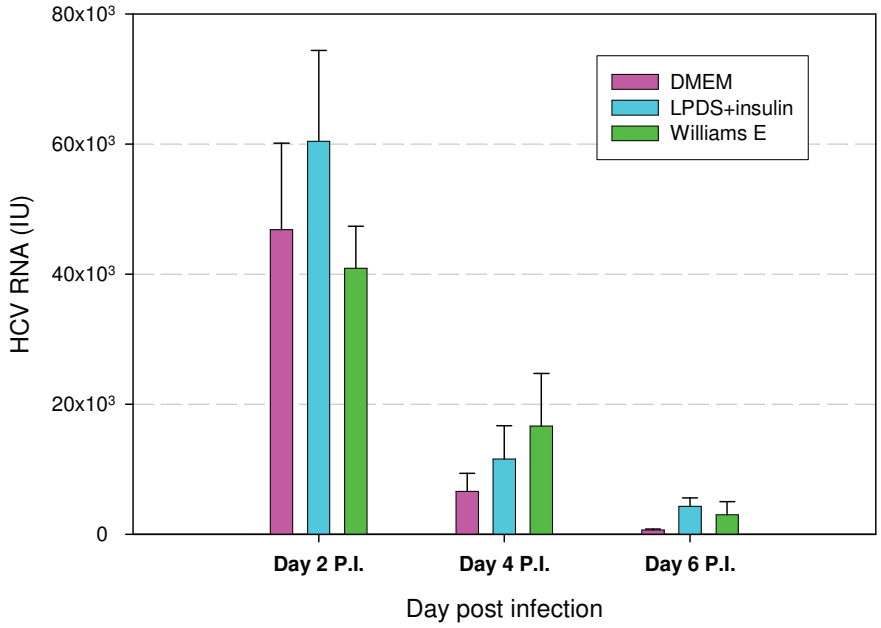


Figure 35: The amount of HCV RNA in LLVP-inoculated Huh 7.5 cells cultured in DMEM, LPDS + insulin, or Williams E medium.
The Huh7.5 cells were exposed to $3X10^6$ IU LLVP for three hours before being washed off with PBS and cultured in the respective media. The cells were harvested on day 2, 4 and 6 post-infection. The amount of HCV RNA was quantitated for 20µg total RNA using real time PCR.

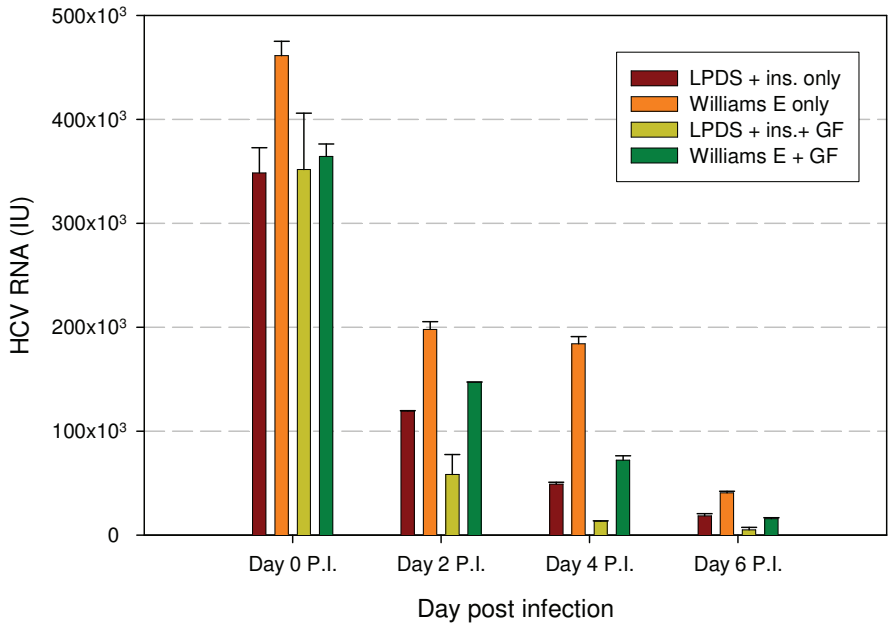


Figure 36: The amount of HCV RNA in LLVP-inoculated primary hepatocytes cultured in LPDS + insulin only, Williams E only, LPDS + insulin + GF, and Williams E + GF.
The primary hepatocytes were exposed to $3X10^6$ IU LLVP for three hours before being washed off with PBS and cultured in the respective media. The cells were harvested on day 0, 2, 4 and 6 post-infection. The amount of HCV RNA was quantitated for 20µg total RNA using real time PCR.

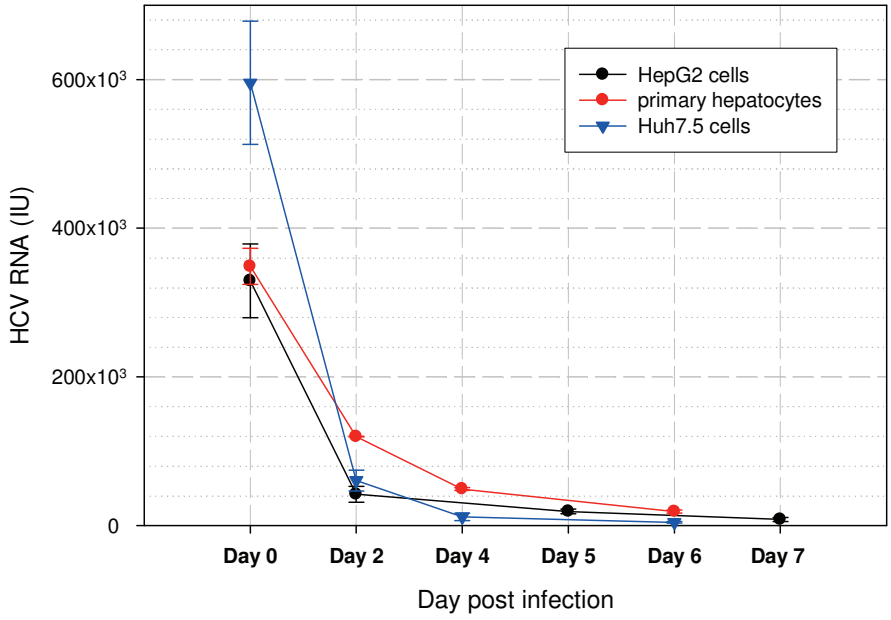


Figure 37: A summary of infectivity assays of LLVP in human HepG2 and Huh7.5 cell lines and in primary hepatocytes on different days post-inoculation.
The cells were treated with LPDS + insulin in EMEM, DMEM and MEM medium respectively.

4.2 The J6/JFH1 system

Robust HCV replication in Huh7.5 cells have been demonstrated by Wakita (Wakita et al., 2005) following transfection of the JFH1 virus, genotype 2a strain in Huh7 cell line. Lindenbach (Lindenbach et al., 2005) have demonstrated more extensive replication of a chimeric virus J6/JFH (genotypes 2a) in Huh7.5 cells to produce infectious viral particles *in vitro*. Both Huh7 and Huh7.5 cells have not been shown to secrete normal host lipoproteins and the involvement of host lipoprotein production pathways in the assembly and release of virus in these systems is unclear. It has been suggested that feeding liver cell lines with fatty acids may stimulate VLDL secretion. We, therefore, decided to attempt to generate infectious LVP from the J6/JFH1 system by feeding the infected cells fatty acid in order to boost host lipoprotein production.

4.3 Production of HCVcc from J6/JFH1 RNA in Huh7.5 cell line

Wild-type pFL-J6/JFH1 DNA, and also mutants pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND DNA were kindly provided by Prof. Charlie Rice from Rockefeller University in the form of plasmid DNA absorbed to a filter paper. The DNA was eluted out from a filter paper in 50µl of TE buffer pH 7.4 and incubated for 30min at room temperature as described in section 2.32.

The eluted DNA was transformed into competent E. coli TG1 bacteria, which was prepared according to the method described in section 2.34, using heat shocked method by placing the DNA in a 42^{o}C waterbath for 90seconds and incubating on ice for 2 minutes. 800µl of the complete SOB medium was then pipetted to each tube of DNA and incubated for 30 minutes in a 37^{o}C waterbath. 200µl of the bacterial

solution from each tube was plated onto an LB + Ampicillin agar plate and incubated at 37^{o}C overnight.

Isolated colonies of transformed bacteria were picked and restreaked on LB + Ampicillin agar plate and incubated at 37^{o}C overnight as described in section 2.35. Cloning was repeated twice with positive clones.

pTM1 plasmid was used as a positive control and water was used as a negative control. The following table 11 shows the number of colonies obtained from the transformation.

DNA dilution	No. of colonies			
	J6/JFH1	**H2476L**	**GND**	**pTM1**
1:2.7	TNTC	TNTC	TNTC	TNTC
1:8	TNTC	TNTC	TNTC	TNTC
1:10	TNTC	TNTC	TNTC	TNTC
1:32	TNTC	TNTC	TNTC	TNTC
1:128	704	1,112	936	TNTC
1:512	29	37	105	TNTC
1:2048	5	1	10	3,296
1:8192	0	0	0	374
1:32768	0	0	1	14
1:131072	0	0	0	0
0*	0	0	0	0
0*	0	0	0	0

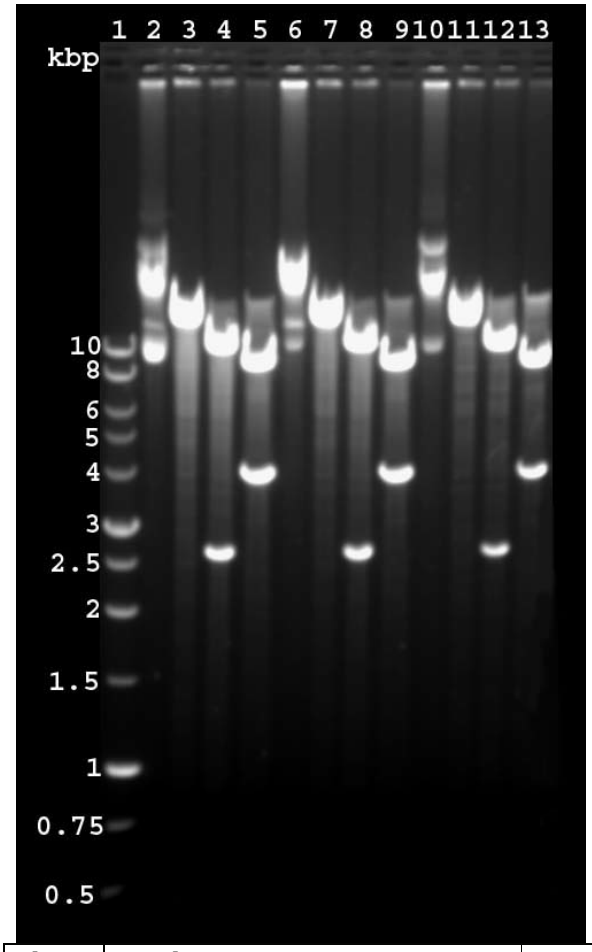
Table 11: A table showing the number of the colonies counted from each transformation experiment.
Different dilutions of pFL-J6/JFH1, pFL-J6/JFH1-H2476L, and pFL-J6/JFH1-GND DNA were used.
TNTC – too numerous to count
* distilled water

Four different colonies from each transformation were picked out, cultured in 5ml volume, DNA purified using QIA Miniprep kit, and size-confirmed using restriction digests (data not shown). Two colonies from each of the initial transformed colonies

giving the right-sized bands were re-cloned, miniprep done, and had the size confirmed again by restriction digests. Eventually one colony of each transformation known as clone 1a (pFL-J6/JFH1), 6a1 (pFL-H2476L), and 10a (pFL-GND) that had been re-cloned was cultured in 500ml volume, DNA was purified using QIA Maxiprep kit (Qiagen), and size-confirmed using restriction digests.

4.4 Restriction digests of pFL-J6/JFH1, pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND plasmids

It is expected that EcoRI will cut the three plasmids only once (figure 14) to produce a linear plasmid of 12,372bp. EcoRI digestion of miniprep DNA pFL-J6/JFH1 (clone 1a), pFL-J6/JFH1-H2476L (clone 6a1), and pFL-J6/JFH1-GND (clone 10a) produced a single band running just above the largest mwm 10,000bp - consistent with this. Digestion with two enzymes EcoRI and XbaI should generate 2 bands of about 10kbp and 2.5kbp. In line with this, digestion of pFL-J6/JFH1, pFL-J6/JFH1-H2476L, and pFL-J6/JFH1-GND with these two enzymes produced a band of ~10kbp and a smaller band running just above the 2.5kbp mwm. Enzymes KpnI and XbaI would be expected to generate 2 bands of about 8kbp and 4kbp. Consistent with this, digestion of the three plasmids produced a large band running just above the 8kbp marker and a second running with the 4kbp marker. However, there were some concerns that because there are only a single base change in the two mutants and the wild-type, there will be a possibility of mix-up that cannot be detected from the restriction digest experiments. Hence, it was important that we checked for these in the clones we had made by sequencing partially the area of the expected mutations.



lane	sample	enzyme
1	1 kbp molecular weight marker	
2	pFL-J6/JFH1 (clone 1a)	no enzyme
3	pFL-J6/JFH1 (clone 1a)	EcoR1 (Invitrogen)
4	pFL-J6/JFH1 (clone 1a)	EcoR1 + Xba1 (Invitrogen)
5	pFL-J6/JFH1 (clone 1a)	Kpn1 + Xba1 (Invitrogen)
6	pFL-J6/JFH1-H2476L (clone 6a1)	no enzyme
7	pFL-J6/JFH1-H2476L (clone 6a1)	EcoR1 (Invitrogen)
8	pFL-J6/JFH1-H2476L (clone 6a1)	EcoR1 + Xba1 (Invitrogen)
9	pFL-J6/JFH1-H2476L (clone 6a1)	Kpn1 + Xba1 (Invitrogen)
10	pFL-J6/JFH1-GND (clone 10a)	no enzyme
11	pFL-J6/JFH1-GND (clone 10a)	EcoR1 (Invitrogen)
12	pFL-J6/JFH1-GND (clone 10a)	EcoR1 + Xba1 (Invitrogen)
13	pFL-J6/JFH1-GND (clone 10a)	Kpn1 + Xba1 (Invitrogen)

Figure 38: A 0.7% TBE agarose gel showing EcoR1, EcoR1 + Xba1, and Kpn1 + Xba1digestions of maxi-prep pFL-J6/JFH1, pFL-J6/JFH1-H2476L, and pFL-J6/JFH1-GND DNA.

4.5 Partial sequencing of pFL-J6/JFH1, pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND plasmids

Partial sequencing was done to confirm the base changes of the mutants compared to that of the wild-type. There are two mutants designed for positive and negative control of J6/JFH1 in the literature; FL-J6/JFH1-H2476L and FL-J6/JFH1-GND respectively. FL-J6/JFH1-H2476L has a point mutation at the 7767th nucleotide (base change) (2476 amino acid; H to L in the NS5B protein). This mutation allows an increase in the efficiency of colony formation compared to the original J6/JFH1. FL-J6/JFH1-GND has a point mutation in the GDD motif of the NS5B protein at the 8618th nucleotide (base change) (2760 aminoacid; D to N). This mutant abolishes the RNA polymerases activity of NS5B. The mutation sites of the two mutants are quite far apart on the NS5B. The primers SI-1 and SI-3 (forward plus strands) and SI-2 and SI-4 (reverse complimentary strands) described in detail in section 2.26 were designed to confirm that the clones were transformed with the correct plasmids.

Reverse complimentary primers SI-5 and SI-6 were designed to allow sequencing from the 5' NTR proximal end of the HCV genome back into the pFL plasmid sequences across the T7 promoter to demonstrate both the correct orientation of the plasmid and the correct alignment of T7 promoter. Sequencing with these primers was carried out as described in section 2.44. Alignment of sequences was done using the megalign (clustal W method) of dnastar software.

2µg of maxiprep pFL-J6/JFH1, pFL-J6/JFH1-H2476L or pFL-J6/JFH1-GND DNA in a 10µl volume each together with 2µM primer pair (SI-1 and SI-2, SI-3 and SI-4 or SI-5 and SI-6 primer pair) in a 5µl volume/primer were sent to Pinnacle in Newcastle University to be sequenced as described in section 2.44. The sequences obtained

were aligned with the published HCV-J6 and HCV-JFH1 sequences as described in section 2.44. Alignment of sequences was done using the megalign (clustal V or W method) of dnastar software. Table 12 below shows the sequencing result.

The alignment of J6/JFH1 **clone 1a (wild-type)** sequence showed a perfect match with the J6/JFH1 wild-type sequence using SI-1, SI-2, SI-3, and SI-4 primers.

The alignment of J6/JFH1 **clone 6a1 (H2476L mutant)** sequence showed perfect match with the J6/JFH1 wild-type sequence except for A to T nucleotide change at the 7767th nucleotide present in both SI-3 and SI-4 sequences.

The alignment of J6/JFH1 **clone 10a (GND mutant)** sequence showed perfect match with the J6/JFH1 wild-type sequence from except for G to A nucleotide change at the 8618th and 8620th nucleotide present in sequences generated by SI-1 and SI-2 primers respectively.

Primer	Location	Plasmid DNA	Readable sequence generated	
Forward **SI-1**	8523-8542	pFL-J6/JFH1	8576-9128	
		pFL-J6/JFH1-H2476L	8585-9134	
		pFL-J6/JFH1-GND	8583-9126**	
Reverse **SI-2**	8734-8754	pFL-J6/JFH1	8199-8697	
		pFL-J6/JFH1-H2476L	8192-8699	
		pFL-J6/JFH1-GND	8192-8701***	
Forward **SI-3**	7659-7678	pFL-J6/JFH1	7710-8222	
		pFL-J6/JFH1-H2476L	7717-8267*	
		pFL-J6/JFH1-GND	7711-8229	
Reverse **SI-4**	7874-7893	pFL-J6/JFH1	7378-7836	
		pFL-J6/JFH1-H2476L	7513-7809*	
		pFL-J6/JFH1-GND	7513-7828	

Table 12: A table of the location of readable sequence generated using SI-1, SI-2, SI-3, and SI-4 primers.
* matched except for A to T nucleotide change at the 7767th nucleotide
** matched except for G to A nucleotide change at the 8618th nucleotide
** matched except for G to A nucleotide change at the 8620th nucleotide

The alignment of J6/JFH1 clone 1a (wild-type), clone 6a1 (H2476L mutant), and clone 10a (GND mutant) sequences using SI-6 primer with J6/JFH1 wild-type sequence from the databank and the T7 promoter sequence showed the expected orientation of the plasmid through the beginning of the J6/JFH1 genome and also the T7 promoter region. The SI-5 primer, however, was too close to the J6/JFH1 start sequence and did not read the T7 promoter region and the J6/JFH1 start sequence.

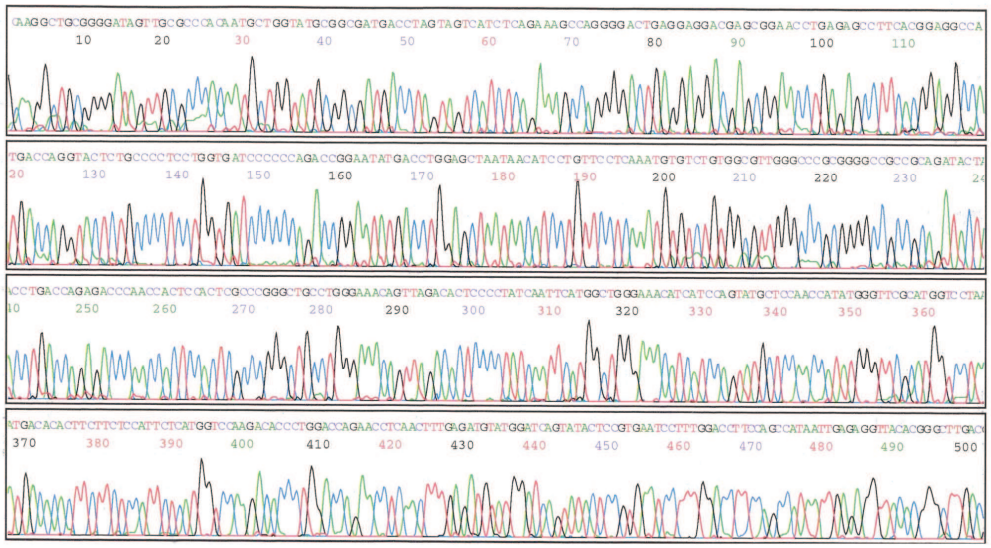
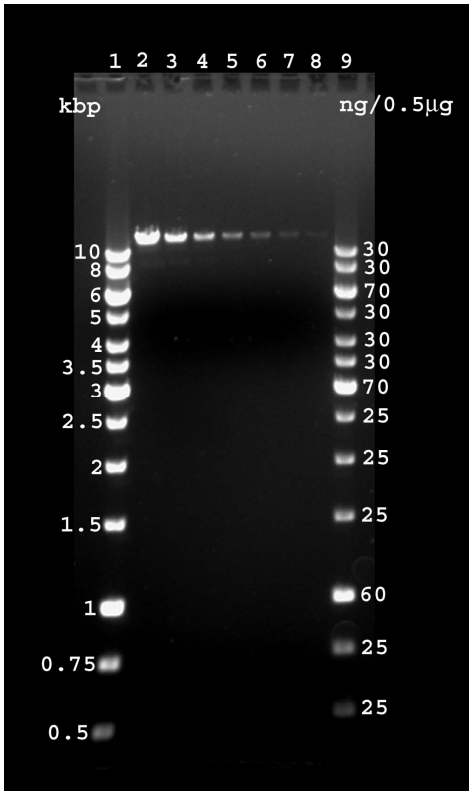


Figure 39: J6/JFH1 clone 1a DNA sequence using the SI-1 primer.
The alignment of J6/JFH1 **clone 1a (wild-type)** sequence using SI-1 primer showed perfect match from 2nd to 554th nucleotide of the sequence which corresponds to the 8576th nucleotide until 9128th nucleotide of the J6/JFH1 sequence from the databank when alignment was made using megalign of the dnastar software.

