Telaprevir as an effective treatment for
genotype 3 Hepatitis C

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Submitted in partial fulfilment of the requirements of the Degree of
Doctor of Philosophy
Statement of originality

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This thesis decides work completed by me with collaborations as listed below:

**Chapter 4**
Clinical trial sites were the Royal London Hospital (Chief Investigator- Professor Graham Foster), St. George’s Hospital, London (Principal Investigator- Dr Daniel Forton), Queens Medical Centre, Nottingham (Principal Investigator- Dr Steve Ryder), Bradford Royal Infirmary (Principal Investigator- Dr Sulleman Moreea)

**Chapter 6**
Next generation sequencing was performed by Dr Ana Da Silva Felipe and Professor John Mclauchlan at the MRC Centre for Virus Research, Glasgow
Publications relating to this thesis

Review article


Peer reviewed abstracts presented as oral presentations at scientific meetings


Peer reviewed abstracts presented as posters at scientific meetings

Abstract

Genotype 3 hepatitis C accounts for 35% of cases of chronic hepatitis C infection in the United Kingdom. At the time this work was commenced there were limited treatment options for patients with genotype 3 hepatitis C with advanced liver disease who had failed treatment with interferon and ribavirin. Approximately 30% of patients with genotype 3 hepatitis C demonstrated a significant virological response on a clinical trial with two weeks of treatment with telaprevir monotherapy.

A novel in vitro capture fusion assay had been developed by our group which enabled assessment of in-vitro hepatitis C virus (HCV) sensitivity to antiviral drugs and indicated that sensitivity to telaprevir could be pre-determined by viral phenotyping.

A multi-centre open label clinical trial was undertaken to evaluate whether the addition of twelve weeks of treatment with telaprevir to standard treatment with 24 weeks interferon and ribavirin was of benefit to patients with advanced liver disease who had previously failed treatment with interferon and ribavirin and whether the ‘capture fusion’ assay could identify patients likely to respond. In addition, we assessed the value of next generation sequencing as a predictor of clinical response.

The main findings were that four out of fourteen patients (29%) achieved an SVR. The capture fusion assay identified two of these four patients as having HCV that was sensitive to telaprevir in vitro. No pre-treatment substitutions were identified on next generation sequencing that correlated with the clinical outcome of treatment.

Trial recruitment was discontinued when sofosbuvir containing all oral regimens became available for patients eligible to participate in the trial. Although telaprevir may be of benefit to a proportion of patients with genotype 3 hepatitis C, advances in the treatment of hepatitis C have resulted in newer, more potent, direct acting antiviral drugs superseding telaprevir.
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Chapter 1: Introduction

1.1 Hepatitis C virus discovery

The hepatitis C virus (HCV) was first identified in 1989, and is a significant cause of chronic liver disease worldwide. Up to 170 million people may be affected (approximately 2 to 3% of the global population). Most hepatitis C infected patients (85%) develop chronic infection, and 20% of these individuals will develop liver cirrhosis after 20 years of infection. This can potentially lead to significant morbidity and mortality, with decompensation of liver disease, hepatocellular carcinoma, and the need for liver transplantation.

1.2 Viral structure and replication

Hepatitis C virus is a positive strand RNA virus in the flaviviridae family with a genome approximately 10,000 nucleotides long. This encompasses an open reading frame (ORF) that is translated via an internal ribosome entry site located in HCV 5’ untranslated region (UTR) resulting in a polyprotein approximately 3000 amino acids long. This polyprotein is processed by cellular (signalase and signal peptidase) and viral proteases (NS2-NS3 and NS3-NS4A) into structural (core, E1, and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (figure 1.1).

The core protein contains two domains. The first domain is involved in RNA binding and nucleocapsid formation, while the second domain is thought to mediate the interaction between the HCV core protein, lipid droplets and the endoplasmic reticulum. The remaining structural proteins encoded by the HCV genome are the envelope glycoproteins E1 and E2. Both E1 and E2 are transmembrane proteins. They interact to facilitate HCV binding to cell surface proteins on hepatocytes to enable viral entry into cells, and fusion of the viral lipid envelope with the host endosomal membrane. The p7 protein can form ion channels in membranes and is from a group of viral proteins called viroporins.
These proteins modulate membrane permeability to facilitate virus entry, assembly or release. The NS2 protein is involved in HCV polyprotein processing and particle formation. It comprises of a membrane domain which is involved in viral particle formation and a protease domain. The protease domain is involved in the cleavage of the viral protein between NS2 and NS3. The HCV NS3 protein is a 70kDa protein which comprises of an N terminal protease (with NS4A acting as a cofactor) and a C terminal helicase. The NS3 protease is involved in viral polyprotein processing and is needed for cleavage of the viral polyprotein at NS3/NS4, NS4/NS5 and NS5A/NS5B. The protease component of NS3 is a serine protease, which forms a chymotrypsin like fold with two β barrel subunits. A zinc ion (Zn$^{2+}$) stabilises the structure with a binding site comprising of Cys 97, Cys 99, Cys 145 and His 149. The catalytic triad of the NS3 protein is formed by His 57, Asp 81 and Ser 139 (figure 1.2). NS4A is involved as a co-factor for the NS3 protease by providing one of the β strands for the N-terminal β barrels of the NS3 protease. The N terminal portion of NS4A is hydrophobic and forms a transmembrane α helix which ensures that the NS3/4A complex is membrane associated. The NS3/4A protease binding site accommodates six amino acids (although...
proteolytic activity is greatest when substrates include 10 amino acids). In addition to its role in processing the HCV polyprotein, the NS3/4A protease also targets host substrates and is involved in modulating the host immune response to HCV infection.

Figure 1.2 A) NS3 domains and catalytic triad B) NS3 crystal structure (amended from Morikawa 2011)

NS4B is an endoplasmic reticulum (ER) membrane associated protein which is involved in remodelling of the ER to form a membranous web\(^1\), which is needed for viral replication. The NS5A protein comprises of three domains and has multiple functions including RNA replication (domains I and II) and viral packaging (domain III)\(^2\). It interacts with several host proteins to achieve these functions, including cyclophilin A and phosphatidylinositol- 4 kinase II\(\alpha\)\(^{23,24}\). The final non-structural
protein is NS5B which is an RNA dependent RNA polymerase (RdRp). The NS5B is similar to other viral RdRp enzymes and has finger, palm and thumb subdomains, with highly conserved RdRp motifs in the palm region. The NS5B protein plays an integral role in RNA replication and therefore viral replication\textsuperscript{25–27}.

1.3 HCV life cycle

HCV virions are 50-80nm in diameter\textsuperscript{28}, with E1 and E2 glycoproteins embedded in the viral membrane surrounding a nucleocapsid composed of the core protein and the HCV RNA genome\textsuperscript{29}. HCV virions associate with low density and very low density lipoproteins in a lipoviral particle\textsuperscript{28}. It is notable that the HCV virion size and density is similar to that of very low-density lipoproteins and this may be due to proteins involved in the processing of lipoproteins also being involved in HCV infectivity, or the composition of HCV virions may enable the virus to evade the host immune response.

1.3.1 HCV cell entry and replication

HCV entry into hepatocytes involves many cellular molecules (an overview of the HCV intracellular lifecycle is shown in figure 1.3). The HCV envelope proteins E1 and E2 play an important part in HCV entry. They are involved in the binding of HCV to cell surface receptors, followed by the fusion of the HCV viral particle and the host cell endosomal membrane. The host cell surface proteins Scavenger Receptor B1\textsuperscript{30} (SR-B1), CD-81\textsuperscript{31}, Claudin-1\textsuperscript{32} and Occludin\textsuperscript{33} have been implicated in HCV cell entry. Due to the close association between HCV and lipoproteins, the low density lipoprotein receptor (LDLR) was thought to have been involved in HCV entry into cells, although this entry pathway does not appear to be involved in HCV infection\textsuperscript{34}. Similarly, the Niemann Pick C1-like protein 1 cholesterol transporter has been identified as being involved in viral entry although it’s role in HCV
entry is unclear\textsuperscript{35}. The E2 protein is thought to bind to CD-81, and interaction occurs between CD-81 and Claudin-1\textsuperscript{36}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{Overview of the intracellular lifecycle of HCV}
\end{figure}

Viral uptake into cells occurs via clathrin mediated endocytosis\textsuperscript{37}, and virions are then transported to early endosomes within cells\textsuperscript{38}. The HCV virion then undergoes membrane fusion in the endosomes and HCV RNA is translated at the endoplasmic reticulum. Following processing of the HCV polyprotein by host proteins (e.g. signal peptidase) and viral proteins (NS2, NS3/4A), HCV proteins associate with the endoplasmic reticulum. NS3/4A, NS4B, NS5A and NS5B form the viral replication complex. NS4B mediated changes in the endoplasmic reticulum occur to create a complex membranous web, and NS5A induces formation of double membrane vesicles\textsuperscript{21}. Host proteins such as phosphotidyl-inositol-4-kinase III, vesicle associated membrane associated proteins and cyclophilin A interact with HCV viral proteins to modulate the structure and lipid composition of the
membranous web. HCV affects the expression of host genes involved in intracellular lipid metabolism, resulting in the intracellular accumulation of lipid to aid HCV viral replication. In addition to the membranous web, lipid droplets (containing triglycerides and cholesterol esters) also play an important role in viral replication and virion assembly.

The HCV core protein interacts with the HCV genomic RNA to form the nucleocapsid. HCV core protein then interacts with lipid drops and results in the recruitment of other viral proteins needed for virion assembly (in particular NS2, NS3, and NS5A). NS2 acts a scaffold co-ordinating the interaction between structural and non-structural proteins to lead to encapsidation of viral RNA.

The production of viral particles is closely linked to the pathway used for production of VLDL. Many apolipoproteins are associated with the HCV viral particles (including apolipoprotein A, B, C and E), although apolipoprotein E is thought to be the most important in affecting viral infectivity.

Following formation of viral particles at the endoplasmic reticulum, virions are transported to the Golgi apparatus and subsequently secreted (in a manner similar to VLDL secretion). Intracellular vesicular trafficking involves proteins such as the Rab GTPases and vesicular associated membrane protein 1 (VAMP1) which facilitate virion transport to the cell membrane and subsequent release of viral particles.

1.4 Epidemiology

The prevalence of hepatitis C infection worldwide is approximately 1.3-3.0%, corresponding to 92-170 million infected individuals. Historically, the major sources of HCV transmission were exposure to contaminated blood or blood products (in particular affecting patients with inherited bleeding conditions who received pooled blood products infected with hepatitis C), use of illicit injectable drugs and nosocomial infections (due to re-use and inadequate sterilisation of needles, particularly in countries such as Egypt and Pakistan), along with the risk of transmission between patients who receive haemodialysis). In Egypt the use of unsterilised needles in the treatment of
endemic shistosomiasis has resulted in a HCV prevalence of over 14%. In the United Kingdom an estimated 215,000 individuals have chronic hepatitis C (approximately 0.4% of the UK population), with genotype 1 and 3 accounting for the majority of cases (45% and 44% respectively). The estimated prevalence of HCV infection in persons who inject drugs is 50%, and this accounts for the majority of current HCV transmission in the UK. Hepatitis C infection has an increased prevalence in particular demographic groups, with people of Asian or British Asian origin having an HCV prevalence of 2.2%, similarly people of Eastern European origin had a prevalence of 3.3% in the UK. Sexual and perinatal transmission of HCV is rare. The routine testing of donated blood since 1991 in the United Kingdom has almost eliminated the transmission of HCV via blood transfusion and products.

1.5 Natural history

The majority (approximately 85%) of patients who are infected with hepatitis C will develop chronic infection. As a result of this, approximately 20-25% of patients with chronic HCV infection will develop liver cirrhosis in the subsequent 20 years. In patients who have liver cirrhosis due to hepatitis C, 4% will develop decompensated cirrhosis every year, and in patients with decompensated cirrhosis annual mortality is approximately 15% without liver transplantation.  

Hepatitis C accounted for 16% of liver transplants undertaken between 1996 and 2012 in the United Kingdom. In addition to decompensation, 1.5-2.5% of patients with HCV related liver cirrhosis will develop hepatocellular carcinoma each year. Patients with concomitant steatohepatitis (both alcohol and non-alcohol related) or hepatitis B or HIV co-infection are at increased risk of liver disease progression. In addition to causing chronic liver disease, hepatitis C infection is also associated with extrahepatic conditions. These include: haematological conditions such as cryoglobulinaemia and non-Hodgkin’s lymphoma; general systemic symptoms including fatigue, arthralgia and fibromyalgia as well as other rare conditions such as vasculitis and porphyria cutanea tarda.
### 1.6 HCV genotypes

Six major genotypes of Hepatitis C have been identified (genotypes 1-6), and recently a seventh has been proposed. The HCV RNA polymerase has a high error rate that results in error prone replication and therefore a variety of Hepatitis C subtypes exist within each of these genotypes (figure 1.4).

![Phylogenetic tree of the 6 major HCV genotypes and subtypes](image)

**Figure 1.4** Phylogenetic tree of the 6 major HCV genotypes and subtypes identified for each genotype (adapted from Simmonds et al. 2005)

The sequence divergence between genotypes is approximately 30%, whilst sequence divergence between subtypes can be up to 20%\(^2\). The distribution of each of these genotypes varies by geographical regions\(^3\) (figure 1.5). Globally, genotype 1 hepatitis C accounts for the greatest proportion of HCV infection (46%), followed by genotype 3 (22%), genotype 2 (13%), genotype 4 (13%), genotype 6 (2%), and genotype 5 (1%), with the remaining 3% comprising of patients with undefined/combination infections. There are, however, significant regional variations with genotype
1 accounting for 62-71% of infections in Europe and North America, whilst genotype 3 accounted for a greater proportion of infections in South Asia (39%).

Figure 1.5 Prevalence of different HCV genotypes in different global regions (adapted from Messina et al. 2015)

In the middle east genotype 4 hepatitis C accounted for 71% of infections, although this is in part due to the high prevalence of genotype 4 hepatitis C infection in Egypt; once Egypt has been excluded the proportion of genotype 4 hepatitis in the middle east is lower (34%)46.

1.7 Immune response to HCV infection

1.7.1 Innate immunity

Innate immune responses are the first line of defence against infection. Interferons (IFNs) are the central cytokines in triggering an antiviral state in cells along with activating other immune cells involved in the innate immune response. Type I IFNs (IFNα and IFNβ) and Type III IFNs (IFNλ1 (IL29), λ2 (IL28A), and λ3 (IL28B)) are produced by cells infected by viruses and by macrophages and dendritic cells. Macrophages and dendritic cells sample material from their surroundings and can detect viral fragments or intact viral particles. Type II IFN (IFNγ) is produced by natural killer cells and
natural killer T cells as part of the innate immune response and by antigen specific T cells (CD4+ Th1 and CD8+ cytotoxic T lymphocytes)\textsuperscript{54,55}. Viral infections are sensed by the toll like receptor (TLR) dependent pathway and by binding of viral RNA to retinoic acid inducible gene-1 (RIG-1) and melanoma differentiation antigen 5 (MDA5)\textsuperscript{56,57}. These pathways result in activation of nuclear factor κβ (NF-κβ) and interferon regulatory factor (IRF) 3 and 7. IRF3 and NF-κβ result in transcription of genes responsible for the Type I and Type III IFN response\textsuperscript{58,59} (figure 1.6).

\textbf{Figure 1.6 Simplified overview of the innate immune response to HCV infection}

The interferons produced bind to IFN receptors (comprising of interferon alpha/beta receptor subunits 1 and 2 (IFNR1 and 2)) which connect to the Jak-STAT pathway and result in transcription of IFN stimulated genes (ISGs). Signalling through the Jak-STAT pathway is regulated by inhibitors such as suppressor of cytokine signalling (SOCS)\textsuperscript{60} and ubiquitin specific peptidase 18 (USP-18)\textsuperscript{61}.
Early on during HCV infection ISG induction is thought to be related to the HCV viral load\textsuperscript{62}. In patients with chronic HCV infection, however, ISG induction varies markedly and is unrelated to viral load. Allelic variants near the IFNλ4 locus are associated with ISG induction\textsuperscript{61}, and it is postulated that the HCV NS3/4A protease cleaves mitochondrial anti-viral signalling protein (MAVS)\textsuperscript{64} and the Toll-IL-1 receptor-domain containing adaptor-inducing IFNβ (TRIF)\textsuperscript{65}. These molecules are involved in the signalling pathways needed for activation of interferon stimulated genes, and by inactivating them HCV manages to interfere with the host innate immune response. Interestingly patients with chronic hepatitis C with an activated interferon system are poor responders to IFNα based treatment. Treatment with IFN in these patients did not increase ISG expression in hepatocytes. USP18 is thought to play an important role in this process\textsuperscript{66}.

Genetic variants near the $IFN\lambda3$ and $IFN\lambda4$ gene have been identified which are associated with response to interferon-based treatment. Paradoxically, the alleles resulting in increased IFNλ4 production are associated with decreased spontaneous clearance of HCV and a lower SVR to treatment with pegylated IFN and ribavirin\textsuperscript{67,68}. This may be a result of increased interferon production exerting selection pressure resulting in the evolution of viral strains resistant to interferon in these individuals.

1.7.2 Innate cellular response

Natural killer (NK) cells are granular lymphocytes which account for the majority of the innate immune cells in the liver. They play an important role in the control of viral infections by either direct cytolytic (e.g. TRAIL or perforin mediated) or non-cytolytic (e.g. IFN and TNFα mediated) pathways\textsuperscript{69}. In chronic hepatitis C, NK cells are activated but may display alterations in phenotype with impaired anti-viral function\textsuperscript{70}. Part of this may be due to a decreased ability of NK cells from patients with chronic hepatitis C to secrete IFNγ.
1.8 Adaptive immune response

1.8.1 T lymphocyte response

Successful immune responses to HCV target a wide range of viral epitopes, likely minimising viral escape options, and are sustained until HCV clearance. In contrast, this broad T cell response often wanes in infections that progress to chronicity. T cell exhaustion is thought to contribute to persistent HCV infection\(^{15}\). Cytolytic activity of T cells is lost early in the development of T cell exhaustion\(^{71}\). Additionally, in chronic HCV, HCV specific CD4+ cells express higher levels of inhibitory co-receptors such as PD-1 which may impair CD8+ T cell function, resulting in impaired T cell proliferation and thereby providing a further mechanism by which the spontaneous HCV clearance rate is reduced\(^{72}\). The high HCV replication rate (up to \(10^{12}\) viral particles per day) in an infected individual, coupled with its error prone RNA polymerase generates a large pool of sequence variation in an infected individual. T cell responses targeting specific epitopes exert selection pressure, favouring variants lacking those epitopes. Viable mutations are those that maintain replicative fitness whilst permitting immunological escape.

1.8.2 B cell and antibody response

Antibody responses to HCV occur several weeks after the onset of viraemia and target structural and non-structural proteins. Neutralising antibodies targeting E1 and E2 develop in most infected patients. Acutely infected patients who spontaneously clear the virus develop broad, cross reactive neutralising antibodies\(^{73}\), while early neutralising antibody responses are weak in patients who progress to chronic infection\(^{74}\). HCV escapes from sterilising humoral immunity by several methods, such as rapid sequence variation, stimulating the production of interfering antibodies\(^{75}\), masking neutralisation epitopes (e.g. by glycosylation) and by concealing itself within lipoviral particles\(^{76,77}\). Many antibody responses towards HCV target the highly variable region near the amino terminus of E2, which may act as a decoy, while conserved neutralisation epitopes may be concealed.
Continuous generation of quasispecies furthermore enables viral escape from neutralising antibody mediated immunity.

High HCV viral replication with the generation of multiple quasispecies along with the ability of the virus to interfere with both the innate and adaptive immune response results in the majority of individuals infected with HCV developing chronic infection. Similarly, these mechanisms affect the response to treatments targeting the host immune response (e.g. IFN based treatment) and may account for the poor success rate of host targeting treatment in certain populations.

1.9 HCV inhibition of host antiviral response

HCV inhibits the host antiviral response via several mechanisms. In addition to their role in viral replication and packaging, many of the proteins that are encoded by the HCV genome also interfere with the host immune response (as demonstrated in figure 1.7).

![Diagram of HCV inhibition of the host antiviral response](image)

Figure 1.7 HCV inhibition of the host antiviral response
1.9.1 HCV core protein

HCV core protein interferes with the interferon signalling pathway and attenuates the host interferon response to HCV. STAT1 is involved in interferon signalling and leads to increased transcription of interferon stimulated genes (ISGs). HCV core binds to STAT1 inhibiting STAT1 phosphorylation and nuclear translocation thereby decreasing the induction of ISGs²⁸.

1.9.2 NS3/NS4A

Once HCV infection is detected, the host interferon response is induced by: pattern recognition receptors, the retinoic acid inducible gene-I (RIG I) and toll like receptor (e.g. TLR-3) pathways. The HCV NS3/4A protease cleaves host proteins such as mitochondrial antiviral signalling protein (MAVS)⁷⁹, and the toll/interleukin receptor domain containing adaptor inducing interferon β (TRIF)⁶⁵. TRIF is an adaptor protein for the RIG-I and TLR3 pathways that are stimulated by host interferons. This leads to inhibition of the interferon mediated immune response. The HCV NS3/4A also inhibits interferon regulatory factor 3 (IRF-3), a downstream component of the RIG-I and TLR3 signalling pathways, thereby further downregulating the transcription of interferon stimulating genes⁸⁰.

1.9.3 NS5A

HCV NSSA protein binds to and inactivates protein kinase R (PKR). This allows viral replication to occur during interferon treatment⁸¹. NSSA also has inhibitory effects on the JAK-STAT signalling pathway⁸². NSSA blocks the interferon stimulated response element (ISRE) promoter and STAT1 phosphorylation.
1.10 History of HCV treatment

1.10.1 Interferon

Interferons are glycoproteins produced in vivo in response to viral infections. Interferons inhibit viral replication through a variety of mechanisms. Interferons were initially noted to decrease serum transaminase levels in the 1980s in patients with non-A, non-B hepatitis. At that time a causative agent hadn’t been identified, and once HCV had been identified it became clear the impact on HCV viraemia was less pronounced than the improvement in liver biochemistry. Interferon monotherapy (with interferon injections three times a week) was noted to normalise ALT levels in up to 40% of patients with hepatitis C within the first three months of treatment. However, interferon monotherapy only achieved sustained virological response rates (with no detectable HCV RNA six months after treatment) of up to 15% with six months of interferon and 25% with 12 months of treatment. These studies led to the recognition that an early response to treatment (which occurred in the majority of patients), did not always correlate with sustained treatment response. This led to the concept of a sustained virological response (SVR) being developed to assess treatment success. An SVR was initially defined as no detectable HCV in serum 24 weeks post completion of treatment. More recently as it has become apparent that most patients who are non-viraemic 12 weeks post treatment progress to achieve an SVR, SVR 12 has been used as an outcome for more recent trials in the treatment of hepatitis C. As the success rates of treatment with interferon monotherapy were poor, adjunctive and alternative treatments were explored from the early 1990s.

1.10.2 Ribavirin

Ribavirin is a synthetic nucleoside analogue which resembles guanosine. It has in vitro activity against several DNA and RNA viruses including flaviviridae. The mechanism of action by which ribavirin inhibits HCV has not been completely determined. Hypotheses that have been proposed
regarding the mechanism of action of ribavirin include inhibition of RNA dependent RNA polymerases (RdRp), inhibition of the 5’ cap structure of viral mRNA and inhibition of the intracellular triphosphate pool. Treatment with ribavirin monotherapy resulted in normalisation of ALT in 40% of patients with HCV but did not markedly lower serum HCV viral levels. Secondly, once treatment with ribavirin was discontinued transaminase levels gradually increased. Therefore, as ribavirin monotherapy was not particularly effective, by the mid-1990s studies began evaluating the efficacy of ribavirin in combination with interferon.

1.10.3 Interferon and ribavirin

The combination of interferon and ribavirin was noted to have a synergistic effect on antiviral activity. Initial studies achieved an SVR rate of 40% in patients who received 12 months of combination therapy. In these studies, it was noted that the SVR rate was much lower in genotype 1 patients (29% with 12 months of treatment) compared to genotypes 2 and 3 (66% and 65% for six and 12 months of treatment). The presence of advanced liver disease was also noted to affect virological response, with fewer patients achieving SVR following 24 and 48 weeks of treatment if bridging fibrosis or cirrhosis was present. Additionally, in these trials of combined treatment with interferon and ribavirin, early treatment response was also noted to predict treatment outcome (with those patients who had a rapid virological response, with undetectable HCV DNA at week four, being more likely to achieve an SVR). In patients who had relapsed following treatment (with undetectable HCV RNA at the end of treatment), retreatment with interferon and ribavirin for six months resulted in a sustained virological response (SVR) of 49% (compared to only 5% in an interferon monotherapy group). In patients who were non-responders to treatment with interferon (with persistent Hepatitis C viraemia during treatment) retreatment with interferon α and ribavirin resulted in SVR rates of 18%. 


1.10.4 Pegylated interferon

The development of Pegylated interferon in 1999 further improved the efficacy of combined interferon and ribavirin treatment. The bonding of polyethylene glycol to interferon increased the half-life of interferon activity, enabling once weekly injections and increasing drug bioavailability. When used as monotherapy, pegylated interferon did not result in markedly improved SVR rates (23% after 48 weeks of treatment), although SVR rates were higher in patients with genotype 2 and 3 HCV with viral loads less than two million copies/mL serum\textsuperscript{93}. The combination of pegylated interferon (1.5µg/kg once weekly) and ribavirin (800mg daily) for 49 weeks resulted in a SVR of 54\%\textsuperscript{94}. In patients with genotype 1 or 4 hepatitis C treated with 48 weeks of peginterferon and ribavirin, the SVR rate in non-cirrhotic patients was 60\%, which dropped to 51\% if bridging fibrosis was present and down to 33\% if patients were cirrhotic. For genotype 2 and 3 patients (treated with 24 weeks of peginterferon and ribavirin) the SVR rate was 75\% if patients were non-cirrhotic, which dropped to 61\% if bridging fibrosis was present and down to 57\% if patients were cirrhotic\textsuperscript{95}. The STEPS trial\textsuperscript{96} evaluated 24 versus 48 weeks of treatment with pegylated interferon and ribavirin in patients with genotype 3 hepatitis C advanced fibrosis or cirrhosis. In this study SVR rates of 48 and 42\% were achieved with 24 and 48 weeks of treatment. In cirrhotic patients this dropped to 46 and 40\% respectively. Rapid virological response at week four was noted to be the only factor significantly associated with SVR\textsuperscript{96}.

For patients that did not achieve an SVR with initial treatment with pegylated interferon and ribavirin retreatment with pegylated interferon and ribavirin was investigated. The EPIC study\textsuperscript{97} evaluated the response to retreatment with pegylated interferon and ribavirin in patients who had previously failed treatment and who had evidence of liver fibrosis (METAVIR F2, F3 or F4). Patients with hepatitis C who had previously relapsed following prior treatment had an overall SVR rate of 23\% for genotype 1 and 57\% for genotype 3. In patients who had previously been non-responders to treatment, the SVR rates were 4\% for genotype 1 and 36\% for genotype 3. This study also identified
that treatment response at week 12 in this population correlated with the likelihood of achieving SVR. 56% of patients with undetectable HCV RNA at week 12 achieved an SVR while only 12% of those patients with detectable HCV RNA proceeded to achieve an SVR. Further studies evaluating retreatment of genotype 3 hepatitis C with pegylated interferon and ribavirin in patients who had previously failed treatment with interferon and ribavirin, resulted in SVR rates of 32% in patients with advanced liver fibrosis (METAVIR F3/F4), compared to 62% in patients with F0-F2 fibrosis.

1.11 Factors affecting treatment response

From these early trials, it was evident that genotype 1 had a lower SVR than genotypes 2 and 3 when treated with pegylated interferon α and ribavirin. High pre-treatment viral load (> 2 million), older patient age and the presence of bridging fibrosis or cirrhosis also adversely affected the likelihood of achieving an SVR. In a retrospective analysis of patients with genotype 3 hepatitis who were treated with pegylated interferon and ribavirin, factors that were associated with a failure to achieve SVR were increased age, increased weight or body mass index, or the presence of diabetes. More advanced fibrosis on biopsy, thrombocytopenia, or the presence of liver cirrhosis were also all associated with a failure to achieve an SVR. Finally, treatment dose reduction or abbreviation of treatment to less than 24 weeks was also associated with a failure to achieve an SVR.

1.11.1 Genetic factors affecting treatment response

It was noted during the 2000s that patients with African ancestry had lower SVR rates than those of European ancestry. Ge et al. identified a polymorphism on chromosome 19 near the interleukin-28B gene which encoded for interferon lambda 3 was associated with a difference in response to treatment with pegylated interferon and ribavirin in patients with genotype 1 hepatitis C. Polymorphisms at locus rs12979860 were associated with differences in treatment outcome. Patients with the CC genotype had a twofold greater likelihood of achieving an SVR compared to
patients with the TT genotype. Tanaka et al identified a further locus (rs8099917) 8 kilobases downstream from the IL-28B gene which was associated with treatment outcome in Japanese patients treated for genotype 1 hepatitis C. Patients homozygous for the major allele (TT) were more likely to achieve an SVR than those heterozygous or homozygous for the minor allele (TG/GG). Subsequent studies demonstrated that the CC genotype at rs12979860 was associated with an increased likelihood of patients achieving a rapid virological response.

Interferon lambda 3 (interleukin-28B) is a type III interferon and is produced in response to viral infection. Like type I interferons its signalling pathway includes JAK and STATs and results in the production of interferon stimulated genes (ISG). A key difference is that IFN-λ results in a more gradual increase in expression of interferon stimulated genes but also inhibits HCV viral replication independently. Studies evaluating the effect of IL-28B genotype on interferon signalling identified that patients with polymorphisms associated with positive treatment outcome had lower ISG expression at the start of treatment, and also had a higher baseline viral load compared to those patients with rs12979860 CT/TT or rs8099917 TG/GG. This pre-existing upregulation of ISGs had an adverse impact on the efficacy of exogenous interferon (such pegylated-interferon), resulting in a decreased likelihood of achieving an SVR.