4.6 Purification of linearized pFL-J6/JFH1 and pFL-J6/JFH1-GND plasmids for in vitro transcription

The XbaI-digested J6/JFH1 (clone 1a) plasmid was purified by running the digested plasmid on 0.7% agarose gel, cutting out the 10kbp band, electro-eluting, and phenol-chloroform extracting / ethanol precipitating (purification 1) as described in section 2.45. The following figure 40 shows the different dilutions of XbaI-digested,

purified J6/JFH1 DNA gave a clean single band of greater than 10kbp and with a concentration of approximately 56µg/ml.

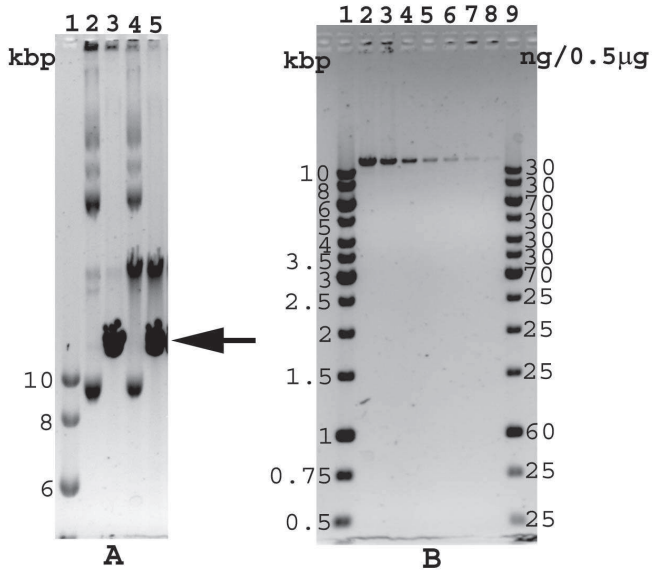


lane	samples	Concentration/Dilution
1	GeneRuler™ 1 kb DNA ladder	1µg/2µl
2	pFL-J6/JFH1 (clone 1a) DNA	1:5
3	pFL-J6/JFH1 (clone 1a) DNA	1:10
4	pFL-J6/JFH1 (clone 1a) DNA	1:20
5	pFL-J6/JFH1 (clone 1a) DNA	1:40
6	pFL-J6/JFH1 (clone 1a) DNA	1:80
7	pFL-J6/JFH1 (clone 1a) DNA	1:160
8	pFL-J6/JFH1 (clone 1a) DNA	1:320
9	GeneRuler™ 1 kb DNA ladder	0.5µg/µl

Figure 40: A serial dilution of XbaI-digested, purified J6/JFH1 DNA run on 0.7% agarose gel. The XbaI-digested J6/JFH1 plasmid was purified by running the digested plasmid on 0.7% TBE agarose gel, cutting out the 10kbp band, electro-eluting, and phenol-chloroform extracted / ethanol precipitated, diluted and re-run on 0.7% TBE agarose gel.

The same XbaI digestion and purification was also done with clone 6a1 (H2476L mutant) and clone 10a (GND mutant). Similar results of one clean band were obtained with clone 6a1 (H2476L mutant) (not shown) but there was a problem with clone 10a (GND mutant) XbaI digestion as the plasmid was only partially successful

(see figure 41A, lane 5 below). To solve this problem, the lower band (arrow) of also greater than 10kbp was excised from the gel, electro-elute, and purified. When re-run on 0.7% TBE agarose gel (see figure 41B below), pFL-J6/JFH1-GND gave a single band of approximately 36µg/ml.



A

lane	sample	enzyme
1	1 kbp molecular weight marker	
2	pFL-J6/JFH1 (clone 1a)	no enzyme
3	pFL-J6/JFH1 (clone 1a)	Xba1 (NEB)
4	pFL-J6/JFH1-GND (clone 10a)	no enzyme
5	pFL-J6/JFH1-GND (clone 10a)	Xba1 (NEB)

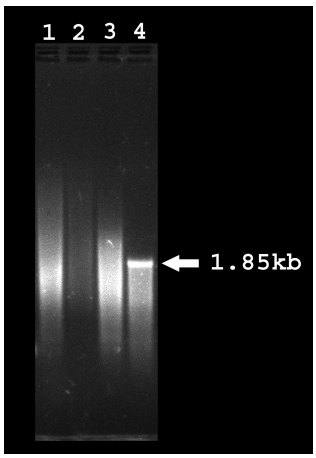
B

lane	samples	Concentration/Dilution
1	GeneRuler™ 1 kb DNA ladder	1µg/2µl
2	pFL-J6/JFH1-GND (clone 10a) DNA	1:5
3	pFL-J6/JFH1-GND (clone 10a) DNA	1:10
4	pFL-J6/JFH1-GND (clone 10a) DNA	1:20
5	pFL-J6/JFH1-GND (clone 10a) DNA	1:40
6	pFL-J6/JFH1-GND (clone 10a) DNA	1:80
7	pFL-J6/JFH1-GND (clone 10a) DNA	1:160
8	pFL-J6/JFH1-GND (clone 10a) DNA	1:320
9	GeneRuler™ 1 kb DNA ladder	0.5µg/µl

Figure 41: A. Comparisons of undigested and XbaI-digested pFL-J6/JFH1 and pFL-J6/JFH1-GND DNA and B. A serial dilution of XbaI-digested and purified J6/JFH1-GND DNA run on 0.7% agarose gel.
XbaI-digested pFL-J6/JFH1-GND DNA (in lane 5) shows two bands (digested and undigested DNA) in figure A. The lower band (shown by arrow in figure A) was excised, electro-eluted, purified and re-run on 0.7% TBE agarose gel (Figure B).

4.7 In vitro transcription of linearized pFL-J6/JFH1 and pFL-J6/JFH1-GND plasmids

In vitro transcription of the purified plasmids of clone 1a (wild-type), clone 6a1 (H2476L mutant), and clone 10a (GND mutant) was carried out as described in section 2.47 to generate positive strand RNA copies of the plasmids. Figure 42 shows the RNAs produced from the *in vitro* transcription of the plasmids linearized and purified as described in section 2.45 and resolved on a glyoxal gel. There was a smear of RNAs around 1.8kb obtained instead of a 9.6kb single band obtained. A different way of preparing a good quality DNA was needed as the RNAs from this experiment could not be use for transfection.



lane	sample
1	J6/JFH1 RNA
2	J6/JFH1-H2476L RNA
3	J6/JFH1-GND RNA
4	pTRI-Xef

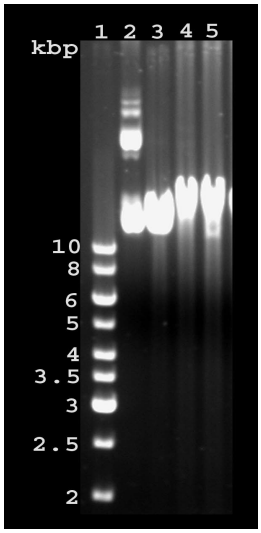
Figure 42: J6/JFH1, J6/JFH1-H2476L, and J6/JFH1-GND RNA transcripts from plasmid DNA purified using purification 1 method.

pTRI-Xef which is 1.85kb in size was used as an *in vitro* transcription positive control and 0.24-9.5kb Invitrogen RNA marker was used as a molecular weight marker (not shown). The RNA was run on a 1% glyoxal agarose gel.

4.8 Purification 2

To solve the problem of partial digestion in pFL-J6/JFH1-GND maxiprep DNA, a double phenol-chloroform purification protocol was carried out with the maxiprep DNA before XbaI restriction digest was done as some kind of protein might have interfered with the digestion. The structure of the plasmid DNA could not be very different from the wild-type pFL-J6/JFH1 DNA as it is unlikely that a point mutation that was far from the XbaI digestion site would make a structural change to the plasmid DNA.

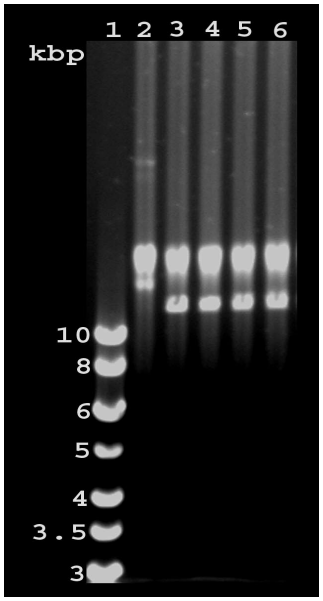
pFL-J6/JFH1-GND maxiprep DNA was pre-purified using double phenol-chloroform extraction as described in section 2.46 without the gel extraction steps and digestion with XbaI was repeated as described in section 2.40. However, pFL-J6/JFH1 did not undergo double phenol-chloroform extraction before the digestion as it could be fully digested without the purification step. The results are presented in figure 43 below. pFL-J6/JFH1 generated one clean band of greater than 10kbp but pFL-J6/JFH1-GND again generated 2 bands both greater than 10kbp. The problem of partial digestion still persisted for pFL-J6/JFH1-GND.



lane	sample
1	GeneRuler^{TM} 1 kb DNA ladder
2	J6/JFH1 undigested
3	J6/JFH1 XbaI (7.5U enzyme / µg DNA)
4	GND undigested
5	GND XbaI (7.5U enzyme / µg DNA)

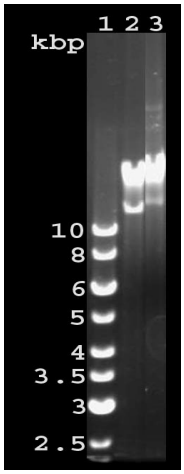
Figure 43: Undigested and XbaI-digested pFL-J6/JFH1 and pFL-J6/JFH1-GND DNA on 0.7% TBE agarose gel.
The pFL-J6/JFH1-GND DNA but not pFL-J6/JFH1 DNA underwent double phenol-chloroform purification before the digestion step (lanes 5 and 3 respectively).

XbaI from 2 different companies (Invitrogen and New England Biologicals) were tested (data not shown). Varying the amount of enzymes used in digestion (figure 44) and increasing the incubation time from 4 hours to 17 hours (figure 45) were also carried out. However, the pFL-J6/JFH1-GND DNA was still partially digested.



lane	sample
1	GeneRuler™ 1 kb DNA ladder
2	GND no enzyme
3	GND XbaI (5U enzyme / µg DNA)
4	GND XbaI (20U enzyme / µg DNA)
5	GND XbaI (50U enzyme / µg DNA)
6	GND XbaI (100U enzyme / µg DNA)

Figure 44: pFL-J6/JFH1-GND DNA digested with varying strength of XbaI and run on 0.7% TBE agarose gel.

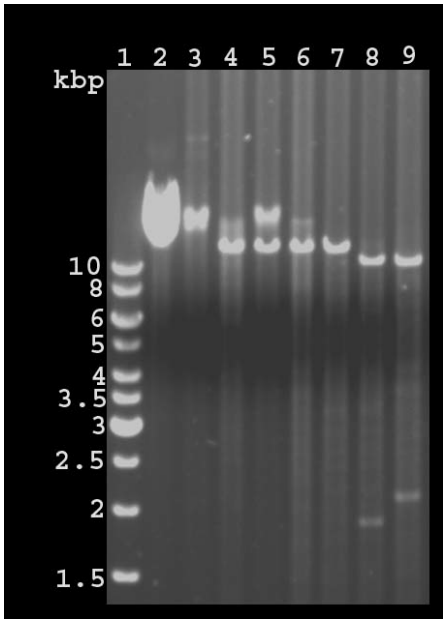


lane	sample
1	GeneRuler™ 1 kb DNA ladder
2	GND XbaI (10U enzyme / µg DNA and incubation time is 17 hours)
3	GND no enzyme

Figure 45: pFL-J6/JFH1-GND DNA digested with XbaI for 17 hours and run on 0.7% TBE agarose gel.

4.8.1 Linearization of pFL-J6/JFH1-GND DNA by double digestion with restriction endonucleases

1.5µl (2µg) maxiprep pFL-J6/JFH1-GND DNA in each reaction was linearized by incubating in 10 units restriction enzymes each: EcoR1, XbaI, XmnI and SspI alone or XmnI+XbaI and SspI+XbaI together as described in material 3 of section 2.39.3 for 4 hours at 37^{o}C. The digests were resolved on a 0.7% TBE agarose gel and the results are presented in figure 46. Whilst XbaI and SspI singly were unable to fully digest the DNA, leaving varying amounts of unlinearized plasmid (lanes 5 and 6 respectively), double digestion with either XmnI+XbaI or SspI+XbaI (lane 8 and 9 respectively) resulted in clean linear DNA at about 10kbp. Even though XmnI (lane 7) gave a clean single band but an XbaI cut is needed as XbaI cuts at the end of the 3'NTR. EcoR1 was included as a single band pFL-J6/JFH1-GND DNA digest control although in this digestion there was an incomplete digestion as well. Using a second restriction enzyme XmnI or SspI that cut in the vector backbone was used to successfully digest pFL-J6/JFH1-GND DNA completely as shown in figure 46.



lane	sample
1	GeneRuler^{TM} 1 kb DNA ladder
2	J6/JFH1 XbaI
3	GND no enzyme
4	GND EcoRI
5	GND XbaI
6	GND SspI
7	GND XmnI
8	GND XmnI + XbaI
9	GND SspI + XbaI

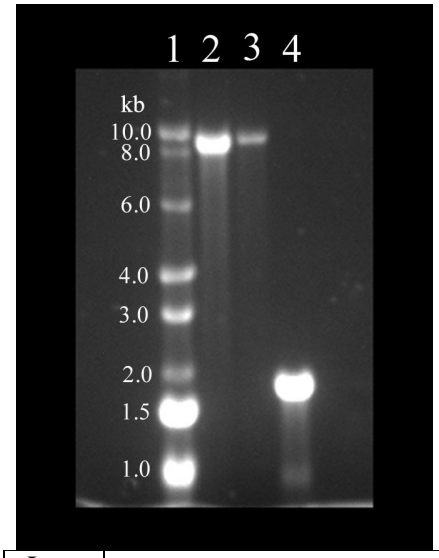
Figure 46: pFL-J6/JFH1-GND DNA digested with restriction endonucleases EcoR1, XbaI, XmnI and SspI alone or XmnI + XbaI or SspI + XbaI together and resolved on a 0.7% TBE agarose gel.

4.9 In vitro transcription using purification 2

Purification 2 method was carried out on a linearized pFL-J6/JFH1 plasmid DNA in an attempt to achieve a successful i*n vitro* transcription. 10.3µl (30µg) maxiprep pFL-J6/JFH1 DNA was linearized by incubating in 150 units of XbaI restriction enzymes as described in material 2 of section 2.39.2 for 4 hours at 37^{o}C. The digested DNA samples were re-digested with 6µl (60U) Mung bean nuclease enzyme at 30^{o}C for 30min to generate blunt-end DNA from the XbaI digest overhang. The sample was treated with 0.2µg/µl Proteinase K and 0.5% SDS at 50^{o}C for 1 hour to digest the proteins. The sample was purified using purification 2 method as described

in detail in section 2.46. *In vitro* transcription was carried out to the 2µg and 1µg of the digested and purified sample as described in section 2.47.

Figure 47 shows different amount of J6/JFH1 RNA on an RNA 1% glyoxal agarose gel showing relatively clean single bands after an *in vitro* transcription of a digested pFL-J6/JFH1 plasmid DNA that was purified using purification 2 method.



Lane		
1	0.5 – 10kb RNA marker (Invitrogen)	
2	J6/JFH1 RNA 1.0 µg	
3	J6/JFH1 RNA 0.3 µg	
4	*In vitro* txn positive control – pTRI-Xef	

Figure 47: Different amount of J6/JFH1 RNA on a 1% glyoxal agarose gel.

4.10 Optimization of in vitro transcription conditions

RNA transcription as described in section 4.9 was carried out for pFL-J6/JFH1 plasmid DNA varying the reaction volumes and incubation times in an attempt to maximise RNA yields. Table 13 shows the different conditions used and the resulting RNA yields. There does not seem to be much difference in RNA yield in condition 1 and condition 2. However, when all the reagents were doubled including doubling the DNA amount but not the incubation time, the RNA yield increased 5 fold. When the

same amount of reagents was used but the time was increased by 17 hours, a 12.6 fold increase was obtained.

DNA amt. at start	Total Reaction vol.	1st T7 amt.	1st incubat. time	2nd T7 amt.	2nd incubat. time	RNA amt.
2µg	20ul	2µl	2hrs	2µl	4hrs	4.78µg
2µg	20ul	2µl	7hrs	-	-	5.46µg
4µg	40ul	4µl	2hrs	4µl	4hrs	23.83µg
2µg	20ul	2µl	2hrs	2µl	22hrs	60.18µg

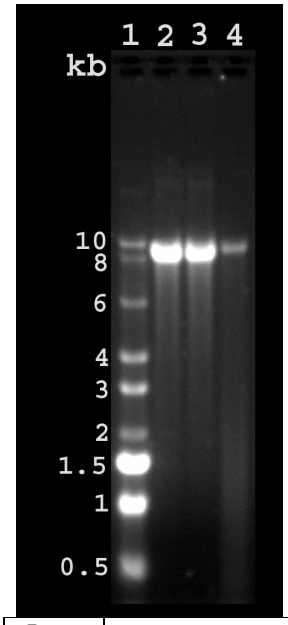
Table 13: *In vitro* transcription optimization of J6/JFH1.
Varying DNA amount, incubation time, T7 polymerase enzyme amount, and reaction volume were tested to maximise RNA yield.

4.11 In vitro transcription of linearized pFL-J6/JFH1, pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND plasmids

In vitro transcription of maxiprep pFL-J6/JFH1, pFL-J6/JFH1-H2476L, and pFL-J6/JFH1-GND DNA as described in section 2.47 was carried out after digestion as described in section 2.40, 2.41, and 2.42 and purification using double phenol-chloroform extraction as described in section 2.46.

Maxiprep of pFL-J6/JFH1 or pFL-J6/JFH1-H2476L (60µg each) and pFL-J6/JFH1-GND DNA (30µg) was digested XbaI (300U) alone and XbaI+XmnI (150U each) respectively. The digested DNA samples were re-digested with Mung bean nuclease enzyme (120U for pFL-J6/JFH1 or pFL-J6/JFH1-H2476L and 60U for pFL-J6/JFH1-GND) at 30°C for 30min. The samples were treated with 0.2µg/µl Proteinase K and 0.5% SDS at 50°C for 1 hour and purified using purification 2 method as described in detail in section 2.46.

Figure 48 shows J6/JFH1 as well as mutants H2476L and GND RNAs all showing relatively clean single RNA bands.



Lane		
1	0.5 – 10kb RNA marker (Invitrogen)	
2	J6/JFH1 RNA	
3	H2476L RNA	
4	GND RNA	

Figure 48: J6/JFH1, J6/JFH1-H2476L and J6/JFH1-GND RNA transcripts from plasmid DNA purified using purification 2 method.
The RNA was run on a 1% glyoxal agarose gel.

4.12 Optimization of electroporation variables

Electroporation optimization was carried out to ensure the best condition for cell survival after electroporating J6/JFH1 and J6/JFH1-GND RNAs into Huh7.5 cells. An increasing number of cells post-electroporation would indicate viability of the cells, an important requisite for the infectivity of the HCVcc virus. To obtain the optimal conditions for the viability of the cells after electroporation, post-electroporation incubation temperature, cuvette size, cell seeding number, and voltage were assessed by stripping the cells with trypsin/versene and counting the cells in each well on a haemacytometer on day 4 and day 7 (with and without sub-culture/passaging the cells on day 4).

4.12.1 Optimization of incubation temperature

The first condition to be optimized is the incubation temperature of Huh7.5 cells directly after electroporation is carried out.

On the day of electroporation, a 60-70% confluent Huh7.5 culture seeded the day before was stripped with EDTA and trypsin, washed and suspended in cold Opti-MEM I medium (Gibco, Paisley, UK) to make 7.5 x 10^6 cells/ml suspension before resuspending them in 400µl cold complete Cytomix buffer (without RNA) and transferring to a 2mm electroporation cuvette. The cells were pulsed immediately at 260V at 960µF. The cells were then either incubated at room temperature for 15min or on ice for 10min before being pipetted into 24ml warm, complete DMEM growth medium and aliquoted 4ml per well in a 6-well-plate and incubated at 37^{o}C, in 5% CO_2 for 24 hours. A more detailed method is described in section 2.50.

The culture medium was replaced with 4ml fresh, warm complete DMEM growth medium and 1) allowed to grow for another 3 days before the cells were stripped with EDTA and trypsin and counted with a haemacytometer or 2) replaced with 4ml fresh, warm complete DMEM growth medium and allowed to grow for another 3 days before being counted again or 3) sub-cultured/passaged on day 4 and allowed to grow for another 3 days before being counted.

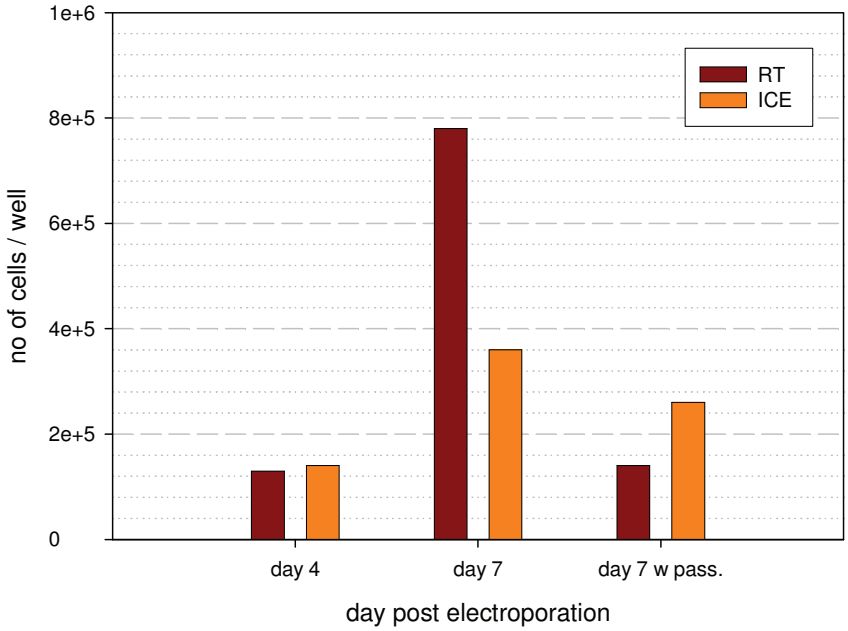


Figure 49: A graph showing the effect of incubation temperature on the viability of Huh7.5 cells post-electroporation.

The results are shown in figure 49. From the graph, the cells fare better achieving 7.8 X 10^5 cells/well compared to 3.6 X 10^5 cells/well at day 7 post-electroporation when incubated at room temperature for 15 minutes before adding warm growth medium to the cells. At day 7 with passage the cells incubated on ice grew marginally better, but only achieved 2.3 X 10^5 cells/well.

4.12.2 Optimization of cuvette size and cell seeding numbers

The best cell numbers to use was also optimized to enhance post-electroporation survival. Optimization was done in both 2mm and 4mm cuvettes with duplicate samples.

On the day of electroporation, a 60-70% confluent Huh7.5 culture seeded the day before was stripped with EDTA and trypsin, washed and suspended in cold Opti-MEM I medium (Gibco, Paisley, UK) to make 7.5 x 10^6 cells/ml, 2 x 10^6 cells/ml, and 5 x 10^5 cells/ml suspension before resuspending them in 400µl cold complete Cytomix buffer (without RNA) and transferring to a 2mm or 4mm electroporation cuvette. The cells were pulsed immediately at 260V at 960µF. The cells were incubated at room temperature for 15min before being pipetted into 24ml warm, complete DMEM growth medium and aliquoted 4ml per well in a 6-well-plate and incubated at 37^{o}C, in 5% CO_2 for 24 hours. A more detailed method is described in section 2.50.

The culture medium was replaced with 4ml fresh, warm complete DMEM growth medium and 1) allowed to grow for another 3 days before the cells were stripped with EDTA and trypsin and counted with a haemacytometer or 2) replaced with 4ml fresh, warm complete DMEM growth medium and allowed to grow for another 3 days before being counted again or 3) sub-cultured/passaged on day 4 and allowed to grow for another 3 days before being counted.

The results are shown in figures 50 and 51. For both cuvettes, the best cell numbers to use is 7.5 X 10^6 cells per cuvette or per sample. Using a 4mm cuvette will yield a higher number of cells surviving; 1.83 X 10^6 cells/well at day 7 compared to that of 2mm cuvette; 1.35 X 10^6 cells/well. This is also true when cells were sub-cultured at day 4 and allowed to grow until day 7 before cell count was made as there were 3.28 X 10^6 cells/ well surviving at day 7 when 4mm cuvette was used as opposed to 1.45 X 10^6 cells/well when 2mm cuvette was used for electroporation.

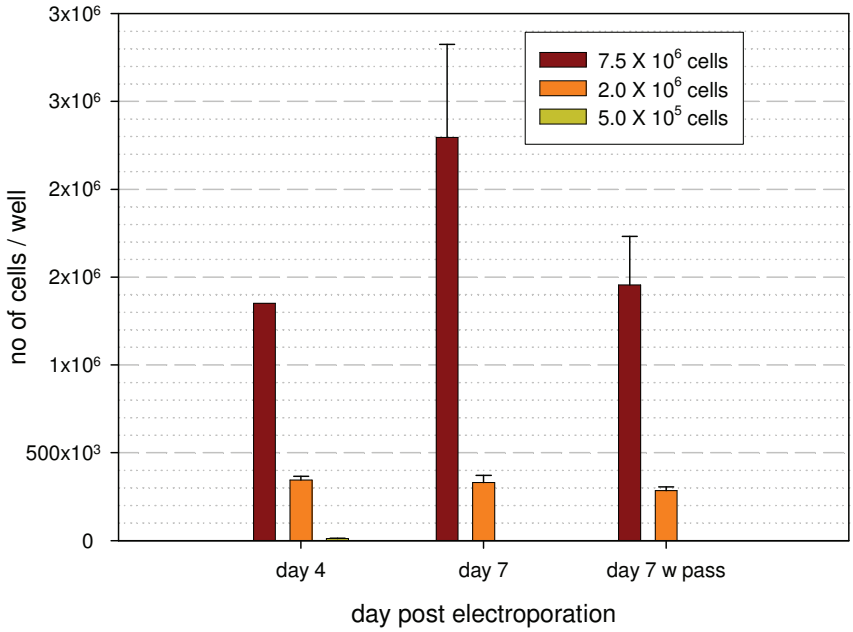


Figure 50: A graph showing the effect of 2mm cuvette and cell seeding numbers on the viability of Huh7.5 cells post-electroporation.

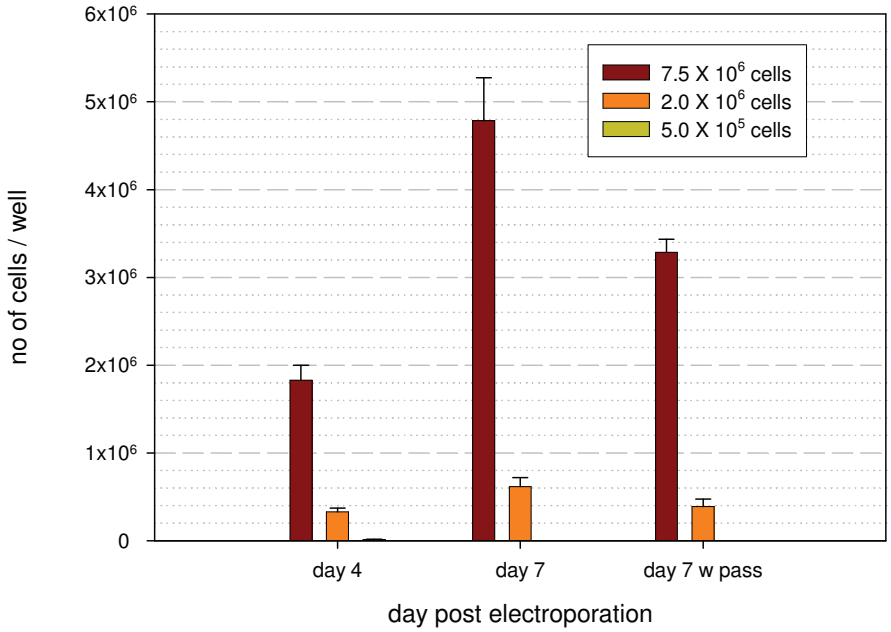


Figure 51: A graph showing the effect of 4mm cuvette and cell seeding numbers on the viability of Huh7.5 cells post-electroporation.

4.12.3 Optimization of cuvette size and voltage

The voltage used during electroporation was also optimized to minimize a high rate of cell death post-electroporation. The voltages tested were 220 volts, 260 volts, and 300 volts. Optimization was done in both 2mm and 4mm cuvettes with duplicate samples.

On the day of electroporation, a 60-70% confluent Huh7.5 culture seeded the day before was stripped with EDTA and trypsin, washed and suspended in cold Opti-MEM I medium (Gibco, Paisley, UK) to make 7.5 x 10^6 cells/ml suspension before resuspending them in 400µl cold complete Cytomix buffer and transferring to a 2mm or 4mm electroporation cuvette. The cells were pulsed immediately at 300V, 260V, or 220V at 960µF. The cells were incubated at room temperature for 15min before being pipetted into 24ml warm, complete DMEM growth medium and aliquoted 4ml per well in a 6-well-plate and incubated at 37^{o}C, in 5% CO_2 for 24 hours. A more detailed method is described in section 2.50.

The culture medium was replaced with 4ml fresh, warm complete DMEM growth medium and 1) allowed to grow for another 3 days before the cells were stripped with EDTA and trypsin and counted with a haemacytometer or 2) replaced with 4ml fresh, warm complete DMEM growth medium and allowed to grow for another 3 days before being counted again or 3) sub-cultured/passaged on day 4 and allowed to grow for another 3 days before being counted.

The results are shown in figures 52 and 53. The percentage of cell survival calculated is the number of cells per well counted on day 4 or day 7 with or without subculture over the initial number of cells aliquoted per well on the day of the electroporation is shown in table 14. There is no significant difference in the voltages use except for the conditions 220V versus 260V at day 7 with sub-culture for the 2mm cuvette and 220V versus 260V at day 7 for the 4mm cuvette. In general however, at day 4, for both 2mm and 4mm cuvettes, there is a higher percentage of cell survival when 220 volts was used in comparison when 260 volts and 300 volts were used. A similar pattern is seen at day 7 although not so clear-cut when the cells were sub-cultured at day 4 and counted on day 7.

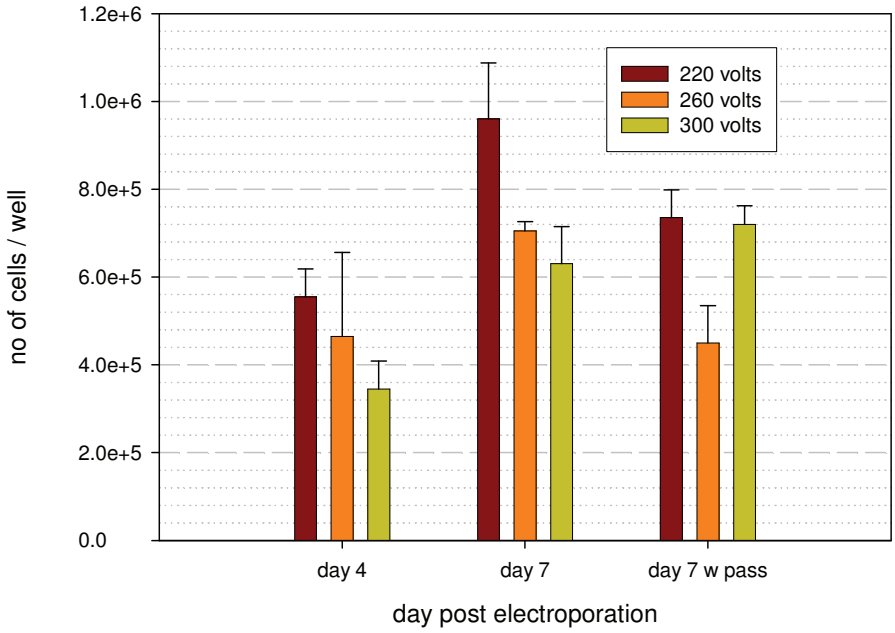


Figure 52: A graph showing the effect of 2mm cuvette and voltage on the viability of Huh7.5 cells post-electroporation.

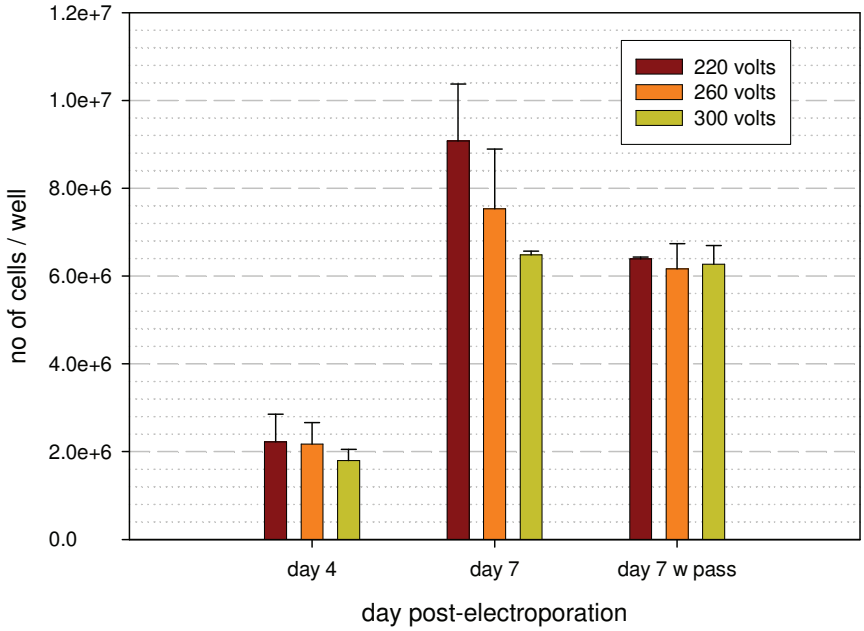


Figure 53: A graph showing the effect of 4mm cuvette and voltage on the viability of Huh7.5 cells post-electroporation.

	2mm cuvette			4mm cuvette		
Voltage (volts)	**Day 4**	**Day 7**	**Day 7 with pass.**	**Day 4**	**Day 7**	**Day 7 with pass.**
220	45	79	60*	178	726**	511
260	38	58	37*	174	602**	493
300	28	52	59	144	518	502

Table 14: Percentage of cell survival post-electroporation in the optimization of cuvette size and voltage.
*significant for $p \leq 0.05$ in paired t-test
**significant for $p \leq 0.05$ in paired t-test

4.12.4 Summary of the electroporation optimization results

From the above optimization experiments, the cell survival rate is best when cells were incubated at room temperature for 15 minutes directly after electroporation, a higher number of cells: 7.5 X 10^6 cells per cuvette were used initially, a lower voltage of 220 volts and a larger width (4mm cuvette) used during the actual electroporation.

4.13 Transfection of J6/JFH1 RNA into Huh7.5 cells

Initially transfection was carried out using a method adapted from the protocol of Kato *et al* (Kato et al., 2006). 10µg of RNA generated from the linearized pFL-J6/JFH1 was transfected into 7.5 X 10^6 Huh7.5 cells per 2mm cuvette using a voltage of 260V according to the protocol described in section 2.51. The electroporated cells were rested for 10min on ice before being pipetted into 20ml warm, complete DMEM growth medium and aliquoted 1ml per well in a 24-well-plate and incubated at 37°C, in 5% CO_2 for 24 hours and followed up to day 10. Figure 54 shows the J6/JFH1 RNA quantification using real time PCR on RNA extracted from the cell supernatants collected each day post-transfection. J6/JFH1 RNA started to increase exponentially after day 6 and reached up to 1.14 X 10^7 IU/mL at day 10.

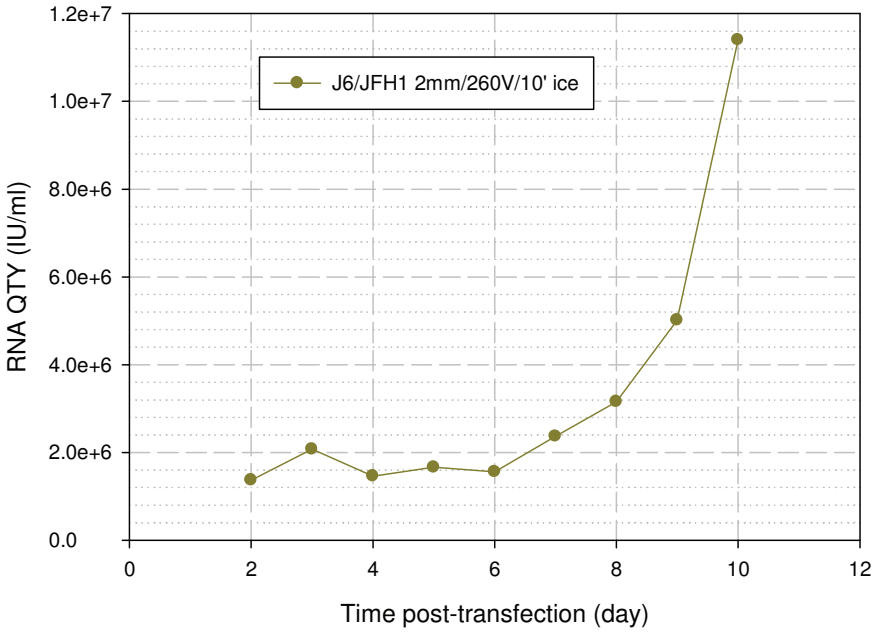


Figure 54: HCVcc RNA output, quantitated by real-time PCR, from the medium after a transfection of J6/JFH1 RNA into Huh7.5 cells.

4.14 Transfection of J6/JFH1 and J6/JFH1-GND RNAs into Huh7.5 cells

This transfection experiment was repeated comparing 2 different conditions. In the first sample, 10µg J6/JFH1 RNA was transfected into 7.5 X 10^6 Huh7.5 cells, electroporation was carried out in a 2mm cuvette with 260 volts current and cells were incubated on ice directly after electroporation. With the second sample, 10µg J6/JFH1 was transfected into 7.5 X 10^6 Huh7.5 cells electroporation was carried out in a 4mm cuvette with 220 volts current and cells incubated at room temperature directly after electroporation. Transfection of 10µg J6/JFH1-GND RNA was also included as a negative control as this mutant does not have the ability to produce HCVcc virus and hence, no increase of HCV RNA was expected. Figure 55 shows the HCVcc RNA quantification using real time PCR on RNA extracted from the cell supernatants collected each day post-transfection and this was carried out until day 15.

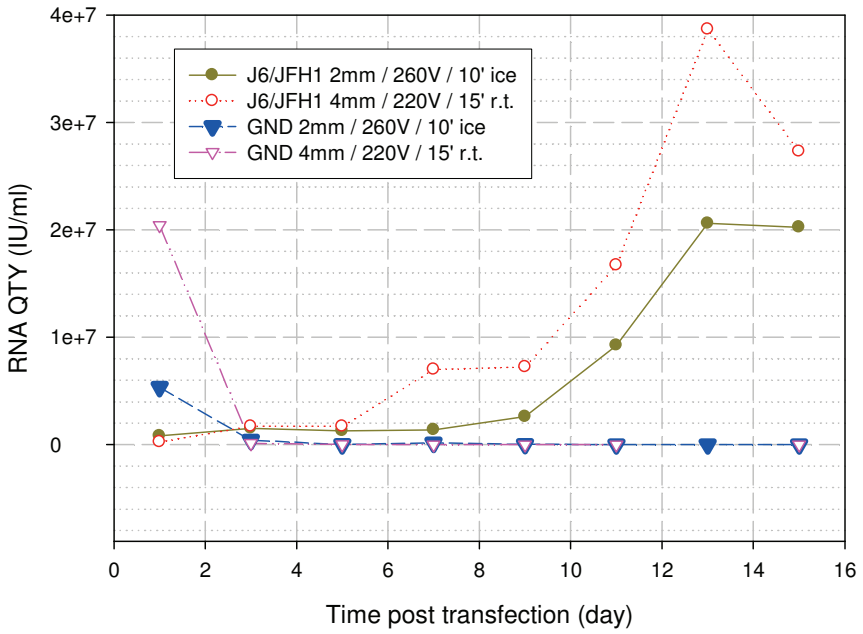


Figure 55: A comparison of HCVcc RNA output from the medium after a transfection of J6/JFH1 and J6/JFH1-GND RNAs into Huh7.5 cells under different electroporation conditions. Two different conditions were tested that is 1)2mm cuvette, 260V current, and 10minute incubation on ice 2) 4mm cuvette, 220V current, and 15minute incubation at room temperature. The RNA was quantitated by real-time PCR.

In this experiment, the rise in RNA output following Kato's condition was somewhat less vigorous by day 8 than in the previous experiment but RNA levels continued to rise to peak at day 13 at 2 X 10^7 IU/ml. Under optimized conditions, an increase in RNA was evident as early as day 7 and the peak at day 13 was almost double that of Kato's conditions.

Transfection with J6/JFH1-GND RNA did not produce any evidence of replication with a decrease and levelling off of HCV RNA after day 1 post-transfection.

4.15 Infection of J6/JFH1 HCVcc in naïve Huh7.5 cells

To confirm that the rise in J6/JFH1 RNA in transfected Huh7.5 cells represents infection with HCVcc, passage of the virus to naïve Huh7.5 cells was attempted. 200µl of frozen, thawed, and centrifuged (1500g) from the medium supernatant of J6/JFH1 RNA-transfected Huh7.5 cells (day 10 post-transfection supernatant of the initial transfection in section 4.16 above) collected was used to infect naïve Huh7.5 cells as described in section infection, propagation and harvest of HCVcc except that the cells were cultured for 5 days. The supernatant was removed, RNA quantification using real time PCR as described in section 2.23 on the extracted RNA from the supernatant was carried out, and cells were scraped to make spots on glass slides as described in section 2.53. When the spots were dried and fixed, they were stained with anti-E2 HCV AP33 mouse monoclonal antibody (1:50 dilution) and anti-core HCV ϕ126 mouse monoclonal antibody (1:1000 dilution). The antibodies were described in section antibodies. Spots of non-infected Huh7.5 cells were also prepared as a negative control.

The result of the RNA output is shown in figure 56. The figure shows there was an increase in the RNA quantity five days after the naïve cells were exposed to the post-transfected supernatant that had been frozen, thawed and centrifuged indicating that J6/JFH1 HCVcc was being produced from the transfection and secreted into the medium which subsequently infected the naïve Huh7.5 cells.

Figure 57 shows a monolayer of dull, reddish non-infected Huh7.5 cells. J6/JFH1 HCVcc-infected Huh7.5 cells probed with anti-E2 HCV AP33 Mab showed foci of intense apple-green immunofluorescence staining against a monolayer of red counter-

stain as shown in figure 58 (an example of a focus). The fluorescence appeared as a nucleo-peripheral ring in an individual cell. Similar results but with greater fluorescence intensity were obtained when J6/JFH1 HCVcc-infected Huh7.5 cells were probed with anti-core HCV ϕ126 antibody in comparison to an anti-E2 HCV AP33 Mab staining as shown in figure 59 although due to technical problem during photography the figures seem to indicate otherwise.

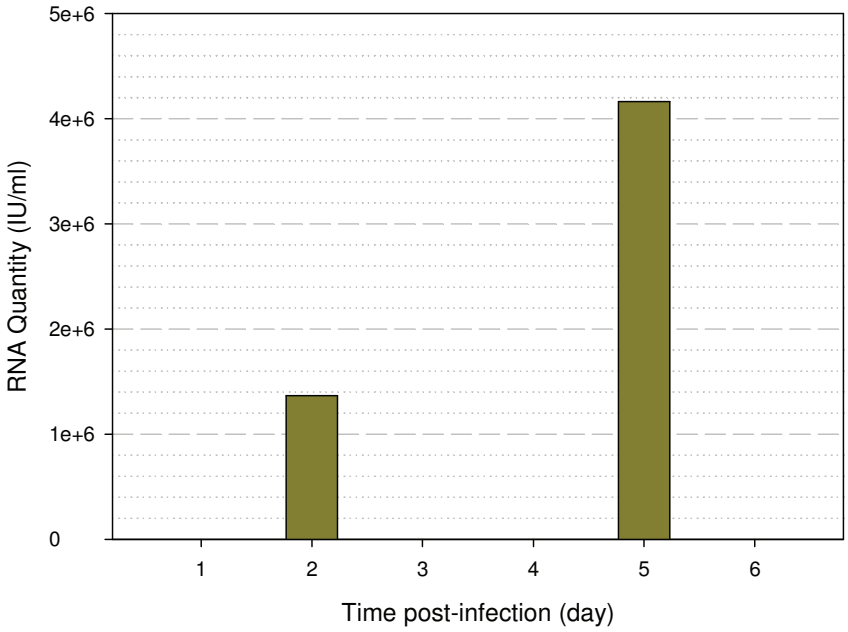


Figure 56: HCVcc RNA output on day 2 and 5 harvested from the medium after an infection of naïve Huh7.5 cells with supernatant of frozen, thawed, and centrifuged (1500g) supernatant from the J6/JFH1 RNA-transfected Huh7.5 cells.
The RNA was quantitated by real-time PCR.