As these observations were noted in patients with genotype 1 hepatitis C, the significance of these polymorphisms were evaluated in genotype 3 hepatitis C. A meta-analysis of trials where IL-28B genotype data was available identified no clear correlation between IL-28B genotype and treatment outcome in genotype 2 and 3 hepatitis C. In a further prospective trial, and in the STEPS trial, IL-28B genotype was not identified as a factor which significantly affected SVR in genotype 3. Holmes et al identified that ISG expression was significantly greater in HCV genotype 1 patients with an IL-28B genotype associated with an adverse outcome compared to HCV genotype 1 patients with the positive IL-28B genotype. In genotype 3 this dichotomy was markedly attenuated, suggesting that there may be HCV genotype dependent variations in ISG expression.
1.12 Interferon and ribavirin side effects and tolerability

1.12.1 Interferon side effects

The broad spectrum of host antiviral mechanisms that are induced by synthetic interferons was also reflected in the side effect profile that was reported from early studies\textsuperscript{88,91}. Generalised symptoms such as chills, fever, myalgia, asthenia, headache and arthralgia were reported in 40-85\% of patients treated. Gastrointestinal symptoms experienced by patients receiving interferon included nausea, anorexia and less frequently diarrhoea. Neuropsychiatric side effects occurred in up to 30\% of patients; insomnia, irritability, decreased libido, decreased concentration and sleepiness were the commonest symptoms reported. Depression was noted in up to 30\% of patients during treatment, and a rarer side effect was interferon triggered seizures\textsuperscript{108}.

Thyroid disease was noted to occur in patients on interferon treatment, with hypothyroidism being the most frequently reported abnormality. In some patients receiving interferon based treatment, thyroid dysfunction that occurred on treatment persisted long-term even once the course of treatment was discontinued or completed\textsuperscript{109}. Dermatological side effects included alopecia, oral lichen planus and flares of psoriasis were also described in individuals receiving treatment\textsuperscript{88,91}.

Haematological side effects are common, with a fall in leukocyte or platelet count noted in over 50\% of patients treated with interferon. Usually these side effects could be monitored with dose reduction of interferon if there was a progressive deterioration of cell counts\textsuperscript{110}. Rare severe thrombocytopenia (Platelet count <50x10^9/L) or neutropenia (<0.7x10^9/L) despite dose reduction necessitated early treatment discontinuation.

1.12.2 Ribavirin side effects

Haemolytic anaemia is the commonest side effect of treatment with ribavirin with an average decrease of approximately 2g/L in patients’ haemoglobin levels observed. In addition to generalised
gastrointestinal symptoms (nausea, anorexia, weight loss and abdominal pain), pruritis, cough, depression and dyspnoea have also been documented as commonly occurring side effects. It is of note that ribavirin is also teratogenic. Patients are therefore advised to use contraceptive precautions during treatment and for seven months after the completion of treatment.

1.13 Direct acting antiviral drugs

Following the identification of the hepatitis C non-structural proteins, research was undertaken to identify their function and whether they were potential therapeutic targets. Despite the lack of a robust in vitro viral replication model, the NS3 protein was identified as a serine protease and predictions were made regarding its catalytic triad (based on the protease structure from other flaviviruses). This led to the development of direct acting antiviral drugs. Following the identification of the first effective hepatitis C NS 3/4A protease inhibitor (the macrocyclic inhibitor BILN-2061 in 2003), further inhibitors of the hepatitis C protease were developed, including telaprevir (VX-950) and boceprevir. At the time of trial conception and during patient recruitment onto the pilot study: Telaprevir in Genotype 3 hepatitis C, telaprevir and boceprevir were the only two direct acting antiviral drugs licenced for use in the treatment of hepatitis C.

1.14 Telaprevir

In early phase I trials in patients with genotype 1 hepatitis C, viral RNA initially dropped rapidly during treatment with telaprevir monotherapy. Following this rapid drop in HCV RNA, a rebound in viral RNA was noted between seven and 14 days due to virological breakthrough. Subsequently, trials using telaprevir in combination with pegylated interferon identified that combination therapy achieved a greater drop in HCV RNA, with 50% of treatment naïve non-cirrhotic genotype 1 patients having undetectable HCV RNA after two weeks of treatment. Following this a further trial evaluated combination treatment with telaprevir, pegylated interferon and ribavirin for 28 days in
genotype 1 treatment naïve patients with hepatitis C. This identified that treatment with all three agents resulted in a greater virological response, with all trial participants having undetectable HCV RNA after 28 days of treatment. Following this, trial participants then continued treatment with a further 44 weeks of pegylated interferon and ribavirin, and 66% achieved an SVR. Even following treatment with all three drugs, two patients experienced virological breakthrough during treatment. These findings, together with the subsequent PROVE-2 study, where treatment with telaprevir, pegylated interferon and ribavirin resulted in an improved SVR (62% with telaprevir, pegylated interferon and ribavirin, compared to 36% for telaprevir and pegylated interferon alone), identified that despite the advent of direct acting antiviral drugs, first generation protease inhibitors would still need to be administered with interferon and ribavirin.

The subsequent ADVANCE, ILLUMINATE and REALIZE trials were the three major phase 3 trials performed evaluating telaprevir in the treatment of genotype 1 hepatitis C. SVR24 rates were 69% and 75% in treatment naïve patients with 24 or 48 weeks in the ADVANCE study compared to 44% with placebo and Peg IFN and ribavirin. ILLUMINATE showed the non-inferiority of 24 weeks of treatment. REALIZE included treatment experienced patients, with higher SVR24 rates in previous relapsers (83-88%) compared with null responders (29-33%) and cirrhotic patients (25-27%). These trials also confirmed that patients with prior non-response to treatment, high pre-treatment viral load or advanced fibrosis had a lower SVR rate.

1.15 Telaprevir in genotype 3 hepatitis C

Following the initial promising results for treatment of genotype 1 hepatitis C, telaprevir was evaluated in the treatment of genotypes 2 and 3. This trial demonstrated a more rapid decline in HCV viral load in a small cohort of patients with genotype 2 hepatitis C treated with telaprevir monotherapy or triple therapy (telaprevir, pegylated interferon and ribavirin) compared to those treated with pegylated interferon and ribavirin alone. In genotype 3 hepatitis C, early virological
response to telaprevir was attenuated. Only two of eight patients in the telaprevir monotherapy arm had a virological response with a greater than 2 log drop in HCV RNA within the first three days of treatment. The median decline in HCV RNA at day three was -0.54 log. At the end of 24 weeks treatment (24 weeks of pegylated interferon/ribavirin or two weeks telaprevir/pegylated interferon/plus 22 weeks pegylated interferon/ribavirin or two weeks telaprevir followed by 24 weeks of pegylated interferon/ribavirin), SVR rates were 44% (4 of 9), 67% (6 of 9) and 50% (4 of 8) respectively. This preliminary study suggested that telaprevir may be of benefit to some patients, although there were no pre-treatment factors identified in this small cohort which predicted response to telaprevir.

1.16 Adverse events with telaprevir treatment

The commonest side effects from adding telaprevir to interferon and ribavirin were increased rates of fatigue, gastrointestinal side effects (nausea and diarrhoea), pruritis, skin rashes and anaemia. The increase in side effects experienced resulted in an increased number of patients in the telaprevir containing arms discontinuing treatment early (with an 8-12% increase in treatment discontinuation compared to patients in the control arm receiving treatment with pegylated interferon and ribavirin)\textsuperscript{122–125}. In addition to adverse events, drug-drug interactions were also noted for telaprevir, as drug metabolism is via the CYP3A pathway. Therefore, concomitant administration of medications which induced or inhibited CYP3A enzymes could result in lower telaprevir plasma concentration, and decreased efficacy or increased plasma concentration and risk of toxicity.

Summary

The results from these landmark trials with telaprevir demonstrated that it is an effective direct acting antiviral drug for patients with genotype 1 hepatitis C (in particular those who were treatment naive and did not have advanced liver disease). With regards to other genotypes, in particular genotype 3, the preliminary trials suggested that a cohort of patients with genotype 3 HCV may be
telaprevir sensitive, and therefore could potentially benefit from the addition of telaprevir to their treatment regimen. One of the challenges would be identifying which subgroup of patients with genotype 3 hepatitis C were most likely to benefit from treatment with telaprevir. If the addition of telaprevir could be targeted to those patients most likely to respond to treatment, this could improve their treatment outcome whilst minimising drug exposure and risk of adverse events to those patients who would be unlikely to have any benefit from telaprevir being added to their treatment regimen.

1.17 Boceprevir

In addition to telaprevir, boceprevir is another protease inhibitor developed for the treatment of hepatitis C. SPRINT-2 and RESPOND-2\textsuperscript{126,127} were the key studies relating to boceprevir in the treatment of genotype 1 hepatitis C. In SPRINT-2 a four-week lead-in of pegylated interferon (PegIFN) and ribavirin was followed by either 24 weeks of boceprevir, PegIFN and ribavirin; or 44 weeks of all three medications depending on treatment response between weeks eight and 24. The SVR24 rates were 67% and 68% in these groups compared to 40% in the placebo arm. RESPOND-2 involved treatment experienced patients, also with a four-week lead in, followed by 32 or 44 weeks of treatment with all three drugs. Patients in the arm that received 32 weeks of treatment were given an additional 12 weeks of PegIFN and ribavirin if HCV RNA was detectable at week 8 on treatment. The SVR rates were 59% and 66% respectively. Prior relapsers had better SVR rates (69% and 75% respectively) compared to null responders (40% and 52% respectively). As with telaprevir, patients with advanced fibrosis had worse SVR rates (50% for treatment naïve patients with METAVIR F3-4), as did black patients (SVR 42%-53%). The commonest side effects encountered with boceprevir were anaemia and dysgeusia, followed by nausea, diarrhoea and neutropenia.

Subsequently, boceprevir was also evaluated in the treatment of genotype 2 and 3 hepatitis C\textsuperscript{128}. Boceprevir monotherapy achieved a modest drop in HCV RNA after 14 days of treatment (1.7 log
decrease in genotype 3 hepatitis C) in a small cohort of patients. This further supported the possibility that protease inhibitors may be of use in the treatment of some patients with genotype 3 hepatitis C.

1.18 NS3 resistant variants

From the earliest studies into first generation protease inhibitors (in the treatment of genotype 1 hepatitis C), resistance associated substitutions were identified. Many of these (e.g. V36A, T54A, R155Q, A156V/T, D168V/A) were noted to confer resistance to multiple different protease inhibitors. In exploratory studies evaluating telaprevir in patients with genotypes 2 and 3 resistance associated substitutions were investigated. Patients with genotype 3 HCV had no mutations at baseline associated with decreased telaprevir susceptibility. Patients who developed virological breakthrough on telaprevir monotherapy were noted to have the T54A and R155K variants (which conferred resistance to telaprevir in patients with genotype 1 HCV). Despite this, following treatment with pegylated interferon and ribavirin, all three patients had undetectable HCV RNA at the end of treatment and one patient achieved an SVR. Additional variants were also identified in patients at the end of the telaprevir monotherapy phase of the trial or at virological breakthrough. These included T47A/T, A67A/V, K92K/N, A98A/T, S101A/T and P146S, although none of these had a significant correlation with treatment outcome. Similarly, it was noted that at position 36 of the NS3 protein, which is a site for G1 resistance associated variants, wild-type genotype 3 Hepatitis C virus contained leucine, compared to valine in genotype 1, although the impact of this substitution on telaprevir sensitivity was unclear.

1.19 Other direct acting antiviral drugs

During the work described in this thesis several new direct acting antiviral drugs were being evaluated in clinical trials. The key drugs in development at that time were sofosbuvir, ledipasvir,
daclatasvir and simeprevir, and these drugs have since completed their evaluation and have become key components of current antiviral regimes for hepatitis C.

1.19.1 NS5B inhibitors

The NS5B inhibitor sofosbuvir was one of several new direct acting antiviral drugs that were evaluated in clinical trials from 2010 onwards. Sofosbuvir was relatively unique in being a nucleotide polymerase inhibitor which conferred an improved side effect burden compared to interferon and protease inhibitors. Initially sofosbuvir was tested in combination with pegylated interferon and ribavirin. In the NEUTRINO study patients with treatment naive genotype 1 hepatitis C received 12 weeks of sofosbuvir, interferon and ribavirin and achieved a sustained virological response rate of 89%. The ELECTRON study suggested that in treatment naïve patients with genotype 2 or 3 hepatitis C, the addition of pegylated interferon did not confer any treatment advantage to 12 weeks of sofosbuvir and ribavirin (with an SVR24 of 100% in all groups), similarly all patients with genotype 2 or 3 hepatitis C who received eight weeks of sofosbuvir, PegIFN and ribavirin achieved an SVR12. These findings suggested that interferon free treatment regimens would potentially be possible. In the subsequent FISSION study, patients with treatment naïve genotype 2 or 3 hepatitis C received 12 weeks of sofosbuvir and ribavirin (which was demonstrated to being non-inferior to pegylated interferon and ribavirin) and achieved an SVR of 56% in genotype 3. The FUSION study explored treatment with sofosbuvir and ribavirin for 12 or 16 weeks in patients who had previously failed treatment with interferon. The SVR rates were only 37 and 63% respectively in patients with genotype 3 hepatitis C. In those with cirrhosis these rates dropped further (19% vs 61%) with 12 and 16 weeks of sofosbuvir and ribavirin.

Despite the paradigm shift in treatment of hepatitis C that the development of sofosbuvir heralded, by early 2014 there were still limited interferon free treatment options available for patients with genotype 3 hepatitis C who had failed previous treatment with interferon and had liver cirrhosis. At
this time the BOSON study\textsuperscript{134} was underway to evaluate the efficacy of sofosbuvir, PegIFN, and ribavirin in the treatment of patients with genotype 2 and 3 hepatitis C who had failed previous treatment, including patients with cirrhosis. The work described in this thesis took place against a background of relatively few treatment options for patients with genotype 3 hepatitis C with advanced liver disease following previously unsuccessful treatment with interferon and ribavirin.

1.19.2 NS5A inhibitors

Following the clinical trials investigating sofosbuvir, two NSA inhibitors were developed in quick succession: daclatasvir and ledipasvir

Daclatasvir was initially investigated in the treatment of patients with genotype 1 hepatitis C. In initial preliminary studies in patients with genotype 1 hepatitis C without cirrhosis, SVR rates of 83\% were achieved with 48 weeks of treatment along with pegylated interferon and ribavirin\textsuperscript{135}. A further study in treatment naïve genotype 1 patients achieved SVR rates of 90\% following 24 weeks of treatment with daclatasvir, pegylated interferon and ribavirin. For patients with genotype 3 hepatitis C, 16 weeks of daclatasvir, pegylated interferon and ribavirin achieved an SVR of 69\%\textsuperscript{136}. This further confirmed that interferon-based treatment for patients with genotype 3 hepatitis C could potentially be abbreviated.

Due to the rapid speed at which new direct acting antiviral drugs were being developed, combinations involving all oral direct acting antiviral drugs were also explored, to avoid exposure to interferon (along with the associated side effects this conferred). The combination of sofosbuvir and daclatasvir was evaluated in non-cirrhotic patients with HCV genotypes 1-3. For previously untreated patients SVR 24 rates were 92\% in genotype 2 and 89\% in genotype 3 hepatitis C with 24 weeks of treatment with sofosbuvir and daclatasvir (with or without ribavirin). In genotype 1 patients
(including patients who had previously failed treatment with interferon/ribavirin and a protease inhibitor) SVR rates of 98% were achieved\textsuperscript{137}.

While studies evaluating the all oral combination of sofosbuvir and daclatasvir were underway, trials evaluating sofosbuvir and another NS5A inhibitor, ledipasvir, were being performed. In the LONESTAR trial\textsuperscript{138}, treatment naïve non-cirrhotic genotype 1 patients achieved SVR rates of 95% with 8 or 12 weeks of sofosbuvir and ledipasvir, and the addition of ribavirin resulted in an SVR of 100% following eight weeks of treatment. Of 22 patients with liver cirrhosis and genotype 1 infection, 21 (95%) achieved an SVR\textsubscript{12} following 12 weeks of treatment with sofosbuvir and ledipasvir with or without ribavirin. The ION 1, 2 and 3 studies confirmed the efficacy of this regimen in larger cohorts of patients, achieving SVR rates of 95% and 99% in genotype 1 patients with 12 and 24 weeks of treatment\textsuperscript{139–141}. By mid-2014, trials were underway evaluating the efficacy of sofosbuvir with ledipasvir in patients with genotype 3 hepatitis C\textsuperscript{142}. Treatment naïve patients only achieved an SVR of 64%, whilst treatment experienced patient achieved an SVR of 82%.

Despite the advent of high potency direct acting antiviral drugs which enabled SVR rates of 95% to be achieved in patients with genotype 1 hepatitis C (including those with cirrhosis and patients who had previously failed treatment with interferon and/or protease inhibitors), these findings had not been replicated in patients with genotype 3 hepatitis C by 2014.

1.19.3 NS3 inhibitors

Simeprevir was another NS3/4A protease inhibitor that was being developed. Twelve weeks of simeprevir, pegylated interferon and ribavirin followed by a further 12 or 36 weeks of pegylated interferon and ribavirin was effective in the treatment of genotype 1 hepatitis C, achieving SVR rates of up to 86% in treatment naïve patients\textsuperscript{143}. The subsequent QUEST-1 and QUEST-2\textsuperscript{144,145} trials further evaluated this treatment regimen in treatment naïve patients, achieving SVR rates of 80%
and 81%. Trials were undertaken to evaluate the efficacy of simeprevir in genotype 3 hepatitis C, but no significant drop in HCV RNA was noted in any trial participants (n=8) treated with simeprevir monotherapy for one week\textsuperscript{146}. Sequence analysis of trial patients with genotype 3 hepatitis identified amino acid substitutions (V36L and D168Q) which conferred a 1 and 100 fold decrease in sensitivity to simeprevir in vitro\textsuperscript{147}.

1.20 Summary of Hepatitis C treatment in 2013

At the time this work was commenced, the standard of care treatment for patients with genotype 1 and 4 hepatitis C was either telaprevir or boceprevir with pegylated interferon and ribavirin. The standard of care treatment for patients with genotype 2 and 3 hepatitis C remained pegylated interferon and ribavirin. All oral treatments were being developed at this time, but none had been licenced for use. During the course of the work described here, the treatment options available for patients with hepatitis C advanced rapidly. By the time this work was completed, many of the treatments described above had been superseded and replaced by newer and more effective treatment regimens. The impact of these newer treatments will be reviewed in further detail in the discussion.

1.21 Hepatitis C replication models

1.21.1 Cell lines permitting HCV replication

The Human hepatoma cell line Huh 7 was discovered in 1982\textsuperscript{148} and has been used extensively in the development of in vitro HCV replication assays. Yoo et al.\textsuperscript{149} created a putative full length clone of the Hepatitis C virus in 1995. This strategy was adopted after failed attempts to create a cell line that permitted direct viral replication from HCV patient plasma. HCV RNA was transcribed from a cDNA clone of HCV\textsuperscript{3}. The transcribed RNA was transfected into Huh 7 cells. HCV RNA levels in the cell culture supernatant were variable and transient, with factors including the addition of foetal bovine serum to cell culture media and the cell cycle phase of the Huh 7 cells affecting HCV replication.
Theilman et al.\textsuperscript{150} evaluated infection of Huh 7 cells and Hep G2 cells with serum from HCV infected patients and noted minimal viral replication, even with modifications of the cell culture media used (including the addition of polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO), both of which increased the efficiency of in-vitro infection with other viruses, and modestly increased HCV infection in the short term). For the first decade following identification of the hepatitis C virus, the lack of an efficient in vitro HCV replicon system hampered further research into the understanding of the HCV life cycle and the development of effective direct acting antiviral drugs. Several developments at the turn of the twenty-first century allowed substantial progress to be made on both those fronts.

1.21.2 Huh 7.5 cells

Blight et al.\textsuperscript{151} developed the pre-existing Huh 7 cell line further in 2002. In order to select cells that were more permissive to HCV replication, Huh 7 cell clones that permitted subgenomic HCV RNA replication without adaptive mutations were treated with IFN-α. Of these clones, the Huh 7.5 cell line permitted much greater viral replication, resulting in a 33-fold increase in the number of neomycin resistant colonies formed.

1.22 Replicons

1.22.1 Con-1

Viral replicons are self-replicating viral RNAs that do not form complete virions but which can be stably expressed in cell culture systems using selectable markers. A number of groups attempted to generate HCV replicons that could be used to model HCV replication in tissue culture models. Lohmann et al.\textsuperscript{152} were the first to successfully create a subgenomic replicon system in 1999 which allowed the propagation of modified HCV genomes (replicons) in vitro. The replicon they developed
was derived from the consensus sequence of genotype 1b HCV cloned from the liver of a patient with chronic HCV infection. This genome was modified by replacing the HCV genome from the core to NS2, or from the core to p7 with the neomycin phosphotransferase gene (neo), and the IRES of the encephalomyocarditis virus, which would direct translation of HCV sequences from NS2 or NS3 up to NS5B (figure 1.8).

Once transfected into the human hepatoma (Huh) cell line, HCV RNA replication occurred and was maintained, although the number of Huh 7 cells infected was small (only 1 in $10^6$ cells demonstrated HCV replication in vitro). It was subsequently noted that the efficiency of the replicon system depended on the development of cell culture adaptive mutations in the HCV replicon sequence, in particular in the NS5A region\textsuperscript{153}, and by engineering specific variants (e.g. replacing a serine residue at position 2204 in the NS5A protein with isoleucine) the percentage of Huh 7 cells infected rose to 10%. Huh 7 cell lines which permitted in vitro replication of subgenomic HCV were then treated with interferon α to ‘cure’ them of HCV. A higher proportion of these cured cells were able to support HCV replication in vitro\textsuperscript{152} (Blight 2002).

1.22.2 JFH-1

A genotype 2a replicon was created by Kato et al.\textsuperscript{154} in 2003 where the HCV genome was recovered from a patient with fulminant hepatitis. The initial subgenomic replicon was created following a method similar to that used in the development of the Con-1 replicon. The JFH-1 subgenomic replicon was noted to have a greater colony forming efficacy than Con-1, and was able to replicate in vitro without adaptive mutations (although adaptive mutations which enhanced in vitro replication were noted in NS5A and NS5B).
In vitro HCV replication models

Subsequently Zhong et al.\textsuperscript{155} evaluated HCV replication when Huh 7 cells were transfected with the full length JFH-1 genome (in contrast to the subgenomic replicons previously used). They identified that 70-80% of cells demonstrated JFH-1 replication 72 hours post transfection. JFH-1 secretion from transfected cells increased rapidly five days post transfection, and secretion continued at a high level for the following 7 days before gradually decreasing. When naïve Huh 7 cells were inoculated with supernatant from JFH-1 transfected cells, JFH-1 was noted to infect these cells 48 hours after
incubation. Infectivity of cell culture derived JFH-1 was evaluated by inoculating a chimpanzee with supernatant from JFH-1 transfected cells. HCV RNA was detected in the chimpanzee’s serum two weeks after inoculation, and remained detectable until five weeks post inoculation. When HCV RNA from the infected chimpanzee was sequenced, it was identical to the JFH-1 strain. The ability for JFH-1 to replicate in vitro and produce infectious viral particles was a key breakthrough, as it facilitated research into the HCV life cycle from viral entry to replication and viral particle formation (figure 1.8).

1.23 Capture fusion assay

The in vitro HCV replication systems described above proved useful in understanding the HCV lifecycle and paved the way for the development of the direct acting antiviral drugs discussed earlier in this chapter. As direct acting antiviral drugs (DAAs) became available (initially NS3 protease inhibitors) these replicons were used to identify drug resistant variants. Although they provided an invaluable tool, there were limitations to this replicon system. The process of identifying drug resistance was laborious as it involved the cloning of every variant HCV sequence from patients who did not respond to drug treatment into the replicon to identify drug resistance. Secondly, the replicon systems were only representative of genotype 1 and 2 hepatitis C, with no stable replicon model for genotype 3 HCV. As a result, in vitro phenotyping of viral resistance to HCV was limited.

A ‘capture fusion’ model of HCV replication was therefore developed by the Foster laboratory in 2013\(^{156}\) (figure 1.9). This was based on the observation that HCV was present in peripheral blood mononuclear cells (PBMCs)\(^{157}\), although there is uncertainty as to whether HCV replicates in PBMCs in vitro\(^{158}\).
Dendritic cells had been implicated in cell-cell infection with HIV\textsuperscript{159}, therefore a model where HCV infected PBMCs were fused with human hepatoma cell lines was developed to assess whether HCV replication occurred in vitro\textsuperscript{156}. This demonstrated in vitro replication of patient derived HCV, including in patients with genotype 3 HCV. Patients infected with direct acting antiviral drug (DAA) resistant HCV were also correctly identified. This provided a useful tool in evaluating the in vitro sensitivity of patient derived HCV to DAA drugs. In particular the capture fusion assay was accurate in predicting which patients with genotype 3 HCV infection were likely to experience a >2log drop in HCV viral load on treatment with telaprevir monotherapy, despite no resistance associated substitutions having been identified in genotype 3 HCV to protease inhibitors\textsuperscript{125,156}. These data suggested that it might be possible to identify drug sensitive genotype 3 variants that might be effectively treated with the available direct acting antiviral drugs (e.g. telaprevir).

**Figure 1.9 Schematic of capture fusion assay**
1.24 Work leading up to this thesis

By late 2013 there were still limited treatment options available for patients with genotype 3 hepatitis C and advanced liver disease, who had previously failed treatment with interferon and ribavirin. At that time the only direct acting antiviral drugs licenced for treatment of hepatitis C in the UK were telaprevir and boceprevir. As the preliminary in vitro and clinical studies with telaprevir in genotype 3 hepatitis C suggested that a proportion of patients may benefit from treatment, the telaprevir in genotype 3 hepatitis C trial was formulated to provide these patients with an alternate treatment option that might benefit a subset of patients. Given the previous work showing that patients with genotype 3 HCV who responded to telaprevir did not contain obvious sequence motifs that predicted the outcome of therapy, we speculated that viral sequencing was unlikely to predict treatment outcome. Since the capture fusion assay had correctly predicted the response to telaprevir in a limited clinical study we speculated that the use of this assay would correctly identify patients with genotype 3 HCV who were likely to respond to treatment with telaprevir. The purpose of this work was to examine these hypotheses.

1.25 Hypotheses

1) A subset of patients with genotype 3 HCV and cirrhosis will benefit from treatment with pegylated interferon, ribavirin and telaprevir.

2) Differences in sensitivity to telaprevir can be detected by viral phenotyping using the capture-fusion assay.

3) Differences in sensitivity to telaprevir identified by viral phenotyping may be used to identify patients with genotype 3 HCV who are likely to benefit from treatment with telaprevir.
1.25.1 Experimental approach

To investigate these hypotheses a clinical trial of pegylated interferon, ribavirin and telaprevir in patients with genotype 3 HCV and cirrhosis who had failed to respond to a course of pegylated interferon and ribavirin was devised, initiated and managed. The trial included an evaluation of viral sensitivity to telaprevir using the capture fusion assay.

During recruitment to the trial, alternative treatment regimens for patients with genotype 3 HCV were developed and licensed (chiefly sofosbuvir) and given the side effects associated with telaprevir treatment it was decided that it was unethical to proceed with the trial to completion. The study was therefore terminated early to protect patients from harm.
Chapter 2: Laboratory Methods

The laboratory methods used in the thesis are described below, in particular the methods related to the capture/monocyte fusion assay and the laboratory methods used in assay optimisation.

2.1 PBMC collection and CD 14 separation

1. Collect 30-50 ml blood in 9ml heparinised tubes.

2. Add 15ml Ficoll Paque to 50ml Falcon tube.

3. Add 15-25ml ml of blood to two 50ml Falcon tubes and dilute with RPMI to 35mls total volume.

4. Carefully layer RPMI/blood mix on top of Ficoll.

5. Centrifuge at 1500rpm for 20mins with brakes off.

6. After centrifugation carefully aspirate PBMC layer.

7. Add PBS to make up to 30mls then spin for 5mins at 1500rpm with brakes on.

8. Dilute to 5mls with RPMI/PBS and count cells with haemocytometer.

9. Spin cells for 5mins at 1500rpm with brakes on.

10. Add Freezing media (FBS and 20% DMSO) to make up a concentration to 1x10^7 cells/ml.

11. Place cells in cryotube and place in Cryofreezing container and place in -80°C freezer.

12. After 24 hours transfer to Liquid nitrogen storage.

CD 14 separation

As per Miltenyi Biotec protocol for CD14 microbeads.

1. Defrost PBMCs if necessary.

2. Add RPMI to make PBMCs up to 10ml.
3. Centrifuge at 200g for 5mins.

4. Discard supernatant.

5. Add 80µl MACS buffer to PBMCs.

6. Add 20µl CD14 MACS beads to PBMCs.

7. Incubate in fridge for 15mins.

8. Prepare MACS column on magnet, on MACS stand.

9. Add 500µl MACS buffer to column.

10. Once PBMC incubation complete, make up to 500µl with MACS buffer.

11. Add PBMC mix to MACS column and collect effluent (containing CD14- cells).

12. Add 3 sets of 500µl MACS buffer to column and continue to collect CD14- effluent.

13. Remove MACS column from magnet and place in new 15ml Falcon tube.

14. Move magnet away from hood.

15. Flush though with 3x 500µl MACS buffer with plunger and collect effluent (containing CD 14+ve cells).

Proceed to step 9 of HCV infection and fusion of monocytes protocol.
2.2 Cell Fusion Protocol

Start from step 1 for THP-1 Capture fusion or from step 9 for PBMC fusion.

Day 1

1. Plate THP-1 cells at density of 1x10^6 cells/mL in RPMI/adds.

2. Add PMA to final concentration 200ng/ml and IFNγ to 10ng/ml, swirl mix.

3. Incubate at 37°C for 18-24 hours.

Day 2

4. Majority of cells will be adherent. Remove supernatant/non-adherent cells, gently wash adherent cells x 3 with RPMI and replace medium with RPMI + 2% FCS.