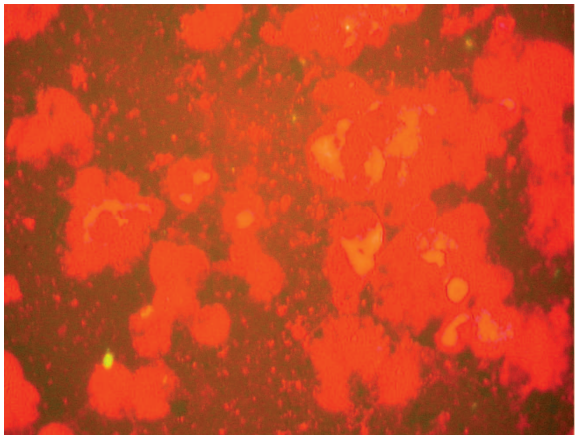


Figure 57: Immunofluorescence assay of non-infected Huh7.5 cells.

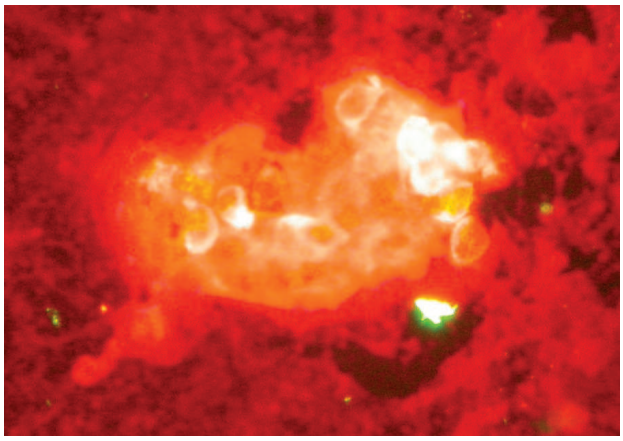


Figure 58: Immunofluorescence assay of J6/JFH1 HCVcc-infected Huh7.5 cells probed with anti-E2 HCV AP33 Mab.
White colours are produced by too much fluorescence causing over-exposure of the image.

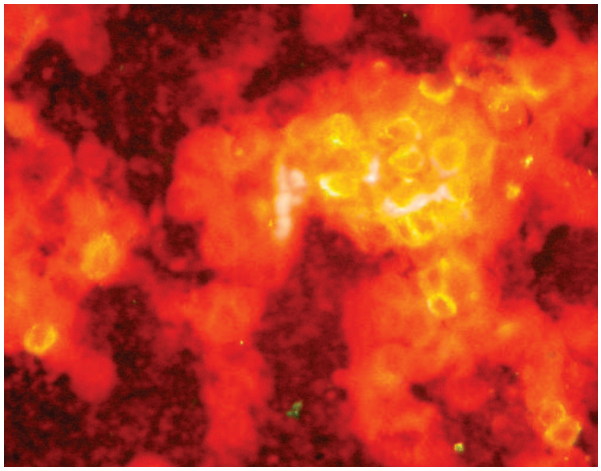


Figure 59: Immunofluorescence assay of J6/JFH1 HCVcc-infected Huh7.5 cells probed with anti-core HCV ϕ126 antibody

We went on to study the growth curve of HCVcc under different conditions such as varying the amount of the FCS and also seeding the cells at different densities to get the optimal growth curve for HCVcc as a high titre of HCVcc is needed for the iodixanol gradient and gel filtration experiments.

4.16 Effect of serum on replication of HCVcc

A growth curve was carried out to compare if the HCVcc grew better in 10% FCS or 3% FCS.

200µl of frozen, thawed, and centrifuged (1500g) from the medium supernatant of J6/JFH1 RNA-infected Huh7.5 cells (kindly provided by Jane McKeating from Birmingham University) topped up with 800µl 3% FCS DMEM growth medium was used to infect naïve Huh7.5 cells seeded at 4 x 10^5 cells/10ml the day before infection in a T_{25} flask and cultured in 10ml of either 3% FCS DMEM growth medium or 10% FCS DMEM growth medium as described in section 2.52 and the cells were cultured for 5 days. The supernatant was removed and RNA quantification using real time PCR as described in section 2.23 on the extracted RNA from the supernatant was carried out.

The results are presented in figure 60. At day 5, the amount of HCV RNA found in the supernatant of 3% FCS growth medium is 1.6 X 10^6 IU/mL which is 94 fold higher compared to 1.7 X 10^4 IU/mL of RNA found in that of 10% FCS growth medium as shown in the following graph.

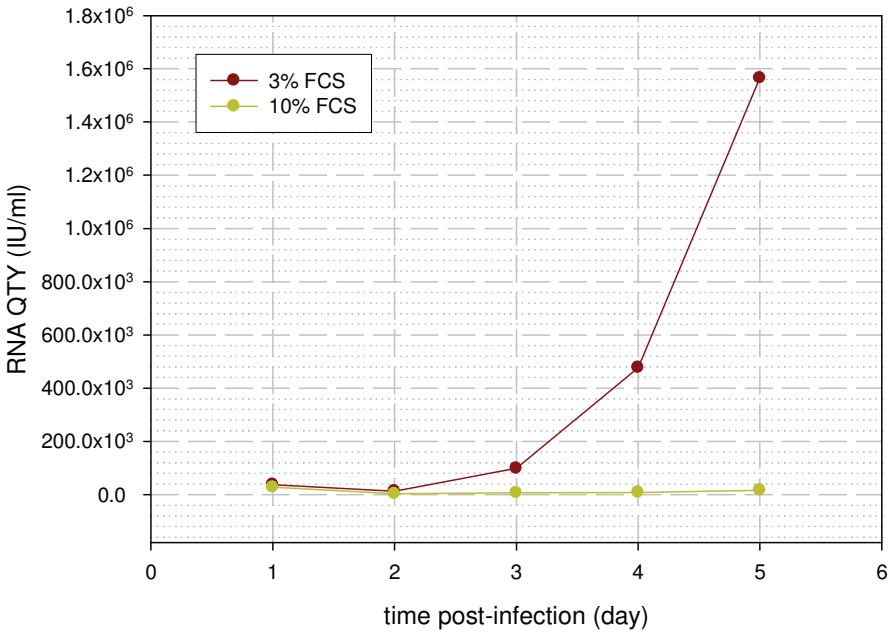


Figure 60: HCVcc RNA outputfrom the medium harvested after a J6/JFH1-RNA infection of naïve Huh7.5 cells cultured in either 3% FCS DMEM growth medium or 10% FCS DMEM growth medium.
The inoculum was a frozen, thawed, and centrifuged (1500g) supernatant from the J6/JFH1 RNA-infected Huh7.5 cells. The RNA was quantitated by real-time PCR.

4.17 Effect of seeding density on replication of HCVcc

Different seeding densities of $2X10^4$ cells/mL, $4X10^4$ cells/mL, and $1X10^5$ cells/mL of Huh7.5 cells were compared to get a sustained high quantity of HCVcc RNA over a long period.

200µl of frozen, thawed, and centrifuged (1500g) from the medium supernatant of J6/JFH1 RNA-infected Huh7.5 cells (pass 4 of the initial transfection experiment) topped up with 800µl 3% FCS DMEM growth medium was used to infect naïve Huh7.5 cells seeded at 2 X 10^4 cells/mL, 4 X 10^4 cells/mL, or 1 X 10^5 cells/mL the day before infection in a T_{25} flask and cultured in 10ml 3% FCS DMEM growth

medium as described in section 2.52 and the cells were cultured for 31 days. The supernatant was removed and RNA quantification using real time PCR as described in section 2.23 on the extracted RNA from the supernatant was carried out.

The results are presented in figure 61. The highest seeding density of 1 X 10^5 cells/mL Huh7.5 cells did not produced the highest amount of RNA at its peak compared to the other 2 seeding densities of 2 X 10^4 cells/mL and 4 X 10^4 cells/mL, nor does it produced the highest sustained HCVcc RNA over a long period of up to 27 days. The lowest seeding density of 2 X 10^4 cells/mL Huh7.5 cells comparatively produced a higher RNA quantity at the peak of its growth curve than that of 1 X 10^5 cells/mL seeding density and it also sustained higher amount of RNA quantity after its peak compared to that of the cells seeded at 4 X 10^4 cells/mL although the peak was highest and came up earliest for the cells seeded at 4 X 10^4 cells/mL.

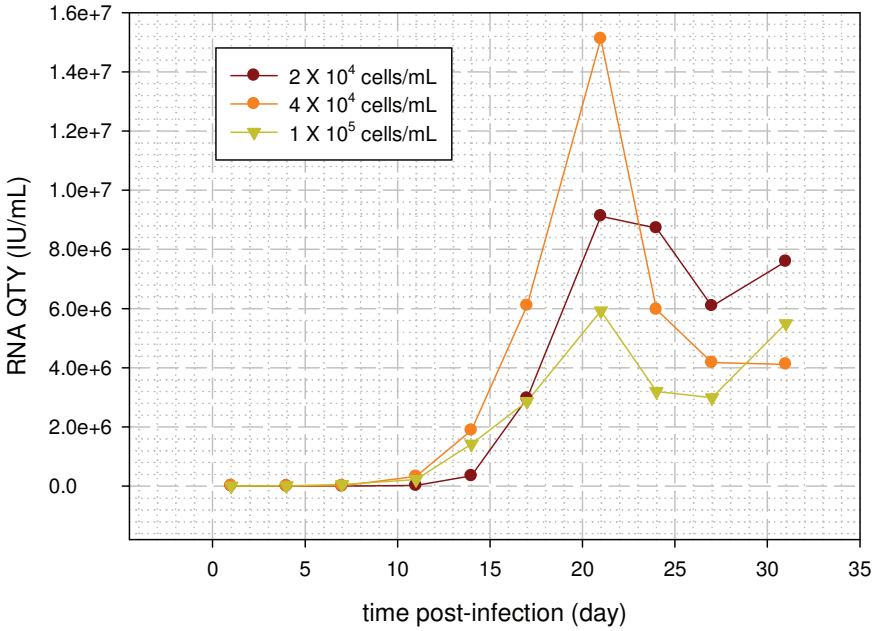


Figure 61: HCVcc RNA output from the medium harvested after a J6/JFH1-RNA infection of naïve Huh7.5 cells seeded at different densities.
The cells were seeded at 2 X 10^4 cells/mL, 4 X 10^4 cells/mL, and 1 X 10^5 cells/mL. The inoculum was a frozen, thawed, and centrifuged (1500g) supernatant from the J6/JFH1 RNA-infected Huh7.5 cells. The RNA was quantitated by real-time PCR.

Chapter 5

5 Results III

5.1 Production of very low density lipoproteins by HepG2 and Huh7.5 cells treated with oleic acid

HCV infection in humans is tied closely to lipid metabolism. There have been reports showing that viral proteins interact with host cell proteins involved in lipid metabolism intracellularly (see section 1.18.1). LVP seem to be hybrid particles in which hepatitis C virions are closely associated with host VLDL (Nielsen et al., 2008). Oleic acid has previously been shown to enhance VLDL secretions by HeG2 cells (Arrol et al., 2000). However, it would be of great interest to study the replication of J6/JFH1 in cells secreting VLDL and so the effect of OA on VLDL secretion was assessed on HepG2, Huh7, and also on Huh7.5 cells of which Huh7 cells and its derivatives are the only cells supportive of robust HCV replication in culture.

5.2 Effect of oleic acid concentration on Huh7.5 cell growth

The following initial experiment was done to study the effect of different concentrations of oleic acid on Huh7.5 cell growth.

$1X10^5$ cells/well Huh7.5 cells were plated in each well of a 6-well-plate and were grown in DMEM growth medium containing 10% FCS for 2 days. The culture medium was replaced with 4ml of 5% FCS DMEM growth medium containing 450µl of 4mM, 2mM, 0.5mM of 10X oleic acid stock solution, or BSA stock solution only for 6 days replacing the medium every 2 days. Duplicate culture was made for each condition mentioned. The cells were monitored by noting the morphology. On day 8, propidium iodide staining was carried out to determine the number of cell death in

FACS analysis. FACS analysis was recorded at time 0, 5, 10, and 20 minutes after propidium iodide was added. A detailed method is described in section 2.16.

From figure 62, the lowest percentage of cell death is when the cells were grown at 0.4mM OA as recorded by FACS at the time lapse of 5minute, 10 minutes and 20minutes.

This indicates that oleic acid is positively beneficial for Huh7.5 cell survival. For studies in which apoB and apoE output is to be assessed by Western blotting, the cells must be grown in serum free medium with oleic acids as there may be cross reaction between human and bovine apolipoproteins. No problems of cellular toxicity were observed when cells were cultured in oleic acid without foetal calf serum.

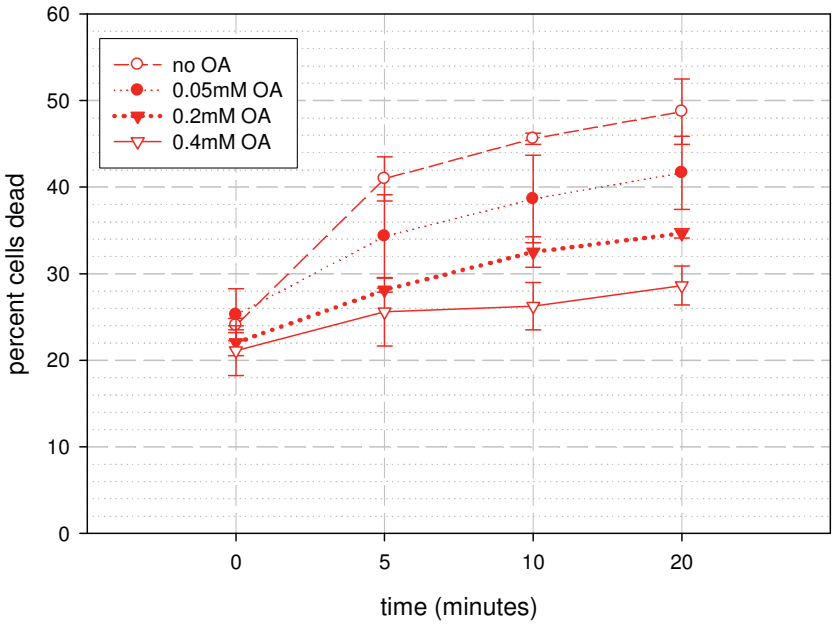


Figure 62: Percentage of cell death analysed using FACS at time 0, 5, 10, and 20 minutes after propidium iodide was added.
Huh7.5 cells were cultured for 6 days in 5% DMEM growth medium containing 0.05mM, 0.2mM, 0.4mM or no oleic acid.

5.3 Western blots on low density lipoproteins from HepG2 and Huh7.5 cells treated with oleic acid

The following experiments were done in an attempt to show production of low and very low density lipoproteins by HepG2 and Huh7.5 cells when treated with oleic acid.

5.3.1 Coomassie staining of low density lipoproteins from HepG2

0.7 X 10^6 cells/well of HepG2 were seeded in 10% FCS EMEM growth medium and incubated for five days at 37^{o}C in 5% CO_2 replacing the medium at one or two days intervals. The medium was then replaced with EMEM serum free medium and BSA with or without oleic acids and incubated for another 2 days. The supernatant was harvested and analysed by potassium bromide sequential ultracentrifugation to separate $\leq$1.006g/ml g/ml and <1.063g/ml fractions which, in serum, contain LDL and VLDL respectively as described in section 2.56. These fractions are concentrated to 140µl volumes using a Vivaspin 2 ml concentrator before delipidation / protein precipitation using methanol / chloroform extraction as detailed in section 2.57. The fractions were then run on SDS-PAGE and the gels were stained with Coomassie blue as described in sections 2.59 and 2.61 respectively.

The results are presented in figure 63 and show a ~549kDa band present in the <1.063g/ml fractions of HepG2 cells treated with BSA plus oleic acids but not BSA alone as indicated by the molecular weight position labelled by the arrow. The band has a similar molecular weight to that of apoB-100 lipoprotein resolved from serum derived LDL. No putative apoB band was detected in the $\leq$1.006g/ml fractions.

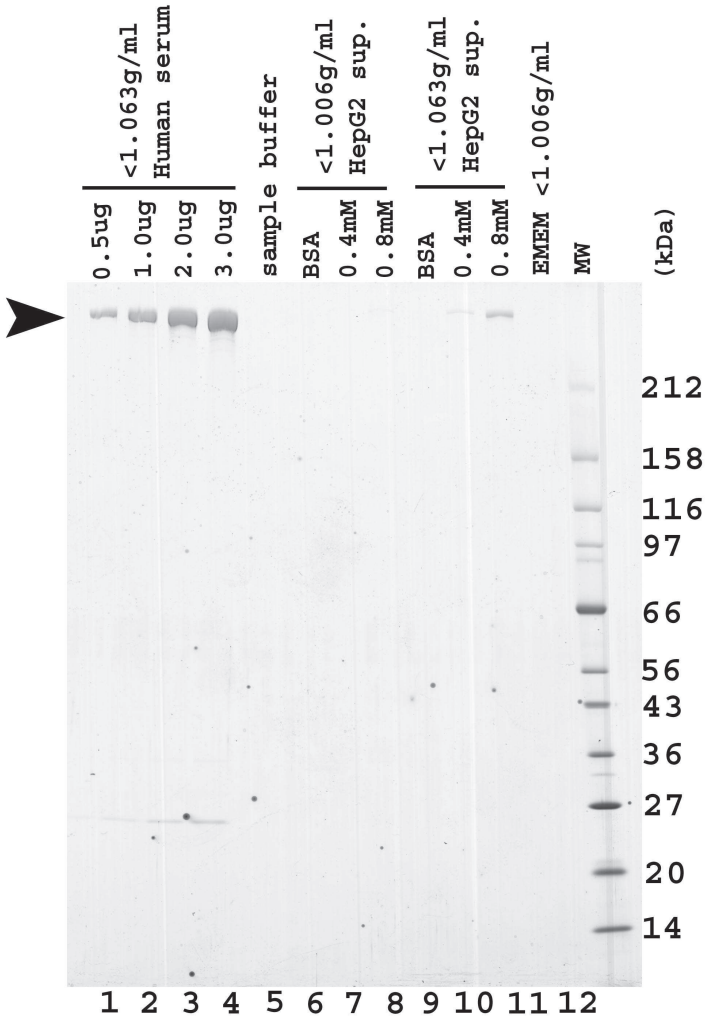


Figure 63: A Coomassie-stained acrylamide gel showing protein bands of 550kDa in size.
The position of 550kDa is indicated by the arrow head. The samples include <1.063g/ml fractions (lanes 1-4) of human serum with known amount of apoB-100 protein, <1.006g/ml fractions (lanes 6-8) and <1.063g/ml fractions (lanes 9-11) of HepG2 cell culture supernatants, and negative control EMEM medium only. HepG2 cells were treated without oleic acid (1.5% BSA) or with oleic acid (0.4mM and 0.8mM concentration).

5.3.2 Western blotting of low density lipoproteins from HepG2

The above experiment (section 5.3.1) was repeated but the gel was not Coomassie-stained but Western blotted (method described in section 2.60) using anti-apoE antibody (DAKO, Ely, UK). For comparison, a Western blot of known <1.006g/ml (VLDL) and <1.063g/ml (LDL) fractions of the human serum was prepared under

similar conditions. The results are presented in figure 64 (lanes 1-4) showing that apoE lipoproteins are found in the VLDL but not LDL fractions of the human serum. The results of the <1.006g/ml (VLDL) and <1.063g/ml (LDL) fractions of HepG2 supernatants are presented in lanes 6-11 and show that apoE lipoprotein is visible in both <1.006g/ml and <1.063g/ml fractions.

The same blot as in figure 64 was stripped (method described in section 2.62) and stained with anti-apoB100 antibody (DAKO, Ely, UK). For comparison, a Western blot of known ≤1.006g/ml (VLDL) and <1.063g/ml (LDL) fractions of the human serum was prepared. The results for the latter are presented in figure 65 (lanes 1-4) showing that apoB lipoproteins are found in both the VLDL and LDL fractions of the human serum although not seen in the lower amount (10ng) VLDL fraction. This could be due to the low amount of VLDL and some proteins might have been lost in the harsh condition of the stripping procedure. The resolved HepG2 supernatant is presented in lanes 6-11 and shows a ~549kDa band in the <1.063g/ml fractions of HepG2 cells treated with BSA plus oleic acids but not BSA alone. The band is not present in ≤1.006g/ml fractions.

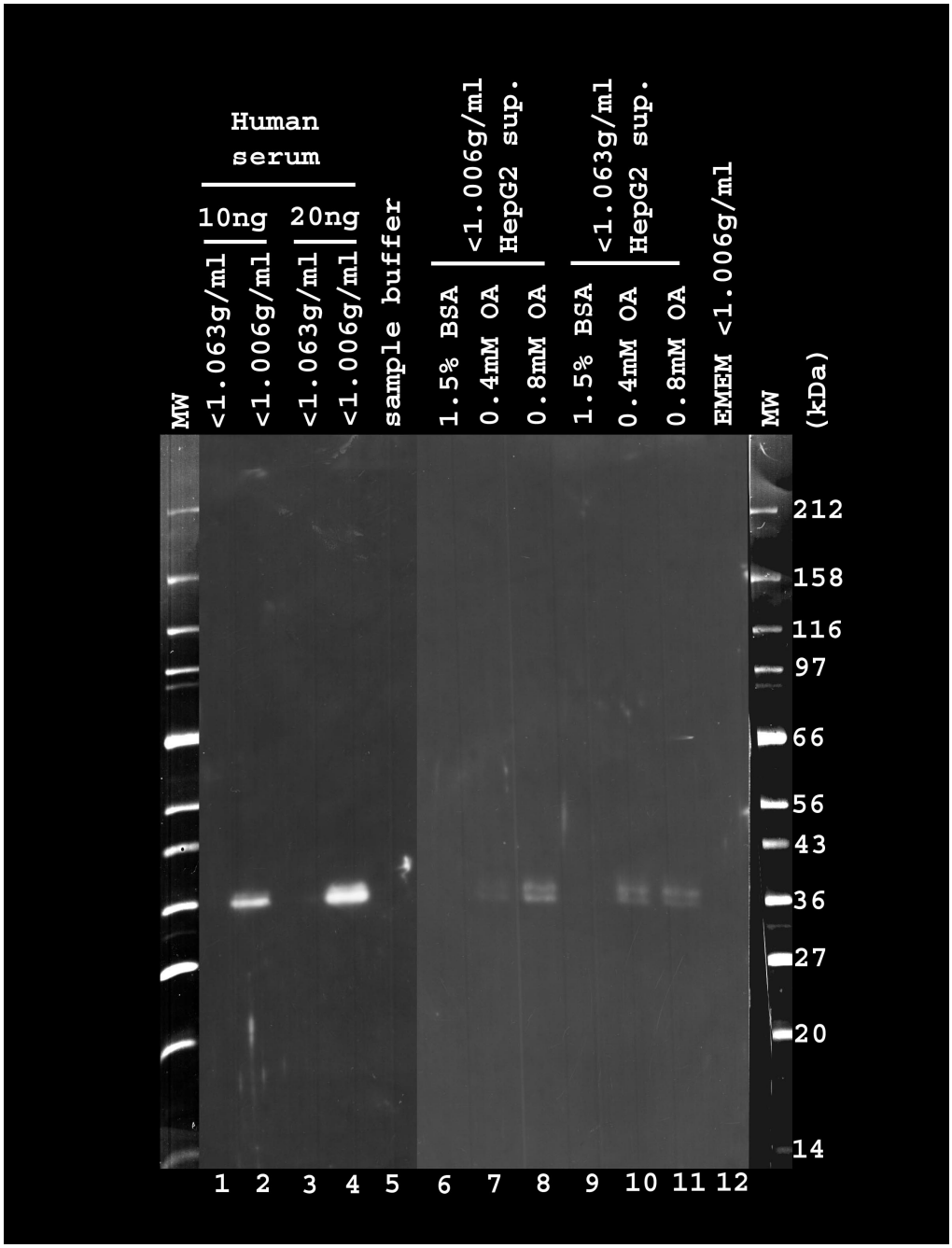


Figure 64: A Western blot of <1.063g/ml (LDL) and ≤1.006g/ml (VLDL) fractions from human serum and HepG2 cell culture supernatants that were purified via potassium bromide sequential ultracentrifugation and probed with anti-apoE antibody.

Lanes 1&3: <1.063g/ml fractions (10ng and 20ng respectively) and lanes 2&4: ≤1.006g/ml fractions (10ng and 20ng respectively). Lane 5: sample buffer only. Lanes 6-8: ≤1.006g/ml fractions and lanes 9-11 : <1.063g/ml fractions. Lanes 6&9: 1.5% BSA treatment, lanes 7&10: 0.4mM OA treatment, lanes 8&11: 0.8mM OA treatment, and lane 12: EMEM medium only.

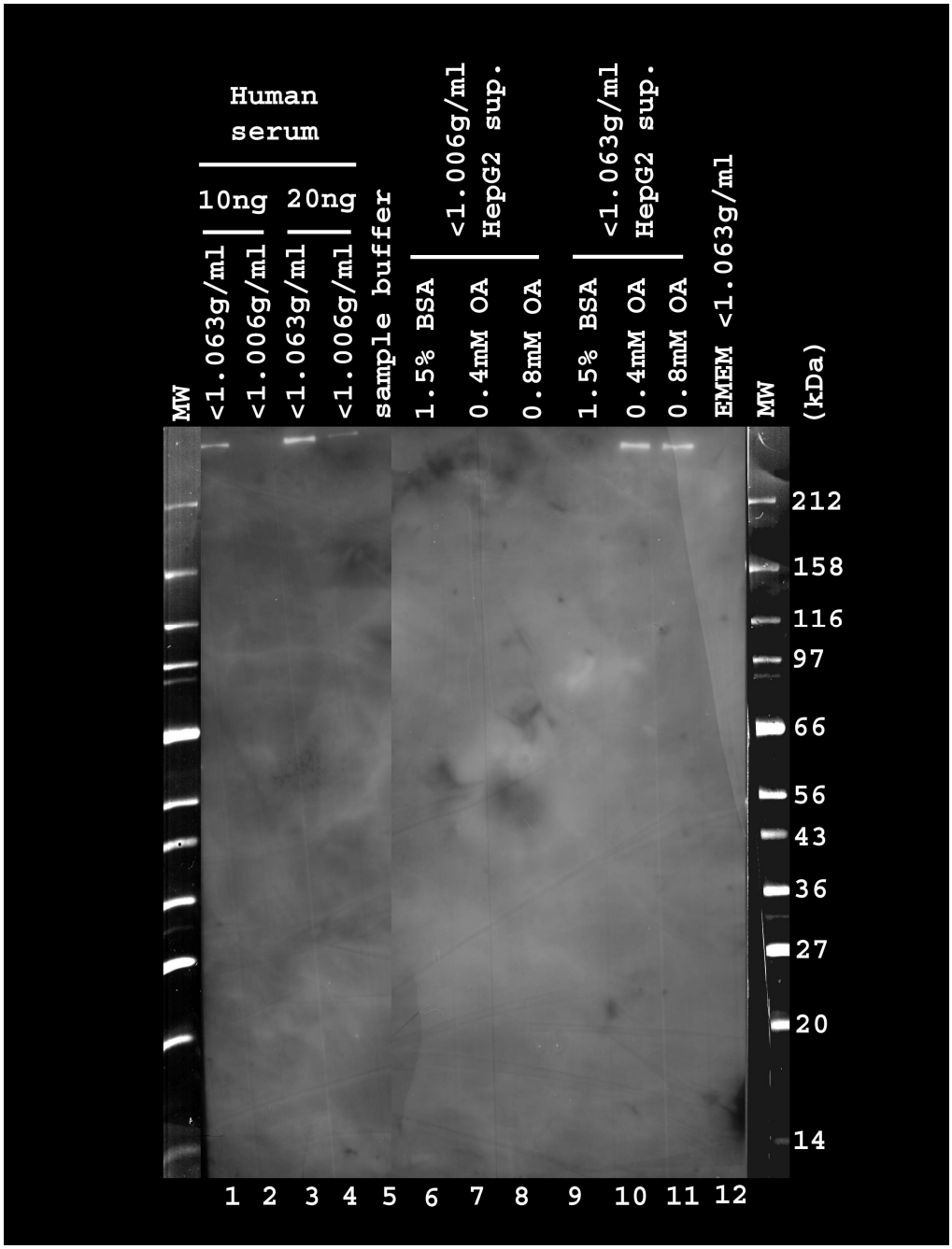


Figure 65: A Western blot of <1.063g/ml (LDL) and ≤1.006g/ml (VLDL) fractions from human serum and HepG2 cell culture supernatants that were purified via potassium bromide sequential ultracentrifugation and probed with anti-apoB100 antibody.
Lanes 1&3: <1.063g/ml fractions (10ng and 20ng respectively) and lanes 2&4: ≤1.006g/ml fractions (10ng and 20ng respectively). Lane 5: sample buffer only. Lanes 6-8: ≤1.006g/ml fractions and lanes 9-11 : <1.063g/ml fractions. Lanes 6&9: 1.5% BSA treatment, lanes 7&10: 0.4mM OA treatment, lanes 8&11: 0.8mM OA treatment, and lane 12: EMEM medium only.

5.3.3 Coomassie staining of low density lipoproteins from Huh7.5

A similar experiment to that in section 5.3.1 was carried out using Huh7.5 cells and the results (Coomassie blue staining) are presented in figure 66 showing a ~549kDa band in both $\leq$1.006g/ml and <1.063g/ml fractions of Huh7.5 cells treated with BSA plus oleic acids but not BSA alone. Also, a ~36kDa band was present in $\leq$1.006g/ml fractions of Huh7.5 cells treated with BSA plus oleic acids but not BSA alone. This corresponds well to the molecular weight of apoE lipoprotein.

The alleged apoB (~550kDa) and apoE (~36kDa) bands of substantial amount from the Coomassie stain were cut out from the gel and sent for matrix assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry. Results from MALDI-TOF analysis of the Coomassie stained gel (figure 66, lane 8) were compared against the most related known proteins from the databank. The ~550kDa band showed a probability score of 196 (scores greater than 64 are significant where $p<0.05$) with human apolipoprotein B-100 precursor and the mass given was 515kDa. The ~36kDa band showed a probability score of 92 (scores greater than 77 are significant where $p<0.05$) with human apolipoprotein E precursor and the mass given was 36kDa.

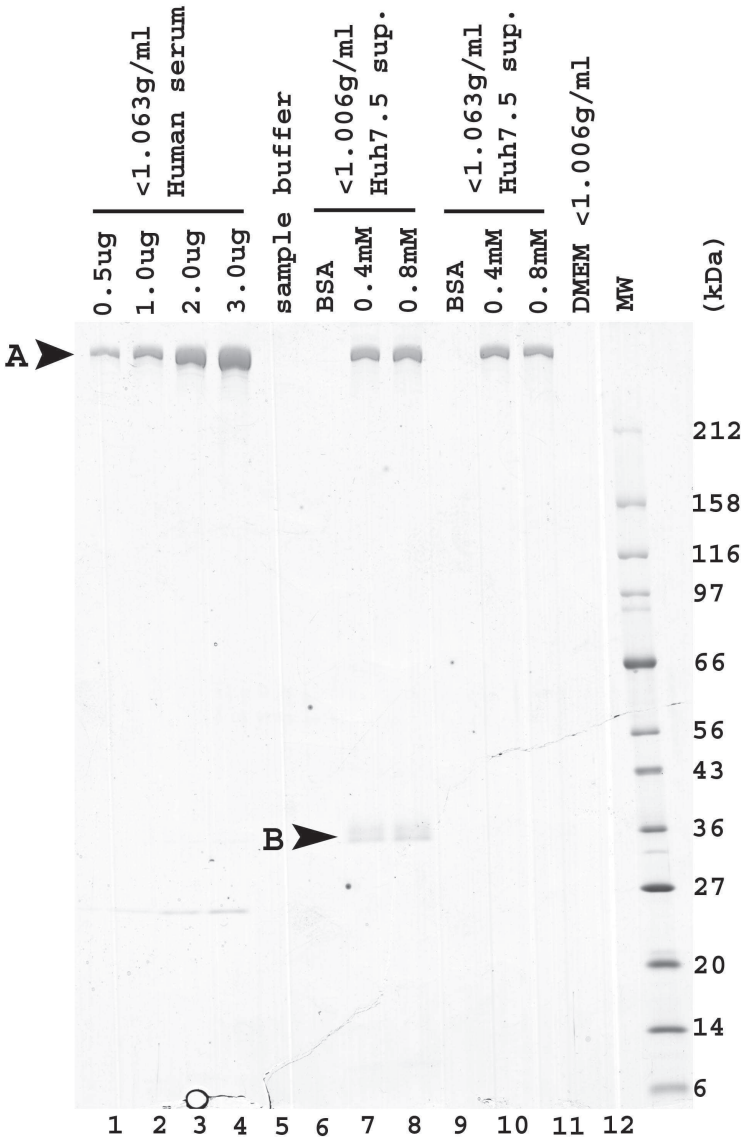


Figure 66: A Coomassie-stained acrylamide gel showing protein bands of 550kDa and 36kDa in size.
The positions of the 550kDa and 36kDa bands are indicated by arrow A and B respectively. The samples include <1.063g/ml fractions (lanes 1-4) of human serum with known amount of apoB-100 protein, <1.006g/ml fractions (lanes 6-8) and <1.063g/ml fractions (lanes 9-11) of Huh7.5 cell culture supernatants, and negative control DMEM medium only. Huh7.5 cells were treated without oleic acid (1.5% BSA) or with oleic acid (0.4mM and 0.8mM concentration).

5.3.4 Western blotting of low density lipoproteins from Huh7.5

A Western Blot using anti-apoE antibody (DAKO, Ely, UK) was also carried out on a similar gel described above. The results are presented in figure 67 showing both

≤1.006g/ml and <1.063g/ml fractions have apoE lipoproteins bands in Huh7.5 cells treated with oleic acid. The bands in the ≤1.006g/ml fractions are much stronger than those in the <1.063g/ml fractions. The ApoE concentration was also at a much higher concentration than that of ≤1.006g/ml fractions from HepG2 supernatants. There was a low amount of apoE lipoproteins found in the <1.063g/ml fractions.

The same blot as in figure 67 was stripped (method described in section 2.62) and stain with anti-apoB100 antibody. The results are presented in figure 68. A ~549kDa band is found in both ≤1.006g/ml and <1.063g/ml fractions in Huh7.5 cells treated with oleic acid.

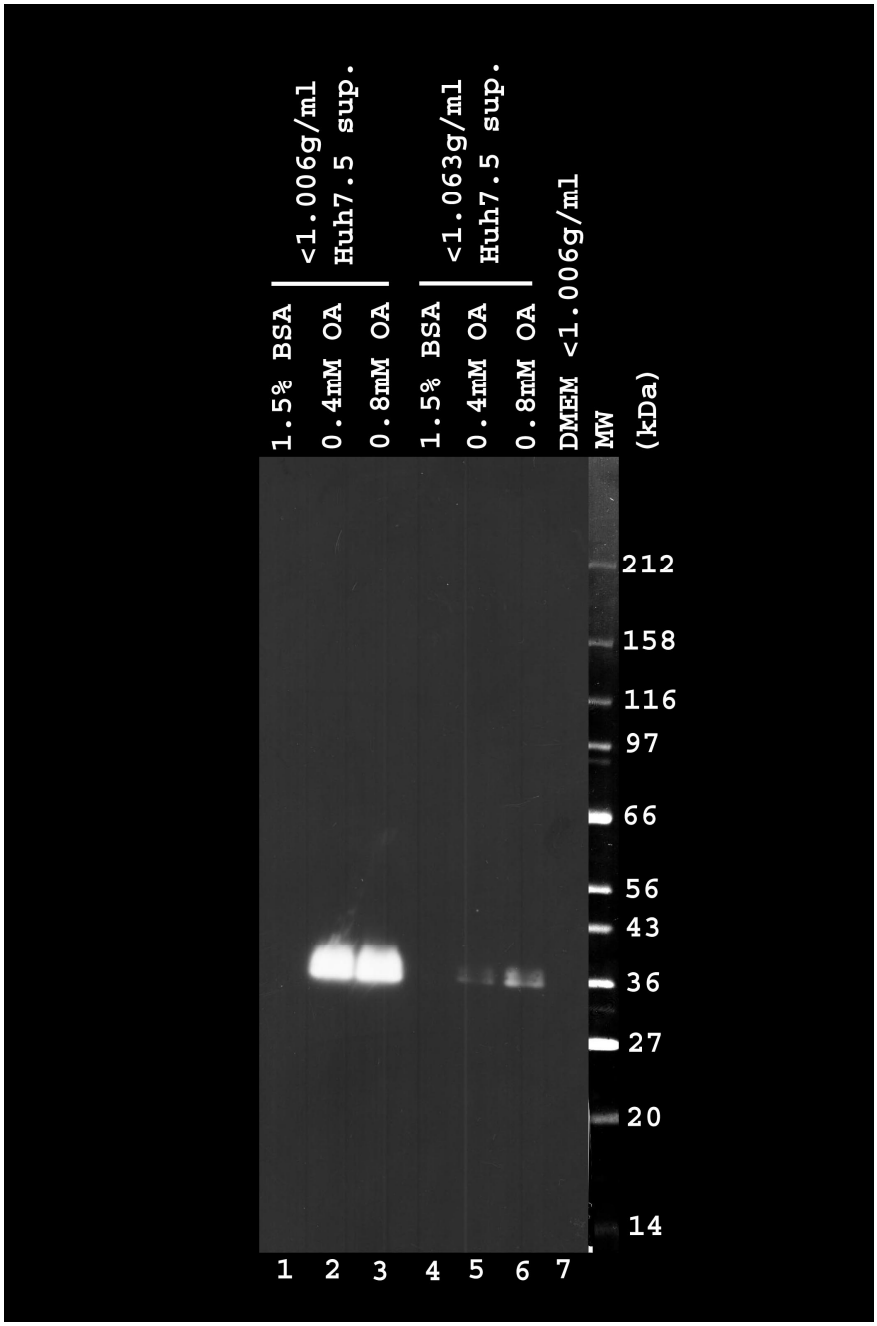


Figure 67: A Western blot of ≤1.006g/ml and <1.063g/ml fractions from Huh7.5 cell culture supernatants that were purified via potassium bromide sequential ultracentrifugation and probed with anti-apoE antibody.

Huh7.5 cells were treated with different concentration of oleic acid. Lanes 1-3: ≤1.006g/ml fractions and lanes 4-7 : <1.063g/ml fractions. Lanes 1&4: 1.5% BSA treatment, lanes 2&5: 0.4mM OA treatment, lanes 3&6: 0.8mM OA treatment, and lane 7: DMEM medium only.

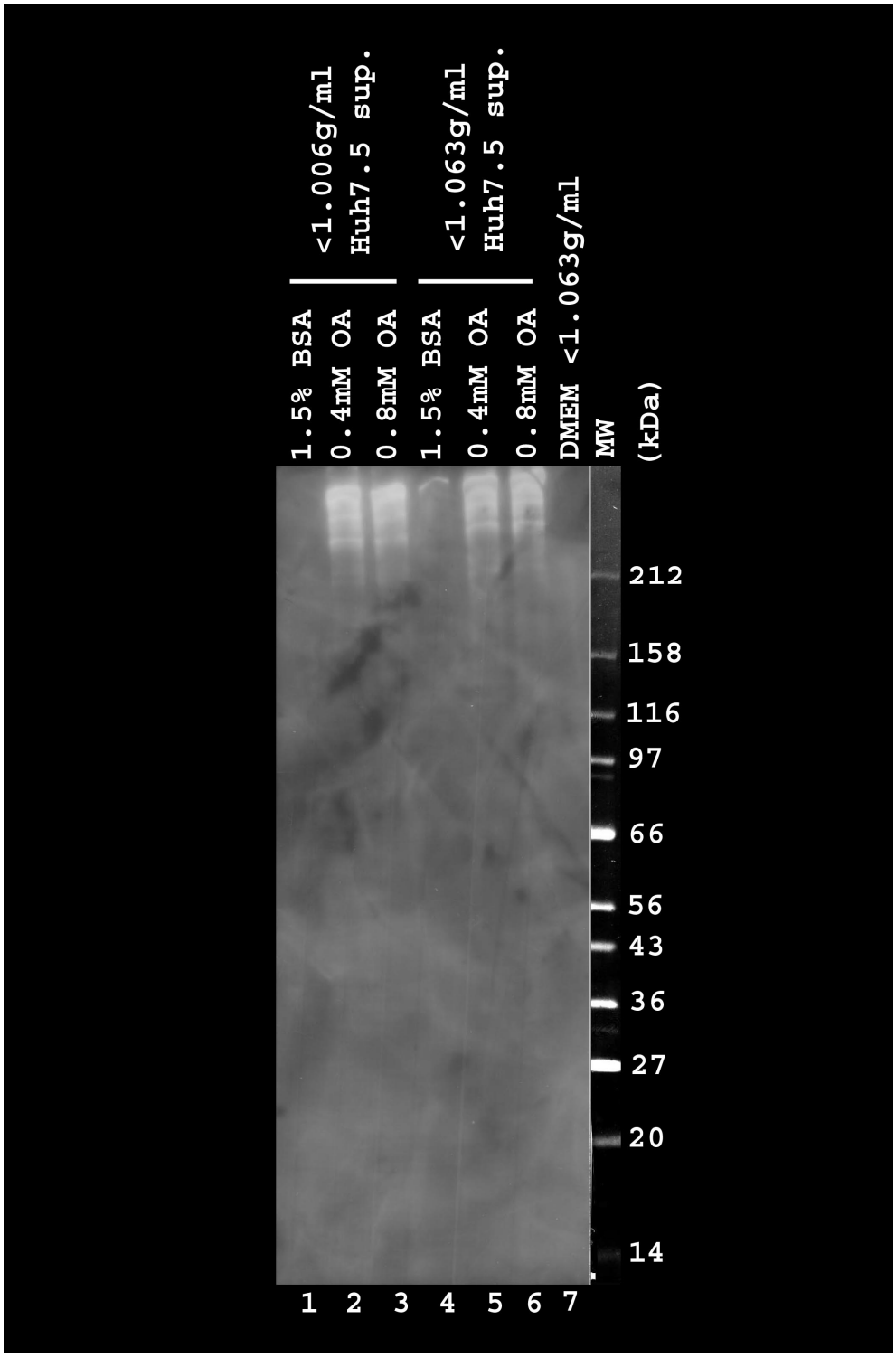


Figure 68: A Western blot of ≤1.006g/ml and <1.063g/ml fractions from Huh7.5 cell culture supernatants that were purified via potassium bromide sequential ultracentrifugation and probed with anti-apoB antibody.

Huh7.5 cells were treated with different concentration of oleic acid. Lanes 1-3: ≤1.006g/ml fractions and lanes 4-7 : <1.063g/ml fractions. Lanes 1&4: 1.5% BSA treatment, lanes 2&5: 0.4mM OA treatment, lanes 3&6: 0.8mM OA treatment, and lane 7: DMEM medium only.

5.3.5 Western blotting of very low density lipoproteins 1, 2, and 3 from Huh7 and Huh7.5

The putative VLDL released by Huh7.5 cells cultured in oleic acid was further characterized. The VLDL fraction was separated into VLDL 1, 2, and 3 fractions and Western blotted with anti-apoB100 and anti-apoE antibodies. For comparison, Huh7 cells, which are less susceptible to HCVcc replication, were included in this experiment to test the hypothesis that secretion of VLDL might explain the increase in susceptibility in the Huh7.5 line.