5. Add HCV serum of known viral load at ratio of 1 HCV copy/cell.

6. Incubate at 37°C overnight.

Day 3 - Fusion

7. Remove supernatant, wash cells x 3 with RPMI.

8. Remove adherent cells into fresh RPMI using cell scraper (x2, washing with RPMI after each scrape).

9. Add equal number of Huh 7.5 cells (to monocytes or PBMCs) and pellet the cells together in a 15ml Falcon tube (3min at 1500rpm usually adequate).

10. Remove supernatant completely and fuse with pre-warmed PEG 1500- add PEG (100µl per million total cells) over 1 min, with gentle stirring. Transfer to 37°C for 90 secs. Remove from incubator and slowly dilute out the PEG in 10ml pre-warmed DMEM/adds. Add the first 1ml over 1 minute, the second 2ml over a further minute and the remainder dropwise.

11. Incubate at 37°C for 5 minutes.
12. Pellet the fused cells (5min at 1000 rpm) and resuspend in an appropriate volume of DMEM/adds to plate at a density of $10^5$cells/ml (e.g. $1\times10^5$ cells/well of 6 well plate in 1ml medium/well). Leave to settle at 37°C overnight.

**Day 4- Drug inhibition**

13. Add inhibitory drugs at appropriate concentrations per well (drug dilution mix containing DMEM/2%FCS/0.05% added to “no drug” wells).

**Day 6- 3 days post- fusion**

14. Aspirate medium from each well and replace with fresh medium and drug.

**Day 8- 5 days post- fusion**

15. Aspirate medium from each well. Dissolve adherent cells in 1ml TRIzol reagent per well or 350µl Buffer RLT.

### 2.3 RNA extraction

Follow steps below or alternatively Qiagen RNeasy Mini-kit protocol (enclosed).

**RNA extraction**

**TRIzol step**

1. Remove supernatant from 6 well plates.

2. Wash each well with 1ml PBS.

3. Aspirate PBS.

4. Add 1ml TRIzol per well.

5. Aspirate contents of well into 1.5ml Eppendorf tube.
RNA precipitation and extraction

1. Add 200µl chloroform per Eppendorf tube.

2. Shake well for 15 seconds.

3. Incubate at room temperature for 2-3 mins.

4. Centrifuge for 15 minutes at 12,000g at 4°C.

5. Remove 400µl from aqueous (upper) phase and transfer to new 1.5ml Eppendorf.

6. Add 500µl isopropanol to each sample.

7. Incubate at room temperature for 10 mins.

8. Centrifuge for 10 minutes at 12,000g at 4°C.

9. Remove supernatant from Eppendorf tubes.

10. Add 1ml 70% Ethanol to each sample.

11. Briefly vortex each sample.

12. Centrifuge for 5 mins at 7500g at 4°C.


14. Air dry pellet for 5-10 mins.

15. Resuspend RNA in 50µl water.

2.4 RNA quantification:

Omit steps 1-4 of following protocol if on column DNase reaction performed when using RNeasy spin columns. Proceed to add 2µl RNA sample to 200µl TE buffer and aliquot 100µl duplicates to 96 well plate and proceed from step 5.

1. DNase treatment. 2µl RNA in 10µl DNase reaction with 1µl DNase, 1µl 10x buffer and 6µl water (Promega).

2. Incubate at 37°C for 30 minutes.

3. Quantification of DNase-treated RNA using the RiboGreen assay (Invitrogen) according to manufacturer’s instructions for high range protocol:

1. Dilute 5µl of each DNase treated RNA sample (in duplicate) in 100µl TE buffer in 96well white plates, dilute high range standards according to protocol and add to plate.

5. Dilute RiboGreen reagent in 1 in 200 TE buffer and add 100µl to each well.

6. Incubate 2-5mins at room temperature in the dark.

7. Read on fluorescence plate reader (standard fluorescence settings).

8. Use standard results to construct standard curve then interpolate sample concentrations. Back calculate to account for dilution steps (if dilutions are done as above then interpolated value= Yng/mL; corrected value= Yx410ng/ml. Then divide by 1000 to give ng/µl).

2.5 Real time quantitative polymerase chain reaction (RT-qPCR)

Set up RT-qPCR reaction using Quantitect Viral Nucleic Acid Detection Kit (Qiagen) according to manufacturer’s recommendations in 20µl volumes. Commercial HCV primer/probe (Taqman, Applied Biosystems) is used at 1µl per reaction. Each RNA sample is measured in triplicate in RT-qPCR. Aim to use 100-200ng/RNA per reaction.
1. Create mix for samples with 4µl 5x Mastermix, 1µl Primer probe, 0.2µl 100x Quantitect Viral RT Mix, Sample volume (to enable 100-200ng RNA per reaction) and water to make up to 20µl.

2. Create mix for two blanks (with 5µl water instead of sample).

3. Make serial dilutions of RNA standard (stock is quantified JFH-1 RNA at 2x10^5 copies/µl). Include standards in duplicate 10 copies/reaction- 10^5 copies/reaction. Dilute 2µl of 2x10^5 JFH-1 in 18µl water, and perform serial 1 in 10 dilutions to obtain JFH-1 standards at 2x10^4, 2x10^3, 2x10^2, 20, and 2copies. Use 5µl per reaction.

4. Run on RotorGene at recommended cycling conditions (50°C for 20 mins, followed by 95°C for 5 mins then 35 cycles of 95°C for 15 seconds, and 60°C for 45 seconds).

2.5.1 PCR Data analysis

1. Select data file on RotorGene thermocycler.

2. Click on autoscale (below graph).

3. Click analysis (on tabs along top).

4. Choose quantitation (Cycling A green) and click show.

5. Click auto-find threshold on menu bar on bottom right.

6. Review that blanks are negative.

7. Right clinic on Quantitative Cycling A green table on bottom left and select export to Excel.

8. In Microsoft Excel divide the calculated copies of RNA by the total RNA at the start of the reaction to give RNA concentration in copies of HCV per nanogram of RNA.

9. Multiply by 1000 to calculate copies per microgram of RNA.
10. Calculate the average copies of HCV RNA per sample (as each sample was performed in triplicate).

11. Calculate HCV RNA per sample as a percentage of average value for no drug inhibition samples.

### 2.6 Laboratory serum viral load quantification

As per QIAamp Viral RNA Handbook: Purification of Viral RNA (Spin Protocol).

1. Pipette 560µl of prepared buffer AVL containing carrier RNA into a 1.5ml microcentrifuge tube.

2. Add 140µl plasma to the microcentrifuge tube. Mix by pulse vortexing for 15 secs.

3. Incubate at room temperature for 10 mins.

4. Briefly centrifuge.

5. Add 560µl 100% ethanol to the sample and mix by pulse vortexing for 15 secs. After mixing, briefly centrifuge the tube to remove drops from the lid.

6. Add 630µl of the solution from step 5 to the QIAamp mini column (in a 2ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000g (8000rpm) for 1 minute. Place the QIAamp into a clean 2ml collection tube, and discard the tube containing the filtrate.

7. Carefully open the mini-column and repeat step 6.

8. Open the QIAamp mini column and add 500µl of buffer AW1. Close the cap and centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp mini column in a clean 2ml collection tube and discard the tube containing the filtrate.

9. Carefully open the QIAamp mini column and add 500µl of buffer AW2. Close the cap and centrifuge at full speed (20,000g/14,000rpm) for 3 mins.
10. Place the QIAamp mini column in a new 2ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp mini column in a clean 1.5ml microcentrifuge tube. Discard the old collection tube containing the filtrate. Open the column and add 60µl of buffer AVE equilibrated to room temperature. Close the cap and incubate at room temperature for 1 min. Centrifuge at 6000g (8000rpm) for 1 min.

12. Proceed to RNA quantification and QPCR subsequently, or store RNA at -20°C or -70°C.

2.7 Capture fusion assay optimisation methods

2.7.1 Cell Colouring Protocol

Reagent Preparation

- DMSO (dimethylsulfoxide)
- PBS
- Cell tracker dyes need to be reconstituted to a final concentration of 10mM in DMSO and diluted to a working concentration of 0.5-25µM in serum free medium. (Use at 10µM). Warm to 37°C.

Colouring Controls:

- 1 no-colour control for each cell line (negative control).
- 1 no-colour control for each cell line for fusion.

Procedure - Colouring

1. For adherent cells aspirate media and replace with 1ml of the pre-warmed CellTracker dye working solution and incubate for 30 mins at 37°C. (For Huh7.5 cells this will be the BODIPY Green Dye solution, for THP1 cells this will be the Orange dye solution).
a. For Cells in suspension: harvest by centrifugation, pour off supernatant and gently re-suspend ~10^6 cells in dye working solution, incubate cells as above.

2. Replace dye-working solution with fresh pre-warmed medium and incubate for 30mins at 37°C.
   a. Centrifuge and re-suspend in fresh medium if in solution.

3. Wash with PBS and replace with medium (RPMI for THP-1, DMEM for huh7.5).

4. Carry out Fusion as per protocol (step 9). (Keep at least 1 well of each coloured cell line to act as a control).

5. After fusion fix cells in 2% paraformaldehyde.

Controls for FACs

- Un-coloured for huh7.5 and THP-1 (2 wells).
- Non-fused coloured huh7.5 and THP1 (2 wells).
- Fusion with un-coloured cells.
- Fusion with coloured cells (1 well).

Dye reconstitution

BODIPY (279.55 g/mol)

- Reconstituted to 10mM therefore need to dilute 10µM.

Orange (Mw = 550.44 g/mol)

- Come in 50 µg aliquots and need to be reconstituted to 10mM see below:

  i.e. Vol= 50 µg/(0.01 M[10 mM] x 550.44 g/mol) = 9.08 µl
2.7.2 Preparation of samples for Flow Cytometry

1. Aim for $1 \times 10^5$-$5 \times 10^5$ cells per sample.

2. For adherent cells, wash with 1mls PBS, then repeat, following this add 1ml PBS to well on tissue culture plate.

3. Scrape cells from well and transfer to 15ml Falcon, wash both sides of scraper and well with 1ml PBS each then add 1ml PBS to well.

4. Repeat 3.

5. Centrifuge sample at 1700 rpm for 5 mins.


7. Resuspend samples in 1ml PBS.

8. Divide sample into 500µl aliquots and place in FACS tube.

9. Centrifuge at 1500 rpm for 5 minutes.

10. Discard supernatant.

11. Add 100µl of FACS buffer and antibody.

12. Incubate on ice in the dark for 1 hour.

13. Add 1ml FACS buffer to each tube.

14. Centrifuge at 1500 rpm for 5 minutes.

15. Discard supernatant.

16. Add 500µl 4% PFA and resuspend for 10 mins.

17. Centrifuge at 1500 rpm for 5 minutes.

18. Resuspend in 500µl PBS.
2.7.3 Transfection protocol

1. Prepare 5x10^5 cells per well on 6 well plate.

2. Use 5 or 10µg plasmid DNA and dilute in 100µl Optimem.

3. Add 5µl Fugene to 95µl Optimem.

4. Leave mixtures in 3 and 4 got 40 minutes at room temperature, then mix together and leave for 15 minutes.

5. Wash cells (culture plate) with optimum twice.

6. Add 600µl Optimem to each well.

7. Add transfection mixes and incubate at 37°C for 1.5 hours.

8. Add 3ml DMEM (10% FBS) and incubate for 24 hours.

9. Add media containing antibiotic to select transfected cells after 24 hours.

10. Leave cells to grow for 4-6 weeks, changing antibody selection media every 2-3 days.

2.7.4 RNA extraction from tissue cultured cells

1. If under 1x10^7 cells harvest with RLT buffer (350µl per well).

2. Add 350µl 70% ethanol to lysate and mix well.

3. Transfer 700µl of sample to a RNeasy mini spin column placed inside a 2ml collection tube and centrifuge for 15 secs at >8000g. Discard the flow through.

4. Add 700µl buffer RW1 to spin column. Centrifuge for 15 secs at >8000g. Discard flow through.

5. Add 500µl buffer RPE to spin column. Centrifuge at >8000g for 15 secs. Discard flow through.

6. Add 500µl buffer RPE to spin column, centrifuge at >8000g for 2 mins.
7. Place spin column in a new collection tube. Centrifuge at full power for 1 min.

8. Place spin column in a new 1.5ml microcentrifuge tube. Add 30-50µl RNase-free water directly to spin column membrane. Close the lid and centrifuge for 1min at >8000g to elute the RNA.


2.7.5 Protocol to create cDNA from cellular RNA

1. Use 1µg mRNA per reaction.

2. Add 0.5µg of primer (random primer) per 1µg of mRNA in <14µl of H₂O.

3. Heat for 5 mins at 70°C to melt secondary structure. Cool immediately on ice the spin briefly.

4. Add 10µl of each dTP (dATP, dTTP, dCTP and dGTP) at 10mM concentration to 60µl H₂O.

5. Add the following components to the primer/mRNA mixture to create a reaction volume of 25µl:

   - M-MLV 5x buffer 5µl
   - DTP mix (from 4) 5 µl
   - 200 Units M-MLV RT 1µl
   - Sample (1µg RNA) and primer
   - H₂O to make up to 25µl

6. Mix and incubate at 37°C for 60 mins.

7. Calculate cDNA concentration using nanodrop.
2.7.6 SYBR green protocol to assess relative expression of cellular genes

1. Create primer mixture (20µl each of forward and reverse primer for gene) and 160µl H_2O.

2. Add the following components to the primer/mRNA mixture to create a reaction volume of 25µl:

   - 2µl primer mix
   - 12.5µl SYBR green reagent
   - 250ng cDNA per reaction
   - H_2O to make up to 25µl

3. Run samples on rotor-gene:

   - 95°C for 10 mins
   - 40 cycles at 95°C for 10 secs, 58°C for 15 secs, and 72°C for 5 secs
   - 72°C for 90 secs
   - Ramp temperature from 72°C to 95°C by 1°C every 5 secs

4. Calculate relative expression of gene of interest using

   \[ 2^{-\Delta\Delta CT} = 2^{-(\text{cycle threshold housekeeping gene} - \text{cycling threshold gene of interest})} \]

2.7.7 Immunohistochemistry protocols

1. Place coverslips in 24 plate.

2. Plate 5x10^4 cells in each well.

3. Remove culture medium from well.

4. Add 350µl 10mM NH_4Cl (incubate for 10 mins at room temperature).
5. Remove NH₄Cl then add 350µl Triton X-100 0.05% and incubate for 5 mins on ice.

6. Remove Triton then add 350µl 5% V/V goat serum in PBS 0.1% Triton.

7. Prepare parafilm for incubation with primary antibody (label and tape down to lab bench).

8. Add primary antibody in 1:100 dilution (dilute with PBS 0.1% Triton and 5% Goat serum), 40µl per slide and place coverslip face down on parafilm.

9. Incubate for 1 hour at room temperature.

10. Wash x 2 with PBS.

11. Add secondary antibody 1:1000 dilution 40µl per coverslip and incubate for 1 hour at room temperature.

12. Wash x 2 with PBS.

13. Dip briefly in H₂O then 70% ethanol.

14. Mount with Prolong Gold and DAPI.

15. Leave in dark for 12 hours.

2.7.8 50% Tissue culture infective dose (TCID 50) Assay

**Day 1 – seed cells**

Plate Huh7 cells in a density of 3000 cells/100µl/well in a 96 well plate.

**Day 2 – infection**

Take cells to BSL3.

Collect the supernatant of cells containing viral particles and centrifuge (6 well plates containing electroporated Huh7 cells).
Add 50µl supernatant (containing JFH-1) per well (1 condition is tested in 12 different wells).

Make 8 serial dilutions of 1/3.

Incubate for 3 days.

**Day 5 – immunofluorescence**

Discard the cell medium.

Submerge the plate and the lid in methanol.

Transfer to plastic box, spray with ethanol.

**Immunofluorescence:**

- Wash 3 times with PBS A
- Incubate with primary antibody for 1 hour at RT (sheep NS5A 1/1000 in PBS A with 1% FCS), the antibody is already diluted 1:10
- Wash 3 times with PBS A
- Incubate with secondary antibody for 30 to 40 minutes at RT (AlexaFluor 488 donkey anti-sheep 1/1000 in PBS/FCS)
- Wash 3 times with PBS A
- Counterstain with DAPI (1:1000) for 5 min
- Wash 3 times in PBS and store in PBS, read and score positive cells on time-lapse microscope

**2.7.9 Western Blot**

**Protein extraction**

1. Initial part of protocol is as per RNAeasy mini kit (steps 1 & 2).

2. Add 700µl of sample to spin column placed within a collecting tube.
3. Centrifuge for 15 secs at >8000g.

4. Collect the flow through (as this contains protein).

5. Place 900µl acetone in an empty 1.5ml microcentrifuge tube.

6. Add 300µl flow through to each tube.

7. Set centrifuge to 4°C (Can store at 20°C if RNA extraction needs to be completed (as per protocol)).

8. Centrifuge protein sample at 14,000rpm for 15 min at 4°C.


10. Add 100µl 100% ethanol to pellet.

11. Spin at 14,000rpm for 5mins at 4°C.

12. Aspirate 100µl supernatant.

13. Resuspend in 100µl WCL buffer (1ml Whole cell lysis buffer + 10µl PMSF (100mM) + 40µl protease inhibitor (25x)).


Bicinchoninic acid assay (BCA assay)

1. The BCA is used to calculate protein concentration.

2. Dilute standards (Bovine serum albumin) BSA in PBS at 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0.0625mg/ml and use a negative control (PBS only).

4. Will need total of 12 wells in 96 well plate for standards then, duplicates for each protein concentration (usually do: undiluted, 1:10, 1:100).

5. Add 200µl of mixture in 3 to each well in 96 well plate and add 25µl of sample or standard to each well.

6. Incubate at 37°C for 30mins.

7. Read on plate reader (on absorbance setting at 570nm).

8. Create standard curve and calculate concentration of samples.

**Protein electrophoresis**

1. Aim for 5µg protein per lane.

2. Make up protein and WCL to 22.5µl per lane in Eppendorf.

3. Add 2.5µl DTT (1M) and 5µl loading buffer (6x laemmli) per lane (total 30µl per lane).

4. Heat to 70°C for 5 mins.

5. Assemble electrophoresis kit.

6. Remove gel cassette from packaging and wash cassette with tap water, remove tap strip at bottom of gel.

7. Place gel cassette and spare cassette holder in tank with text facing outwards and clamp in place.

8. Add MOPS-SDS 1x running buffer to space between both cassettes to above lip on cassette.

9. Remove comb from gel and wash wells with running buffer.

10. If no leak to remainder of tank add running buffer (MOPS-SDS 1x) to halfway up tank.

11. Add 3µl ladder to first well of gel and 28µl of samples to subsequent wells.
12. Run gel at 200V for 35 mins.

Gel transfer

1. Add transfer buffer (TRIS/glycine and 10% methanol) to wadding and blotting paper (cut to size).

2. Unpack nitrocellulose membrane and add to kit above to soak in transfer buffer.

3. Place several sheets of wadding and blotting paper to solid gel holder (for gel transfer) with black side (negative) of gel holder facing down.

4. Dismantle gel casing, keep gel with side towards text on casing facing down.

5. Cut off bottom right corner of gel (opposite side to ladder).

6. Remove bottom of gel with loading reagent, and top of gel with wells and stacking gel using a palette knife.

7. Place membrane on top of gel.

8. Transfer gel and membrane to gel holder and place on top of wadding/blotting paper. Add further layer of blotting paper on top of membrane and several layers of wadding, and place lid for gel holder.

9. Recycle running buffer in tank (MOPS-SDS 1x).

10. Rinse tank with water.

11. Place gel holder (with wadding, blotting paper, membrane and gel) in tank and clamp in place.

12. Ensure gel holder has sufficient transfer buffer.

13. Place water in outer segment of tank.

14. Place tank in bucket of ice.
15. Run at 25V for 1 hour.

Western blot

1. Place membrane in shallow container and add Ponceau’s solution (this will highlight the protein bands on membrane).

2. Cut membrane into strips (in pairs/groups of control vs test conditions).

3. Place strips in 50ml falcon tube and wash with 1x TBS.

4. Add 5ml blocking solution (39ml 5% PVP in PBS and 1ml FBS) and place on roller to block for 1 hour at 4°C.

5. Add primary antibody at 2µl per falcon tube and incubate overnight at 4°C.

6. Wash membrane with 1x TBS and 0.2% Tween 20mls and repeat 3x.

7. Prepare secondary antibody 1µl to 10mls blocking solution (see step 4), and add 5ml after 3rd wash.

8. Incubate for 1 hour.

9. Wash as per step 6.

10. Prepare enhanced luminescence (ECL) kit. Add 2mls of Pierce ECL mixture A to 2mls of mixture B.

11. Add mixture in 10 to gel facing side membrane and incubate for 5 mins.

12. Turn face down onto cling film and wrap in clingfilm.

13. Take to dark room and place on photographic paper.

Chapter 3: Capture fusion assay optimisation

Background

One of the aims of the telaprevir in genotype 3 trial, was to prospectively assess in-vitro sensitivity of patient derived HCV from trial participants to telaprevir. As the trial was designed to evaluate treatment in patients with advanced fibrosis or cirrhosis, it was anticipated that HCV viral loads in these patients would be lower than previously used in the capture fusion assay. Furthermore the capture fusion assay was only successful with 80% of patient serum samples which was regarded as suboptimal for a clinical assay. In order to overcome the challenges posed by using patient serum samples with low HCV viral load, and to improve the proportion of patient samples that could be accurately phenotyped using the capture fusion assay, a number of modifications of the Huh 7.5 cell line were evaluated to determine whether viral replication in the assay could be improved.

3.1 Huh 7.5 Cells cultured in Human serum

Steenbergen et al.\textsuperscript{160} hypothesised that HCV infection was not only dependent on the presence of human hepatocytes but also required human serum factors. Their group identified that humanisation of mouse lipoprotein profiles enabled successful HCV infection of chimeric mouse livers (which had been transplanted with human hepatocytes). Huh 7.5 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with either 10% Foetal Bovine serum (standard Huh 7.5 cell culture conditions) or DMEM with 2% Human Serum. Cells cultured in Human serum demonstrated a change in morphology with increased expression of hepatocyte tight junction proteins and HCV entry receptors, and also a marked increase in JFH-1 replication, and increased infectivity of virus produced in cells grown under the new culture conditions. We therefore hypothesised that Huh7.5 cells grown in human serum would increase viral replication in the capture fusion assay.
3.1.1 Methods:

Huh 7.5 cells cultured in DMEM with 10% FBS and penicillin/streptomycin were trypsinised, the trypsin was inactivated with DMEM. The cells were then centrifuged at 300g for 5 minutes. The cells were then resuspended in PBS and counted. Cells were divided into $1 \times 10^6$ cell aliquots and placed in separate 15 ml falcon tubes. Cells were centrifuged again at 300g for 5 minutes then resuspended in either DMEM with 10% FBS or DMEM 2% HS (with penicillin/streptomycin) and plated in T75 culture flasks with 20mls media. Media was changed every 72 hours and cells were passaged once they approached confluence. Huh 7.5 cells in DMEM and 10% FBS were passaged every 72 hours. The Huh 7.5 cells cultured in 2% HS took longer to approach confluence and were first passaged after 10 days, and then every 72 hours. After at least 21 days of culture in 2% Human serum cells were used in experiments.

3.1.2 Results

3.1.2.1 Cell RNA and protein expression

After cells had been passaged in DMEM with Human Serum for 21 days, cells were plated out in six well plates at $1 \times 10^6$ per well and harvested after 24 hours and genomic RNA was extracted. PCR was performed with SYBR green to evaluate the relative expression of mRNA for HCV cell entry proteins. The fold change of mRNA expression was calculated by the Pfaffl method\textsuperscript{161}, and the results shown are relative to cells cultured in FBS (figure 3.1). These experiments demonstrated that culturing Huh 7.5 cells in human serum resulted in (non-significant) increased expression of occludin and claudin (as previously observed by Steenbergen et al), but also resulted in a non-significant increase in CD 81 expression whilst a rise in SR-B1 mRNA levels was not observed (which was in contrast with the Steenbergen paper).
3.1.2.2 Immunohistochemistry

In order to evaluate whether increases in mRNA resulted in translated protein, immunohistochemistry was performed to evaluate the expression of cell surface proteins involved in HCV cell entry. 5x10^4 cells (cultured in either 10% FBS or 2% HS) were seeded onto coverslips in 24 well plates, and incubated for 24 hours. These cells were fixed with 4% PFA, and immunohistochemical analysis was performed (as described in Methods). Image J software was used to calculate the relative immunofluorescence of tissue staining.
Figure 3.2: Immunohistochemistry of Huh 7.5 cells cultured in standard conditions (10% FBS) (l) or 2% HS (r), A) Albumin B) CD81 C) Claudin D) E cadherin
Immunohistochemistry (figure 3.2) suggested increased expression of CD-81 and E-cadherin in cells passaged in DMEM with 2% Human serum (as noted in the original paper). Having identified potential differences in the expression of cell surface proteins involved in HCV cell entry, experiments assessing JFH replication were performed to evaluate whether the noted increase in these proteins resulted in increased HCV replication.

3.1.2.3 HCV replication in vitro- JFH

Huh 7.5 cells cultured in 10% FBS and 2% HS were seeded onto a 12 well plate at a concentration of $1 \times 10^5$ cells per well. JFH-1 from a common stock was added at an MOI of 0.01, and the cell culture media for these cells was replaced after 4 hours with fresh media (containing 10% FBS or 2% HS respectively). Cells were cultured initially for 3 or 5 days and harvested with 500 microlitres of TRIZOL. JFH-1 RNA was extracted from these samples (as described in Methods) and HCV RNA was quantified. Initial experiments (figure 3.3) demonstrated non-significant increased JFH replication by approximately 1 log both at day 3 and day 5 post infection. Subsequently a time-course experiment was performed to evaluate the rate of increase in JFH RNA. This demonstrated that JFH-1 replication in Human serum cells occurred at a lower rate over the first 24 hours then increased by day 6. This may be a result of a lower rate of cell division (as noted by Steenbergen) affecting viral replication over the first 24 hours, with increased JFH-1 replication subsequently noted at days 3 and 6. The findings from these experiments suggested that there may be a marginal increment in JFH-1 replication when using Huh 7.5 cells cultured in 2% Human serum.
3.1.2.4 Infectivity of JFH-1 produced in Huh7.5 cells cultured with 2% Human Serum

The infectivity of JFH-1 produced in Huh7.5 cells cultured in FBS or HS was evaluated by a Tissue Culture infectious dose assay (TCID 50). This evaluated the infectivity of serial dilutions of JFH-1 produced under the two different tissue culture conditions, and enabled calculation of the number of 50% infectious doses of virus contained per ml of viral supernatant. The TCID$_{50}$ results for the cells cultured in FBS and HS are demonstrated below (figure 3.4), with an example of the NS5A immunohistochemistry used to calculate the TCID$_{50}$ value. These data confirm the observation that Huh 7.5 cells grown in human serum are more permissive to HCV replication. It is unclear whether this is due to increased internal cellular replication or changes in viral entry.
3.1.2.5 Colour fusion data

In order to evaluate whether there was any effect of the phenotypic changes in Huh 7.5 cells cultured in 2% Human Serum on the efficiency of PEG mediated cell fusion, experiments were performed to assess if there was any difference in cell fusion between Huh 7.5 cells cultured in FBS and those cultured in Human serum. Huh 7.5 cells (cultured in FBS or Human Serum) were labelled with CellTracker orange CMRA, and THP-1 cells were labelled with CellTracker violet. The figure below (figure 3.5) demonstrates the effective cell labelling of both Huh 7.5 and THP-1 cells. As the THP-1 cells had been stimulated prior to co-culture a population of THP-1 cells adherent to Huh 7.5 cells was noted with both the cells cultured in FBS and those cultured in HS. This made quantification of effective cell fusion challenging. Given the difficulties in interpreting these experiments and as cell fusion alone is not an accurate surrogate marker of viral replication in the capture fusion assay further experiments were performed to evaluate if Huh 7.5 cells cultured in Human Serum permitted greater viral replication in the capture fusion assay.
Figure 3.5. Flow cytometry data. A) Huh 7.5 cells cultured with 10% FBS: negative control (l), with CellTracker orange (r) B) Huh 7.5 cells cultured with 2% HS: negative control (l), with CellTracker orange (r) C) THP-1 cells: negative control (l), with CellTracker violet (r) D) Huh 7.5 FBS cells fused with THP-1 cells: uncoloured cells (l), with Huh 7.5 FBS cells labelled with CellTracker orange and THP-1 cells labelled with CellTracker violet E) Huh 7.5 HS cells fused with THP-1 cells: uncoloured cells (l), with Huh 7.5 FBS cells labelled with CellTracker orange and THP-1 cells labelled with CellTracker violet.
3.1.2.6 Capture fusion data

As the objective of using new and modified cell lines was improved HCV replication in the capture fusion assay, paired capture fusions were performed using Huh 7.5 cells cultured in FBS and HS and THP-1 cells infected with HCV infected serum (figure 3.6). Serum from three Genotype 3 HCV patients was used and drug inhibition was assessed by evaluating sensitivity to a single concentration of telaprevir. Telaprevir was used as this would be the drug of interest in the telaprevir in genotype 3 clinical trial.

![HS and FBS data](image)

**Figure 3.6.** Capture fusion data comparing Huh 7.5 cells cultured in FBS or Human serum. 3 different HCV containing serum samples were used and sensitivity to drug inhibition was assessed using telaprevir.

Whilst Huh 7.5 cells cultured in human serum demonstrated a marked increase in viral replication in the capture fusion assay for one HCV serum sample (SD029), the HCV copy number per microgram
of RNA was similar for both the Huh 7.5 cells cultured in FBS and those cultured in 2% HS for the remaining two HCV serum samples.

3.1.3 Conclusions

Huh 7.5 cells cultured in 2% Human serum demonstrate phenotypic changes compared to those cultured in 10% FBS. They demonstrate altered expression of cell surface proteins involved in tight junction formation (some of which are also involved in HCV cell entry). The replication of JFH-1 virus is greater in Huh 7.5 cells cultured in Human serum compared to those cultured in FBS. Viral replication in the capture fusion assay was greater in one patient serum sample containing HCV, with no difference between the cell lines when another two different HCV containing patient serum samples were used. These data suggested that culturing Huh7.5 cells in human serum alone would not consistently augment HCV viral replication in the capture fusion assay. Therefore Huh7.5 cells cultured in Human Serum would be unlikely to provide a solution enabling HCV replication (in the capture fusion assay) from low viral copy number samples. Alternative strategies were subsequently pursued to optimise in vitro viral replication.

3.2 SEC14 L2

3.2.1 Background

One of the limitations in evaluating hepatitis C in-vitro was the need for sub-genomic replicons to acquire or contain adaptive mutations in order to replicate in cell culture conditions. This was thought to represent a lack of essential host factors or presence of inhibitory factors in the cell lines used (such as Huh 7 derived cells). In order to overcome this block Saeed et al. transduced Huh 7.5 cells with lentivirus libraries containing a range of human cDNA. These transduced cells were electroporated with G418 selectable HCV sub-genomic replicons, which usually require at least 2 adaptive mutations for replication. Multiple colonies harbouring replicons with parental sequence.
cDNA sequence analysis from these colonies identified the same gene product, SEC14L2 (tocopherol associated protein 1 (TAP-1), supernatant protein factor 1 (SPF1)).

Huh 7.5 cells stably expressing SEC14L2 were transfected with wild type replicons from genotypes 1a, 1b, 2a, 3a, 4a, and 5a, and stably expressed wild type viral sequence. Patient derived HCV had not previously resulted in persistent and productive infection in cultured hepatoma cells. When SEC14L2 cells were inoculated with 1a, 1b and 3a patient sera with high viral titres viral replication was detected in vitro.

3.2.2 SEC14L2 mechanism of action

SEC14L2 is a cytosolic lipid binding protein family member, which is ubiquitously expressed in human tissues. SEC14L2 has 3 domains, an N terminal CRAL-TRIO domain that binds to small lipophilic molecules, a SEC14 like domain that contains a hydrophobic ligand binding pocket, and a C-terminal GOLD domain that facilitates protein-protein interactions. All 3 domains were needed for HCV replication. The functions of SEC14L2 include regulation of the PI3K/Akt signalling pathway, cholesterol synthesis, and vitamin E metabolism. Neither the PI3K/Akt pathway nor cholesterol synthesis related roles of SEC14L2 affected HCV replication.

SEC14L2 acts as a Vitamin E (tocopherol) binding protein. Vitamin E acts as a lipid soluble antioxidant, inhibiting lipid peroxidation and enhanced HCV replication. HCV isolates resistant to lipid peroxidation (e.g. JFH-1) showed no change in viral replication irrespective of SEC14L2 expression. When SEC14L2 cells were cultured in medium containing lipid depleted FBS (including vitamin E) HCV replication was no longer supported. This effect was reversed by vitamin E supplementation.

3.2.3 Results

SEC14L2 cells were kindly provided by Professor Peter Simmonds (Oxford). A Western blot was performed to confirm the presence of SEC14L2 (figure 3.7). Following on from the previous work
with Huh 7.5 cells cultured in Human serum, SEC14L2 cells were cultured in 10% FBS and 2% HS. Viral replication in the SEC14L2 cells was then assessed.

As HCV replication had been shown in SEC14L2 cells using patient serum samples containing HCV from genotypes 1 and 3 (by Saeed et al.) the cells were evaluated to assess if viral replication was observed with serum from patients infected with Genotype 3 HCV with a range of viral loads, and at different Magnitudes of infection (MOI). SEC14L2 cells were seeded at 1x10^5 per well in 6 well plates and after 24 hours were incubated with patient serum containing HCV for 4 hours. The cell culture medium was removed after 4 hours and fresh medium was added. Samples were harvested at Day 0 (4 hours post incubation with HCV), Day 1, Day 3, Day 5 and Day 7 post infection. Although HCV replication did occur over 5 days with one patient serum sample containing HCV (see figure 3.8), no viral replication was seen in SEC14L2 cells (cultured in either FBS or in human serum) with any other patient serum samples containing HCV. This may potentially be as a result of either the viral titres used, be due to the lipid profile and vitamin E levels of patient derived HCV, or alternatively reflect the need for replication enabling mutations when HCV from patient sera is used.
As robust viral replication from patient serum containing HCV was not observed following direct incubation with the SEC14L2 cells further experiments were undertaken to evaluate the utility of the SEC14L2 cells (cultured with 10% FBS) in the monocyte fusion assay. Four HCV genotype 3 patient monocyte samples were chosen from patients with serum viral loads of 1x10^6-6x10^6. 1x10^6 patient monocytes were fused with 1x10^6 Huh7.5 cells and a further 1x10^6 monocytes were fused with 1x10^6 SEC14L2 cells (figure 3.9). These experiments demonstrate that SEC14L2 cells facilitate a non-significant increase in HCV viral replication in the monocyte fusion assay in samples with high serum viral loads including patients with liver cirrhosis. Therefore, SEC14L2 cells were used alongside Huh 7.5 cells in subsequent experiments designed to optimise HCV replication in the monocyte/capture fusion assay.
Figure 3.9. A-D) HCV replication 5 days after fusion of monocytes from 4 different HCV patients with Huh 7.5 and SEC14L2 cells E) Pooled data from all 4 monocyte fusion experiments
3.3 Transfection of Huh 7.5 and SEC14L2 cells with a construct expressing ICAM bound Protective Antigen Domain 4

In addition to evaluating modifications to Human hepatoma cells and their effect on viral replication, experiments were undertaken to optimise monocyte-hepatocyte fusion. One of the limitations of the capture fusion assay/monocyte fusion assay is the indiscriminate fusion of hepatocytes and monocytes. The hypothesis that increased monocyte-hepatocyte fusion would increase viral replication was tested by further modifying Huh 7.5 and SEC14L2 cells. Previous work undertaken by our group in collaboration with Dr Angray Kang involved transfecting Huh 7.5 cells with plasmids expressing anti-HCV E2 antibodies, anti-LDL antibodies, and LDLR fused to ICAM and assessing their effect on HCV infection in Huh 7.5 cells. None of these synthetic receptors increased JFH or Con-1 infection of Huh7.5 cells. In addition to these synthetic receptors a further plasmid had been developed by Dr Kang to potentially increase hepatocyte/monocyte fusion. The pEGFP-C3 plasmid was used with the EGFP cut out from the Nhel to the Xbal restriction sites. The EGFP sequence was replaced by a leader sequence, Haemagglutinin tag, and Protective Antigen Domain 4 (PAd4).

Figure 3.10. pEGFP-C3 plasmid with EGFP cut out from Nhel to Xbal and replaced with ICAM leader sequence followed by HA tag, and PAd4-ICAM sequence
(from *Bacillus anthracis*) fused to ICAM (figure 3.10). Protective antigen domain 4 was selected as it is a ligand for capillary morphogenesis gene 2 (CMG-2) which is expressed in monocytes (with increased expression of CMG-2 upon differentiation of monocyte cell lines into macrophages using PMA)\textsuperscript{163}. We speculated that overexpression of this gene would enhance approximation of monocytes and Huh7.5 cells leading to improved cell fusion and thereby enhanced viral replication in the capture-fusion assay. Huh 7.5 cells and SEC14L2 cells were transfected with the plasmid (as described in the methods section) and placed under G418 selection. Expression of PAd4-ICAM was assessed by immunohistochemistry with a Tumour endothelial marker 8 (TEM-8)–Red fluorescent protein (RFP) labelled antibody (see figure 3.11). TEM-8 is an alternative receptor for PAd4 (which is predominantly expressed by vascular endothelial cells). Expression of PAd4-ICAM was confirmed by flow cytometry and cell sorting (using a mouse anti haemagglutinin tag primary antibody and 488 goat anti mouse secondary antibody), cells from the highest decile of PAd4-ICAM expression were selected to form stable cell stocks of Huh7.5 PAd4 and SEC14 PAd4 cells (figure 3.12).
Figure 3.11. Huh 7 and Huh 7 SEC14 cells transfected with PAd4. Immunohistochemistry with RFP labelled TEM8 A. Huh 7.5 PAd4 0.5µg B. Huh 7.5 PAd4 1µg C. Huh 7 SEC14 PAd4 1µg (G418) D. Huh7 SEC14 PAd 4 1µg (G418/puro) E. Huh 7.5 (negative control)
Figure 3.12. Flow cytometry data of A) Huh 7.5 cells (negative control) B) Huh 7.5 cells with anti-HA primary and Alexa Fluor 488 secondary antibody C) Huh 7.5 PAd4 cells (negative control) D) Huh 7.5 PAd4 cells with anti-HA primary and Alexa Fluor 488 secondary antibody.
Huh 7.5 PAd4 cells were then evaluated in the monocyte fusion assay and compared to Huh 7.5 cells to assess if transfection with PAd4 improved viral replication following monocyte fusion (using 6 samples from genotype 3 HCV patients with high serum viral loads $1 \times 10^6$-$6 \times 10^6$). HCV replication occurred with all 6 samples in Huh 7.5 PAd4 cells, and only in 4 samples in with Huh 7.5 cells. Data from the four samples where replication occurred with both cell lines comparing viral replication is shown in figure 3.13.

![Figure 3.13. A-D) Monocyte fusion data for 4 different HCV patient monocyte samples E) Pooled data from all 4 experiments](image)
Huh 7.5 cells transfected with PAd4 demonstrated a non-significant increase in HCV replication in vitro in the monocyte fusion assay. Although the increase in HCV replication seen with Huh 7.5 PAd4 cells was non-significant, this increase in viral replication combined with the more consistent viral replication observed in Huh 7.5 PAd4 (6/6 samples compared to 4/6 with Huh 7.5 cells) justified evaluating these cells in additional assay optimisation experiments.

Further experiments evaluating HCV replication in vitro were undertaken using monocytes from HCV infected patients with low serum HCV viral load. The three cell lines used for these experiments

![Figure 3.14. A-D) Comparison between SEC and SEC14 PAd4 cells with 4 different HCV patient monocyte samples (with low serum viral load) E) Pooled data from all four samples](image)

Figure 3.14. A-D) Comparison between SEC and SEC14 PAd4 cells with 4 different HCV patient monocyte samples (with low serum viral load) E) Pooled data from all four samples
were SEC14L2 cells, Huh 7.5 PAd4 cells and SEC14L2 PAd4 cells. 4 monocyte samples from genotype 3 HCV patients with cirrhosis were used. The serum HCV viral loads of the samples used ranged from 2x10^4 to 1x10^6 IU/ml. SEC14 L2 cells were initially compared with SEC14L2 PAd4 cells (figure 3.14). HCV viral replication was non-significantly increased in monocyte fusion experiments using SEC14 PAd4 cells compared to SEC14 cells, Huh 7.5 PAd4 and SEC14 PAd4 cells were also compared (figure 3.15) in the monocyte fusion assay to determine which cell line would be most effective to be used in the monocyte fusion assay with samples from the TIG3 trial which included several patients with low serum HCV viral loads.

Figure 3.15. A-D) Comparison between Huh7.5 PAd4 and SEC14 PAd4 cells with 4 different HCV patient monocyte samples (with low serum viral load) E) Pooled data from all four samples
The SEC14 PAd4 cells demonstrated significantly greater HCV replication than Huh 7.5 PAd4 cells, and increased viral replication compared to SEC14 cells. Therefore, SEC14 PAd4 cells were used for subsequent monocyte and capture fusion experiments related to the TIG3 trial.

3.4 Conclusions

The optimisation experiments that were undertaken highlighted the challenges faced in achieving a robust in-vitro HCV replication system. As one of the aims of the telaprevir in genotype 3 trial was to evaluate in vitro sensitivity of patient derived HCV to telaprevir, assay optimisation could only focus on adaptations to the hepatoma cell lines used. Three different adaptations were evaluated, culturing hepatoma cells in human serum, using hepatoma cells transfected with SEC14L2 cDNA, and using cells transfected with PAd4. The first two adaptations attempted to address potential factors which inhibited replication of patient derived HCV in vitro, whilst the final adaptation was more specifically targeted at potentially improving the efficacy of monocyte-hepatoma cell fusion in the capture fusion assay. Although modest changes were noted with the use of human serum and SEC14L2 cells these adaptations alone did not result in a marked increase in patient derived HCV replication in vitro. The SEC14L2 cells transfected with PAd4 demonstrated increased HCV replication in the capture fusion assay, including in patient samples with lower viral loads. These cells were therefore also used in the telaprevir in genotype 3 trial to assess in-vitro telaprevir sensitivity.
Chapter 4: Telaprevir in patients with Genotype 3 Hepatitis C: Pilot clinical study to evaluate efficacy and predictability of therapy in patients who have failed to respond to pegylated interferon and ribavirin.

4.1 Background

Genotype 3 hepatitis C is the second commonest genotype of HCV in the United Kingdom accounting for 44% of HCV in England\(^{48}\). The majority of treatment naïve, non-cirrhotic patients respond to treatment with Pegylated Interferon and Ribavirin. In Genotype 3 HCV patients with cirrhosis the success rates are less than 60%\(^{96}\) and patients that fail to eradicate the virus are at risk of complications of chronic liver disease, and at the time of trial inception (prior to sofosbuvir becoming available) there were no alternative treatments for these Genotype 3 cirrhotic patients who had previously failed treatment with interferon and ribavirin.

Telaprevir formed the standard of care treatment for patients with genotype 1 HCV, achieving SVR rates of >75%\(^{122,124}\). Telaprevir is given with pegylated interferon and ribavirin for a total duration of 12 weeks and the interferon-based therapy is then continued for a further 12 or 36 weeks, depending upon pre-treatment characteristics. Telaprevir in combination with pegylated interferon and ribavirin was shown to be effective even in patients who had previously failed to respond to therapy with pegylated interferon and ribavirin. Patients who demonstrated an early virological response were more likely to achieve an SVR, as no patients with a HCV viral load of >1000 at week 4 on treatment achieved a SVR. Similarly, only 25% of patients with a HCV viral load between 100 and 1000 at week 4 achieved a SVR\(^{164}\).
Clinical trials with telaprevir monotherapy in patients with Genotype 3 HCV demonstrated that most patients had a very modest decline in HCV viral load (0.5 log), but a minority (~30%) had a drop in HCV RNA >2 log\textsuperscript{125}. Previous work from our group\textsuperscript{156} showed that patients with Genotype 3 HCV who responded to telaprevir monotherapy had demonstrable sensitivity to telaprevir in our ‘capture-fusion’ assay. This led to the hypothesis that for patients with Genotype 3 HCV who had no reasonable alternative treatment options therapy with pegylated interferon, ribavirin and telaprevir may allow some patients to respond to treatment. We further hypothesised that patients likely to respond to triple therapy with pegylated interferon, ribavirin and telaprevir could be identified by pre-treatment assessment of sensitivity to telaprevir using the ‘capture-fusion’ assay. At the time this trial was conceived and initiated there were no available treatment alternatives to pegylated interferon and ribavirin for patients with Genotype 3 HCV and for patients with cirrhosis who had failed to respond to this medication the standard of care was ‘no treatment’. During the course of this trial alternative medications for the treatment of Genotype 3 HCV became available and at that time we determined that it was no longer appropriate to enrol patients to the trial, which was therefore discontinued early.

4.2 Trial objectives and outcomes

Primary objective: To determine whether patients with genotype 3 HCV and cirrhosis who have relapsed following therapy with PegIFN and RBV will achieve a sustained virological response (SVR) if treated with telaprevir, PegIFN and RBV.

Secondary objective: To determine whether pre-treatment viral phenotyping predicts the response to therapy with telaprevir in patients with G3 HCV and cirrhosis.
Primary outcome: The proportion of patients with a sustained virological response (SVR) 12 weeks after end of treatment (SVR12). SVR 12 is defined as undetectable HCV RNA on a blood sample taken between 12 and 18 weeks after the end of treatment measured using a sensitive, validated polymerase chain reaction (PCR) assay with a lower limit of quantification of at least 30 IU/ml. End of treatment is defined as date of final dose of any IMP (whichever is the latest date).

Secondary outcomes:

- The proportion of patients who are phenotypically poorly responsive to telaprevir (defined as virus with a poor response to telaprevir in vitro i.e. an IC$_{50}$ of >10 μMol in an in vitro assay) who achieve early and late virological clearance.

- The proportion of patients with a sustained virological response 24 weeks after the last dose of PegIFN and RBV (SVR24). SVR 24 is defined as undetectable HCV RNA on a blood sample taken between 24 and 30 weeks after the final dose of PegIFN and ribavirin measured using a sensitive, validated polymerase chain reaction (PCR) assay with a lower limit of quantification of at least 30 IU/ml.

- The proportion of patients who have undetectable HCV RNA (measured using a sensitive, validated polymerase chain reaction (PCR) assay with a lower limit of quantification of at least 30IU/ml) after 1,2,3 and 4 weeks of therapy with PegIFN, RBV and telaprevir.
4.3 Contributions

I co-authored the study protocol (with assistance from Professor Graham Foster and Ailsa Wetherall), and subsequently managed the research and ethics committee and Medicines and Health Regulatory Authority submissions. I devised the study laboratory protocols and standard operating procedures to ensure the laboratory-based component of the trial was compliant with good clinical practice principles. I initiated the different trial sites (with assistance from Ailsa Wetherall) and then monitored trial progress at all the participating sites (with assistance from Adrienne Richards). I uploaded case report forms onto the Discovere database which was used for data collection from the trial. At the Royal London site, I was involved in patient identification and recruitment, along with the management of patients on the clinical trial. I was responsible for data collection, data management and analysis of the trial outcomes. I performed and analysed all of the ‘capture-fusion’ analyses.

4.4 Study Design

Patients who fulfilled the study inclusion and exclusion criteria received open label treatment with 12 weeks of Telaprevir 1125mg twice daily, PegIFN alfa 2a 180 µg once weekly and ribavirin 400mg twice daily followed by 12 weeks of PegIFN alfa 2a 180 µg once weekly and ribavirin 400mg twice daily. Samples were taken for viral phenotyping prior to treatment commencing and at 8 weeks on treatment (figure 4.1). Strict ‘stopping rules’ were included in the trial to ensure that futile therapy was identified and therapy discontinued as soon as it was clear that therapy was ineffective.
Figure 4.1 Schematic of Study Protocol with treatment stopping rules based on virological response and samples collected for in-vitro drug sensitivity phenotyping
4.5 Study patient selection

Patients attending outpatient clinic at study sites who were deemed suitable for this study were identified by their consultant and were contacted to ascertain if they wished to participate in the study. Those who were willing to participate were provided with a patient information sheet about the study. After study subjects had reviewed the information (for at least 24 hours after receiving the information sheet) those that still wished to participate were consented and enrolled onto the study.

The inclusion criteria for the study included patients with low haemoglobin levels, low neutrophil counts and low platelet counts who were excluded from treatment with telaprevir according to the drug license. However, since licensing of telaprevir substantial real-world experience had shown that such patients could be treated safely and as such patients had very limited treatment options (and therefore an urgent unmet need for treatment) at the time of study recruitment they were deemed suitable candidates for the study and approval from the MHRA for their inclusion in this study was requested and given. Hence the study involved ‘off label’ use of telaprevir in genotype 3 HCV in patients who had characteristics that excluded their treatment according to the telaprevir license (which was only for patients with genotype 1 HCV).

The study aimed to recruit 30 patients, with competitive recruitment across all study sites. This number trial participants would enable further evaluation of the findings noted in the previous telaprevir monotherapy trial and the preliminary capture fusion findings (as described in the primary and secondary outcomes for the trial). The sample size of 30 was calculated based on guidance from the National Institute for Health Research, Research Design Service (as there was only limited data for the efficacy of pegylated interferon and ribavirin in the retreatment of genotype 3 hepatitis C in patients with cirrhosis). The inclusion and exclusion criteria for trial participants were designed to
ensure that treatment with telaprevir, PegIFN and ribavirin was appropriate (i.e. the patient had limited treatment options and a delay in treatment was likely to have harmful effects), and that the risk of serious adverse events was minimised by early discontinuation of therapy in patients where a response to treatment was thought improbable based on the early virological response.

4.6 Inclusion criteria

• Age ≥18 years of age and ≤70 years old.
• Advanced fibrosis - defined as a liver biopsy within 2 years showing an Ishak fibrosis score of >4 OR radiological evidence of cirrhosis (ultrasound scan or fibroscan reading >10.6kPa).
• Previous therapy with pegylated interferon and ribavirin for at least 24 weeks with undetectable HCV RNA at the end of therapy and detectable HCV RNA six months after treatment cessation.
• Chronic genotype 3 HCV infection, RNA positivity with genotype 3 infection confirmed at a local laboratory.
• HBsAg negative and no clinical evidence of co-infection with HIV.
• Platelet count >50,000 cells/mm³ (support with eltrombopag is permitted).
• Neutrophil count >600 cells/mm³.
• All female patients of childbearing potential and all males with female partners of childbearing potential must be prepared to use two forms of effective contraception (combined) during treatment and 6 months after treatment end.
• Able and willing to give informed consent and able to comply with study.

4.7 Exclusion Criteria

• Evidence of other cause of significant liver disease – serum ferritin >1000, biochemical evidence of Wilson’s disease, autoantibody titres in excess of 1:160.
• Poorly controlled diabetes that, in the investigators opinion, precludes therapy.
• Severe retinopathy that, in the opinion of the investigator, precludes therapy.
• Evidence of ascites seen on previous liver ultrasound.

• Haemoglobin concentration <11 g/dL in females or <12 g/dL in males or any patient with an increased risk for anaemia (e.g., thalassemia, sickle cell anaemia, spherocytosis, history of gastrointestinal bleeding) or for whom anaemia would be medically problematic.

• Albumin levels <35 G/L.

• Females who are pregnant or breast-feeding.

• History of severe psychiatric disease, including psychosis and/or depression, characterized by a suicide attempt, hospitalization for psychiatric disease, or a period of disability as a result of psychiatric disease within the last 2 years.

• History of immunologically mediated disease (e.g., inflammatory bowel disease, idiopathic thrombocytopenic purpura, lupus erythematosus, autoimmune haemolytic anaemia, scleroderma, severe psoriasis (defined as affecting >10% of the body, where the palm of one hand equals 1%, or if the hands and feet are affected), rheumatoid arthritis requiring more than intermittent nonsteroidal anti-inflammatory medications for management.

• Other on-going serious medical condition in the opinion of the investigator that would prohibit treatment.

• Poorly controlled thyroid dysfunction that, in the investigators opinion, precludes therapy.

• History of major organ transplantation with an existing functional graft.

• History of severe pre-existing cardiac disease, including unstable or uncontrolled cardiac disease in the previous 6 months.

• History or laboratory testing showing evidence of a haemoglobinopathy.

• Concomitant administration with active substances that are highly dependent on CYP3A for clearance and for which elevated plasma concentrations are associated with serious and/or life-threatening events. These active substances include alfuzosin, amiodarone, bepridil, quinidine, astemizole, terfenadine, cisapride, pimozide, ergot derivatives (dihydroergotamine, ergonovine, ergotamine, methylergonovine), lovastatin, simvastatin, atorvastatin, sildenafil or tadalafil (only
when used for treatment of pulmonary arterial hypertension) and orally administered midazolam or triazolam.

- Concomitant administration with Class Ia or III antiarrhythmics, except for intravenous lidocaine.
- Concomitant administration of INCIVO with active substances that strongly induce CYP3A e.g. rifampicin, St John’s wort (*Hypericum perforatum*), carbamazepine, phenytoin and phenobarbital and thus may lead to lower exposure and loss of efficacy of INCIVO (telaprevir).

For patients that were enrolled onto the trial stringent ‘stopping rules’ were applied to minimise exposure to trial medication in patients who were unlikely to benefit from treatment. These stopping rules were based on data from clinical trials evaluating telaprevir in genotype 1 HCV. Virological response at week 4 was predictive of treatment outcome in these trials so similar criteria were applied to this trial. The virological stopping criteria were as follows (figure 4.1):

- If the viral load after 4 weeks of therapy with PegIFN, Ribavirin and telaprevir was >1000 IU/ml, therapy would be abandoned and the patient would be deemed a non-responder to therapy.
- If the viral load at week 8 was greater than 1000 IU/ml OR the viral load had not declined by more than 3 logs from the pre-treatment viral load, therapy would be abandoned and the patient deemed a non-responder to therapy.

In addition to virological criteria determining whether treatment should be discontinued, patient safety criteria were also included which would result in treatment discontinuation. These criteria were:

1. Patients would be withdrawn from the study if there was evidence of systemic drug toxicity which, in the opinion of the investigator, modified the risk: benefit ratio in favour of harm.
2. Request of the participant or discretion of the investigator.


3. Inability or participant’s failure to comply with the protocol requirements.

4. Pregnancy in a trial participant or their partner.

The trial protocol received approval from North East London Research Ethics Committee in November 2013, with Medicines and Healthcare Products Regulatory Agency approval in June 2014. The first trial participant was screened on the 7th of July 2014. Fourteen patients were recruited at the four participating sites (The Royal London Hospital; Queen’s Medical Centre, Nottingham; St George’s Hospital, London; and the Bradford Royal Infirmary), with the final trial participant enrolled on the 6th of November 2014. Trial recruitment was terminated early as sofosbuvir based all oral regimens became available on the early access programme in England and it was expected that patients with compensated cirrhosis would eligible for those treatments in the subsequent 12 months.

4.8 Results

Trial Participant demographic data

Fourteen patients were recruited onto the trial. There were ten male patients and four female patients. The median age of trial participants was 49 years (range 32-63 years). Seven patients were Asian, six were Caucasian and one patient described their ethnicity as other (table 4.1). All patients had advanced liver fibrosis or cirrhosis secondary to Hepatitis C, and all had relapsed following previous treatment with interferon and ribavirin. Thirteen out of the fourteen patients had additional co-morbidities; diabetes was the commonest co-morbidity (n=7), followed by hypertension (n=4), dyspepsia (n=4), skin conditions (n=3), depression (n=3), hypothyroidism (n=2), previous hysterectomy (n=2), insomnia (n=1), hay fever (n=1). The co-morbidities for each individual trial participant are listed in table 4.2, with medications they were taking shown in table 4.3. The medications taken by trial participants at screening correlated with their medical history.
### Demographic data (at screening)  

<table>
<thead>
<tr>
<th>Demographic data (at screening)</th>
<th>n=14</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>47 (32-63)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10 (71%)</td>
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<tr>
<td>Female</td>
<td>4 (29%)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
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</tr>
<tr>
<td>Caucasian</td>
<td>6 (43%)</td>
</tr>
<tr>
<td>Asian</td>
<td>7 (50%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (7%)</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>7 (50%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3 (21%)</td>
</tr>
<tr>
<td>Skin Conditions</td>
<td>3 (21%)</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Depression</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Previous hysterectomy</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Insomnia</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Hay fever</td>
<td>1 (7%)</td>
</tr>
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</table>

**Table 4.1. Demographic characteristics of Study participants at screening**

**Table 4.2 Co-morbidities for each trial participant**
<table>
<thead>
<tr>
<th>Medications at Baseline visit</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
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<tr>
<td><strong>Diabetic medication</strong></td>
<td>n (%)</td>
<td>Antidepressants</td>
<td>n (%)</td>
<td>Analgesia</td>
</tr>
<tr>
<td>Metformin</td>
<td>5 (36%)</td>
<td>Citalopram 1 (7%)</td>
<td>Paracetamol 2 (14%)</td>
<td></td>
</tr>
<tr>
<td>Gliclazide</td>
<td>1 (7%)</td>
<td>Sertraline 1 (7%)</td>
<td>Co-codamol 2 (14%)</td>
<td></td>
</tr>
<tr>
<td>Glimepiride</td>
<td>1 (7%)</td>
<td>Amitriptyline 1 (7%)</td>
<td>Tramadol 1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ibuprofen 1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antihypertensive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>medication</td>
<td></td>
<td></td>
<td>Antacids</td>
<td>Buprenorphine 1 (7%)</td>
</tr>
<tr>
<td>Ramipril</td>
<td>3 (21%)</td>
<td>Lansoprazole 3 (21%)</td>
<td>Methadone 1 (7%)</td>
<td></td>
</tr>
<tr>
<td>Losartan</td>
<td>1 (7%)</td>
<td>Omeprazole 1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gaviscon 1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thyroid replacement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
<td>Antihistamines</td>
<td></td>
</tr>
<tr>
<td>Levothyroxine</td>
<td>2 (14%)</td>
<td>Chlorpheniramine 1 (7%)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Fexofenadine 1 (7%)</td>
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</tbody>
</table>

Table 4.3 Medications taken by trial participants at their baseline visit

Five diabetic patients were on medication (metformin), of these five two were also on a second diabetic medication (glimepiride (n=1) and gliclazide (n=1)). Four patients with hypertension were on antihypertensive medications (ramipril n=3 and losartan n=1). The mean blood pressure for trial participants at their baseline visit was 131/79 mmHg (range 110-165/59-107), along with a pulse rate of 83 beats per minute (range 67-98). The four patients with dyspepsia were on medication for this (lansoprazole n=3, omeprazole n=1, and gaviscon n=1). One patient was on both lansoprazole and gaviscon. Mean results (and range) at baseline (as demonstrated in figure 4.2) were: HCV viral load $2.5 \times 10^6$ IU/L ($1 \times 10^5-1 \times 10^7$); alanine transaminase levels of 104.5 IU/L (33-366), bilirubin 10 µmol/L (6-20), sodium 138 mmol/L (133-142), creatinine 62 µmol/L (35-78), haemoglobin 145.6 g/L (104-167) and platelet count 139 x10⁹/L (53-243).
Figure 4.2. Baseline results: a) HCV viral load b) ALT c) Bilirubin d) Sodium e) Creatinine f) Haemoglobin g) Platelets

4.9 Patient Outcomes

Of the 14 patients that commenced treatment, 13 completed the trial (until they met one of the predetermined trial endpoints). Three patients did not achieve a sufficient virological response at week 4 so their treatment was discontinued. All 10 of the remaining trial participants achieved the virological response necessary at week 8 to continue on treatment. One participant had virological breakthrough on treatment at week 12. Nine patients completed 24 weeks of treatment, of these four achieved an SVR 24 and five relapsed. One patient withdrew from the trial after two weeks of treatment due to adverse events. An overview of the overall outcomes for trial participants is shown in figure 4.3. The baseline demographics and laboratory results of patients with each trial outcome.
(SVR, relapse, treatment stopped at week 4, virological breakthrough and withdrawal from the trial) are listed in table 4.4, and figure 4.4