0.7 X 10^6 cells/well of Huh7 and Huh7.5 were seeded in 4ml 10% FCS DMEM growth medium and incubated for five days at 37^{o}C in 5% CO_2 replacing the medium at one or two days intervals as described in section 2.63. The medium was then replaced with DMEM serum free medium and BSA with (0.4mM oleic acid) or without oleic acids and incubated for another 2 days. 48ml supernatant was harvested, pooled, concentrated, and fractionated by the method described in section 2.58. Each fraction was concentrated to 280µl volume using a Vivaspin 6ml concentrator. 40µl of each fraction was then run on SDS-PAGE as described in section 2.59 and Western blotted as described in section 2.60 with anti-apoB100 and anti-apoE antibodies (DAKO, Ely, UK).

Figure 69 below shows that anti-apoB recognizes mainly the ~550kDa at VLDL-3 fraction and figure 70 shows that anti-apoE recognizes the ~36kDa again at VLDL-3 fraction in both Huh7 and Huh7.5 cells. This result does not support the hypothesis that the crucial difference between Huh7.5 cells and its parent cell line is the ability to secrete VLDL.

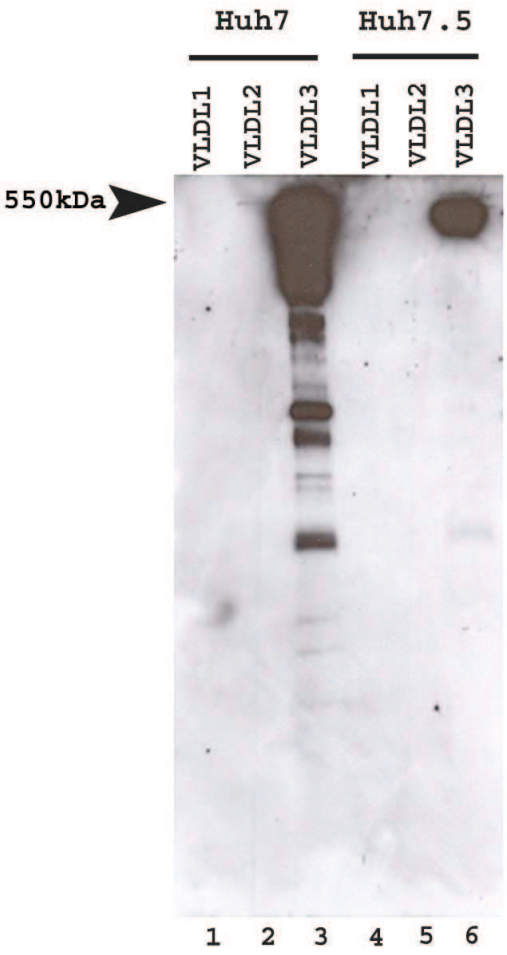


Figure 69: A Western blot of VLDL-1, VLDL-2, and VLDL-3 fractions from Huh7 and Huh7.5 cell culture supernatants after cells' treatment with 0.4mM OA probed with anti-apoB antibody. VLDL-1 is in lanes 1 and 4, VLDL-2 is in lanes 2 and 5, and VLDL-3 is in lanes 3 and 6. The VLDL fractions were purified via flotation ultracentrifugation.

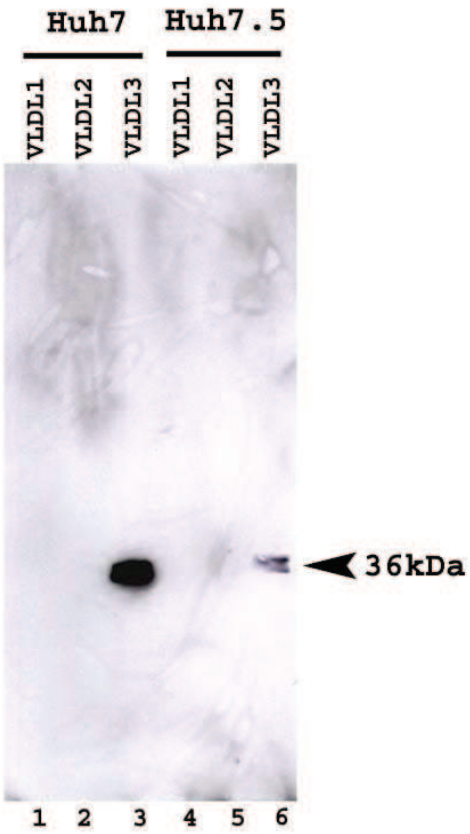


Figure 70: A Western blot of VLDL-1, VLDL-2, and VLDL-3 fractions from Huh7 and Huh7.5 cell culture supernatants after cells' treatment with 0.4mM OA probed with anti-apoE antibody. VLDL-1 is in lanes 1 and 4, VLDL-2 is in lanes 2 and 5, and VLDL-3 is in lanes 3 and 6. The VLDL fractions were purified via flotation ultracentrifugation.

5.4 Infection and culture of the J6/JFH1 HCVcc in Huh7.5 cells in the presence or absence of oleic acid – an optimization

To study the growth curve of the HCVcc under oleic acid treatment, the following experiment was carried out. The experiment was also designed to optimize the conditions for the culture of the HCVcc in Huh7.5 cells in the presence or absence of oleic acid.

2×10^4 cells/ml per well of naïve Huh7.5 cells was seeded the day before infection in 1ml 10% DMEM growth medium and incubated at 37°C, in 5% CO_2 as described in

section 2.52 except that the cells were incubated until day 11 post-infection and the supernatant harvested for RNA samplings on day 1, 3, 5, 7, 9, and 11. The inoculum used was a passage 4 of J6/JFH1 RNA-transfected Huh7.5 cells with HCVcc RNA quantity of 1.8 X 10^5 IU per well and the cell culture medium used until day 11 was 3% FCS DMEM growth medium. On day 11, the cell's culture medium was replaced with 1ml of 1) 3% FCS DMEM growth medium + 1.5% BSA, 2) 3% FCS DMEM growth medium + 0.4mM oleic acid, 3) DMEM serum free medium + 1.5% BSA or 4) DMEM serum free medium + 0.4mM oleic acid. The supernatant was harvested on alternate day until day 16 post-infection.

The results are presented in figure 71. The highest increase of HCVcc RNA is found in supernatant of infected cells grown in serum free medium with oleic acid after day 11. There is very little HCV RNA detected during the first eight days of the culture presumably because there is very little replication taking place as there are very little core and non-structural proteins are available in the first few days. It has also been reported that only a small portion of the HCV particles released is infectious whilst the main portion of the released particles lack infectivity (Miyanari et al., 2007). On day 16, there is a 2.6 fold increase of HCVcc RNA in supernatant of infected cells grown in serum free medium with oleic acid (8.2 X 10^6 IU/ml) compared to that in supernatant of infected cells grown in serum free medium without oleic acid (3.2 X 10^6 IU/ml) and a 2 fold increase compared to that in supernatant of infected cells grown in medium containing 3% FCS with oleic acid (4.1 X 10^6 IU/ml).

The above experiment was similarly repeated twice using a pooled inoculum of varying passages of the alleged J6/JFH1 HCVcc from snap-frozen, thawed and

centrifuged supernatant samples as described in section 2.52. The HCVcc RNA quantity of 1.8 X 10^5 IU per well was used. On day 11, the cell's culture medium was replaced with 1ml of 1) DMEM serum free medium or 2) DMEM serum free medium + 1.5% BSA + 0.4mM oleic acid. The supernatant was harvested on alternate day until day 16 post-infection.

The results are compiled and presented in Figure 72. There is a general consensus that when HCVcc-infected Huh7.5 cells were treated with oleic acid, there is an increase of HCVcc RNA compared to when they were not treated with oleic acid. There is also a trend that the HCVcc RNA quantity decreases following a very high increase of HCVcc RNA from previous days of infection.

From figures 71 and 72, the best condition is serum free medium with oleic acid added.

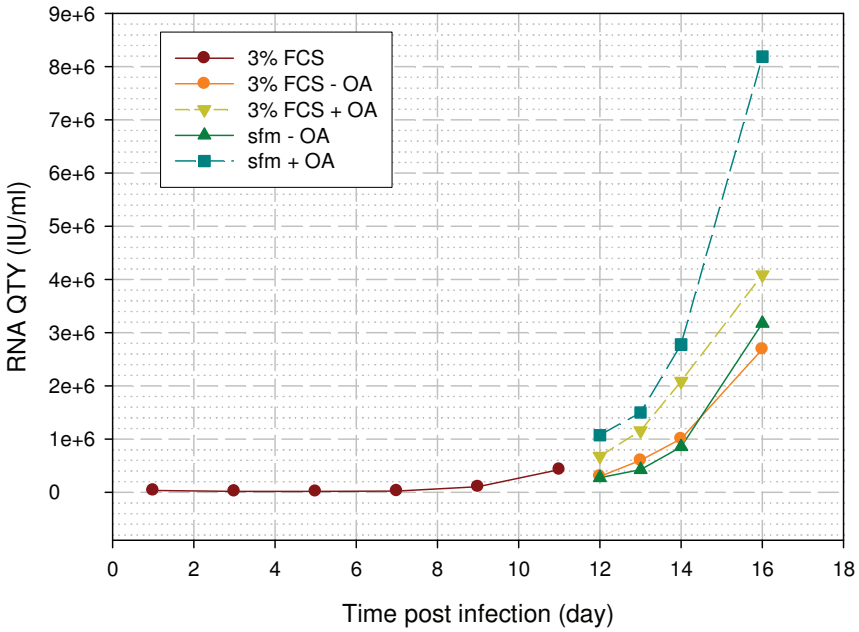


Figure 71: Comparison of HCVcc RNA output after a J6/JFH1-RNA infection of naïve Huh7.5 cells cultured in the different types of medium with or without oleic acid treatment.

The infected cells were cultured in 1)3% FCS DMEM growth medium, 2)3% FCS DMEM growth medium + 0.4mM oleic acid, 3)DMEM serum free medium or 4)DMEM serum free medium + 0.4mM oleic acid. The inoculum used was a frozen, thawed, and centrifuged (1500g) supernatant from pass 4 of J6/JFH1 RNA-transfected Huh7.5 cells. The RNA was quantitated by real-time PCR.

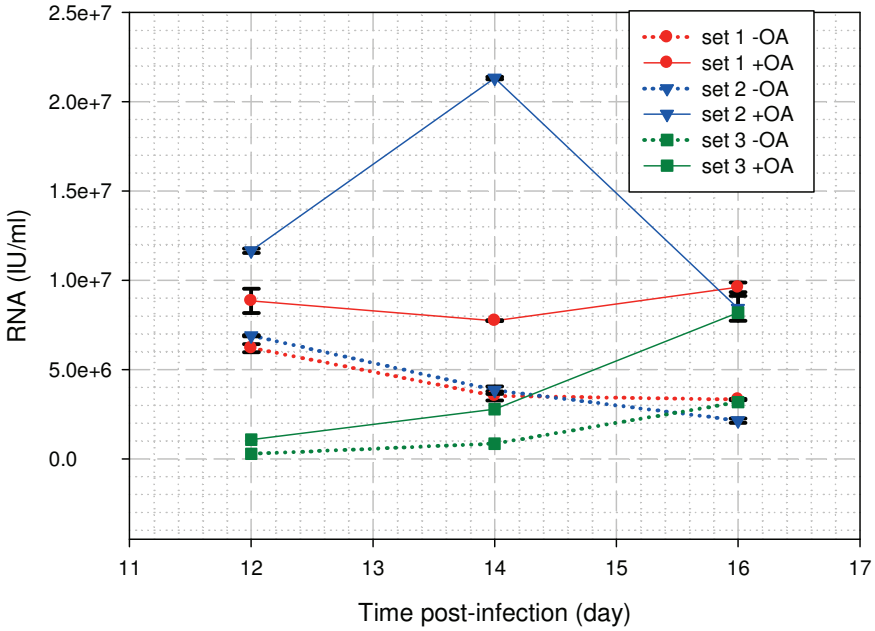


Figure 72: Compilation of 3 sets of similar experiments depicting HCVcc RNA output when naïve Huh7.5 cells were infected with HCVcc.
The infected cells were cultured in either serum free medium + 1.5% BSA (–OA) or serum free medium + 0.4mM oleic acid (+OA). The RNA was quantitated by real-time PCR.

5.5 Characterization of the 'LVP' production from J6/JFH1 HCVcc infected and oleic acid treated Huh7.5 cells

5.5.1 Density of 'LVP'

The following experiment was done to determine and compare the density of 'LVP' produced from HCVcc infected Huh7.5 cells with or without oleic acid treatment using an iodixanol density gradient.

2 x 10^4 cells/ml per well of naïve Huh7.5 cells was seeded in a 24-well-plate the day before infection in 1ml 10% DMEM growth medium and incubated at 37^{o}C, in 5% CO_2 as described in section 5.4. A pooled inoculum of varying passages of the

alleged J6/JFH1 HCVcc from snap-frozen, thawed and centrifuged supernatant samples with HCVcc RNA quantity of 1.8 X 10^5 IU per well was used. The cells were cultured in 3% DMEM growth medium and incubated until day 11 post-infection and the supernatant harvested for RNA samplings on day 1, 3, 5, 7, 9, and 11 and replaced with fresh medium as required. On day 11, the medium was replaced with either 1)DMEM serum free medium + 1.5% BSA or 2) DMEM serum free medium + 0.4mM oleic acid was added to each well. The supernatant was harvested on alternate day until day 16 post-infection. 5ml of day 16 post-infection supernatant samples were harvested, pooled, and concentrated to 0.5ml volume and applied onto an iodixanol gradient as described in detail in section 2.64. The 27 fractions (0.5ml per fraction) collected were analysed for density using a digital refractometer (Atago, Japan) and HCVcc RNA quantity using real-time PCR.

The results are presented in figure 73. In the absence of oleic acid treatment, HCVcc RNA (2.3 X 10^6 IU/fraction) peaks at fraction 12 at density 1.146g/ml as shown in the figure. In the presence of oleic acid treatment, there is a shift of the HCVcc RNA (1.8 X 10^7 IU/fraction) peak to a lower density of 1.135g/ml (fraction 14).

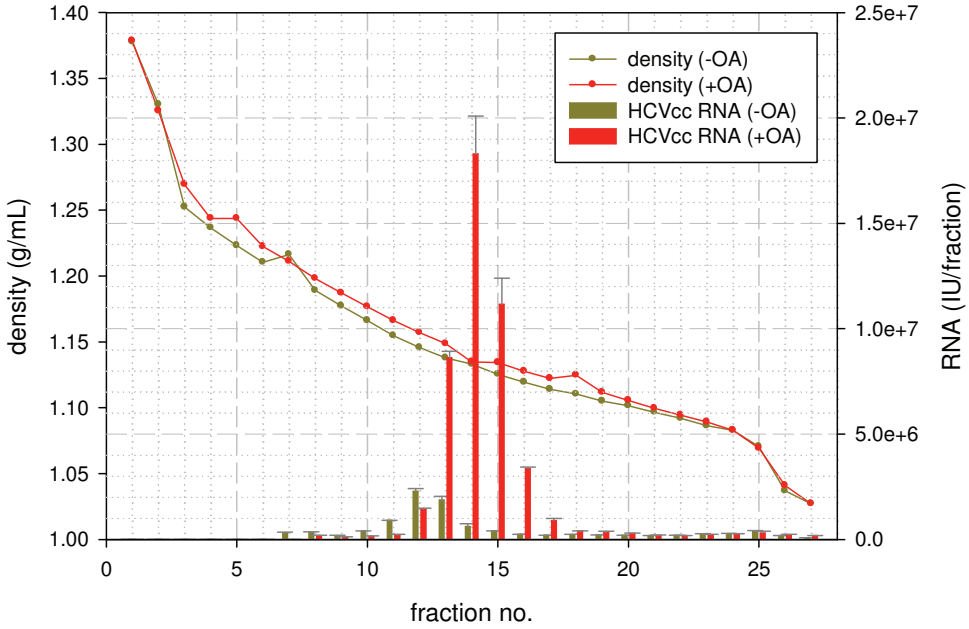


Figure 73: The density of 'LVP' produced from HCVcc infected Huh7.5 cells with or without oleic acid treatment using an iodixanol density gradient.
HCVcc RNA for each fraction was quantitated by real-time PCR.

5.5.2 Size of 'LVP'

The following experiment was done to compare the size of 'LVP' produced from HCVcc infected Huh7.5 cells with or without oleic acid treatment to that of chylomicron, VLDL 1, VLDL 2, IDL, and LDL using a gel filtration column.

The 'LVP' production from J6/JFH1 HCVcc infected and oleic acid treated Huh7.5 cells was similarly carried out as in section 2.64. However, 30ml of day 16 post-infection supernatant samples were harvested, pooled, and concentrated to 4ml volume and injected on to the gel filtration column as detailed in section 2.65. 600µl of each of the collected and pooled 24 fractions were analysed for HCVcc RNA quantity using real-time PCR.

The results are shown in figure 74. In the absence of oleic acid treatment, HCVcc RNA (1.6 X 10^7 IU/fraction) peaks at fraction 17 at size between VLDL 1 and VLDL 2 as shown in the figure. In the presence of oleic acid treatment, the peak of HCVcc RNA (3.4 X 10^7 IU/fraction) is also found in fraction 17. To date, there has been no report of the size of HCVcc in the literature.

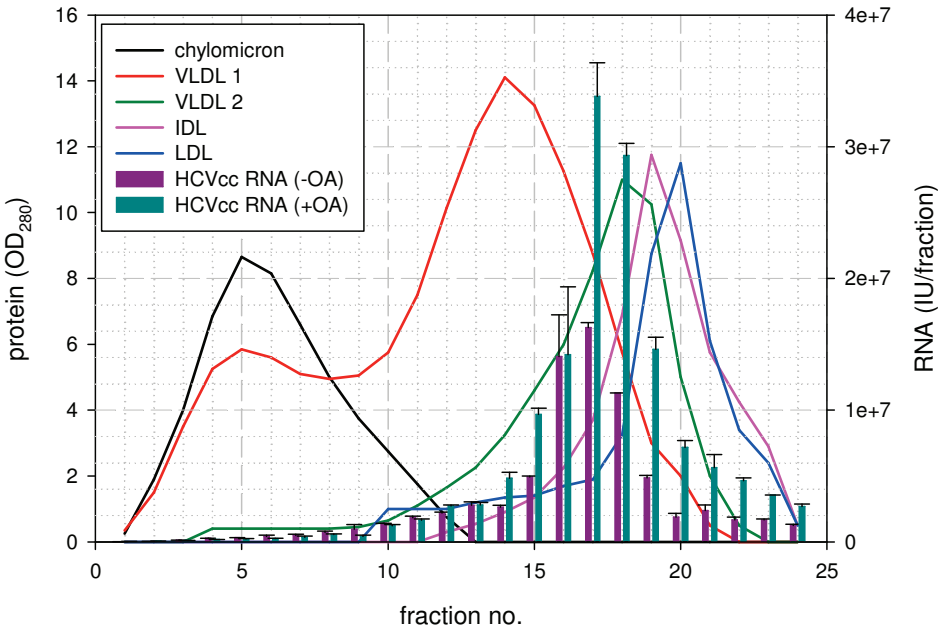


Figure 74: The size of 'LVP' produced from HCVcc infected Huh7.5 cells with or without oleic acid treatment using a gel filtration column.
The size of 'LVP' as depicted by the HCVcc RNA quantity was compared against the sizes of chylomicron, VLDL1, VLDL2, IDL, and LDL. The RNA for each fraction was quantitated by real-time PCR.

Chapter 6

6 Discussion

6.1 Studies on the binding of liver-derived HCV LVP and host lipoproteins to HepG2 cells

The ability of HCV LVP from the liver-macerate (LLVP) of an HCV genotype 1a infected patient who had common variable immunodeficiency, to bind and subsequently infect hepatocyte-derived cell lines and primary hepatocytes was initially investigated.

A number of receptors used by HCV to bind and gain entry into cells particularly hepatocytes or hepatocyte-derived cell lines had been suggested by others. These include receptors used by host lipoproteins to enter cells such as LDLr and SR-B1. A number of recent studies of HCVcc or HCVpp indicate that this is the case. Zeisel *et al* (Zeisel et al., 2007) using the HCVcc system showed that SR-B1 is crucial for the entry of HCV into host cell. Binding of HCVcc to Huh7.5 cells was specifically inhibited in a dose dependent manner by antibodies to the extracellular loop of SR-B1. In addition, infectivity of HCVcc was reduced by down regulation of SR-B1 expression by an siRNA technique (Zeisel et al., 2007).

Monazahian *et al* (Monazahian et al., 1999) have shown that the adsorption of HCV RNA-carrying material (HCVrcm) to human fibroblasts *in vitro* was inhibited completely by the addition of >200μg/ml of purified LDL and that HCV could bind to COS-7 cells which normally do not bind HCV after being transfected with a vector containing human LDLr. In another study, endocytosis of HCV from infected human sera was shown to be mediated by LDLr as demonstrated by the direct correlation of

LDL receptor activity with that of the endocytosed virus, complete inhibition by anti-LDLr antibody, inhibition with anti-apoB and anti-apoE antibodies, by biochemical inhibitors of LDL endocytosis, and lack of viral endocytosis via LDLr was provided by cells known to have no LDLr (Agnello et al., 1999).

Endocytosis of serum derived HCV RNA, measured by in situ hybridisation, correlated with expression of LDL receptor G4 (B cells), HepG2, and Daudi cells (Agnello et al., 1999). Agnello *et al* also showed that endocytosis could be inhibited in a dose-dependent manner when the cells were pre-incubated with anti-LDLr antibody and, with high enough concentration, complete inhibition was achieved. They also showed that 25–100μg/ml of LDL or VLDL completely inhibited HCV endocytosis whereas HDL concentration of up to 200μg/ml did not (Agnello et al., 1999). Using confocal microscopy, low density HCV and LDL were shown to localize together on the cells surface, low density but not intermediate density HCV particles were shown to bind to lymphoid cells (MOLT-4) and fibroblasts expressing the LDLr, and using anti-LDLr antibodies, the binding correlated with LDLr expression (Wunschmann et al., 2000).

Uptake of HCV via SR-B1 and LDLr may occur indirectly following interaction of these receptors with host lipoproteins if HCV is associated with the host lipoprotein. There have been a number of studies that have described an association of HCV with LDL/VLDL in the serum of patients infected with HCV (Prince et al., 1996, Thomssen et al., 1992, Thomssen et al., 1993, Nielsen et al., 2004). Serum β-lipoprotein was associated with the HCV RNA at the density of 1.03g/ml – 1.08g/ml but not with HCV RNA with higher density of 1.12g/ml – 1.20g/ml (Thomssen et al.,

1992). In some patients, HCVrcm could be immunoprecipitated together with anti-β-lipoproteins or with anti-immunoglobulins or with both or with neither (Thomssen et al., 1993). Using a column chromatographic procedure to fractionate plasma containing HCV, Prince *et al* found that HCV RNA was detected (by PCR) primarily in the VLDL fraction although virus associated with LDL could have also been eluted together with the VLDL fraction (Prince et al., 1996). In another study using iodixanol gradients by Nielsen *et al*, all virus from a patient S6 serum was found at a density below 1.13g/ml and the virus with density below 1.10g/ml could be precipitated out with anti-apoB100 polyclonal antibody showing that the HCV of low density was associated with LDL/VLDL (Nielsen et al., 2004).

Association with host lipoprotein may increase the infectivity of HCV as Hijikata *et al* have shown that HCV from a patient with high *in vivo* infectivity in chimpanzee was principally found in fractions of density 1.063g/ml while HCV with low *in vivo* infectivity was found principally in fractions >1.063g/ml (Hijikata et al., 1993b).

Martin (Martin, 2005) suggested that HCV LLVP may also be taken up via LDLr as they bind better to LPDS/insulin treated cells than hydroxyl 25-OH treated cells and that this correlates with raised LDLr expression. Also binding could be blocked by pre-incubation with LDL. If HCV is binding via the interaction of the host lipoprotein moiety of the LLVP to LDLr, it should be inhibited by anti-apoB and anti-apoE antibodies.

6.2 Anti-apoB-100 and anti-apoE antibodies do not block binding of LLVP

Host lipoproteins bind to LDLr via apoB100 and apoE and this can be blocked with anti-apoB100 and anti-apoE antibodies. Binding of LDL to the LDLr is mediated by apoB (Brown and Goldstein, 1986) and VLDL by apoE (Chappell et al., 1993). The LDLr is able to bind apoB100 in LDL but it binds apoE in VLDL with a higher affinity (Windler et al., 1980). Another study showed that in hypertriglyceridemic subjects, the binding of VLDL1 to isolated LDLr is mediated through the thrombin-accessible apoE and that it binds via potentially dissociable apoE rather than non-transferable apoB (Brown et al., 1986).

Monoclonal antibody (Mab) 1D7 which is specific for human apoE could block binding of lipid-associated apoE to the LDLr (Maurice et al., 1989) and anti-apoB Mabs that are specific for a region that covers a thrombin cleavage site at residue 3249 of apoB could totally inhibit LDL binding to the LDLr (Milne et al., 1989).

A study made by Andre *et al* (Andre et al., 2002) showed that binding of LVP derived from the serum could also be blocked by anti-apoB and anti-apoE antibodies. In a similar study Agnello *et al* found that both anti-apoB and anti-apoE were required for maximal blocking for the endocytosis of VLDL whereas anti-apoB alone was enough to block LDL endocytosis. Using these conditions, 65% inhibition of HCV (from infected serum) endocytosis was achieved (Agnello et al., 1999). In contrast, in the HCVpp (genotypes 1a) system, anti-apoE but not anti-apoB could weakly inhibit the infectivity when monoclonal antibodies to the receptor binding sites of ApoB and ApoE were preincubated with the HCVpp (Bartosch et al., 2003a).

In this study, the ability of polyclonal rabbit anti-apoB-100 antibody or a combination of anti-apoB-100 and anti-apoE antibodies to block the uptake of LLVP into the HepG2 cells was initially investigated. Contrary to what was expected, instead of anti-apoB100 and anti-apoE blocking the uptake of LLVP into the HepG2 cells, anti-apoE was without effect and anti-apoB100 actually enhanced the uptake of LLVP. In studies made by Maurice, Milne, Bartosch and Andre, monoclonal antibodies were used instead of polyclonal antibodies. Anti-apoB100 antibody and anti-apoE antibody used were polyclonal rabbit antibody against human apoB-100 and purified rabbit immunoglobulin against human apoE respectively. Whilst the polyclonal anti-apoB-100 was shown to be efficient at reducing binding of LDL to HepG2 cells, no equivalent data for the efficacy of the anti-apoE antibodies was available.

The failure of anti-apolipoprotein antibodies to block binding of LLVP suggests that LLVP may not be binding to LDLr, or LDLr is not the main or only receptor used in the binding process, or that LLVP bind to LDLr by an apolipoprotein independent manner. These results, however, paralleled the increase in oxLDL uptake into HepG2 cells when it was exposed to anti-apoB100 antibody.

A possible reason for the opposing result here to that of studies with serum-derived LVP could be the source or nature of the HCV particle as serum-derived LVP may have undergone different additional 'processes' than that of liver-derived LLVP.

The failure of anti-apoB100 and anti-apoE to block binding of LLVP could be due to other receptors being used in the binding of LLVP. LLVP may be more analogous to HCVcc than to serum LVP which means that SR-B1 may actually play a more

important role in binding as SR-B1 has been demonstrated to act as a receptor for HCVcc. Two anti-SR-B1 monoclonal antibodies, 3D5 and C167, efficiently blocked HCVcc infection of Huh7.5 hepatoma cells in a dose-dependent manner; hence showing that SR-B1 was directly involved in HCV infection. 3D5 and C167 could bind to conformation-dependent SR-B1 determinants and inhibit soluble E2 interaction with SR-B1 (Catanese et al., 2007). A study by von Hahn *et al* shows that SR-B1 is an important component of the cellular HCV receptor complex because oxLDL, an SR-B1 ligand, and not native LDL was found to strongly inhibit HCVpp and HCVcc entry (von Hahn et al., 2006). Incubation of J6/JFH1 with oxLDL (10µg/ml), which is an SR-B1 ligand, for 1 hour at 37^{o}C before infection led to a marked reduction in the percentage of infected cells and using limiting dilution assays in the presence of increasing amount of oxLDL, a dose-dependent inhibition was shown suggesting that SR-B1 is an important component of the cellular HCV receptor complex (von Hahn et al., 2006).

Scarcelli *et al* (Scarselli et al., 2002) showed that E2 binding to human hepatoma cell lines do not depend on CD81 as previously proposed but instead SR-B1 was found to be responsible for E2 binding as proven by the fact that only CHO cells transfected with the human SR-B1 acquired the ability to bind E2 but not mouse SR-B1 or the SR-B1 related human CD36. HVR1 was also found to be essential for E2-SR-B1 interaction as HVR1 deletion mutants were unable to bind to SR-B1 expressing cells. Similar findings were made by Bartosch *et al* (Bartosch et al., 2003b). By using receptor competition assays, they found that anti-SR-B1 polyclonal antibodies against SR-B1 ectodomain that could block binding of soluble E2 could prevent HCVpp (genotypes 1a and 1b) infectivity in Huh7 cells. They also showed that 9/27 monoclonal antibody, which binds to C-terminal part of H77 HVR1 sequence and

specifically blocks binding of genotype 1a E2 to SR-B1, could neutralize efficiently in a dose-dependent manner the infectivity of genotype 1a HCVpp but not 1b.

To date, it looks as if HCVpp and HCVcc requires CD81, CLDN1 and SR-B1. SR-B1 may act as a primary receptor involved in the early part of the entry process (Evans et al., 2007) whereas CD81 and CLDN1 may be involved in the post-binding steps (Evans et al., 2007, Koutsoudakis et al., 2006, Cormier, 2004).

6.3 DiI-oxLDL uptake by HepG2 cells under different drug treatments

That anti-apoB100 antibody enhances the uptake of LLVP suggests that LLVP is not binding to LDLr. Martin has shown that LLVP binding can be blocked by pre-incubation with both LDL and oxLDL (Martin, 2005). Thus, LLVP may mimic binding of oxLDL rather than LDL.

Whilst LDL binds both to the LDL receptor (Hajjar, 1997) and scavenger receptor B1 (SR-B1) (Steinbrecher, 1999), oxidized LDL binds to SR-B1 but not to the LDL receptor (Hajjar, 1997). Thus, LLVP and oxLDL may bind via SR-B1. Von Hahn et al showed that oxLDL was not a receptor blocker as the binding of soluble E2 to SR-B1 or CD81 was not affected by oxLDL but oxLDL incubation could change the biophysical properties of HCVpp (von Hahn et al., 2006). He suggested that there is a ternary interaction of oxLDL with both virus and target cells and thus, concluded that oxLDL which is an SR-B1 ligand is a potent cell entry inhibitor for HCV.

LDL receptor expression can be reduced with hydroxycholesterol (25-OH) (Srivastava et al., 1995, Peng et al., 1996) and increased by removal of low density lipoproteins from the medium and addition of insulin (Agnello et al., 1999, Wade et al., 1989, Wade et al., 1988) and Martin (2005) has shown that these treatments have a similar effect upon LLVP binding. The effect of these conditions on the expression of SR-B1 is not clear, the hypothesis that LLVP and oxLDL are binding via SR-B1 predicts that these conditions will have a similar effect on oxLDL uptake.

As expected from the findings made by Martin (Martin, 2005), there was a significant decrease of DiI-LDL uptake by HepG2 cells when cells were pre-incubated in hydroxycholesterol, whereas, there was a significant increase in mean intensity when cells were cultured in LPDS plus insulin. However, there was little change in the DiI-oxLDL uptake by HepG2 treated with hydroxycholesterol, although, there was a significant increase in mean intensity when cells were treated with LPDS plus insulin. These results show that oxLDL does not behave in exactly the same was as LDL.

DiI-LDL was taken up more efficiently than DiI-oxLDL as shown by the 20-fold difference in the mean fluorescence intensity when the cells are treated with LPDS plus insulin since the DiI labelling of both LDL and oxLDL was similar. These findings suggest that either LPDS plus insulin upregulates SR-B1 to lower levels than LDL-r or that binding via LDLr is much more efficient than that via SR-B1. It cannot be excluded that the rise in oxLDL uptake in insulin treated cells could be due to residual uptake by LDLr. It is unlikely that the rise in oxLDL uptake was actually the uptake of residual LDL if the oxidation process of LDL, which destroys apoB100, was complete. The oxLDL preparation used followed that described by Lopes-Virella

et al which react optimally and consistently with defined oxLDL antibodies (Lopes-Virella et al., 2000) and aggregation, which is a characteristic of extensively oxidized LDL, was removed following the method of Hoff *et al.* (Hoff et al., 1993).

6.4 Effect of anti-apoB100 antibody on the binding of DiI-LDL and DiI-oxLDL

The increased binding of ox-LDL in cultures incubated in LPDS and insulin, designed to upregulate LDLr expression suggests that oxLDL may be taken up by residual binding of oxidised apoB100 to LDLr rather than by the SR-B1 receptor through recognition of oxidized ligands on the surface of oxLDL particle. If so, binding should be blocked with anti-apoB100 antibody. This did not prove to be the case as, although the result was not significant, pre-incubation of DiI-oxLDL with anti-apoB100 antibody increased binding of DiI-oxLDL to HepG2 cell. A similar treatment of DiI-LDL resulted in a significant 98% reduced binding. These results suggest that binding of oxLDL is not apoB100 dependent and therefore quite different from binding of LDL. This leaves as an alternative explanation that oxLDL binds to LPDS plus insulin treated cells via SR-B1 and that this receptor is also being increased in expression by the treatment. This possibility was tested by quantitative FACS analysis of anti-SR-B1 and anti-LDLr antibodies to cells cultured in LPDS plus insulin and those cultures in 2, 5 hydroxycholesterol as discussed in the next section.

6.5 Effect of LPDS plus insulin and 2,5 hydroxycholesterol on the expression of LDLr and SR-B1 on HepG2 cells and non-hepatic cells

In order to compare the effect of insulin on SR-B1 expression with the previously determined effect on LDLr expression and LVP binding, SR-B1 and LDLr

expressions were quantitated by FACS analysis with anti-SR-B1 and anti-LDLr antibody respectively on normal untreated HepG2 cells grown in 10% Foetal Calf Serum, cells grown in LPDS plus insulin and cells grown in FCS plus hydroxylcholesterol using FITC-staining of HepG2 cells for FACS. Contrary to our hypothesis, SR-B1 was significantly down-regulated by both LPDS plus insulin 1.2-fold and by hydroxycholesterol 1.4-fold in comparison to SR-B1 expressed in normal growth medium with 10% FCS. No previous studies on the effect of LPDS plus insulin or hydroxycholesterol on SR-B1 expression could be found. LDLr was significantly up-regulated 2.2-fold by LPDS plus insulin which was similar to the findings of others (Agnello et al., 1999, Wade et al., 1989, Wade et al., 1988) and down-regulated 1.3-fold by hydroxycholesterol in comparison to LDLr expressed in normal growth medium with 10% FCS which is also in agreement with the finding of others (Srivastava et al., 1995, Peng et al., 1996, Dashti, 1992).

Vero cells (monkey kidney cells) are non-hepatocyte derived cell which has LDLr, CD81 and GAGs present at the surface of the cell and is able to bind and replicate HCV (Germi et al., 2002). However, there has so far been no evidence on the expression of SR-B1 in Vero cells. An experiment was carried out to see if see if SR-B1 is also expressed in Vero cells and if LPDS plus insulin affects the expression of LDLr and SR-B1 in non-hepatocyte derived cells as it does in hepatocyte derived cells liver specificity. Our results showed that LDLr expression was increased in HepG2 cells but not in Vero cells which contradicts the findings of Germi who confirmed the role of LDLr as HCV receptor in Vero cells as anti-LDLr mAb reduced HCV binding by 60% and both LDL and VLDL which bind to LDLr, inhibit HCV adsorption (Germi et al., 2002). In our study, similar results were obtained with anti-

SR-B1 showing an increase in SR-B1 expression in HepG2 cells but not in Vero cells. Binding of LVP to Vero cells was higher than that to HepG2 cells (Martin, 2005) and hence, does not correlate with SR-B1 and LDLr as shown in the results above.

SR-B1 expression in HepG2 cells was slightly lowered by insulin treatment in the first experiment and slightly raised in the second suggesting that this receptor is not affected by insulin. These experiments, therefore, do not confirm the predictions for the hypothesis that LLVP and oxLDL, bind to insulin treated HepG2 cells via SR-B1.

6.6 Effect of suramine wash on DiI-ox-LDL bound to HepG2 cell surface

Martin (Martin, 2005) demonstrated that, like LDL, binding of LLVP was sensitive to suramine suggesting that it involved cell surface glycoasaminoglycans. Suramine sensitivity/resistance of oxLDL binding compared to that of LDL was therefore, tested. By confocal microscopy and FACS assessment, our results confirm the observations made by (Martin, 2005) where DiI-LDL bound at $0^{o}C$ was removed after a suramine wash. However, the binding of DiI-oxLDL at both $0^{o}C$ and $37^{o}C$ was very much lower than DiI-LDL and the effect of suramine washing was difficult to gauge by confocal microscopy. By FACS analysis, the removal of DiI-oxLDL from cells at both $0^{o}C$ and $37^{o}C$ was not significant. Thus unlike LLVP, DiI-oxLDL does not appear to be bound to glycosaminoglycans.

6.7 Effect of anti-E2 HCV antibody and anti-HVR-1 monoclonal antibody blocking binding of LLVP

The components believed to act as ligands for the HCV receptors include HCV envelope glycoproteins, E1 and E2 which play an important role in the entry of HCV into host cells. In a review made by Tellinghuisen and Rice (Tellinghuisen and Rice, 2002), E2 was thought to be responsible for the binding to the putative host-cell receptor (s). Deletion of HVR1 of E2 has been shown to reduce HCVpp infectivity in cell culture (Bartosch et al., 2003b, Callens et al., 2005) thus, supporting a major role of HVR1 in host cell entry. In another study using HCVpp, it has been suggested that the particle binding to SR-B1 depends upon the E2 glycoprotein (Bartosch et al., 2003b).

Scarselli et al (Scarselli et al., 2002) originally identified SR-B1 as the putative receptor to HCV because it binds soluble E2. Interaction between soluble E2 and SR-B1 has been shown to be specific (Scarselli et al., 2002, Barth et al., 2005). However, no direct interaction between SR-B1 and E1E2 heterodimer has been reported to date.

Monoclonal or polyclonal antibodies against linear as well as conformational epitopes of E2 have been shown to inhibit cellular binding of HCV-LP binding, entry of HCVpp, and infection of HCVcc. Other groups had found that antibodies directed against the E2 protein are able to neutralize cell entry of HCVpp (Bartosch et al., 2003a, Keck et al., 2008). Mab 9/27 which is specific for the amino acids 396-407 within the C-terminus of HVR1 specifically neutralized HCVpp or HIV-HE1E2 (HIV pseudotype bearing HCV strain H native E1 and E2 glycoproteins) (Hsu et al., 2003). The H35 and H48 monoclonal antibodies which target conformational epitopes on E2 could reduce infectivity of HCVpp genotype 1a by up to 70% (Bartosch et al., 2003a).

An E2-specific human mAb C1 was able to neutralize HCVcc in a dose-dependent manner (Lindenbach et al., 2005). Monoclonal antibodies 3D5 and C167 against conformational human SR-B1 and are able to inhibit the interaction of soluble E2 with SR-B1 and were able to block HCVcc infection of Huh7.5 cells in a dose dependent manner (Catanese et al., 2007).

In order to assess the role of E2 in the binding of liver derived HCV LVP to HepG2 cells, polyclonal anti-E2 antibody and a monoclonal antibody to the E2 hypervariable region were tested for their ability to block LVP binding. The anti-HVR-1 3C7-C3 monoclonal antibody used in this study recognized a truncated natural E2 protein (H661) and HCV-LP (1a), both derived from strain H (Cerino et al., 2001) and both this and the polyclonal anti-E2 antibody have previously been shown to bind to S6 HCV in an immunoprecipitation experiment (Nielsen et al., 2006). In our results, there was no significant difference between the binding of LLVP to HepG2 cells following treatment with normal goat IgG or goat anti-E2 HCV antibody. Similarly, treatment of LLVP with anti-HVR-1 HCV E2 monoclonal antibody produced no significant change in the binding compared to anti-RSV-F monoclonal antibody.

From the results above, neither polyclonal antibody against E2 nor anti-HVR1 monoclonal antibody against the hypervariable region of E2 blocks binding of LVP to HepG2 cells. This suggests that E2 is not involved in binding but it cannot be excluded that E2 is involved in the binding of LLVP to HepG2 but these particular antibodies do not affect the binding site.

6.8 Conclusions concerning binding of S6 LLVP to HepG2 cells

Martin (Martin, 2005) found that liver derived lipo-viro-particle (LLVP) binding to HepG2 cells could be inhibited by both LDL and ox-LDL. I have shown that binding of LLVP to HepG2 resembles binding of oxidised low density lipoprotein (oxLDL) more closely than that of low density lipoprotein (LDL). Thus, whereas anti-apoB100 antibodies blocked uptake of LDL by HepG2 cells, the same antibody actually enhanced the uptake of both oxLDL and LLVP. As both LDL (Steinbrecher, 1999) and ox-LDL (Hajjar, 1997) bind to SR-B1, this observation prompted the hypothesis that LLVP, like oxLDL, bind to SR-B1 to enter and infect cells. This hypothesis predicts that the established properties of LLVP binding to HepG2, including increased uptake by insulin treated cells and removal by suramine will be shared by the oxLDL/SR-B1 system.

However, comparisons between oxLDL and LLVP binding demonstrated that SR-B1 expression and oxLDL binding did not parallel LDLr expression in cells treated with OH-cholesterol or LPDS and insulin and that, unlike LLVP, oxLDL binding was not reversed by suramine. Further anti-E2 HCV antibody and anti-HVR-1 monoclonal antibody fail to block the binding of LLVP suggesting that the E2 glycoprotein, which is responsible for binding HCVcc to SR-B1, does not mediate binding of LLVP.

Recent experiments carried out by Nielsen *et al.* indicate that LVP semi-purified on iodixanol gradients may contain viral RNA containing microsomal membrane particles created during the maceration of the liver (Nielsen et al., 2008). He showed that the HCV RNA from the liver eluted as a broad peak from Toyopearl gel filtration

column with the highest peak of about 6.5 x 10^7 IU with most of the HCV in the liver found within membranes which has a diameter of $\geq$100nm. In light of this, it is not clear whether the HCV RNA bound to HepG2 cells in the binding studies presented here represents the binding of LVP or microsomal membrane fragments.

Binding of liver derived HCV 'LVP' from liver macerate to HepG2 cells, which appears to be mediated via host cell receptor-ligand interactions, may not be biologically relevant and may not lead to infection in the cell. An infection system, in which the ability of adsorbed virus to penetrate and initiate infection in the cells can be monitored, is required to investigate whether binding of LVP via host cell ligands can lead to infection.

6.9 Determination of HCV RNA quantity upon inoculation of HCV 'LVP' from liver macerate to HepG2 and Huh7.5 cell lines and primary hepatocytes

Since HCV was first identified, many attempts had been made to infect cells with HCV *in vitro*. In 1997, cDNA clones of HCV which were functional were first constructed and with these clones, chimpanzees became infected after an intrahepatic transfection with the recombinant viral RNA (Kolykhalov, 1997, Yanagi et al., 1997) but the genomes failed to replicate *in vitro*. Following this, it became possible to select for HCV RNA replication in cell culture by creating HCV replicons that were able to express a drug-stable gene (Lohmann, 1999) but culture adaptive mutation was required for efficient replication (Blight, 2000). In 2005, Wakita *et al. (Wakita et al., 2005)* reported robust replication in Huh7 cells of a full length clone of HCV genotype 2a strain of virus derived from a case of fulminant hepatitis. A number of

authors demonstrated improved replication of this virus and its derivatives in lines of Huh7 cells (Huh7.5) which were selected for replication HCV sub-genomic replicons and then cured of replicon by interferon alpha treatment (Lindenbach et al., 2005, Zhong, 2005).