**Figure 4.3. Overview of trial participant outcomes**
<table>
<thead>
<tr>
<th></th>
<th>SVR N=4</th>
<th>Relapse N= 5 (%)</th>
<th>Break-Through N= 1 (%)</th>
<th>Stopped at Week 4 N=3 (%)</th>
<th>Withdrew N=1 (%)</th>
</tr>
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<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Gender</strong></td>
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</tr>
<tr>
<td>n (%)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4 (100)</td>
<td>5 (100)</td>
<td>0</td>
<td>1 (33)</td>
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<tr>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>1 (100)</td>
<td>2 (67)</td>
<td>1 (100)</td>
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<tr>
<td><strong>Ethnicity</strong></td>
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<td></td>
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<tr>
<td>n (%)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>1 (25)</td>
<td>4 (80)</td>
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<td>1 (33)</td>
<td>0</td>
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<tr>
<td>Asian</td>
<td>3 (75)</td>
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<td>1 (100)</td>
<td>1 (33)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (33)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (75)</td>
<td>1 (20)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2 (40)</td>
<td></td>
<td>1 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin Conditions</td>
<td>1 (25)</td>
<td>2 (40)</td>
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<tr>
<td>Hypothyroidism</td>
<td>1 (20)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Depression</td>
<td>2 (20)</td>
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<td></td>
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<tr>
<td><strong>HCV RNA</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Log IU (SD)</td>
<td>6.2 (0.44)</td>
<td>5.2 (0.27)</td>
<td>5.23</td>
<td>5.67 (0.94)</td>
<td>7.05</td>
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<tr>
<td><strong>ALT</strong></td>
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</tr>
<tr>
<td>IU (SD)</td>
<td>138.5 (153.4)</td>
<td>102.6 (37.4)</td>
<td>142</td>
<td>69.3 (35.1)</td>
<td>46</td>
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<tr>
<td><strong>Bilirubin</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>mmol (SD)</td>
<td>8.75 (3.4)</td>
<td>9.6 (4)</td>
<td>10</td>
<td>12.3 (6.7)</td>
<td>6</td>
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<tr>
<td><strong>Haemoglobin</strong></td>
<td></td>
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</tr>
<tr>
<td>g/L (SD)</td>
<td>151 (12)</td>
<td>152 (16)</td>
<td>132</td>
<td>147 (13)</td>
<td>104</td>
</tr>
<tr>
<td><strong>Platelets</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Giga/L (SD)</td>
<td>131 (41)</td>
<td>145 (61)</td>
<td>114</td>
<td>153 (76)</td>
<td>125</td>
</tr>
</tbody>
</table>

Table 4.4 Baseline demographics of patients with different trial outcomes
Figure 4.4 Demographic and Laboratory results at baseline for patients in different treatment outcome groups a) Age b) HCV RNA c) ALT d) Sodium e) Haemoglobin f) Platelets
4.9.1 Results from Baseline to Week 4 on treatment

After one week on treatment the viral loads of trial participants had declined by a mean of 2.05 log10 IU to 88,596 IU, the virological decline seen in groups of patients with different outcomes is shown in figure 4.5. The alanine transaminase (ALT) levels of trial participants had improved to a mean of 40.4 IU/L (SD 13.6), with different changes in each group of patient outcomes (figure 4.5). Haemoglobin and platelet counts remained stable (figure 4.5) at a mean value of 131 g/L. Only one trial participant reported adverse events in the first week of treatment (headaches, fatigue, difficulty sleeping, itchy skin and decreased appetite). Two patients were commenced on high energy dietary supplements, one was commenced on paracetamol, and one patient was commenced on saxagliptin for diabetes (in addition to metformin).
Figure 4.5 Week 1 on treatment biochemistry results (l) and change from baseline (r) a) HCV RNA b) ALT, c) Bilirubin d) Haemoglobin e) Platelets
At week two a further decline in viral load was noted to a mean of 37,598 IU (a drop of 3.16 log from baseline) (figure 4.6) with ALT levels had changed to a mean of 89.3 IU/L (SD 129.9), with a median value of 38 IU/L, which represented a mean drop in ALT of 13.6 IU (figure 3.6). At this point a decrease in haemoglobin was noted in some trial participants (figure 4.6) to a mean of 131.9 g/L (SD 15.9) which was a mean drop of 13.6 g/L (SD 17.14) and one participant withdrew from the trial at this point due to adverse events (abdominal pain, nausea and pruritis). Five other trial participants had also reported adverse events (flu symptoms, lethargy, low mood, maculopapular rash, nausea, and palpitations) by week 2 on treatment. By week two on treatment one further patient had been commenced on paracetamol as needed and calogen.
Figure 4.6 Week 2 on treatment biochemistry results (l) and change from baseline (r) a) HCV RNA b) ALT, c) Bilirubin d) Haemoglobin e) Platelets
At week 4, the mean viral load of all trial participants had dropped further to a mean of 648.5 IU (a mean of drop in HCV viral load of log 3.39). The four patients who achieved an SVR had a mean viral load of 130 IU/ml, the 5 patients who relapsed had a mean viral load of 332 IU/ml while three patients (mean HCV RNA 2078 IU/ml) did not achieve a sufficient virological response (HCV RNA <1000) and had their treatment discontinued (figure 4.7). There was a significant difference in the viral loads of patients who achieved an SVR or relapse compared to those that stopped treatment at week 4 (p=0.03 and p=0.05 respectively). Mean ALT levels remained at 95 IU/ml (median 35.5 IU/ml, SD 123 IU/ml) although the majority of patients were noted to have an improvement in ALT from baseline. A further drop in haemoglobin was also noted to a mean of 129 g/L (SD 16) with the haemoglobin levels and changes from baseline shown for each different patient outcome group. (figure 4.7).
Figure 4.7. Week 4 on treatment biochemistry results (l) and change from baseline (r) a) HCV RNA b) ALT, c) Bilirubin d) Haemoglobin e) Platelets
The trends for HCV viral load and serum biochemistry from baseline to week 4 on treatment are shown in figure 4.8. This shows that all groups of patients irrespective of trial outcome had a progressive drop in HCV viral load from their baseline visit to week 4 on treatment. The haemoglobin and platelet levels of trial participants also dropped from baseline visit to week 4 on treatment (by a mean of 20.2 g/l haemoglobin and 49 giga/l platelets). Liver biochemistry changes

Figure 4.8 Trend of HCV viral load and biochemistry in trial participants from baseline visit to week 4 on treatment: a) HCV viral load b) Haemoglobin c) Platelets d) ALT e) Bilirubin
were also noted over the first 4 weeks of treatment, with the mean ALT of trial participants decreasing by 15 IU/L and bilirubin rising by 4.7 \( \mu \text{mol/L} \). Trial participants had experienced further adverse events by week 4 (nausea, musculoskeletal pain, skin symptoms (rash/itching) \( n=5 \)), flu like symptoms \( n=4 \), Sore throat, dyspnoea, irritability \( n=3 \), lethargy, headaches \( n=2 \), proctalgia, insomnia, altered taste \( n=1 \)). The patient that withdrew from the trial was commenced on metoclopramide, cyclizine, and esomeprazole for their symptoms between week 2 and 4 on the trial. Four patients received treatment for pruritis and rash: loratidine and betnovate \( n=1 \), E45 cream \( n=1 \), oilatum \( n=1 \), diprobase and chlorpheniramine \( n=1 \). Patients were also commenced on ferrous sulphate for anaemia \( n=1 \), lansoprazole for dyspepsia \( n=1 \), paracetamol for leg pain \( n=1 \), and ondansetron for nausea \( n=1 \).

4.9.2 Results from week 4 to week 12 on treatment

The HCV viral load of trial participants who achieved a viral load of \(<1000 \text{ IU} \) at week four (and therefore continued treatment) declined further from week 4 to week 8 to a mean of 10 IU/ml (with all trial participants having a viral load \(<30 \)) figure 4.9. As all remaining trial participants had a viral load of \(<100 \text{ IU} \) at week 8, all 10 continued on treatment. Haemoglobin and platelet counts had stabilised at the levels seen at week 4 with only small changes from week 4 to week 8 (a mean of 7.7 g/L rise in haemoglobin and 15.1 giga/L rise in platelets were noted). ALT levels had improved further (mean drop 37 IU/L (although SD was 92 IU/L)) and bilirubin levels remained stable with only a mean of 2.78 \( \mu \text{mmol/L} \) increase noted. From week four to eight all trial participants experienced further adverse events. These included gastrointestinal symptoms (vomiting-nausea \( n=6 \), decreased appetite \( n=2 \), altered taste \( n=2 \), proctalgia \( n=1 \), constipation \( n=1 \)), musculoskeletal symptoms (muscular pain \( n=3 \), abdominal pain \( n=1 \), cramps \( n=1 \)), respiratory symptoms (cough \( n=3 \), dyspnoea \( n=1 \)), neuropsychiatric symptoms (insomnia \( n=2 \), dizziness \( n=2 \), headache \( n=1 \), paraesthesia \( n=1 \), visual disturbance \( n=1 \), irritability \( n=1 \), anxiety \( n=1 \), depression \( n=1 \),
Figure 4.9 Week 8 on treatment HCV RNA (l) and change from week 4 (r)

skin symptoms (rash n=4, itching n=2, and hair loss n=1) and other symptoms (lethargy n=4, dysuria n=2, flu like symptoms n=2, weakness n=1, temperature n=1, sweats n=1, sore throat n=1). One patient was noted to have biochemical abnormalities (anaemia and thrombocytopenia which were identified between weeks 4 to 8). Trial participants commenced additional medication for certain adverse events: three patients with nausea needed medication (ondansetron n=2, cyclizine n=1); three patients with rashes/pruritis commenced treatment (cetirizine n=2, topical cream n=3); two patients commenced medication for musculoskeletal pain (paracetamol n=1, and co-codamol n=1); two patients with a cough/sore throat were given antibiotics (amoxicillin n=1, clarithromycin n=1) and one commenced simple linctus; one patient commenced omeprazole for dyspepsia; one patient commenced anusol for proctalgia; and one patient commenced darbepoetin for anaemia.
At week 12 ten trial participants had completed 12 weeks of treatment with Telaprevir, pegylated interferon and ribavirin. Only three of the 10 trial participants had detectable RNA at this point (figure 4.10). Two participants had HCV RNA <15 IU/ml, and one <30 IU/ml. One of the trial participants was noted to have HCV RNA <15, having previously had undetectable HCV RNA levels. On repeat testing this individual was noted to have virologic breakthrough and their treatment was discontinued. Haemoglobin, platelet counts and liver biochemistry of trial participants remained stable from week 4 to 12 (figure 4.10) with only a mean 3 g/L change in haemoglobin, a mean 11.9 giga/L change in platelets, an ongoing improvement in ALT (mean decrease of 53 IU/L) and mean 3.4 µmol/l change in bilirubin. At week 12 further patients had experienced adverse events: rash (n=5), nausea (n=2), poor concentration (n=2), insomnia (n=1), and diarrhoea (n=1). Four patients commenced additional treatment for rashes (cetirizine n=1, chlorpheniramine n=1, sudacrem n=1, and cetraben n=1); one patient commenced cyclizine and gaviscon for nausea; and one patient started corsodyl mouth wash for mouth ulcers.
Figure 4.10. Week 12 on treatment biochemistry results (l) and change from week 4 (r) a) HCV RNA b) haemoglobin, c) platelets d) ALT e) bilirubin
4.9.3 Week 12-24 on treatment

The remaining nine trial participants continued on the trial with a further 12 weeks of Pegylated Interferon and Ribavirin. At week 16 all trial participants bar one had undetectable HCV RNA. This patient subsequently relapsed post treatment. Trial participants’ biochemistry remained stable from week 12 to week 16 on treatment. Mean haemoglobin was 129 g/L, platelet count was 80 giga/L, ALT was 90.7 IU/L and bilirubin was 10.7 µmmol/L. Between weeks 12 and 16, patients experienced several more adverse events: two developed a rash, one developed a dental abscess, one developed thrush, and one patient developed an elevated GGT (attributed to alcohol consumption). Two patients commenced treatment for their rash (one patient started cetirizine and diprobase, and one commenced betnovate cream); the patient with thrush commenced on canesten; and the patient with a dental abscess was treated with metronidazole.

At week 20 all participants bar the one that had detectable HCV RNA at week 16 had undetectable HCV RNA. Trial participants’ biochemistry remained stable from week 16-20. Haemoglobin remained at 129 g/L, platelet count was 86 giga/L, ALT was 106.6 IU/L and bilirubin 11 µmmol/L. Two patients had developed further adverse events: One developed a rash, and one developed paraesthesia. Two patients commenced treatment for rashes (chlorpheniramine n=1, and E45 cream n=1).

At the end of treatment all patients who achieved an SVR had an undetectable HCV viral load, while 2 of the 5 patients who relapsed had detectable HCV RNA (figure 4.11). Mean haemoglobin at this point was 128 g/L, platelet count was 76 giga/L, ALT 88.9 IU/L and bilirubin 11 µmmol/L. Adverse events occurred between weeks 20 and 24: one trial participant developed chest pain and a rash, one developed a cough, one developed a urinary tract infection, one developed insomnia, and another developed muscular back pain. Treatment was commenced with trimethoprim for the patient with a UTI, and benylin for the patient with a cough.
Figure 4.11 End of treatment biochemistry results (l) and change from week 12 (r) a) HCV RNA b) ALT c) bilirubin d) haemoglobin e) platelets
4.9.4 Post treatment

At week 12 post treatment four of nine patients had a detectable viral load (figure 4.12), with a mean HCV viral RNA of log 5.6 IU/ml. Mean haemoglobin had increased to 145.9 g/L, as had the mean platelet count which was now 141 giga/L. Mean ALT had risen to 128.9 IU/L and mean bilirubin was 8.3 µmol/l. Two further adverse events were noted: rectal bleeding (n=1), and dysuria (n=1).

At week 24 post treatment only the 4 patients who achieved an SVR and one patient who relapsed (HCV viral load log 5.9 IU/ml) remained on the trial (figure 4.13). Mean haemoglobin had remained at 149.6 g/L, mean platelets remained at 135 giga/L, mean ALT was 48 IU/L and mean bilirubin was 10. Post treatment further trial participants experienced adverse events: one had developed muscular back pain and sciatica, one developed eczema on both shins, one developed increased urinary frequency and rectal bleeding, one developed cramps, and one developed a coryzal illness. The patient with eczema was commenced on hydrocortisone cream, and the patient with sciatica was commenced on co-codamol and gabapentin.
Figure 4.12. Post treatment week 12 biochemistry results (l) and change from end of treatment (r) a) HCV RNA b) ALT c) bilirubin d) haemoglobin e) platelets
4.10 Adverse events

There was only one serious adverse event during the trial. This occurred after one of the trial participants had been screened but prior to their baseline visit. The patient was admitted for under 24 hours with confusion, agitation and aggression. This was thought to be secondary to alcohol and substance misuse, and resolved spontaneously. That individual subsequently tolerated treatment as per trial protocol and completed 24 weeks of treatment. Treatment on the trial was tolerated relatively well with only one patient withdrawing from the trial due to treatment side effects. This may be because the trial participants had previously tolerated treatment with interferon and ribavirin. Despite all patients except one reaching a trial endpoint for treatment, the combination of telaprevir, interferon and ribavirin resulted in all patients experiencing adverse events during treatment (table 4.5). The most frequently reported adverse events were skin related (n=28), followed by gastrointestinal (n=24), neuropsychiatric (n=24), general/systemic (n=22), musculoskeletal (n=12), respiratory (n=6), urological/gynaecological (n=6), biochemical abnormalities (n=4), oral (n=3), and other categories (n=2). The breakdown of each of those
Table 4.5 Adverse events experienced by trial participants

<table>
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<th>Symptom</th>
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<th>% of trial participants</th>
<th>Symptom</th>
<th>Number</th>
<th>% of trial participants</th>
<th>Symptom</th>
<th>Number</th>
<th>% of trial participants</th>
</tr>
</thead>
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<td>7</td>
</tr>
<tr>
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</table>

categories is shown in table 4.6. Skin related side effects were: rash (n=21), pruritis (n=5), with periorbital oedema and hair loss each experienced by one trial participant. Gastrointestinal adverse events were: nausea (n=10), vomiting (n=3), loss of appetite (n=3), dyspepsia/reflux (n=3), constipation (n=1), diarrhoea (n=1), proctalgia (n=1), rectal bleeding (n=1), and abdominal pain (n=1). Neuropsychiatric adverse events were: insomnia (n=6), dizziness (n=4), paraesthesia (n=3), memory impairment (n=2), visual disturbance (n=2), dysgeusia (n=2), depression (n=2), irritability (n=1), ear pain (n=1), and anxiety (n=1). General/systemic adverse events experienced were: fatigue (n=7), coryzal symptoms (n=6), pyrexia (n=4), headache (n=3), and weakness (n=2). Musculoskeletal adverse events were: musculoskeletal pain (n=12). This was divided into: generalised musculoskeletal pain (n=5), lower limb pain (n=3), back pain (n=3), and chest pain (n=1). Respiratory adverse events were: cough (n=5), dyspnoea (n=1). Urological/gynaecological adverse events were: dysuria (n=3), urinary tract infection (n=1), vaginal discomfort (n=1), vaginal thrush (n=1). Biochemical abnormalities noted were: raised bilirubin (n=1), raised gamma GT (n=1), anaemia (n=1), and thrombocytopenia (n=1).
Table 4.6 Adverse events episodes by body system

Oral/dental adverse events were: dental abscess (n=1), mouth ulcers (n=1), and sore mouth (n=1).

The remaining adverse events were: foot injury after treading on a nail (n=1), and injection site reaction (n=1).

The majority of these adverse events occurred early during treatment, of a total of one hundred and thirty-one adverse effects noted, thirty-eight were recorded during the first 4 weeks of treatment (with nineteen of these occurring during the first fortnight of treatment). A further fifty-eight adverse events occurred by week eight on treatment, and only a further thirteen adverse events were recorded by week twelve on treatment. Fourteen further adverse events were recorded from
weeks twelve to twenty-four on treatment. Eight adverse events commenced after patients completed treatment.

The majority of the adverse events experienced were mild (n=98, 75%), 32 were moderate (24%) and only one adverse event was graded as severe. The severe adverse event was for a patient who had chest pain at week twenty on treatment. That patient attended their local emergency department and was discharged after having blood tests taken and an ECG performed. This was not thought to be due to the trial medications, and the patient was not commenced on any additional regular medication. Unfortunately, their symptoms persisted until the end of follow up although they did not need any additional medications or treatment. The majority of adverse events (n=94, 72%) experienced by trial participants resolved by the end of the trial follow up period (twenty-four weeks post treatment). The median duration of adverse events experienced was three weeks (standard deviation 8.1 weeks). Thirty-seven (28%) of adverse events experienced were still ongoing at the end of treatment. Six of these were adverse events experienced after patients had completed treatment. The adverse events still experienced at the end of trial follow up were gastrointestinal (n=9, (dyspepsia n=3, loss of appetite n=2, nausea n=2, rectal bleeding n=1 and diarrhoea n=1)), neuropsychiatric (n=8, (insomnia n=3, depression n=1, dizziness n=1, irritability n=1, paraesthesia n=1, ear pain n=1)), skin (n=7, (pruritis n=4, rash n=3)), musculoskeletal pain (n=5), general/constitutional side effects (n=4, (fatigue n=2, coryzal symptoms n=1, pyrexia n=1), biochemical abnormalities (n=3, (anaemia n=1, thrombocytopenia n=1, abnormal liver biochemistry n=1)) and urogynaecological (n=1, (dysuria n=1).

Two of the adverse events experienced (low haemoglobin and coryzal symptoms) were thought to be definitely due to the IMP. Eighteen adverse events were thought to be probably related to the IMP (rash n=5, fatigue n=3, nausea n=2, insomnia n=2, coryzal symptoms n=1, pyrexia n=1, dyspepsia n=1, irritability n=1, poor concentration n=1, pruritis n=1). Of the remaining adverse events experienced 47 were possibly related to the IMP, and 61 were unlikely to be, or were not related to the IMP. With regards to action taken for adverse events, one patient temporarily discontinued all
three IMPs due to nausea, and one patient had both their ribavirin and interferon dose decreased for anaemia and thrombocytopenia respectively. Twenty-seven adverse events resulted in the patient receiving an additional medication, these were (rash n=8, dyspepsia n=3, pruritis n=2, musculoskeletal pain n=2, insomnia n=2, vomiting n=2, urinary tract infection n=1, vaginal discomfort n=1, cough n=1, proctalgia n=1, pyrexia n=1, coryzal symptoms n=1). Fourteen adverse events needed additional action taken: six patients were monitored by their local HCV nurses and needed a prescription (these were for headache n=2, nausea n=1, mouth ulcers n=1, rash n=1, vaginal candidiasis n=1); four adverse events were monitored by the local HCV nurses. One referral was made the local dermatologists for assessment of a rash. One patient had an additional ECG during the trial. One patient was seen in the emergency department for chest pain then discharged (without needing admission). One final patient had their dose of interferon and ribavirin reduced due to thrombocytopenia and anaemia (as described above).

Twelve patients needed additional medication for treatment of adverse events experienced during the trial. A total of 59 additional medications were taken by trial participants (see table 4.7). The most frequent indication for additional medication was skin symptoms (n=22: rash n=14, pruritis n=7, general skin care n=1), this was followed by gastrointestinal symptoms (n=12: nausea n=8, dyspepsia n=3, proctalgia n=1), musculoskeletal pain (n=5), dietary supplementation as an adjunct to treatment (n=4), general symptoms (n=3: coryzal symptoms n=1, headache n=1, sore throat n=1), respiratory symptoms (n=3: cough n=3), glycaemic control (n=2), oral symptoms (n=2: mouth ulcer n=1, dental abscess n=1), urological/gynaecological symptoms (n=2: vaginal candidiasis n=1, urinary tract infection n=1), anaemia (n=2), insomnia (n=1), injection site reaction (n=1). The medications prescribed were topical creams (n=15), antihistamines (n=8), antiemetics (n=7), analgesia (n=7), dietary supplements (n=4), antibiotics (n=4), antacids (n=4), anaemia (n=2), medication for glycaemic control (n=2), cough medicine (n=2), mouthwash (n=1), anal cream (n=1), sleeping tablets (n=1) and antifungal medication (n=1). The majority of medications (55/59) were commenced while patients
Table 4.7 Additional medication commenced during trial

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were on treatment with trial medication (the median time point at which trial participants commenced additional medications was 5 weeks after baseline visit (standard deviation 9.8 weeks)). The mean duration of treatment was 5 weeks, and 44% (24/55) of additional medications that were commenced during the trial were stopped by the end of trial follow up. Of the total of 59 medications commenced during the trial 35 were still being taken at the end of trial follow up. Just under half of these were for skin complaints (n=15, and included 10 topical moisturising/barrier creams, 4 antihistamines were also still being taken at the end of the trial, and one trial participant was still on hydrocortisone cream), nine medications were still being taken for gastrointestinal side
effects (5 were antiemetics and 4 were antacids), four patients were still needing analgesia for musculoskeletal symptoms at the end of the trial, and two patients were on additional diabetic medication. Two trial participants remained on nutritional supplements at the end of the trial, one patient was still using topical cream for an injection site reaction, another was still taking paracetamol for coryzal symptoms, and a final patient was still needing zopiclone for insomnia.

Although there were no serious adverse events whilst patients were receiving treatment as part of the trial, the adverse event profile was consistent with previous data on treatment with telaprevir, interferon and ribavirin. This treatment combination resulted in a range of previously documented symptoms, and resulted in almost all the trial participants needing medication to alleviate at least some of their symptoms. As would be expected with this treatment combination, skin related side effects, general/constitutional adverse events, and gastrointestinal adverse events were frequently experienced. Despite the frequency of symptoms, all trial participants except one, completed treatment as per the trial protocol.

4.11 Discussion

All patients bar one completed participation on the trial until a virological endpoint was achieved. The overall SVR rate in this population who had failed to respond to pegylated interferon and ribavirin was 29% (four patients). Three patients did not achieve a sufficient viral response at week 4 to continue treatment and one patient withdrew before week four due to adverse events. Of the remaining patients on one patient had virological breakthrough before the end of treatment (this was between weeks 12 and 24 on treatment, after they had completed twelve weeks of treatment with telaprevir). The number of patients achieving an SVR was comparable to previous trials evaluating retreatment with pegylated interferon in patients with genotype 3 hepatitis C and advanced fibrosis or cirrhosis who had previously failed treatment with interferon and ribavirin$^{98,165}$. As the number of patients enrolled onto the trial was lower than expected and the overall number
that achieved an SVR was only 4 patients, it is hard to determine the impact of adding telaprevir to pegylated interferon and ribavirin. Furthermore, the absence of an untreated control arm (treated with pegylated interferon and ribavirin alone) makes assessment of the additional value of telaprevir problematic. During the course of the clinical trial, many other treatment regimens were concurrently being evaluated in clinical trials, including several all oral treatment regimens. The prospect of all oral treatment regimens becoming available in the next few years undoubtably affected recruitment onto the trial. It is of note that none of the trial participants were initially eligible for the early access programme, therefore the trial provided a treatment option with possible additive benefit to pegylated interferon and ribavirin to a group of patients who had a limited range of treatment readily accessible at the time of trial recruitment.

The development of sofosbuvir, and first generation NS5A inhibitors revolutionised the treatment of hepatitis C, providing all oral regimens which had excellent efficacy in treating genotype 1 hepatitis C. Further protease inhibitors (grazoprevir and paritaprevir) were also developed, although early trials with the combination of grazoprevir, elbasvir and ribavirin only resulted in SVR rate of 45 and 57% in non-cirrhotic genotype 3 patients. Subsequent newer protease inhibitors glecaprevir and voxilaprevir demonstrated pan-genotypic activity rendering first generation protease inhibitors (such as telaprevir and boceprevir) obsolete. The rapid improvements in this field render the use of telaprevir obsolete and it is unlikely that the approach outlined here will be pursued further.

Treatment was tolerated well despite the side effects experienced by trial participants. This was consistent with the side effect profile from treatment with telaprevir, interferon and ribavirin. No serious adverse events occurred on treatment and only one patient withdrew from the trial due to treatment related side effects. It should be noted that the patient cohort included in this trial were
highly ‘self-selected’. They had all undergone previous treatment with pegylated interferon and ribavirin and were therefore aware of the adverse events this treatment entailed and were willing to tolerate these. It is unclear whether a group of treatment naïve patients would have tolerated treatment in a similar manner.

Although the findings of this trial have been superseded by the availability newer all oral direct acting antiviral drug regimens, protease inhibitors are now are a mainstay in the standard of care treatment of patients with genotype 3 hepatitis C in the United Kingdom, but given the high incidence of side effects in this study we would not recommend further studies in this population with telaprevir based regimens.
Chapter 5: Telaprevir in Genotype 3 Hepatitis C: Capture fusion results

5.1 Methods

Initial capture fusion experiments were performed (as described in Methods) using THP-1 monocytes. $1 \times 10^6$ THP-1 cells were aliquoted onto 6 well plates and stimulated with phorbol myristate acetate (PMA) and interferon γ for 24 hours. Patient serum containing hepatitis C (taken at the baseline visit during the trial) was added to the THP-1 cells at a magnitude of infection of one (i.e. one IU of virus per THP-1 cell). Patient serum was blinded using an independent laboratory scientist prior to addition to THP-1 monocytes to eliminate bias in the interpretation of capture fusion data. Cells were incubated with patient serum for 24 hours. These cells were then fused with $1 \times 10^6$ Huh 7.5 hepatocytes as described in Methods. The fused cells were divided into 20 aliquots and plated out in 6 well plates with 1ml of culture medium (DMEM with 10% FBS). 24 hours post fusion telaprevir was added at four different concentrations ($10 \mu M$, $1 \mu M$, $0.1 \mu M$, $0.01 \mu M$ and no drug). The dose range was selected based on previous work by Cunningham et al.\textsuperscript{156} which enabled identification of HCV from patient serum which was sensitive or insensitive to telaprevir in vitro. Samples which were telaprevir sensitive had an IC$_{50}$ of less than 1mM while insensitive samples had an IC$_{50}$ of >10mM. Cell culture medium was changed four days post fusion, and telaprevir was added to the new medium. Experiments were harvested five days post fusion in 1 ml of TRIzol reagent. RNA extraction was performed as previously described, with total RNA quantified using RiboGreen reagent, and viral RNA calculated by real time quantitative PCR. Drug inhibition of HCV viral replication from each serum sample was quantified by representing HCV RNA detected for each drug concentration as a percentage of hepatitis C in the sample without drug. Dose response curves and the half maximal inhibitory concentrations (IC$_{50}$) were calculated by using GraphPad software. Statistical analysis was performed using unpaired t-tests for pooled samples and IC$_{50}$ values. Mann Whitney U tests were used for statistical analysis of HCV viral load and telaprevir sensitivity in vitro.
5.2 Capture Fusion results

The capture fusion results for the patients in the TIG 3 trial are shown in figures 5.1-5.2. Capture fusion data was obtained for 10 samples and dose response curves and IC₅₀ values were calculated using GraphPad software. Insufficient HCV viral RNA was detected in four samples (including in the aliquots without drug inhibition) to calculate dose response curves. Three samples were telaprevir sensitive in vitro with IC₅₀ values of 0.13, 0.03 and 8x10⁻⁹ µM (figure 5.1), albeit with wide confidence intervals. Repeated analysis of samples was precluded by a limited amount of patient serum, which may have allowed more robust data to be generated.

Figure 5.1 Samples from patients on the telaprevir in genotype 3 HCV trial that are sensitive to telaprevir in the capture fusion assay.
For seven patients no clear response to telaprevir could be seen (figure 5.2). The increase in HCV RNA seen in some patients at the highest concentrations of drug may reflect cell death due to toxicity and we regard this as artefactual. The lack of any clear dose response curve led us to conclude that these patients were telaprevir ‘insensitive’ and the failure to demonstrate reduction of HCV RNA at drug concentrations that were non-toxic precluded an accurate assessment of the IC50.
Figure 5.2. Samples from patients on the telaprevir in genotype 3 HCV trial that are insensitive to telaprevir in the capture fusion assay.