In our attempt to have an HCV infectious system going *in vitro* for HCV binding study, we set out to inoculate two hepatoma cell lines, HepG2 and Huh7.5 and primary hepatocytes with HCV RNA of S6b LLVP and monitored the HCV RNA quantity for 7 days post inoculation but our attempt failed to demonstrate any increase in HCV RNA although there is a possibility that incubation of the cells for a longer period might produce evidence of virus replication. Studies of the virus JFH1 which has been shown to replicate in Huh7 and Huh7.5.1 cells show HCV replication decreasing in the first week but increasing thereafter (Zhong, 2005). It would, therefore, be interesting to monitor cultures inoculated with S6b LLVP over a longer period.

Using a strand-specific RT-PCR, Fournier et al showed replication of HCV serum samples in primary adult human hepatocyte as assessed by the intracellular negative-strand RNA which appeared at day 1 post–infection peaking at day 3 and day 5 (15-fold amplification) followed by a decrease until day 14 (Fournier et al., 1998). In another study using a competitive RT-PCR and human fetal hepatocytes, the highest level of replication was detected 30 days' post-infection with 5.5 x 10^3 RNA copies per ml for cell-free virus and 38,650 RNA copies per 6 x 10^5 infected cells (Iacovacci et al., 1997). Ito *et al* monitored HCV RNA until day 14 and found that the RNA increased from day 1 to day 4 and falling off subsequently in IMY-N9 cells developed

from a fusion of human hepatocytes with HepG2 cell but not in the parent HepG2 cells (Ito et al., 2001). Five clones of MT-2 cells were found to have intracellular positive-stranded RNA detected until 21-30 days post-infection, peaking at day 4 until day 11 at least (Mizutani et al., 1996). In a kinetic study of HCV replication in long term cultures of human hepatocytes, positive-strand RNA was detectable after day 8 and day 12 post-infection in cells whereas secreted viral RNA became detectable at day 10 gradually increasing during the 3 months of culture with RNA quantities ranging from 955 at day 12 post-infection to about $60x10^3$ ge/ml at day 90 (Rumin et al., 1999).

It is still unknown why serum-derived HCV replicates poorly in primary human hepatocytes and hepatoma cells *in vitro* (Burlone and Budkowska, 2009). Bartenschlager *et al* (Bartenschlager, 2000) had difficulty trying to produced HCV from cells transfected with RNA transcribed from a cDNA copy of the viral genome. Successful replication of full length genome replicons failed to produce infectious virus particles in culture (Blight, 2002, Pietschmann, 2002).

Investigations to date have shown that different *in vivo* sources of HCV behave differently in attaching to HepG2 cells. Serum derived virus is in short supply and may be complexed with antibody. S6 liver derived virus is complex and it has not proved possible to determine the biological significance of the observed binding patterns. An infectious LVP is required to study the productive attachment pathways. The only infectious HCV systems to date are JFH1 and J6/JFH in Huh7.5 cells with the later being the highest yielding system available as reviewed in section 1.10.2. There are similarities between serum derived HCV and J6/JFH HCVcc as the density

of the virus particles is heterogeneous with peak infectivity found in the lower density fractions (Lindenbach et al., 2005) and there is a similarity in the assembly of JFH1 HCVcc with the production of VLDL as HCVcc assembly depends on microsomal transfer protein (MTP) and apoB100 (Gastaminza et al., 2008). Huang et al (Huang et al., 2007b) also showed a close association between HCV production and VLDL assembly demonstrating that an inhibitor of MTP and siRNA inhibition of apoB, both of which are necessary for VLDL assembly, caused reduction in HCV production. The lower density fractions of HCVcc in Huh7.5 cells may thus represent infectious LVP and their study could allow the elucidation of the mechanism of attachment and binding of LVP to hepatocytes.

6.10 The J6/JFH1 system

When this work began, the HCV subgenomic replicon had been widely used in the HCV studies. This was followed by robust HCV replication in Huh7.5 cells demonstrated by Wakita (Wakita et al., 2005) following transfection of the JFH1 virus, genotype 2a strain in the Huh7 cell line. Lindenbach (Lindenbach et al., 2005) have demonstrated more extensive replication of a chimeric virus J6/JFH (genotypes 2a) in Huh7.5 cells to produce infectious viral particles *in vitro*. Huh7 cells are human hepatoma cell line that has the ability to secrete VLDL (Higashi et al., 2002, Higashi et al., 2003, Lalanne et al., 2005) but the involvement of host lipoprotein production pathways in the assembly and release of virus in these systems is unclear. It has been suggested that feeding liver cell lines with fatty acids may stimulate VLDL secretion. Oleate increases the secretion of VLDL by 425% in HepG2 cells (Dashti and Wolfbauer, 1987) and in Caco-2 cells, there is a 55-fold increase in the concentration of VLDL (Dashti et al., 1990). We, therefore, decided to attempt to

generate infectious LVP from the J6/JFH1 system by feeding the infected cells fatty acid in order to boost host lipoprotein production.

Attempts made to reproduce the J6/JFH1 system in our laboratory encountered a few problems. Initially, the transformation of the wild-type pFL-J6/JFH1 DNA, and also mutants pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND DNA, which were kindly provided by Prof. Charlie Rice, went smoothly enough as the transformation were successful and we were able to get the said DNA. Initially, the plasmids were checked for correct sizes by using restriction enzymes that would cut at the appropriate sites as labelled on the pFL-J6/JFH1 restriction map kindly supplied together with the plasmids. The predicted fragment sizes by the restriction enzymes used are; EcoRI digest should produce a band of about 12kbp, EcoRI and XbaI double digest should generate 2 bands of about 10kbp and 2.5kbp, and KpnI and XbaI double digest should generate 2 bands of about 8kbp and 4kbp. Restriction enzyme of the miniprep DNA from the three different clones gave the sizes predicted.

Partial sequencing of NS5B region (from 7659 until 7893 nucleotides using SI-3 and SI-4 primers and from 8523 until 8754 nucleotides using SI-1 and SI-2 primers) as well as the 5' NTR region (22 until 111 nucleotides using SI-5 and SI-6 primers) of the pFL-J6/JFH1 and the two mutants pFL-J6/JFH1-H2476L and pFL-J6/JFH1-GND was successfully done to confirm the base changes of the mutants compared to that of the wild-type, to make sure there had not been any mix-up of the three different clones, and to demonstrate both the correct orientation of the plasmid and the correct alignment of T7 promoter.

Attempts were then made to to transcribe HCV RNA from pFL-J6/JFH1 and pFL-J6/JFH1-GND plasmids. No further work was done with pFL-J6/JFH1-H2476L. A problem arose during the initial purification of linearized plasmids for in vitro transcription which was done by running the XbaI-digested plasmids on 0.7% agarose gel, cutting out the 10kbp band, electro-eluting, and phenol-chloroform extracting / ethanol precipitating (purification 1). XbaI digestion of pFL-J6/JFH1 DNA gave a relatively clean single band but pFL-J6/JFH1-GND reproducibly gave an incomplete digestion which was unexpected. This problem remains unexplained as the structure of the plasmid pFL-J6/JFH1-GND DNA, which differs only from the wild type by a single point mutation should not be very different from the wild-type pFL-J6/JFH1 DNA as it is unlikely that the point mutation that is far from the XbaI digestion site could make a structural change to the plasmid DNA.

Purification of pFL-J6/JFH1-GND DNA before XbaI digestion, to remove impurities left during the maxiprep digestion which might cause the incomplete digestion, failed to rectify the problem. Attempts to completely digest pFL-J6/JFH1-GND DNA by using XbaI enzyme from a different company, increasing the incubation time, and increasing the enzymes concentration also failed.

Only after doing a double digestion of pFL-J6/JFH1-GND DNA with XmnI + XbaI or SspI + XbaI enzymes was it possible to fully digest the DNA. This suggests that during the maxiprep digestion, the structure of pFL-J6/JFH1-GND DNA was such that the XbaI digestion site was hidden from XbaI enzyme and was exposed only when linearization was first done by a different enzyme such as XmnI and SspI.

Gel purification produced a single clean DNA band for both pFL-J6/JFH1 and pFL-J6/JFH1-GND plasmids but these products yielded only a smear of RNAs around 1.8kb in the *in vitro* transcription reaction instead of a 9.6kb single band representing the full genome of HCV. The adoption of the purification 2 method after the XbaI digestion of pFL-J6/JFH1 DNA plasmid resulted in the full *in vitro* transcription of pFL-J6/JFH1 DNA.

The transcribed RNA was electroporated into Huh7.5 cells which were screened at intervals post-electroporation for HCV RNA by qRT-PCR. Following optimization of the electroporation/transfection conditions, there was an increase in HCV RNA output for J6/JFH1 discernable from day 7 but not for J6/JFH1-GND RNA transfection. As the mutant with J6/JFH1-GND genome has a defective RNA polymerases activity of NS5B, it would not be expected to synthesize new RNAs after the transfection.

These results may be compared to those of Lindenbach *et al* (Lindenbach et al., 2005), who, using qRT-PCR, could detect 359 $\pm$ 62 relative amount (to that of J6/JFH1-GND) of J6/JFH1 HCV RNA as early as 48 hours using only 1μg of RNA transcripts per transfection. We could detect 2.5 X 10^3 relative amount only at day 7 using 10μg of RNA transcripts. Using Northern hybridization, Wakita *et al* could only detect degraded input JFH1 RNA 12h after transfection but full length JFH1 RNA was detected 24h post-transfection and this remained detectable up to 72hours but none from JFH1/GND replication-incompetent mutant (Wakita et al., 2005).

That the HCV RNA detected in the supernatant from day 7 was indeed infectious HCVcc was established by infection of naïve Huh7.5 cells with supernatants from transfected cultures. HCVcc RNA output increased a few days after the frozen, thawed, and centrifuged supernatant sample of the J6/JFH1 transfected Huh7.5 cells was used to inoculate naïve Huh7.5 cells

To demonstratre de novo viral protein synthesis as well as genome replication Lindenbach showed that NS5A expression could be transferred to naive Huh7.5 cells by the FL-J6/JFH-transfected culture media after the media from these cultures were clarified by centrifugation and filtration (Lindenbach et al., 2005). In our studies, an indirect immunofluorescence experiment using anti-E2 HCV AP33 and anti-core HCV ϕ126 monoclonal antibodies showed foci of intense apple-green immunofluorescence staining in a fixed monolayer of red counter-stained Huh7.5 cells showing E2 glycoproteins and core proteins of HCVcc RNA being translated and produced in the Huh7.5 cells.

A growth curve carried out to compare if the HCVcc grew better in 10% FCS or 3% FCS showed that a lower percentage serum was beneficial. 3% FCS in the medium increasingly enhanced the HCVcc production up to 94 fold at day 5 compared to that containing 10% FCS. This was however done once only. FCS is made up of pooled material containing undefined factors from different source which may have different influences on HCV RNA replication and infection (Abe et al., 2007). Other authors have found that increased concentration of FCS favour replication of HCV replicons. Thus Abe *et al* reported that HCV RNA replication in OR6, a genome-length HCV RNA (strain O of genotype 1b) with Renilla luciferase as a reporter in Huh7 cells,

luciferase activity, which correlated with HCV RNA replication, increased with increasing percentage of FBS in the growth medium (Abe et al., 2007). Delipidation of FCS also has also been reported to have a detrimental effect on its ability to support HCV replicon replication. Thus Huang et al (Huang et al., 2007a) showed that the HCV RNA of HCV replicons (Huh7-K2040 cells) in Huh7 cells in delipidated FCS decreased about three fold compared to in 10% FCS. The contrast between these studies and the results reported here suggests that higher concentrations of serum inhibit elements of the infectious cycle not shared by the replicon and the full length infectious virus, such as assembly, release and cell-to cell transmission.

6.11 Production of very low density lipoproteins by HepG2 and Huh7.5 cells treated with oleic acid

LVP seem to be hybrid particles in which hepatitis C virions are closely associated with host VLDL (Nielsen et al., 2008). It has been shown in earlier studies that Huh7 cells are able to produce VLDL (Higashi et al., 2002, Higashi et al., 2003, Lalanne et al., 2005). In Caco-2 cell (human-derived intestine cell line), oleate increased the production of VLDL by 55-fold (Dashti et al., 1990). Oleic acid could also stimulate VLDL production in HepG2 cells (Arrol et al., 2000). Here, we have set out to increase production of VLDL by feeding oleic acids to Huh7.5 cells, which are the only cell type capable of supportive robust HCV replication in culture. Initially however, it was important to assess the toxicity of oleic acid in cell cultures. Huh7.5 cells were chosen for toxicity studies. Oleic acid with a concentration of up to 0.4mM was positively beneficial for Huh7.5 cell survival and there was no observable toxic effect on Huh7.5 cells when grown in serum free medium with 0.4mM and also 0.8mM oleic acid.

Next, VLDL secretion was monitored following oleic acid treatment of both Huh7.5 and HepG2 cells. Triglyceride lipoproteins secreted by hepatocytes can be divided into two forms - LDL, of density between 1.019-1.063g/ml, which contain apolipoprotein apoB100 but not apoE, and the larger, less dense VLDL of 0.95-1.006g/ml which contain both apolipoproteins (Gotto et al., 1986, Ginsberg, 1998). Accordingly the output of oleic acid treated and control cells was fractionated by iodixanol density gradient centrifugation and characterised using Coomassie Blue staining and Western blotting to detect apoB100 and apoE output, confirmed by MALDI-TOF analysis.

When treated with BSA plus oleic acid, HepG2 cells were found to secrete apoB100 proteins only in fractions with the density of LDL (1.063g/ml) but not in the VLDL fractions (<1.006g/ml). Trace amounts only of apoE proteins were detected by Western blot in both fractions. Treatment with BSA alone did not produce any bands in either fraction.

However, in Huh7.5 cells, both apolipoproteins were found in the VLDL fractions of cultures treated with oleic acid but not in control cultures treated with BSA. In the LDL fraction, a strong band of apoB100 but only trace amounts of apoE proteins was seen.

This data suggests that oleic acid treated Huh7.5 cells produce a VLDL-like particle containing both apoB-100 and apoE lipoproteins with a density of <1.006g/ml. HepG2 cells produced an LDL-like particle containing an apoB-100 lipoprotein which

is consistent with previous observations that apoB accumulates in the tissue culture medium when HepG2 cells were treated with 0.4mM oleic acid (Arrol et al., 2000).

6.12 Band identification in very low density lipoproteins 1, 2, and 3 from Huh7 and Huh7.5

To further characterize the putative VLDL release by Huh7.5 cells cultured in oleic acid, the VLDL fraction was further characterized by separation into VLDL 1, 2, and 3 fractions with Western blotting for apoB100 and apoE. In these experiments, we used Sodium Chloride flotation ultracentrifugation to separate VLDL1 ($S_f > 100$), VLDL2 ($S_f > 60$) and VLDL3 ($S_f > 20$) with VLDL 1 being the lowest density and the largest VLDL. *In vivo,* hepatocytes secrete both VLDL1 or VLDL2 but not VLDL3 (Olofsson and Boren, 2005).

In our experiments, both Huh7.5 and Huh7 cells produced apoB-100 and apoE lipoproteins in the VLDL 3 (the highest density VLDL) fraction but not in VLDL1 and VLDL2 fractions. Fatty acids stimulate the VLDL synthesis and secretion in a manner dependent on the amount and types of fatty acids available (Homan et al., 1991). Oleic acid has only one cis double bond which probably makes the formation of VLDL structure more compact and, hence, denser in comparison to other unsaturated fatty acids for example arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which has more cis double bonds. Our experiments show that addition of oleic acid to the medium of Huh7.5 cells induces synthesis of particles with the characteristics – density and apolipoprotein content – of VLDL3. This system is thus a promising area for further investigation on the genesis of LVP *in vitro*.

6.13 Characterization of HCVcc by density and size

6.13.1 Infection and culture of the J6/JFH1 HCVcc in Huh7.5 cells in the presence or absence of oleic acid

J6/JFH1 HCVcc infected Huh7.5 cells were found to have a somewhat greater amount of HCVcc RNA in the culture supernatant when the infected cells were grown in 3% FCS medium with oleic acid compared to medium without oleic acid. When cells were cultured in serum free medium in the absence of oleic acid, yields of viral RNA were similar to cultures with 3% serum but, in the presence of oleic acid, yields were increased over two fold. This result is in accordance with the finding of Kapadia and Chisari (Kapadia and Chisari, 2005) using SfiI HCV full-length replicon (genotype 1b) in Huh7 cells. They showed that polyunsaturated fatty acids (PUFAs) arachidonic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids inhibited HCV replication whereas saturated lauric, myristic, and palmitic and monounsaturated oleic fatty acids induced HCV replication. The study showed that HCV replication was highest with oleic acid compared to other fatty acids mentioned and 150% higher compared to the mock sample.

In the characterization of the density of J6/JFH1 HCVcc from infected Huh7.5 cells, it was found that in the absence of oleic acid treatment, HCVcc RNA peaks at fraction 12 at density 1.146g/ml. These results are in agreement with the Lindenbach's findings who reported the size of J6/JFH1 HCVcc peak at density 1.13 to 1.14g/ml when HCVcc was harvested from Huh7.5 cells grown in complete DMEM medium (Lindenbach et al., 2005). Gastaminza *et al.* (Gastaminza et al., 2006) found that extracellular particles of the JFH1 HCVcc have a buoyant density that ranges from 1.03g/ml to 1.16g/ml with peak HCVcc RNA at 1.15g/ml. In our experiment, in the

presence of oleic acid treatment, the peak shifted to a lower density of 1.135g/ml. The shift of J6/JFH1 HCVcc to a lower density may reflect the association of HCVcc with VLDL3 secreted by oleic acid treated Huh7.5 cell. If that is the case, it is not clear why there is only a small difference in the density (0.011g/ml) of the HCVcc produced by the oleic treated compared to the non-oleic treated Huh7.5 cell.

If the decrease in density of HCVcc recovered from OA treated cells is associated with association of the viral particles with host lipoprotein or host lipoprotein of increased lipid content and thus lower density, it is expected that there will be a concommitant shift of smaller sized to a bigger sized HCVcc. However, gel filtration analysis revealed no shift in HCVcc RNA peak in oleic acid treated compared to the non-oleic treated infected Huh7.5 cells. The peak of HCVcc occurred somewhere in between VLDL1 and VLDL2 indicating that the viral particle is already rather larger than a VLDL3. It is not clear if the Toyopearl column is capable of resolving the increase in size of HCVcc/VLDL3 aggregation.

6.14 Conclusion

The overarching hypothesis is that HCV complexed with host VLDL as LVP binds to and enters cells via a viral glycoprotein independent mechanism using host receptor: ligand interactions. This hypothesis is difficult to test with LVP purified from clinical material. Virus recovered from serum of infected patients is limited in supply and heterogenous in density and immunoglobulin content and the biological significance of this heterogeneity is unknown. Following early studies by Caroline Martin, I have attempted to study the uptake of liver derived LVP semi-purified from the liver macerate of an agammaglobulinaemic patient by density gradient centrifugation.

Although, Martin has shown that LLVP binds in a similar way to LDL with increased glycosaminoglycan dependent binding to LPDS / insulin treated cells with enhanced LDLr expression, blocked by incubation with excess LDL, my experiments have shown that anti-apoB100 and anti-apoE could not block the uptake of LLVP into the HepG2 cells. As oxLDL, thought to bind to hepatocytes via SR-B1, could also block LLVP binding, I explored the hypothesis that LLVP binds via SR-B1. However, evidence that oxLDL binding was glycosaminoglycan dependent, like LLVP was unconvincing, and there was no evidence of increased SR-B1 expression in LPDS / insulin treated cells which resulted in increased LLVP binding. Further attempts to show that LLVP binding could be blocked by anti-E2 antibodies, reported to block HCV SR-B1 interactions, were negative. LLVP binding studies were beset with difficulties due to two main weaknesses; 1) studies parallel to these by (Nielsen et al., 2008) showed that the predominant HCV RNA containing particle in LLVP preparations is probably a microsomal fraction of infected cells rather than virion 2) the biological relevance of the observed binding assessed by RT-PCR detection of HCV RNA associated with hepatocytes after washing cannot be assessed.

The switch to an assessment of binding by infectivity would circumvent both weaknesses of this system but LLVP were not infectious for either HepG2 cells or Huh7.5 cells. LLVP were therefore abandoned and an attempt was made to develop HCV LVP in cell culture. The subsequent part of the study aimed initially to set up the J6/JFH1 infectious system followed by testing the hypothesis that HCVcc treated with a monounsaturated fatty acid, oleic acid, could result in production of a VLDL associated HCV or an *in vitro* LVP. Oleic acid treatment of Huh7.5 cells resulted in the secretion of lipoprotein particles carrying apoB100 and apoE and of a density

similar to VLDL3. Oleic acid treatment of Huh7.5 cells infected with J6/JFH1 resulted in the secretion of higher concentration of HCVcc RNA and this was associated with a shift in the HCVcc RNA peak towards lower density fraction. The HCVcc RNA was also characterized by size and was found to be larger than the VLDL3 secreted by uninfected Huh7.5 cells. There was, however, no discernible increase in size of the lower density HCV RNA containing particles secreted by oleic acid treated cells and it remains unclear whether the shift in density represents association of HCVcc with host lipoprotein.

6.15 Future Work

Studies using SfiI HCV full-length replicon (genotype 1b) showed that oleic acid, a monounsaturated fatty acid together with saturated (lauric, myristic, and palmitic) fatty acids induced HCV RNA replication as opposed to polyunsaturated fatty acids (arachidonic, eicosapentaenoic acid, and docosahexaenoic acid) (Kapadia and Chisari, 2005). Our study yield similar results when J6/JFH1 infected Huh7.5 were treated with oleic acids. The major problem to be addressed in future work is the full characterization of putative LVPcc produced by oleic acid treated J6/JFH1 infected Huh7.5 in comparison with higher density particles in the same preparations and in untreated infected cells.

In this study, RNA replication peaks in fraction with density of 1.135g/ml with oleic acid treatment and 1.14g/ml in the absence of oleic acids. It would be useful to know if these peaks in RNA replication actually coincides with the highest production of infectious HCVcc and an infectivity assay would provide the means to get this measurement. Particle:infectivity ratios will give some insight into the biological relevance of the shift of the density. Immunoprecipitation studies with anti-

apolipoproteins B100, E and C will determine whether the shifted particle is a hybrid virus / host lipoprotein. Neutralization studies with antibodies to the viral glycoproteins may demonstrate whether or not reduced density is associated with resistance to neutralization. Should these studies confirm that the output of the oleic acid treated J6/JFH1 system do represent LVP binding studies, assessing the role of LDLr and SR-B1 can be resumed.

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