The pooled telaprevir inhibition curves for samples that were sensitive (n=3) and insensitive (n=7) were calculated. There was a significant difference (p=0.009, p=0.01) between the pooled sensitive and pooled insensitive samples at telaprevir doses of 1µM and 10µM (figure 5.3). The IC₅₀ values
obtained for telaprevir sensitive samples were significantly different (p<0.001) to those from telaprevir insensitive samples (figure 5.4).

Having identified telaprevir sensitive and insensitive samples, the serum samples were unblinded to enable correlation with clinical outcome (figure 5.5). Of the three patients with serum HCV that was sensitive to telaprevir in vitro, two achieved an SVR, whilst one stopped therapy at week 4 due to
insufficient virological response. Seven patients had serum HCV that was insensitive to telaprevir in vitro. One of the patients achieved an SVR, one stopped at week 4 on treatment, 4 relapsed post treatment, and one withdrew from the trial at week 2. The sensitivity and specificity of the capture fusion assay in predicting clinical outcome for patients on the TIG 3 assay is shown in table 5.1. The capture fusion assay had a sensitivity of 67% and a specificity of 86%. The corresponding positive predictive value was 67%, with a negative predictive value of 86%. The correlation between in vitro
Table 5.1. The sensitivity and specificity of the capture fusion assay in predicting clinical outcome in the Telaprevir in Genotype 3 clinical trial

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<td>Positive predictive value (67%)</td>
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<td>Sensitivity (67%)</td>
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telaprevir sensitivity and viral load at baseline, week 2 and week 4 visits are shown (figure 5.6) along with the log drop in HCV RNA from baseline to week 2 and 4, and the log drop in HCV RNA from week 2 to 4. There was no significant difference in baseline HCV RNA between those patients who were sensitive to telaprevir and those that were insensitive (p=0.25). Similarly, there was no significant difference between in vitro serum HCV sensitivity and viral load at weeks 2 and 4 (p=0.50, p=0.39). The change in HCV viral load from baseline to week 2 and from baseline to week 4 on treatment was not significantly different (p=0.39, p=0.47). The change in HCV RNA from weeks 2 to 4 on treatment was also not significantly different between patients with serum HCV which was sensitive to telaprevir compared to those with serum HCV insensitive to telaprevir (p=0.19).

The initial capture fusion data demonstrated that three of 10 HCV serum samples were sensitive to telaprevir in vitro. This data is consistent with previous data evaluating genotype 3 HCV sensitivity to telaprevir in vitro. Interestingly two out of three patients who had telaprevir sensitive HCV in vitro achieved an SVR however there was no significant difference in virological response between the patients who had HCV sensitive to telaprevir in vitro and those who had HCV insensitive to telaprevir in vitro.
Figure 5.6. Telaprevir sensitivity in vitro and HCV viral load and change in viral load on treatment: a) Telaprevir sensitivity and baseline HCV RNA b) Telaprevir sensitivity and HCV RNA at week 2 on treatment c) Telaprevir sensitivity and change in HCV RNA from baseline to week 2 on treatment d) Telaprevir sensitivity and HCV RNA at week 4 on treatment e) Telaprevir sensitivity and change in HCV RNA from baseline to week 4 on treatment f) Telaprevir sensitivity and change in HCV RNA from week 2 to week 4 on treatment.
5.3 Monocyte fusion assay data

The data using THP1 cell fusions did not allow an accurate differentiation of responder and non-responder patients. However, four samples did not generate meaningful data and we therefore examined alternative ‘capture-fusion’ approaches to increase the sensitivity of the model and to increase the proportion of samples that generated useful data. As described in Results Chapter 1 (Capture fusion assay optimisation). Huh 7.5 cells were developed to permit greater viral replication in vitro. Once stable cell lines transfected with both SEC14L2 and PAd4 were established these were used for preliminary monocyte fusion experiments which identified increased HCV replication in vitro when this cell line was used. Subsequently the Huh 7.5 SEC14L2 PAd4 cells were used in fusion experiments with monocytes obtained from TIG 3 trial participants at their baseline visit. The rationale behind using monocytes from trial participants was that due to the low baseline serum HCV viral load in several of the patients the serum ‘capture-fusion’ assay data was suboptimal and we speculated that the use of patient monocytes would improve on the quality of the data and perhaps allow patients with very low viral loads to be studied. Once again patient monocytes were blinded by laboratory co-workers prior to fusion to avoid bias during results analysis. The same range of Telaprevir doses as used in the capture fusion assay was evaluated using the method described in Chapter 2. 1x10^6 patient monocytes were fused with an equivalent number of Huh 7.5 SEC14L2 PAd4 hepatocytes. Telaprevir was added at days one and 4 post fusion with samples harvested at day 5 and in-vitro telaprevir sensitivity was calculated (GraphPad). Statistical analysis of the monocyte fusion data was performed using unpaired t-tests for pooled dose response curve analysis, and IC_{50} analysis. Mann Whitney U tests were used for statistical analysis of HCV viral load and telaprevir sensitivity in vitro.

Telaprevir dose response curves were obtained for 13 of the 14 samples. There was insufficient HCV viral replication with monocytes from one patient in the fusion assay. Three samples were sensitive to telaprevir in vitro with IC_{50} values of 0.14, 0.005, and 0.003 µM (fig 5.7). The remaining 10 samples were insensitive to telaprevir in vitro (with IC_{50} values greater than 10µM) (fig 5.8 a-b). Pooled dose
Figure 5.7. Samples sensitive to telaprevir in the monocyte fusion assay.

Figure 5.8a. Samples insensitive to telaprevir in the monocyte fusion assay.
response curves were calculated for monocyte samples that had telaprevir sensitive HCV in vitro and those monocyte samples that were insensitive to telaprevir in vitro (fig 5.9). Pooled HCV replication in vitro was significantly different in patients with monocyte derived HCV sensitive to telaprevir compared to those who were telaprevir insensitive at 1 and 10µM concentrations of telaprevir.
The IC₅₀ values obtained were significantly different for monocyte samples sensitive to telaprevir (p<0.001) compared to those insensitive to telaprevir (figure 5.10).

The monocyte samples were unblinded to enable correlation of the in vitro results with clinical outcome (figure 5.11). Monocyte fusion data was obtained from thirteen of the fourteen patient samples used. Three samples were identified as being telaprevir sensitive. Of these patients one achieved SVR, one relapsed, and one stopped treatment at week 4 due to insufficient virological response. Ten samples were insensitive to telaprevir in vitro. The clinical outcomes for these patients are as follows: three achieved an SVR, four relapsed post treatment, one patient
experienced virological breakthrough on treatment, and two patients stopped treatment at week 4 due to insufficient virological response. This resulted in the monocyte fusion assay having a sensitivity of 25%, a specificity of 78%, a positive predictive value of 33%, and a negative predictive value of 70% (table 5.2).

![Diagram of Monocyte Fusion Results and Clinical Outcome](image)

**Figure 5.11. Correlation between in vitro telaprevir sensitivity in the monocyte fusion assay and clinical outcome.**

<table>
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<th>Negative predictive value (70%)</th>
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<td>Specificity (78%)</td>
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</table>

**Table 5.2. The sensitivity and specificity of in vitro telaprevir sensitivity in the monocyte fusion assay predicting clinical outcome**
The correlation between in vitro telaprevir sensitivity in the monocyte fusion assay and viral load at baseline, week 2 and week 4 visits are shown (figure 5.12) along with the log drop in HCV RNA from:

- **Figure 5.12.** Telaprevir sensitivity in vitro in the monocyte fusion assay and its correlation with HCV viral load and change in viral load on treatment. 
  
  a) Telaprevir sensitivity and baseline HCV RNA 
  
  b) Telaprevir sensitivity and HCV RNA at week 2 on treatment 
  
  c) Telaprevir sensitivity and change in HCV RNA from baseline to week 2 on treatment 
  
  d) Telaprevir sensitivity and HCV RNA at week 4 on treatment 
  
  e) Telaprevir sensitivity and change in HCV RNA from baseline to week 4 on treatment 
  
  f) Telaprevir sensitivity and change in HCV RNA from week 2 to week 4.
baseline to week 2 and 4, and the log drop in HCV RNA from week 2 to 4. There was no significant difference in baseline HCV RNA between those patients who were sensitive to telaprevir and those that were insensitive (p=0.29). Similarly, there was no significant difference between in vitro serum HCV sensitivity and viral load at weeks 2 and 4 (p=0.41, p=0.34). The change in HCV viral load from baseline to week 2 and from baseline to week 4 on treatment was not significantly different either (p=0.23, p=0.34). The change in HCV RNA from weeks 2 to 4 on treatment was also not significantly different between patients with serum HCV which was sensitive to telaprevir compared to those with serum HCV insensitive to telaprevir (p=0.23).

The correlation between the capture fusion results and monocyte fusion results is shown in figure 5.13. Results from both assays were only available for nine of the 14 patients.

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**Figure 5.13. In vitro sensitivity to telaprevir in both the capture and monocyte fusion assays and its correlation with patients’ clinical outcomes on the telaprevir on genotype 3 HCV trial**
Three patients were insensitive in both the capture fusion and monocyte fusion assay. All three of these patients did not achieve an SVR. Of the remaining six, three had HCV sensitive to telaprevir in the capture fusion assay, but not in the monocyte fusion assay. Two of these patients achieved an SVR, while one was stopped at week 4 due to insufficient virological response. The final three patients had HCV sensitive to telaprevir in the monocyte fusion assay, but not in the capture fusion assay. One of these patients achieved an SVR, while one relapsed, and one stopped treatment at week 4 due to insufficient virological response.

The dose response curves for the samples where there was a discrepancy between the capture fusion assay (using patient serum) and the monocyte fusion (using patient monocytes) were compared figure 5.14.

![Figure 5.14. Samples with a discrepancy in telaprevir sensitivity between capture fusion and monocyte fusion](image)

Figure 5.14. Samples with a discrepancy in telaprevir sensitivity between capture fusion and monocyte fusion
Figure 5.14 continued. Samples with a discrepancy in telaprevir sensitivity between capture fusion and monocyte fusion.

Of the six samples where there was a discrepancy a dose response curve could not be calculated in four of these samples A, D-F. In the remaining two samples, B and C (where HCV from patient serum
was sensitive in the capture fusion assay), dose response curves were calculated in the monocyte fusion assay despite the samples being insensitive to telaprevir, although the IC$_{50}$ was greater than 10µM.

Potential hypotheses regarding the discrepancy between the capture fusion and monocyte fusion would include technical factors, in particular as three of the discordant samples (B, D and E) had viral loads below 1x10$^6$. Alternatively, in 3 of the samples HCV derived from monocytes appeared to be sensitive to telaprevir in vitro (D-F) while HCV from the same patient’s serum was not sensitive in-vitro (and no dose response curve could be calculated). Similarly, in one sample (A) HCV from patient serum was sensitive in the capture fusion assay but a dose response curve could not be calculated in the monocyte fusion assay. These findings warranted further investigation, and repeat capture fusion experiments were performed. Unfortunately, there were insufficient monocytes from trial participants to repeat the monocyte fusion experiments. This could have provided an opportunity to undertake next generation sequencing on HCV derived from monocytes and enabled comparison with HCV from patient serum, to determine if there are different in HCV quasispecies present in these two different compartments, and whether this would account for the discrepancy noted in the capture and monocyte fusion results.
5.4 Capture fusion data: Grazoprevir

During the course of the Telaprevir in genotype 3 clinical trial additional antiviral drugs became available. In addition to the NS5B inhibiting drug sofosbuvir, new second generation protease inhibitors also became available which were thought to have greater pan-genotypic activity. Grazoprevir is one of the first of these pan-genotypic protease inhibitors. In order to evaluate whether HCV from patients in the TIG 3 trail was sensitive to grazoprevir, preliminary experiments identifying a dose range which would discriminate between grazoprevir sensitive and insensitive HCV in vitro were performed.

The s52 (genotype 3) replicon was used to assess the inhibitory effect of grazoprevir (a second generation pan genotypic protease inhibitor) on HCV RNA replication. Huh 7.5 cells containing the replicon were seeded in 96 well plates with clear bottoms at 1x10^4 cells/per well. 24 hours later cells were exposed to a range of concentrations of grazoprevir and incubated for 72 hours. Cell lysates were prepared using 1x cell lysis buffer, and firefly luciferase expression was measured with a luciferase assay system (Promega) and BMG plate reader. The s52 replicon assay results are shown (fig 5.15) and identified an IC_{50} value of 0.01µM of Grazoprevir.

![S52 replicon assay graph](image)

**Figure 5.15. S52 replicon assay evaluating inhibition of the S52 genotype 3 replicon by grazoprevir.**
Subsequently capture fusion experiments (using THP-1 monocytes, and Huh 7.5 cells transfected with SEC14L2 PAd4) were performed using serum from G3 HCV patients who had previously failed treatment with interferon and ribavirin. A dose-range from 0.001 to 1µM concentration of grazoprevir was used with four different HCV sera. None of the samples were sensitive to grazoprevir at this dose range (figure 5.16).

![Grazoprevir inhibition curves](image)

**Figure 5.16.** Grazoprevir inhibition curves of HCV replication in the capture fusion assay at a grazoprevir concentration range from (0.001 to 1µM)

Further experiments were performed using a grazoprevir dose range of (0.01 to 10 µM) to evaluate whether the preliminary results were due to the utilisation of a dose range that was too low to discriminate between grazoprevir sensitive and insensitive samples. Eight further samples from patients who had failed treatment with interferon and ribavirin were used and capture fusion data was obtained from all of these samples (figure 5.17). The results from these eight samples fell into two distinct groups. Four samples had dose response curves with dose dependent drug inhibition for which IC\textsubscript{50} values could be calculated. These four samples (number 5, 6, 7 and 10) were designated as
being sensitive to grazoprevir in vitro. The IC_{50} values for these samples were 0.005, 0.073, 3.4, and 4.5µM. The remaining four samples were designated as insensitive to grazoprevir as no dose related drug inhibition was apparent. The pooled grazoprevir dose response curves for samples sensitive to and insensitive to grazoprevir are shown in figure 5.18.

Figure 5.17. Grazoprevir inhibition curves of HCV replication in the capture fusion assay at a grazoprevir concentration range from (0.01 to 10µM)
Pooled HCV replication in vitro was significantly different between samples sensitive to grazoprevir and those insensitive to grazoprevir at concentrations of 1 and 10µM of grazoprevir (p=0.05, p=0.04). The IC$_{50}$ values obtained were significantly different for those samples sensitive to grazoprevir, compared to those insensitive to grazoprevir (p=0.0003) (figure 5.19).
5.5 Repeat capture fusion experiments

The final group of fusion experiments performed as part of the telaprevir in Genotype 3 Hepatitis C trial aimed to evaluate whether drug sensitivity at a single concentration could be used to evaluate if a sample was sensitive to a specific antiviral drug in vitro. The rationale for using a single drug dose was based on the limited supply of patient serum from the clinical trial, as using a single dose could potentially facilitate in-vitro testing of several drugs in a single assay without exhausting the stock of trial participant serum. The experiments were performed using the capture fusion assay (THP-1 monocytes and Huh 7.5 SEC14 PAd4 cells) with blinded HCV containing serum from trial participants at their baseline visit on the trial used to “infect” the THP-1 cells. The concentration of drugs to be used were identified as the lowest concentration of an inhibitory drug which achieved a significant difference in HCV replication values in pooled sensitive and insensitive samples. The inhibitory concentrations used for each drug were as follows: Peg IFN 1 IU/ml, Ribavirin 1µM, Telaprevir 1µM, and Grazoprevir 1 µM. Experiments were harvested using the methods previously described, and results were obtained for all patient samples (figure 5.20a-c). In order to identify which HCV serum samples were drug sensitive and which were insensitive to the single dose of each drug used unpaired t tests with Welch’s correction (GraphPad) were calculated comparing the values for the control sample (with no drug inhibition) against each with drug inhibition. The results of this analysis are shown in table 5.4. Samples with a value of <0.05 were identified as having a significant difference between the no drug control and drug inhibited sample. Using this analysis one sample was noted to be sensitive to Interferon, one sample was sensitive to ribavirin, telaprevir and grazoprevir, and a third sample was only sensitive to telaprevir. The samples were unblinded to enable correlation between clinical outcome and in vitro sensitivity (figure 5.21). The two samples which were sensitive to telaprevir in-vitro both achieved an SVR, while the sample sensitive to interferon in-vitro was from a patient who relapsed post treatment. The sensitivity and specificity of in vitro drug sensitivity predicting SVR or failure to achieve SVR is shown in table 5.5. In vitro drug sensitivity had a sensitivity of 50% in predicting SVR, and specificity of 90%. in vitro sensitivity was
Figure 5.20a. Capture fusion experiments using Huh 7.5 SEC14L2 PAd4 cells and single drug concentration inhibition of HCV replication
Figure 5.20b. Capture fusion experiments using Huh 7.5 SEC14L2 PAd4 cells and single drug concentration inhibition of HCV replication.
Figure 5.20c. Capture fusion experiments using Huh 7.5 SEC14L2 PAd4 cells and single drug concentration inhibition of HCV replication.

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Table 5.4. P values from t-tests comparing samples with drug inhibition at a single concentration with samples without drug inhibition (nil samples).
Figure 5.21. In vitro sensitivity to antiviral drugs in the capture fusion assay with single drug concentration HCV inhibition and correlation with clinical outcome.

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Table 5.5 Sensitivity and specificity of in vitro sensitivity to single concentrations of antiviral drugs and clinical outcome.
compared with the previous capture and monocyte fusion experiments (table 5.6). Two of the samples from patients who achieved an SVR were sensitive to telaprevir in both the capture fusion experiments (original capture fusion assay, and single drug dose capture fusion with Huh 7.5 SEC14L2 cells). The monocyte samples that were sensitive to telaprevir were different samples to those sensitive in the capture fusion assay.

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Table 5.6. In vitro sensitivity to telaprevir in the capture fusion assay, monocyte fusion assay and single drug concentration capture fusion assay
5.6 Discussion

In vitro telaprevir sensitivity was evaluated using the capture fusion and monocyte fusion assays to evaluate whether in vitro drug sensitivity correlated with clinical response. The capture fusion assay identified 3 samples as being sensitive to telaprevir, two of those samples were from patients who subsequently achieved an SVR. Interestingly, there was no significant difference in clinical virological response from baseline visit to week four between patients sensitive to telaprevir in vitro and those insensitive to telaprevir in vitro. These findings could potentially be due to several causes. First of all, they may represent the inability of the capture fusion assay to identify samples that are sensitive to telaprevir. Secondly, because telaprevir was one drug in a three-drug treatment regimen, patients with HCV insensitive to telaprevir in vitro may have had a good clinical response to either interferon or ribavirin. Finally, these findings may be a result of variations in treatment concordance, or variations in adhering to guidance on how telaprevir should be taken (with fatty meals or snacks to aid absorption).

The next group of experiments evaluated telaprevir sensitivity in the monocyte fusion assay and utilised a modified Huh 7.5 cell line (Huh 7.5 SEC14L2 PAd4) which enabled increased HCV replication in-vitro. In-vitro HCV replication was improved in the monocyte fusion assay with results obtained for 13 out of 14 samples. Three samples were noted to be telaprevir sensitive in the monocyte fusion assay, however all three were different samples to those noted to be sensitive to telaprevir in the capture fusion assay. This could potentially be a result of assay variability, and therefore repeat capture fusion experiments were planned to assess assay reproducibility.

Monocytes are known to be reservoirs for HCV, and it may be that the specific intracellular environment in monocytes may exert different selection pressures to that experienced by HCV in serum. This may result in the selection of HCV quasispecies in different body compartments with varying sensitivity to antiviral drugs, and may account for the differences in capture fusion and monocyte fusion telaprevir sensitivity in vitro which were seen in some samples from the same patient.
Telaprevir is a first-generation protease inhibitor and has many side effects which would limit its use as more potent second-generation protease inhibitors become available which also have an improved side effect profile. One of these second-generation protease inhibitors is grazoprevir. Once initial dose range finding experiments were completed, eight genotype 3 HCV samples were tested for grazoprevir sensitivity in vitro. Four (50%) of these samples were sensitive to grazoprevir in vitro. If these findings were demonstrated at a larger scale grazoprevir would indeed be a more effective protease inhibitor in genotype 3 HCV, with 50% of individuals having HCV sensitive to grazoprevir in vitro, compared to 30% having HCV sensitive to telaprevir in vitro.

Finally, the capture fusion results were analysed to evaluate which telaprevir and grazoprevir concentrations enabled discrimination between drug sensitive and insensitive samples. The lowest drug concentration in the capture fusion dose response curve which identified a significant difference between drug sensitive and insensitive groups was used. Single doses of IFN and ribavirin (based on previous work by our laboratory group) were used to test for multidrug sensitivity in a single capture fusion experiment. This strategy was used as the volume of serum obtained from trial participants was limited, and as the trial participants had advanced fibrosis many of them had low serum HCV viral loads. This necessitated the use of large volumes of HCV containing serum (>1ml per experiment for certain samples) to achieve a magnitude of infection of 1 (or to achieve a MOI as close to 1 as possible). This approach enabled repeat capture fusion experiments to assess telaprevir sensitivity in vitro, whilst allowing concurrent evaluation of grazoprevir sensitivity in HCV serum from trial participants. The modified Huh 7.5 SEC14L2 PAd4 cells were used in the capture fusion assay with THP-1 monocytes and dose response data was obtained for all 14 samples. Only two samples were noted to be sensitive to telaprevir. Both these samples had previously been identified in the initial capture fusion experiments. Both those individuals achieved an SVR. Only a single HCV sample was noted to be sensitive to interferon in vitro, and a further sample was noted to be sensitive to ribavirin. It is unclear whether these findings represent a clinical trial population that had previously been treated with interferon and ribavirin and had HCV that was not sensitive to interferon or
ribavirin in vitro, or whether the findings were due to limitations of the assay (with the inter replicate variability limiting the number of samples where statistically significant differences in HCV replication). Only one sample was noted to be sensitive to grazoprevir (p<0.05), this sample was also sensitive to telaprevir. Grazoprevir sensitivity appeared to correlate with telaprevir sensitivity, although there wasn’t a statistically significant difference between grazoprevir inhibition and the no drug control samples in the second sample that was telaprevir sensitive. The t test value for this sample was 0.0598. This finding may have been due to increased variability between replicates with single dose grazoprevir inhibition compared to telaprevir. Alternatively, the pooled IC_{50} values for grazoprevir had a mean of 2.15µM, and it may be that a higher dose of grazoprevir needed to be used.

The results above also highlight the challenges in evaluating a novel in vitro HCV replication model in a ‘real world’ environment. The wide error bars in the dose response curves and for single drug inhibition experiment preclude the use of the assay as a test that could aid clinical decision making in the choice of treatment regimen. The pooled data however is more suggestive of a difference in treatment sensitivity between different samples, and the identification of the same two samples as being sensitive to telaprevir when both sets of experiments were blinded also suggests phenotypic data can be replicated. One of the key challenges would be decreasing the intra assay variability for individual replicates, which could potentially be achieved by increasing the number of replicates for each data point, as four replicates were used for each drug concentration. The limitations to this strategy are firstly the increase in patient serum likely to be needed and secondly it would make an already complex assay even more cumbersome (each capture fusion experiment takes approximately one week including cell preparation, followed by harvesting, RNA extraction and quantification, then PCR). Using single drug concentrations could potentially aid this but would need rigorous quality control, and extensive validation of the appropriate drug concentration to use in order to identify drug sensitivity in-vitro. Finally, it would have been useful to sequence HCV from samples that were deemed insensitive to telaprevir from both drug inhibited samples and the no
drug controls, to evaluate whether any particular resistance associated substitutions were selected by drug inhibition and to compare this to pre and post treatment HCV in patient serum.

To summarise HCV from patients on the telaprevir in genotype 3 hepatitis C was tested for in vitro telaprevir sensitivity using capture fusion and monocyte fusion assays. A new next generation protease inhibitor grazoprevir was also evaluated in the capture fusion assay. Finally, a capture fusion experiment testing HCV inhibition with single concentrations of IFN, Ribavirin, Telaprevir and grazoprevir was performed. This enabled assessment of in vitro drugs sensitivity for multiple drug despite many trial samples having a volume of serum with low HCV viral loads. The samples that were sensitive to telaprevir in the single dose fusion assay were two of the samples identified in the original capture fusion assay, and both trial participants achieved an SVR. Due to the wide error bars in this cohort of patients with low HCV viral load, and the lack of correlation between the in-vitro results and decline in patient viral load over the first four weeks of treatment, the assay is unlikely to be of clinical utility in predicting patient treatment outcome. Secondly as the assay is laborious and time consuming it would be impractical for widespread use. Where it may be of use is in patients who have failed treatment with direct acting antiviral drugs to evaluate whether in-vitro sensitivity correlates with particular resistance associated variants.
Chapter 6: Telaprevir in Genotype 3: Sequencing data

6.1 Background

Hepatitis C exists as a variety of quasispecies in infected individuals. The quasispecies with the greatest replicative fitness form the majority of the viral population, while quasispecies with variants that have lower replicative fitness form minority species. Selection pressure exerted by the host immune system, or by external factors such as direct acting antiviral drugs may select for particular variants. During treatment with direct acting antiviral drugs minority species which confer resistance to a particular drug may be selected, or drug resistant species that occur de-novo due to the poor fidelity of the HCV RNA polymerase may be selected. These drug resistant variants may have poor replicative fitness, as observed by the decline in HCV viral load on treatment, however virological breakthrough on treatment can occur if a DAA resistant variant with high replicative fitness develops, and even low-level viral replication can ultimately lead to post treatment relapse. In addition, certain individuals were noted in early studies to have resistant variants as the dominant species suggesting that at least some of the variants that conferred virological resistance to DAAs could exist without a significant loss of replicative fitness\textsuperscript{169}.

The mechanisms by which resistance develops to protease inhibitors are related to the structure and function of the NS3 protein. The NS3 protein contains two regions: an N-terminal third contains the region responsible for the protease activity of the protein while the C-terminal two thirds form an RNA helicase whose function is unclear. The NS3 protease belongs to the trypsin/chymotrypsin protease superfamily. The enzyme consists of two β barrel domains, flanked by two short α helices. The central hydrophobic region of NS4A forms one β strand in one of the β barrels. A zinc ion stabilises the structure and is essential for its function. The substrate binding site accommodates amino acid sequences 6-10 amino acids long. The consensus cleavage site has cysteine and serine at the scissile bond. The catalytic triad of the protease is serine 139, histidine 57 and aspartate 81. The
oxyanion hole has a backbone of glycine 137 and serine 139. The NS4A protein contributes to the proper positioning of the catalytic triad and substrate.

Most protease inhibitors competitively inhibit the substrate binding site. Initial protease inhibitors were macrocyclic compounds, which were followed by linear peptide like molecules (telaprevir and boceprevir). Many amino acid substitutions contribute to the resistance of NS3 to protease inhibitors. Selection of variants which are drug resistant and capable of replicating efficiently are rapidly selected. It is of note that the locations of the amino acid substitutions conferring resistance to protease inhibitors are often quite far from the substrate binding pocket. One of the hypotheses for this finding is that the van der Waals surface of the inhibitor molecule has a larger footprint than the natural substrate thereby permitting substitutions which are drug resistant without complete loss of protease action. Additional secondary substitutions could then potentially restore viral replicative fitness.

Initial DAA resistant variants were identified with first generation protease inhibitors (the first DAAs evaluated in the treatment of Hepatitis C). HCV resistance associated substitutions were noted in early in vitro analysis of first generation protease inhibitors, and in subsequent clinical trials. As first-generation protease inhibitors were developed using genotype 1 based hepatitis C replicon systems, and their clinical efficacy was much greater in genotype 1, they were used predominantly in the treatment of genotype 1 hepatitis C. Subsequently, there is much data on the development of resistance to protease inhibitors in genotype 1 hepatitis C while data on resistance or sensitivity to protease inhibitors in genotypes 2 and 3 is limited.
6.2 Method

200 µl of plasma from trial patients was sent to the MRC Virology Unit in Glasgow for sequencing (by Dr Ana De Silva Felipe and John McLaughlan). RNA was extracted from plasma using the Agencourt RNAdvance blood kit (Beckman Coulter), eluted into 11 µl of water. The RNA was then reverse transcribed using Superscript III (Invitrogen) with random hexamers and a NEB Second Strand synthesis kit (New England Biolabs) for library preparation using the KAPA library prep kit (KAPA Biosystems) with index tagging for 16 cycles of PCR using KAPA HiFi Hotstart (KAPA Biosystems) and NEBNext Multiplex Oligos (oligonucleotides) for Illumina Index primer sets 1 and 2 (New England Biolabs). Libraries were quantified by Qubit (Thermofisher) and pooled at equimolar concentrations for sequencing on the Illumina Mi Seq platform (Illumina). Following initial quality assessment of the sequencing data as Fastq files using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), Sam files were created by mapping against whole genome HCV reference sequences using Tanoti (http://bioinformatics.cvr.ac.uk/tanoti.php). Sequencing data for individual samples was received in Fasta format and alignments and phylogenetic analysis was performed by myself using Geneious.

6.3 Results

Initial analysis of the samples was performed by aligning the sequences (of the majority species in TIG3 trial participants) using Geneious 9.1 along with a reference G3 HCV sequence (accession no GQ356202). Phylogenetic trees were created using Geneious 9.1. The phylogenetic trees for the complete HCV RNA sequences for trial participants is shown in figure 6.1, along with the phylogenetic tree for the complete HCV protein sequence. Although the phylogenetic tree for the complete HCV RNA sequences demonstrated no clustering of samples based on either clinical response or in vitro sensitivity to telaprevir, the complete translated protein sequence showed clustering of sequences from patients who had their treatment terminated at week 4 for insufficient response and patients who achieved SVR. Regarding in vitro sensitivity samples that were sensitive
Figure 6.1 Phylogenetic trees of samples in the TIG 3 clinical trial. A) Phylogenetic tree of RNA sequence. B) Phylogenetic tree of protein sequence. Samples labelled as per clinical outcome. Those samples sensitive to telaprevir in the capture fusion assay are labelled as sensitive.
in vitro did not cluster together in the same sub clade. Subsequently phylogenetic trees were created for the NS2 and NS3 protein sequences for trial participants (figure 6.2 and 6.3). There was no clustering of sequences from patients who achieved an SVR, had treatment discontinued at week 4 on treatment or who had HCV sensitive to telaprevir in vitro.

6.3.1 NS2 sequencing data

Although NS2 is not a target of telaprevir several sequence motifs were identified which were associated with treatment outcome. NS2/3 cleavage has been implicated in viral replication efficacy\textsuperscript{15} therefore these findings were analysed further. The translated NS2 sequences from the majority species of TIG3 trial participants were compared to identify if any amino acid substitutions were associated with clinical outcome or in vitro sensitivity to telaprevir (based on the capture fusion assay data). Only two substitutions that were potentially associated with clinical outcome were identified (figure 6.4). Each substitution was present in two of the patients who achieved SVR. One was a substitution of threonine for serine at amino acid 2 of the NS2 protein and was present in two individuals who achieved SVR (one of whom had serum HCV sensitive to telaprevir in the capture fusion assay). The second substitution was at amino acid 63 with the substitution of alanine for valine in two patients that achieved SVR. Neither of these samples was sensitive to telaprevir in vitro. Analysis of minor species was also undertaken, with any substitutions accounting for more than 1% of the total viral population analysed (see table 6.1). There was no clear correlation between any of the identified minority species and clinical outcome. Evaluation of the amino acid sequence of samples did not identify any substitutions associated with in vitro telaprevir sensitivity in all samples. Finally, on evaluation of significant minority species (>1% of total next generation reads) no amino acid substitutions associated with in vitro telaprevir sensitivity were noted in majority or significant minority species (table 6.1).
Figure 6.2. NS2 protein sequence phylogenetic tree

Figure 6.3. NS3 protein sequence phylogenetic tree
Figure 6.4. NS2 substitutions associated with clinical outcome (L) S2T and (R) V64A. Both substitutions were observed in patients who achieved SVR.
Table 6.1 minority species (>1% reads) for any amino acid in NS2
6.3.2 NS3 sequencing data

The translated NS3 sequences from the majority species from TIG3 trial participants were compared to identify if any particular substitutions were associated with SVR, treatment discontinuation or in vitro sensitivity to telaprevir. The resistance associated substitutions (RAS) previously identified in genotype 1 HCV patients receiving protease inhibitors are shown in figure 6.5a. The majority species from each patient at all those RAS sites are shown in figures 6.5b and 6.5c. At amino acid position 36 of the NS3 protein all trial participants had leucine substituted for valine. This resistance associated substitution is the reference sequence for Genotype 3 hepatitis C. At positions 41 (glutamine), 43 (phenylalanine), 54 (threonine), 55 (valine), and 56 (tyrosine) all patients had the reference amino acids which conferred sensitivity of genotype 1 hepatitis C to first generation protease inhibitors. At position 80 (glutamine) all patients had the reference amino acid apart from one patient who withdrew from the trial (who had a resistance associated substitution of leucine at this position). At positions 122 (serine), 155 (arginine), 156 (alanine), 158 (valine), and 170 (isoleucine), the majority species from all samples was identical to the reference sequence for genotype 1 and genotype 3 hepatitis C. At position 168 all samples expressed glutamine as the majority species, consistent with the genotype 3 HCV reference sequence. The reference amino acid at position 168 in genotype 1 hepatitis c was aspartic acid, however substitution of glutamine at this position had not been identified as a resistance associated substitution in genotype 1 hepatitis C. At position 175 isoleucine was expressed by the majority species in all trial samples. This was consistent with the reference sequence for genotype 3 hepatitis C, but had been identified as a resistance associated substitution in genotype 1 hepatitis C (M175L). In summary, analysis of the protein sequence expressed by the majority species from trial patients did not identify any substitutions correlating with clinical trial outcome. At V36 and M175 all samples expressed resistance associated substitutions identified in genotype 1 hepatitis C, but these substitutions were the reference sequence for genotype 3 hepatitis C. At amino acid 168 all trial samples had a substitution (which was consistent with the
Figure 6.5. A) NS3 Resistance associated substitutions identified in genotype 1a (l) and 1b (r) Hepatitis C B) Sequencing data for amino acids 31-90 in TIG trial patients with amino acid locations of G1 RAS highlighted C) Sequencing data for amino acids 121-180 in TIG trial patients with amino acid locations of G1 RAS highlighted
reference sequence for genotype 3 hepatitis C) which had not been identified to be a resistance associated substitution in genotype 1 hepatitis C.

As the translated amino acids (from the majority species of HCV) from trial patients did not demonstrate any pattern of known resistance associated substitutions pre-treatment affecting clinical outcome, the minority species at the previously identified resistance associated substitution sites were also analysed (table 6.2). This demonstrated that no minority species at the previously identified RAS sites were present at a frequency greater than 1% apart from the presence of L80Q which was present in 3.71% of reads in a single patient who withdrew from the trial.

Table 6.2 Percentage of next generation sequence reads which identified the majority species and percentage of reads demonstrating minority species (if total number of reads for a minority species was >1%) at locations identified as G1 resistance associated substitution sites.
As there was no association between the previously identified resistance associated substitutions and clinical outcome, further analysis was performed to evaluate whether other NS3 amino acid substitutions correlated with clinical outcome (figure 6.6). Two substitutions were noted to be present in patients achieving SVR: T477A was present in 3 of the 4 patients who achieved an SVR, and was not present in any of the patients that didn’t achieve an SVR; S558A was present in 2 of 4 patients achieving an SVR and in one patient who withdrew from the trial at week 2. Similarly, several substitutions were noted in patients who terminated treatment early due to insufficient virological response: A67V was present in two patients who stopped treatment at 4 weeks, along with one patient who relapsed and a patient who withdrew from the trial. T581M was noted in all patients who stopped treatment at 4 weeks, along with two patients who relapsed, and one who achieved SVR. At A413 those patients who had the reference G3 sequence included all three patients who stopped treatment at week 4 due to insufficient response, one patient who relapsed, one patient who achieved an SVR and one patient who withdrew from the trial.

Minority HCV quasispecies were also evaluated to assess if there was any correlation between the presence of quasispecies at particular location and treatment outcome (table 6.3). At amino acid 67 samples which did not have alanine as the majority species, or as a minority species with a frequency above 1% were three samples with valine at amino acid 67. Of these three samples two stopped treatment at week 4 and one relapsed. At amino acid 125 three individuals had a glycine minority species with a frequency >1%, and two of these also had an arginine minority species with a frequency >1%. The two of these patients with the arginine minority species achieved SVR whilst the other individual relapsed after completing treatment. The HCV samples from the two patients who achieved SVR were both sensitive to telaprevir in vitro.
Figure 6.6 Potential NS3 variants that were associated with SVR (T477A), (S558A) (A-B), and withdrawal of treatment at week 4 due to insufficient response (A67V), (A413T), (T581M) (C-E)
Table 6.3: NS3 minority species (any amino acid substitutions with a frequency >1% in any sample)

At amino acid 176 two patients had asparagine as the majority species (the remainder had serine as the majority species). A further patient had asparagine as a minority species with a frequency of 5.8%. Of the two patients with asparagine as the majority species, one achieved SVR, and the other relapsed post treatment. The patient with asparagine as a minority species also achieved SVR. At amino acid 477 all patients who had threonine as the majority species with an absence of any minority species (>1%) did not achieve SVR (3 stopped treatment at week 4 and two relapsed post treatment).

The phylogenetic tree of the NS3 amino acid sequence from trial patients showed no clustering of samples in clades based on in vitro telaprevir sensitivity. Similarly, no amino acid substitutions in majority species were identified that correlated with in vitro sensitivity to telaprevir. 3 minority species (with a frequency >1% of total sequencing reads) were identified which could potentially be associated with in vitro sensitivity to telaprevir: The presence of arginine as a minority species at amino acid 125 (serine is the majority species) was noted in two patients with HCV sensitive to telaprevir in vitro both of whom achieved an SVR (as previously described); at amino acid 274...
(leucine majority species) the presence of a proline minority species (>1%) was noted in two patients with HCV sensitive to telaprevir in vitro and one sample which was insensitive to telaprevir in vitro. The clinical outcomes were: one patient sensitive to telaprevir in vitro who stopped treatment at week 4 due to insufficient response to treatment, one patient with HCV sensitive to telaprevir in vitro who achieved an SVR, and one patient with HCV insensitive to telaprevir in vitro who relapsed post treatment. At amino acid 381 (leucine majority species) two samples had arginine (as a minority species >1%). Both these samples were sensitive to telaprevir in vitro (one patient stopped treatment after 4 weeks of treatment for insufficient response to treatment, and the other achieved an SVR).

6.3.3 NS4A sequencing data

The NS4A protein sequence was also analysed as NS4A is involved in the proteolytic activity of hepatitis C. There was no clustering of patients based on the amino acid sequence of NS4A that correlated with clinical outcomes (figure 6.7). No amino acid substitutions (in the majority species) were identified that were associated with SVR or termination of treatment at week 4. Analysis of minority species did not yield any association with treatment outcome or in-vitro sensitivity to telaprevir (table 6.4).

There was no clustering of samples based on the amino acid sequences of NS4B and NS5B that correlated with treatment outcome. With the NS5A protein sequence, there was also no clustering of samples based on amino acid sequence (although two samples from patients who achieved SVR were in adjacent subclades). At amino acid 411 three patients expressed arginine (instead of glutamate or glycine) as the majority species and two of these patients achieved SVR, and one withdrew from the trial early. The substitution observed may possibly be related to a potential effect of NS5A on interferon sensitivity.
Figure 6.7. Phylogenetic tree for NS4A protein sequence
Table 6.4. Minority species (>1%) in NS4A

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Figure 6.8. NS5A potential sequence variant associated with SVR (E411R)
6.4 Discussion

Analysis of the phylogenetic tree created based on amino acid sequence homology for trial participants demonstrated clustering of patients based on clinical outcome. Although this suggested that similarities in amino acid sequence for the entire HCV genome were likely to predict clinical outcome, this was not demonstrated for any particular non-structural protein in isolation. Similarly, no single resistance associated substitution was associated with clinical outcome (either relating to stopping treatment after 4 weeks or achieving SVR). Substitutions present in some of the patients who either stopped treatment at 4 weeks or who achieved an SVR were noted. Analysis of minority species (with >1% frequency) did not identify any positions where the presence of a particular amino acid as either a majority species or a significant minority species correlated with clinical outcome. This may be indicative of several substitutions which may affect clinical outcome in combination although the small sample size and lack of post treatment sequencing data limited the ability to identify amino acid substitutions which were associated with early cessation of treatment or relapse. Similarly, with the small number of samples that were sensitive to telaprevir in vitro, no single amino acid substitution was identified which correlated with in vitro telaprevir sensitivity. There were several amino acid positions where the presence of particular minority species appeared to correlate with telaprevir sensitivity. These substitutions would be unlikely to result in in-vitro sensitivity to telaprevir on their own unless they were associated with additional amino acid variants in majority species that were sensitive to telaprevir.

Although these findings are consistent with previous data which attempted to identify resistance associated variants in the NS3 sequence for genotype 3 HCV a more recent trial evaluating grazoprevir in the treatment of genotype 3 HCV identified three resistance associated variants which had a greater than five-fold effect on in vitro sensitivity to grazoprevir in a genotype 3 HCV chimaeric replicon system. These were Y56H, A156G and Q168K. None of the TIG3 trial patients had any of these substitutions at baseline. With the advent of newer protease inhibitors such as glecaprevir and
voxilaprevir for the treatment of genotype 3 hepatitis C including in patients who have failed previous direct acting antiviral treatment hopefully further light will be shed on the resistance associated substitutions that confer resistance of G3 HCV to protease inhibitors and how this pattern differs to genotype 1 HCV.
Chapter 7: Discussion and update

The telaprevir in genotype 3 hepatitis C trial demonstrated the possible utility of telaprevir in treating genotype 3 hepatitis C. Four patients on the trial achieved an SVR, although the rapid progress in the treatment of hepatitis C has resulted in telaprevir being withdrawn and superseded by newer protease inhibitors. This undoubtably affected trial recruitment, which was terminated early once details of the early access programme in England had been finalised. As the cohort of patients recruited onto the trial was small, we are unable to ascertain if there is any benefit in adding telaprevir to pegylated interferon and ribavirin. The impact of the trial on the clinical treatment of hepatitis C will therefore be negligible following the development of multiple all oral pan-genotypic treatment regimens which have now become the standard of care in the treatment of hepatitis C, with high SVR rates even in patients with advanced liver disease (including those that have previously failed treatment). Fortunately, the stringent stopping rules for treatment ensured that patients with a low likelihood of achieving an SVR had limited exposure to treatment. This was reflected in the good safety profile of the clinical trial, despite treating patients with advanced fibrosis and cirrhosis with both telaprevir and interferon.

The process of developing and then managing a clinical trial gave an invaluable insight into understanding the process by which new clinical treatments are developed. Overseeing the development of an electronic trial database (and the subsequent data entry from clinical report forms), along with initiation, monitoring and end of trial visits to participating sites provided experience in ensuring the trial met regulatory and good clinical practice standards.

The telaprevir in genotype 3 hepatitis C trial also included assessment of a novel in vitro HCV replication model. This enabled the testing of serum (and monocyte) HCV from trial participants, and evaluating whether drug inhibition of viral replication was detected in vitro. One of the key challenges faced was achieving robust viral replication in the capture fusion assay from patients with low serum HCV titres. The capture fusion assay identified three patients that were sensitive to
telaprevir in vitro, of whom two achieved an SVR. Due to the low baseline viral load of many patients on the trial, the capture fusion assay was modified to use SEC14L2 cells, which resulted in enhanced in vitro replication of HCV. Subsequently, monocyte fusions were undertaken (due to the limited supply of patient serum) to assess if the modifications to the assay had improved viral replication in vitro, and to confirm the previous findings. Unfortunately, when the monocyte fusion assay was performed the results were discordant, with different samples being identified as telaprevir sensitive. This led to concerns regarding the validity of the assay in identifying samples sensitive to telaprevir in vitro. Further capture fusion experiments (using a single dose of telaprevir) were performed with the SEC14L2 cells, and these identified two of three samples (both of which were from patients who achieved an SVR) previously identified as telaprevir sensitive in the capture fusion assay. As the samples were all blinded until data analysis had been completed, this suggests replicability of the capture fusion assay, and that the assay may be of predictive value. A major limitation of the assay are the wide error bars for individual data points, which preclude the use of the assay as a clinical test. Secondly, the cumbersome nature of the assay and low throughput would also limit its utilisation. A more realistic use for the assay could be in assessing in vitro sensitivity of HCV in patients who have failed direct acting antiviral regimens and correlating this with next generation sequencing data to potentially identify novel resistance associated substitutions.

Next generation sequencing of HCV from trial participants at baseline did not identify any substitutions (or minority species) associated with treatment failure. Similarly, no substitutions were identified which correlated with in vitro sensitivity. This is most likely due to the small sample size of patients in the trial, however it may also reflect a more complex mechanism of resistance for genotype 3 HCV to telaprevir (which could potentially involve several different resistance associated substitutions). In all likelihood, large cohorts of patients who fail treatment would be needed to identify potential resistance associated substitutions that predict response to treatment with telaprevir in genotype 3 hepatitis. It is unlikely that these will be identified due to the changes in hepatitis C treatment since completion of the trial.
7.1 Advances in hepatitis C treatment since 2014

In the 24 months following the initiation of the Telaprevir in Genotype 3 (TIG3) hepatitis C trial, there have been significant advances in the treatment of hepatitis C. Following patient recruitment onto the TIG3 trial, compassionate use of sofosbuvir with ledipasvir and daclatasvir was commenced in 2014 for patients with decompensated liver disease due to hepatitis C in the UK. The ALLY 3 trial had demonstrated that sofosbuvir and daclatasvir for 12 weeks only achieved SVR rates of 63% in patients with genotype 3 HCV and liver cirrhosis\textsuperscript{173}. A subsequent trial evaluating sofosbuvir, daclatasvir and ribavirin for 12 or 16 weeks in patients with liver cirrhosis achieved SVR rates of 83-89\textsuperscript{%}\textsuperscript{174}. Real world data from the expanded access programme in the UK\textsuperscript{175} demonstrated that while the overall rate for SVR was high (for this cohort of patients with predominantly decompensated liver disease), there was a marked difference between outcomes for genotype 1 HCV and genotype 3. The SVR rates for genotype 1 patients treated on the early access programme was 91\% compared to 69\% for genotype 3 patients. In particular, the SVR rate for patients with genotype 3 hepatitis C treated with sofosbuvir and ledipasvir was only 62\% compared to 73\% for those treated with sofosbuvir and daclatasvir.

While the early access programme was underway, second generation NSSA inhibitors were being evaluated in clinical trials. The ASTRAL-3\textsuperscript{176} trial used sofosbuvir and velpatasvir (a second generation NSSA inhibitor) for 12 weeks achieving SVR rates of 97\% in patients with genotype 3 HCV without cirrhosis, with a modest decrease in patients with compensated cirrhosis (91\%). The ASTRAL-4\textsuperscript{177} study also used velpatasvir and sofosbuvir, but demonstrated that patients with genotype 3 hepatitis C and decompensated liver disease had lower SVR rates than their genotype 1 counterparts (SVR 50\% for 12 or 24 weeks sofosbuvir and velpatasvir, SVR 85\% for 12 weeks sofosbuvir, velpatasvir and ribavirin compared to 88\%, 93\%, and 94\% for the equivalent genotype 1a groups). So, although direct acting antiviral drugs had significantly improved outcome for genotype 3 patients with
advanced liver disease (with a further small improvement by adding ribavirin) there remained a significant minority of patients with genotype 3 HCV who did not achieve an SVR.

In order to investigate this variation and to understand the mechanism by which treatment failed for the small proportion of patients that didn’t achieve an SVR, next generation sequencing of patient HCV was undertaken as part of the clinical trials evaluating newer direct acting antivirals. It was noted that particular resistance associated substitutions, especially in the NS5A sequence, resulted in a decreased SVR12 rate. A30K, L31F/M and Y93H were especially implicated in resistance to NS5A inhibitors, while the following resistance associated substitutions were noted in NS5B: N142T, L159F, E237G, L320I, S282 and V321A/IS96T\textsuperscript{176}. Despite the presence of NS5A resistance associated substitutions in 28% of patients (using a sequencing cut off of 15% of sequencing reads) in the POLARIS studies using sofosbuvir and velpatasvir, SVR rates of 97-100% were achieved in genotypes 1,2,4,5 or 6. For genotype 3 however, 12% of patients had NS5A resistance associated substitutions at baseline. In patients with NS5A resistance associated substitutions the SVR rates were lower, 93% compared to those without those substitutions at baseline (98%). In patients with genotype 3 hepatitis C and the Y93H substitution in the NS5A sequence the SVR12 rate dropped further to 86%\textsuperscript{178}, whilst patients in the ALLY-3 trial (who received 12 weeks of sofosbuvir and daclatasvir) with the Y93H substitution only achieved an SVR12 of 54%\textsuperscript{173}.

Whilst the NS5A/NS5B combinations achieved excellent SVR rates, in particular for patients with genotype 1 hepatitis C even with previous treatment failure or advanced liver disease (where SVR rates still remained above 95%), in genotype 3 hepatitis C the data from the ALLY-3 and POLARIS studies along with real world data from the expanded access programme demonstrated that an unmet treatment need still existed for patients with genotype 3 hepatitis C and advanced liver disease or Y93 resistance associated substitutions.
Fortunately, additional treatment regimens were also in development at the time, including some which contained second generation protease inhibitors. Elbasvir (NS5A inhibitor) and grazoprevir (NS3 inhibitor) were evaluated in the treatment of genotype 3 hepatitis C (in treatment naïve non-cirrhotic patients). This combination only achieved SVR rates of 45% with 12 weeks of treatment or 57% with 16 weeks of treatment\textsuperscript{166}. Analysis undertaken to determine whether resistance associated substitutions accounted for virological failure only identified NS3 substitutions (which conferred a >5 fold change in sensitivity) in only 35% of patients with virological failure. NS5A resistance associated substitutions however occurred more frequently, with the Y93H substitution occurring in 76% of patients who experienced virological breakthrough. The C-ISLE study evaluated grazoprevir, elbasvir and sofosbuvir (with or without ribavirin) in the treatment of patients with genotype 3 hepatitis C and cirrhosis. Treatment naïve patients treated with elbasvir/grazoprevir, sofosbuvir and ribavirin for eight weeks achieved an SVR of 91%, whilst treatment with elbasvir/grazoprevir and sofosbuvir for 12 weeks achieved an SVR12 of 96%. In patients who had been treated with interferon and ribavirin previously, treatment with elbasvir/grazoprevir and sofosbuvir for 12 or 16 weeks resulted in SVR12 rates of 94%. In both the trials above, a high proportion of patients had baseline NS3 resistance associated substitutions but these were not necessarily associated with clinical outcome. In the C-ISLE study, despite 50% of patients having detectable NS5A RAS at baseline, the SVR rate was 98% in participants with or without RAS\textsuperscript{179}.

Another regimen that has subsequently become the standard of care treatment for genotype 3 hepatitis C in the United Kingdom is glecaprevir and pibrentasvir. Glecaprevir (ABT-493) is a pan genotypic NS3 inhibitor which maintained activity even against substitutions at 155 and 168 (which conferred resistance against previous generations of NS3 inhibitors). Pibrentasvir (ABT-530) is a NS5A inhibitor with an improved resistance profile to RAS at M28T, A30K and Y93H. The Surveyor II study evaluated this combination in patients with genotype 3 HCV and compensated cirrhosis, achieving SVR rates of 95% in treatment naïve patients following 12 weeks of treatment. In treatment experienced patients who received 16 weeks of treatment three out of four patients
achieved an SVR12 (75%). The combination of ABT-493/ABT-530 and ribavirin achieved an SVR12 of 100% in patients with genotype 3 HCV and cirrhosis (irrespective of prior treatment status)\textsuperscript{167}. In the third phase of the Surveyor II trial, larger numbers of patients were treated with glecaprevir and pibrentasvir. Treatment naïve cirrhotic genotype 3 patients achieved an SVR12 of 98% following 12 weeks of treatment. Treatment experienced non-cirrhotic genotype 3 patients achieved SVR12 of 91% and 95% with 12 and 16 weeks of treatment respectively. An SVR12 of 96% was achieved in treatment experienced genotype 3 patients with cirrhosis following 16 weeks of treatment. Patients with previous sofosbuvir experience achieved an SVR12 of 98%\textsuperscript{180}.

Initial trials evaluating treatment duration with sofosbuvir/velpatasvir and voxilaprevir (a pan genotypic NS3 inhibitor) achieved SVR12 rates of 94% following eight weeks of treatment in treatment naïve genotype 3 HCV patients with cirrhosis. Treatment experienced genotype 3 HCV patients with cirrhosis were given 12 weeks of treatment and also achieved SVR rates of 94%\textsuperscript{181}. Subsequent trials evaluated this combination for 12 weeks in NS5A experienced patients (POLARIS-1), and also in patients exposed to any DAA except NS5A inhibitors (POLARIS-4). In POLARIS-1 genotype 3 NS5A experienced patients achieved an SVR12 of 95%, with an SVR12 rate of 93% in patients with cirrhosis. In POLARIS-4 an SVR rate of 96% was achieved in genotype 3 patients treated with a non-NS5A DAA regimen (predominantly sofosbuvir based treatment)\textsuperscript{182}. In POLARIS-3 SVR12 rates of 96% were achieved with both 12 weeks of sofosbuvir/velpatasvir and eight weeks of sofosbuvir/velpatasvir and voxilaprevir in DAA naive patients with genotype 3 hepatitis C and liver cirrhosis\textsuperscript{168}.

As these newer, pangenotypic (and better tolerated) protease inhibitors became available, first generation protease inhibitors were superseded and therefore withdrawn from usage. As a result telaprevir was withdrawn from use in Europe in September 2016. Some of the challenges faced in the Telaprevir in Genotype 3 clinical trial still remain, however. The limited efficacy of grazoprevir/elbasvir in genotype 3 further demonstrated that protease inhibitors appeared to only
confer benefit in a proportion of patients. Interestingly, there were no significant NS3 RAS that were identified that conferred resistance to treatment in patients with genotype 3 HCV. This contrasts with the NS5A resistance associated substitutions which are well documented with both initial NS5A inhibitors (daclatasvir, ledipasvir and elbasvir) along with newer NS5A inhibitors (velpatasvir and pibrentasvir). With the most recent NS3 containing regimens, the high efficacy of treatment results in a small number of patients who have virological failure or relapse post treatment. Subsequently, predicting patients who may fail treatment or identifying combinations of RAS that confer resistance is challenging. As the sequencing data from the telaprevir in genotype 3 trial demonstrated, in a small cohort of samples the likelihood of identifying RAS which confer NS3 resistance in genotype 3 is limited. Whilst there may be certain RAS which appear to correlate with clinical outcome, it is difficult to extrapolate firm conclusions when other variables may be present (such as sensitivity to additional drugs in a treatment regimen, pharmacokinetic variables and treatment adherence). This highlights the need for an effective in vitro system to enable accurate phenotyping of patient derived genotype 3 HCV to direct acting antiviral drugs. This data then allows a greater number of samples to be tested and may improve the ability to detect RAS which confer resistance to a single DAA (which may not be apparent when patients are treated with multi-DAA combinations).

### 7.2 Advances in cell culture HCV replication systems

Whilst the rapid development of multiple new and effective direct acting antiviral drugs continued, research was being undertaken to improve the in vitro models available to evaluate genotype 3 hepatitis C in vitro. The aim was to move away from the existing in vitro replication systems that were either based on genotype 1 (Con1) or 2 (JFH-1) to those that would facilitate improved understanding of drug sensitivity in-vitro for genotype 3 hepatitis C.

Saeed et al. created a sub-genomic replicon from a genotype 3 hepatitis C isolate (S310). S310 was derived from patient serum (from a genotype 3 patient with post-transplant recurrence of HCV) which demonstrated infectivity of primary human hepatocytes. A sub-genomic replicon was then
created based on this isolate which replicated in vitro. Those clones that demonstrated in vitro replication developed cell culture adaptive mutations. Kim et al. subsequently developed an in vitro genotype 3 replication system based on the S310 replicon, this system enabled viral replication and the production of viral particles in cell culture. The infectivity of the genotype 3 S310 was approximately 10% of JFH-1. Interestingly, in vitro infection with S310 resulted in increased intracellular lipid droplet formation, in keeping with the increased incidence of steatosis in patients infected with genotype 3 hepatitis C. Unfortunately, the S52 and S310 subgenomic replicons did not replicate well (even in modified hepatoma cells) when reporter genes (e.g. luciferase) were added to enable testing of drug sensitivity.

In addition to attempting to develop a genotype 3 replicon, research was undertaken to evaluate why clinical isolates of HCV replicated poorly in cultured hepatoma cells. Huh 7.5 cells were transfected with human cDNA and then transfected with subgenomic replicons lacking adaptive mutations. This enabled the identification of specific cDNA which augmented in vitro replication. The SEC14L2 cDNA permitted replication of subgenomic replicons without adaptive mutations. SEC14L2 was postulated to increase in vitro HCV replication by enhancing vitamin E mediated inhibition of lipid peroxidation. SEC14L2 also enabled replication of HCV from patient serum in Huh 7.5 cells, although high viral loads were needed to achieve this (5.98x10⁶ to 3.2x10⁷).

Despite these advances, there is still no readily available cell culture system which permits patient derived HCV replication in vitro. In order to assess patient derived genotype 3 HCV sensitivity to specific drugs a chimeric replicon would need to be created with the majority and significant minority species from a particular patient. The capture fusion model allowed an efficient method of assessing patient derived HCV sensitivity to particular antiviral drugs. With the addition of SEC14L2, which augmented in vitro HCV replication, this enabled genotype 3 HCV from patients with low viral loads to replicate in vitro. This was of particular relevance to the telaprevir in genotype 3 hepatitis C trial. As patients recruited to the trial had advanced fibrosis or cirrhosis, their viral loads were
generally lower than those than would be expected in non-cirrhotic HCV patients (and lower than the patient HCV viral loads which replicated in SEC14L2 cells). The capture fusion assay permitted in vitro viral replication of HCV from multiple different patients in the telaprevir in genotype 3 trial, and enabled phenotypic testing of HCV sensitivity to telaprevir.

The capture fusion assay has been used since the completion of the telaprevir in genotype 3 study to evaluate the in vitro sensitivity of patient derived HCV to a novel antiviral compound (SB9200)\textsuperscript{186}. Further work undertaken by our group used the capture fusion assay to assess in vitro sensitivity of patient derived HCV to sofosbuvir in patients who had either achieved SVR or relapsed post treatment with sofosbuvir. This enabled identification of the A150V substitution in the NS5B protein which was associated with a decreased SVR rate.

As the number of patients who fail treatment has decreased, identifying the mechanisms of treatment failure becomes more challenging. The capture fusion assay remains a useful tool in assessing in vitro sensitivity of patient derived HCV to antiviral drugs. As the current first line treatment for genotype 3 HCV in the UK is glecaprevir (a NS3 inhibitor) and pibrentasvir, the capture fusion assay could be used to evaluate HCV sensitivity from patients who have failed this regimen to other protease inhibitors such as voxilaprevir which forms part of the “rescue” treatment (voxilaprevir/sofosbuvir/velpatasvir) for patients that have had treatment failures. In addition to assessing in vitro drug sensitivity, this may allow the assay to be used prospectively to potentially predict treatment outcome, and furthermore enable identification of the resistance associated substitutions and mechanisms which confer HCV genotype 3 resistance to protease inhibitors.
7.3 Summary

Whilst telaprevir may be of use in the treatment of genotype 3 hepatitis C, the rapid progress in hepatitis C treatment over the last four years has rendered it obsolete, resulting in it being withdrawn from clinical use (as more potent pan-genotypic protease inhibitors have become available). The small number of patients who enrolled onto the trial had limited treatment options available to them at the time of trial recruitment, and strict stopping rules minimised exposure to treatment in patients where SVR was unlikely. The capture fusion assay was investigated as a tool which could predict treatment response. Low patient HCV viral load posed a challenge in achieving robust in vitro viral replication. The capture fusion assay was modified to enhance viral replication, using SEC14L2 cells and the PAd4 construct. Despite this, intra-assay variability precludes its use as a clinical tool. It may have a utility in identifying drug sensitivity and correlation with next generation sequencing data may help identify resistance associated substitutions. The next generation sequencing undertaken on trial participants’ baseline HCV did not identify any substitutions associated with treatment response. There were no substitutions identified which directly correlated with in vitro sensitivity to telaprevir either. This is unsurprising due to the small patient cohort involved in the trial.

7.4 Future work

As telaprevir has been withdrawn from use, further clinical work using it will not be undertaken. The focus of research would shift to newer protease inhibitors used in genotype 3 hepatitis C such as glecaprevir and voxilaprevir. The capture fusion assay may be of use in assessing patient derived HCV sensitivity to either (or both) of these drugs, and correlating in vitro sensitivity to treatment outcome and next generation sequencing to identify relevant resistance associated substitutions which may affect treatment outcome for genotype 3 patients treated with regimens containing a protease inhibitor.
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TITLE OF THE PROTOCOL:
Telaprevir in patients with genotype 3 HCV: pilot clinical study to evaluate efficacy and predictability of therapy in patients who have failed to respond to pegylated interferon and ribavirin

Short title/Acronym: Genotype 3 Hepatitis C: Therapy with Telaprevir

Sponsor: Queen Mary, University of London

Representative of the Sponsor: Gerry Leonard
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Phone: 020 7882 7260
Email: sponsorsresp@bartshealth.nhs.uk

Funders: Janssen UK

REC reference: 13/LO/1473

EudraCT reference: 2013-003729-27

Emergencies or Serious Adverse Event (Refer to Section 7.4 of the protocol)

Contact: 24 Hour Emergency No: 020 7377 7000 (Switchboard) and ask to be put through to The Gastroenterology Registrar on call

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The clinical study as detailed within this research protocol (Version 3.4 dated 06 June 2016), or any subsequent amendments, involves the use of an investigational medicinal product and will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996), Principles of GCP, and the current regulatory requirements, as detailed in the Medicines for Human Use (Clinical Trials) Regulations 2004 (UK S.I. 2004/1031) and any subsequent amendments of the clinical trial regulations.

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Chief Investigator Site:
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- Blizard Institute
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- 4 Newark St
- London
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Signature and Date:
The clinical study as detailed within this research protocol (Version 3.4 dated 06 June 2016), or any subsequent amendments, involves the use of an investigational medicinal product and will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996), Principles of GCP, and the current regulatory requirements, as detailed in the Medicines for Human Use (Clinical Trials) Regulations 2004 (UK S.I. 2004/1031) and any subsequent amendments of the clinical trial regulations. This is a pilot study with a statistical analysis conducted by the Chief Investigator.

Statistician Name: Graham Foster

Statistician Address:

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Centre for Digestive Diseases
Blizard Institute
Queen Mary University of London
4 Newark St
London
E1 2AT

Signature and Date:
The clinical study as detailed within this research protocol (Version 3.4 dated 06 June 2016), or any subsequent amendments, involves the use of an investigational medicinal product and will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996), Principles of GCP, and the current regulatory requirements, as detailed in the Medicines for Human Use (Clinical Trials) Regulations 2004 (UK S.I. 2004/1031) and any subsequent amendments of the clinical trial regulations.

Principal Investigator Name:

Principal Investigator Site:

Signature and Date:
### STUDY SUMMARY/SYNOPSIS

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<td><strong>Methodology</strong></td>
<td>Multi-site, pilot study evaluating the impact of Telaprevir in patients with genotype 3 HCV who have failed to respond to pegylated interferon and ribavirin therapy</td>
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<td><strong>Study Duration</strong></td>
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<td>To determine whether a proportion of patients with genotype 3 HCV who have failed to respond to pegylated interferon and ribavirin respond to re-treatment with a telaprevir containing regime</td>
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<td><strong>Number of Subjects/Patients</strong></td>
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<td><strong>Main Inclusion Criteria</strong></td>
<td>People with genotype 3 HCV who have advanced fibrosis and who have failed to respond to therapy with pegylated interferon and ribavirin.</td>
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<td><strong>Statistical Methodology and Analysis</strong></td>
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## Glossary of Terms and Abbreviations

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<td>Adverse Event</td>
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<td>Adverse Reaction</td>
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<td>Participant</td>
<td>An individual who takes part in a clinical trial</td>
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1. Introduction

1.1 Background

Chronic infection with genotype 3 hepatitis C virus (HCV) is common [1]. The majority of those who are infected respond to therapy with current anti-viral agents (pegylated interferon (PegIFN) and ribavirin (RBV)) [1]. However, in patients with genotype 3 HCV and cirrhosis, response rates are less than 60%, with many patients relapsing when therapy is withdrawn [1]. Patients with genotype 3 HCV and cirrhosis who have failed to respond to antiviral therapy are at high risk of complications of HCV (chiefly hepatocellular carcinoma) [2] and there are no effective treatments. In patients with genotype 1 HCV the protease inhibitor telaprevir improves the response to therapy [3] and in patients who have relapsed following pegylated interferon and ribavirin the response to therapy with telaprevir, pegylated interferon and ribavirin is impressive, with sustained virological response rates of >80% [4]. Clinical trials with telaprevir monotherapy in patients with genotype 3 HCV show a relatively poor response with a mean reduction in viral load of 0.5 log (compared to 3-4 log in patients with genotype 1 HCV) [5]. However, within the cohort of patients treated with telaprevir monotherapy a proportion (approximately 30%) showed a significant response with a reduction of HCV RNA of >2 logs. Given that patients with genotype 1 HCV who have relapsed after pegylated interferon and ribavirin therapy are very sensitive to re-treatment with telaprevir containing regimes we speculate that the modest antiviral effect of telaprevir in patients with genotype 3 HCV may be sufficient to induce a response in a proportion of subjects. We have recently developed a viral replication model which allows virus from patients with HCV to be cultured in vitro and its sensitivity to anti-viral agents assessed. We have shown that different genotype 3 isolates have differing sensitivity to telaprevir and we speculate that the probability of response may be determined by pre-treatment assessment of viral sensitivity to telaprevir. We now propose to examine these possibilities in a clinical trial.

1.2 Investigational Medicinal Products

This trial involves 3 licensed medications:

**IMP1** – Telaprevir (INCIVO) 375 mg film coated tablets to be taken orally.

**IMP2** - 40 Kd Pegylated interferon alfa 2a (Pegasys) 180 µg in pre-filled syringe/pen for subcutaneous injection

**IMP3** - Ribavirin (Copegus) 200 mg tablets to be taken orally.

1.3 Pre-Clinical Data

Telaprevir

*Animal toxicology and/or pharmacology*

In rats and dogs, telaprevir was associated with a reversible reduction of red blood cell parameters accompanied by a regenerative response. In both rats and dogs, AST/ALT elevations were observed in most studies, of which the increase in ALT in rats was not normalised after recovery. Histopathological findings in the liver were similar in both rat and dog studies, of which not all were fully resolved after recovery. In rats (but not in dogs), telaprevir caused degenerative changes in testes which were reversible and did not affect fertility. In general, exposure levels in relation to human values were low in animal pharmacology and toxicology studies.
Carcinogenesis and mutagenesis
Telaprevir has not been tested for its carcinogenic potential. Neither telaprevir nor its major metabolite caused damage to DNA when tested in the standard battery of mutagenesis assays, in the presence and absence of metabolic activation.

Impairment of fertility
Telaprevir had no effects on fertility or fecundity when evaluated in rats.

Embryo-foetal development
Telaprevir readily crosses the placenta in both rat and mouse giving a foetal:maternal exposure of 19 - 50%. Telaprevir did not have any teratogenic potential in rat or mouse. In a fertility and early embryonic development study in rats, an increase in non-viable conceptuses was observed. Dosing of the animals did not result in any exposure-margin when compared to human exposure.

Excretion into milk
When administered to lactating rats, levels of telaprevir and its major metabolite were higher in milk compared to those observed in plasma. Rat offspring exposed to telaprevir in utero showed normal body weight at birth. However, when fed via milk from telaprevir-treated dams, body weight gain of rat pups was lower than normal (likely due to taste aversion). After weaning, rat pup body weight gain returned to normal.

Pegylated interferon alfa 2a
The non-clinical toxicity studies conducted with Pegasys were limited due to species specificity of interferons. Acute and chronic toxicity studies have been carried out in cynomolgus monkeys, and the findings observed in peginterferon dosed animals were similar in nature to those produced by interferon alfa-2a.

Reproductive toxicity studies have not been performed with Pegasys. As with other alpha interferons, prolongation of the menstrual cycle was observed following administration of peginterferon alfa-2a to female monkeys. Treatment with interferon alfa-2a resulted in a statistically significant increase in abortifacient activity in rhesus monkeys. Although no teratogenic effects were seen in the offspring delivered at term, adverse effects in humans cannot be excluded.

Pegasys plus ribavirin
When used in combination with ribavirin, Pegasys did not cause any effects in monkeys not previously seen with either active substance alone. The major treatment-related change was reversible mild to moderate anaemia, the severity of which was greater than that produced by either active substance alone.

Ribavirin
Ribavirin is embryotoxic and/or teratogenic at doses well below the recommended human dose in all animal species in which adequate studies have been conducted. Malformations of the skull, palate, eye, jaw, limbs, skeleton and gastrointestinal tract were noted. The incidence and severity of teratogenic effects increased with escalation of the dose. Survival of foetuses and offspring is reduced.

Erythrocytes are a primary target of toxicity for ribavirin in animal studies, including studies in dogs and monkeys. Anaemia occurs shortly after initiation of dosing, but is rapidly reversible upon cessation of treatment. Hypoplastic anaemia was observed only in rats at the high dose of 160 mg/kg/day in the subchronic study.
Reduced leucocyte and/or lymphocyte counts were consistently noted in the repeat-dose rodent and dog toxicity studies with ribavirin and transiently in monkeys administered ribavirin in the subchronic study. Repeat-dose rat toxicity studies showed thymic lymphoid depletion and/or depletion of thymus-dependent areas of the spleen (periarteriolar lymphoid sheaths, white pulp) and mesenteric lymph node. Following repeat-dosing of dogs with ribavirin, increased dilatation/necrosis of the intestinal crypts of the duodenum was noted, as well as chronic inflammation of the small intestine and erosion of the ileum.

In repeat dose studies in mice to investigate ribavirin-induced testicular and sperm effects, abnormalities in sperm occurred at doses in animals well below therapeutic doses. Upon cessation of treatment, essentially total recovery from ribavirin induced testicular toxicity occurred within one or two spermatogenic cycles.

Genotoxicity studies have demonstrated that ribavirin does exert some genotoxic activity. Ribavirin was active in an in vitro Transformation Assay. Genotoxic activity was observed in in vivo mouse micronucleus assays. A dominant lethal assay in rats was negative, indicating that if mutations occurred in rats they were not transmitted through male gametes. Ribavirin is a possible human carcinogen.

Administration of ribavirin and peginterferon alfa-2a in combination did not produce any unexpected toxicity in monkeys. The major treatment-related change was reversible mild to moderate anaemia, the severity of which was greater than that produced by either active substance alone.

1.4 Clinical Data

These medications are licensed and NICE approved for the treatment of genotype 1 HCV.

Telaprevir, in combination with peginterferon alfa and ribavirin, is indicated for the treatment of genotype 1 chronic hepatitis C in adult patients with compensated liver disease (including cirrhosis):
- Who are treatment-naïve;
- Who have previously been treated with interferon alfa (pegylated or non-pegylated) alone or in combination with ribavirin, including relapers, partial responders and null responders (see sections 4.4 and 5.1).

In the Phase 3 studies, none of the patients with HCV RNA > 1,000 IU/ml at either week 4 or week 12 achieved SVR with continued peginterferon alfa and ribavirin treatment. In treatment-naïve patients in the Phase 3 studies, 4/16 (25%) patients with HCV RNA levels between 100 IU/ml and 1,000 IU/ml at week 4 achieved SVR. In patients with HCV RNA between 100 IU/ml and 1,000 IU/ml at week 12, 2/8 (25%) achieved an SVR.

Special populations

Renal impairment
There are no clinical data on the use of INCIVO in HCV patients with moderate or severe renal impairment (CrCl ≤ 50 ml/min). In HCV-negative patients with severe renal impairment, no clinically relevant change in telaprevir exposure was observed. Therefore, no dose adjustment is recommended for telaprevir in HCV patients with renal impairment.

There are no clinical data on the use of telaprevir in patients on hemodialysis.

Hepatic impairment
Telaprevir is not recommended in patients with moderate to severe hepatic impairment (Child-Pugh B or C, score ≥ 7) or decompensated liver disease. Dose modification of telaprevir is not required when administered to hepatitis C patients with mild hepatic impairment (Child-Pugh A, score 5-6).

**Severe rash**
Severe rashes have been reported with telaprevir combination treatment. In placebo-controlled Phase 2 and 3 trials, severe rash (primarily eczematous, pruritic and involving more than 50% body surface area) was reported in 4.8% of patients who received telaprevir combination treatment compared to 0.4% receiving peginterferon alfa and ribavirin. 5.8% of patients discontinued telaprevir alone due to rash events and 2.6% of patients discontinued telaprevir combination treatment for rash events compared to none of those receiving peginterferon alfa and ribavirin.

In placebo-controlled Phase 2 and 3 trials, 0.4% of patients had suspected Drug Rash with Eosinophilia and Systemic Symptoms (DRESS). In telaprevir clinical experience, less than 0.1% of patients had Stevens - Johnson syndrome. All of these reactions resolved with treatment discontinuation. DRESS presents as a rash with eosinophilia associated with one or more of the following features: fever, lymphadenopathy, facial oedema, and internal organ involvement (hepatic, renal, pulmonary). It may appear at any time after start of treatment, although the majority of cases appeared between six and ten weeks after the start of treatment with telaprevir.

**Anaemia**
In placebo-controlled Phase 2 and 3 clinical trials, the overall incidence and severity of anaemia increased with telaprevir combination treatment compared to peginterferon alfa and ribavirin alone. Haemoglobin values of < 10 g/dl were observed in 34% of patients who received telaprevir combination treatment and in 14% of patients who received peginterferon alfa and ribavirin. Haemoglobin values of < 8.5 g/dl were observed in 8% of telaprevir combination treatment compared to 2% of patients receiving peginterferon alfa and ribavirin. A decrease in haemoglobin levels occurs during the first 4 weeks of treatment, with lowest values reached at the end of telaprevir dosing. Haemoglobin values gradually improve after completion of telaprevir dosing.

**Cardiovascular**
Results of a study conducted in healthy volunteers demonstrated a modest effect of telaprevir at a dose of 1,875 mg every 8 hours on the QTcF interval with a placebo-adjusted maximum mean increase of 8.0 msec (90% CI: 5.1-10.9). Exposure at this dose was comparable to the exposure in HCV-infected patients receiving a dose of 750 mg telaprevir every 8 hours plus peginterferon alfa and ribavirin. The potential clinical significance of these findings is uncertain.

**The use of telaprevir in combination with peginterferon alfa-2b**
The Phase 3 studies were all conducted with peginterferon alfa-2a in combination with telaprevir and ribavirin. There is no data using telaprevir in combination with peginterferon alfa-2b in treatment-experienced patients and limited data in treatment-naïve patients. Naïve patients treated with either peginterferon alfa-2a/ribavirin (n = 80) or peginterferon alfa-2b/ribavirin (n = 81) in combination with telaprevir, in an open label study, had comparable SVR rates. However, patients treated with peginterferon alfa-2b experienced more frequent viral breakthrough, and were less likely to meet the criteria for shortened total treatment duration.

One published study Foster et al (5) has shown limited activity in patients with Genotype 3 HCV and the purpose of this pilot study is to determine whether or not this activity is
sufficient to justify further clinical trials in a subset of patients with minimal alternative treatment options.

**Hepatic impairment**
Telaprevir has not been studied in patients with severe hepatic impairment (Child-Pugh C, score ≥ 10) or decompensated liver disease and is not recommended in these populations. Telaprevir has not been studied in HCV-infected patients with moderate hepatic impairment (Child-Pugh B, score 7-9). In HCV negative patients with moderate hepatic impairment, reduced exposure to telaprevir was observed.

**Summary of the safety profile**
The overall safety profile of telaprevir is based on all available pooled Phase 2 and 3 clinical trial data (both controlled and uncontrolled) containing 2,641 patients who received telaprevir combination treatment.

The incidence of adverse drug reactions (ADRs) of at least moderate intensity (≥ Grade 2) was higher in the telaprevir group than in the placebo group. During the telaprevir /placebo treatment phase, the most frequently reported ADRs of at least Grade 2 in severity in the telaprevir group (incidence ≥ 5.0%) were anaemia, rash, pruritus, nausea, and diarrhoea. During the telaprevir/placebo treatment phase, the most frequently reported ADRs of at least Grade 3 in the telaprevir group (incidence ≥ 1.0%) were anaemia, rash, thrombocytopenia, lymphopenia, pruritus, and nausea.

**Clinical virology studies**
In Phase 2 and 3 clinical trials of telaprevir, treatment-naïve and prior treatment-failure patients with predominant telaprevir resistant variants at baseline (pre-treatment) were rare (V36M, T54A and R155K < 1% and T54S 2.7%). Predominant baseline resistant variants at baseline (pre-treatment) were rare (V36M, T54A and R155K < 1% and T54S 2.7%). Predominant baseline resistance to telaprevir does not preclude successful treatment with telaprevir, peginterferon alfa, and ribavirin. The impact of predominant telaprevir-resistant variants at baseline is likely greatest in patients with a poor interferon response, such as prior null responders.

A total of 215 of 1,169 patients treated with a T12/PR regimen in a Phase 3 clinical trial had on-treatment virologic failure (n = 125) or relapse (n = 90). Based on population sequencing analyses of HCV in these 215 patients, the emergence of telaprevir-resistant HCV variants was detected in 105 (84%) virologic failures and in 55 (61%) relapsers, and wild-type virus was detected in 15 (12%) virologic failures and in 24 (27%) relapsers. HCV sequencing data were not available for 16 (7%) patients. Sequence analyses of the telaprevir-resistant variants identified substitutions at 4 positions in the NS3-4A protease region, consistent with the mechanism of action for telaprevir (V36A/M, T54A/S, R155K/T, and A156S/T/V). On-treatment virologic failure during telaprevir treatment was predominantly associated with higher-level resistant variants, and relapse was predominantly associated with lower-level resistant variants or wild-type virus.

Patients with HCV genotype 1a predominately had V36M and R155K single and combination variants, while patients with HCV genotype 1b predominately had V36A, T54A/S, and A156S/T/V variants. This difference is likely due to the higher genetic barrier for the V36M and R155K substitutions for genotype 1b than genotype 1a. Among patients treated with telaprevir, on treatment virologic failure was more frequent in patients with genotype 1a than with genotype 1b and more frequent in prior null responders than in other populations (treatment-naïve, prior relapsers, prior partial responders; see section 5.1, Clinical Experience, Efficacy in Previously Treated Adults).
Follow-up analysis of INCIVO-treated patients who did not achieve an SVR showed that the population of wild-type virus increased and the population of telaprevir-resistant variants became undetectable over time after the end of telaprevir treatment. Of a combined 255 treatment-naïve and previously treated patients from Phase 3 studies 108, 111, and C216 in whom telaprevir-resistant variants had emerged during treatment, 152 (60%) patients no longer had resistant variants detected by population sequencing (median follow-up of 10 months). Of the 393 resistant variants detected in the 255 patients, 68% of NS3-36, 84% of NS3-54, 59% of NS3-155, 86% of NS3-156, and 52% of NS3-36M+NS3-155K variants were no longer detected.

In a follow-up study of 98 treatment-naïve and prior treatment-failure patients who were treated with a INCIVO regimen in a Phase 2 or Phase 3 study and did not achieve SVR, telaprevir-resistant variants were no longer detected in 85% (83/98) of patients (median follow-up of 27.5 months). Clonal sequencing analysis of a subset of patients who had wild-type HCV by population sequencing (n=20), comparing the frequency of resistant variants before the start of telaprevir treatment and at follow-up, showed that the HCV variant population in all patients had returned to pre-treatment levels. The median time for telaprevir-resistant variants to become undetectable by population sequencing was longer for variants NS3-36 (6 months), NS3-155 (9 months) and NS3-36M+NS3-155K (12 months) predominantly observed in patients with genotype 1a than for variants NS3-54 (2 months) and NS3-156 (3 months) predominantly observed in patients with genotype 1b.

Clinical efficacy and safety
The efficacy and safety of INCIVO in patients with genotype 1 chronic hepatitis C were evaluated in three Phase 3 studies: 2 in treatment-naïve patients and 1 in previously treated patients (relapsers, partial responders, and null responders). Patients in these studies, 108, 111 and C216, had compensated liver disease, detectable HCV RNA, and liver histopathology consistent with chronic hepatitis C. Unless otherwise indicated, INCIVO was administered at a dosage of 750 mg every 8 hours; the peginterferon alfa-2a dose was 180 μg/week, and the ribavirin dose was 1,000 mg/day (patients weighing < 75 kg) or 1,200 mg/day (patients weighing ≥ 75 kg). Plasma HCV RNA values were measured using the COBAS® TaqMan® HCV test (version 2.0), for use with the High Pure System. The assay had a lower limit of quantification of 25 IU/ml. In the description of Phase 3 study outcomes below, SVR, considered virologic cure, was defined based on the HCV RNA assessment in the study week 72 visit window, using the last measurement in the window. In the case of missing data within the week 72 window, the last HCV RNA data point from week 12 of follow-up onwards was used. In addition, the limit of quantification of 25 IU/ml was used to determine SVR.

Efficacy in treatment-naïve adults
Study 108 (ADVANCE)
Study 108 was a randomised, double-blind, parallel-group, placebo-controlled, Phase 3 study conducted in treatment-naïve patients. INCIVO was given for the first 8 weeks of treatment (T8/PR regimen) or the first 12 weeks of treatment (T12/PR regimen) in combination with peginterferon alfa-2a and ribavirin for either 24 or 48 weeks. Patients who had undetectable HCV RNA at weeks 4 and 12 received 24 weeks of peginterferon alfa-2a and ribavirin treatment, and patients who did not have undetectable HCV RNA at week 4 and week 12 received 48 weeks of peginterferon alfa-2a and ribavirin treatment. The control regimen (Pbo/PR) had a fixed treatment duration of 48 weeks, with telaprevir-matching placebo for the first 12 weeks and peginterferon alfa-2a and ribavirin for 48 weeks.

The 1,088 enrolled patients had a median age of 49 years (range: 18 to 69); 58% of the patients were male; 23% had a body mass index ≥ 30 kg/m2; 9% were Black; 11% were Hispanic or Latino; 77% had baseline HCV RNA levels ≥ 800,000 IU/ml 15% had bridging fibrosis; 6% had cirrhosis; 59% had HCV genotype 1a; and 40% had HCV genotype 1b. The
SVR rate for the T8/PR group was 72% (261/364) ($P < 0.0001$ compared to Pbo/PR48 group)

**Study 111 (ILLUMINATE)**

Study 111 was a Phase 3, randomised, open label study conducted in treatment-naive patients. The study was designed to compare SVR rates in patients with undetectable HCV RNA at weeks 4 and 12 who were treated with INCIVO for 12 weeks in combination with peginterferon alfa-2a and ribavirin for either 24 weeks (T12/PR24 regimen) or 48 weeks (T12/PR48 regimen). Patients with undetectable HCV RNA at weeks 4 and 12 were randomised at week 20 to receive either 24 weeks or 48 weeks of peginterferon alfa-2a and ribavirin treatment. The primary assessment was an evaluation of non-inferiority, using a margin of -10.5% of the 24-week regimen compared to the 48-week regimen in patients with undetectable HCV RNA at weeks 4 and 12. The 540 enrolled patients had a median age of 51 years (range: 19 to 70); 60% of the patients were male; 32% had a body mass index $\geq 30$ kg/m²; 14% were Black; 10% were Hispanic or Latino; 82% had baseline HCV RNA levels $> 800,000$ IU/ml; 16% had bridging fibrosis; 11% had cirrhosis; 72% had HCV genotype 1a; and 27% had HCV genotype 1b.

A total of 352 (65%) patients had undetectable HCV RNA at weeks 4 and 12. In patients who had undetectable HCV RNA at weeks 4 and 12, there was no additional benefit to extending peginterferon alfa-2a and ribavirin treatment to 48 weeks (difference in SVR rates of 2%; 95% confidence interval: -4%, 8%).

**Study 110**

Study 110 was a phase II randomised, double-blind, placebo-controlled study conducted in patients with chronic genotype 1 HCV/HIV co-infection who were treatment-naive for hepatitis C. Patients were either not on antiretroviral therapy (CD4 count $\geq 500$ cells/mm3), or had stable controlled HIV (HIV RNA $< 50$ copies/ml, CD4 count $\geq 300$ cells/mm3) being treated with efavirenz or atazanavir/ritonavir in combination with tenofovir disoproxil fumarate and emtricitabine or lamivudine. Patients were randomised to 12 weeks of INCIVO (750 mg every 8 hours if taken in combination with atazanavir/ritonavir, tenofovir disoproxil fumarate, and emtricitabine or lamivudine OR 1125 mg every 8 hours if taken in combination with efavirenz, tenofovir disoproxil fumarate, and emtricitabine) or placebo. All patients received peginterferon alfa-2a and ribavirin for 48 weeks. Fifty-five out of 60 patients received ribavirin at a fixed dose of 800 mg/day and the remaining 5 patients received a weight-based ribavirin dose. At baseline, 3 (8%) subjects had bridging fibrosis and 2 (5%) subjects had cirrhosis in the T12/PR48 arm. In the Pbo/PR arm, 2 (9%) subjects had baseline bridging fibrosis and no subjects had baseline cirrhosis. The response rate in the Pbo/PR arm was higher than that seen in other clinical studies of peginterferon bitherapy (historical SVR rates $< 36$%).

**Efficacy in previously treated adults**

**Study C216 (REALIZE)**

Study C216 was a randomised, double-blind, placebo-controlled, Phase 3 study conducted in patients who did not achieve SVR with prior treatment with peginterferon alfa-2a and ribavirin or peginterferon alfa-2b and ribavirin. The study enrolled prior relapsers (patients with HCV RNA undetectable at end of treatment with a pegylated interferon-based regimen, but HCV RNA detectable within 24 weeks of treatment follow-up) and prior non-responders (patients who did not have undetectable HCV RNA levels during or at the end of a prior course of at least 12 weeks of treatment). The non-responder-population was comprised of 2 subgroups: prior partial responders (greater than or equal to 2 log10 reduction in HCV RNA at week 12, but not achieving HCV RNA undetectable at end of treatment with a peginterferon and ribavirin) and prior null responders (less than 2 log10 reduction in HCV RNA at week 12 of prior treatment with peginterferon and ribavirin).
Patients were randomised in a 2:2:1 ratio to one of three treatment groups: simultaneous start (T12/PR48): INCIVO from day 1 through week 12; delayed start (T12(DS)/PR48): INCIVO from week 5 through week 16; Pbo/PR48: placebo through week 16. All treatment regimens had a 48-week duration of peginterferon alfa-2a and ribavirin treatment.

The 662 enrolled patients had a median age of 51 years (range: 21 to 70); 70% of the patients were male; 26% had a body mass index ≥ 30 kg/m2; 5% were Black; 11% were Hispanic or Latino; 89% had baseline HCV RNA levels > 800,000 IU/ml; 22% had bridging fibrosis; 26% had cirrhosis; 54% had HCV genotype 1a; and 46% had HCV genotype 1b. SVR rates for the T12(DS)/PR group were 88% (124/141) for prior relapsers, 56% (27/48) for prior partial responders, and 33% (25/75) for prior null responders. For all populations in the study (prior relapsers, prior partial responders, and prior null responders), SVR rates were higher for the T12/PR group than for the Pbo/PR48 group across subgroups by sex, age, race, ethnicity, body mass index, HCV genotype subtype, baseline HCV RNA level, and extent of liver fibrosis.

Study 106 and Study 107
Study 106 was a randomised, double-blind, placebo-controlled, Phase 2 study that enrolled patients who had failed prior treatment with peginterferon alfa-2a and ribavirin or peginterferon alfa-2b and ribavirin. Among prior relapsers in the T12/PR24 treatment group who had undetectable HCV RNA at weeks 4 and 12 of treatment, the SVR rate was 89% (25/28) and the relapse rate was 7%.

Study 107 was an open label, rollover study for patients who were treated in the control group (placebo, peginterferon alfa-2a, and ribavirin) of a Phase 2 study of telaprevir and who did not achieve SVR in the Phase 2 study. Among prior relapsers in the T12/PR24 treatment group who had undetectable HCV RNA at week 4 and 12 of treatment, the SVR rate was 100% (24/24).

Use of peginterferon alfa 2a or 2b
Two types of peginterferon alfa (2a and 2b) were studied in the Phase 2a open label, randomised study C208 in treatment naive patients.

All patients received 12 weeks of INCIVO in combination with the peginterferon alfa/ribavirin standard therapy. Patients were randomised to 1 of 4 treatment groups:
- INCIVO 750 mg every 8 hours with peginterferon alfa-2a 180 μg/week and ribavirin 1,000 or 1,200 mg/day
- INCIVO 750 mg every 8 hours with peginterferon alfa-2b 1.5 g/kg/week and ribavirin 800 or 1,200 mg/day
- INCIVO 1,125 mg every 12 hours with peginterferon alfa-2a 180 g/week and ribavirin 1,000 or 1,200 mg/day
- INCIVO 1,125 mg every 12 hours with peginterferon alfa-2b 1.5 g/kg/week and ribavirin 800 or 1,200 mg/day

Peginterferon alfa-2a/peginterferon alfa-2b and ribavirin were used according to their relevant Summary of Product Characteristics. At week 12, INCIVO dosing ended and patients continued on standard therapy only. 73.8% (59/80) of patients in the pooled peginterferon alfa-2a group met the criteria (undetectable HCV RNA at week 4 through week 20) for the shortened 24 week peginterferon/ribavirin treatment duration versus 61.7% (50/81) of patients in the pooled peginterferon alfa-2b group.

*Side effects observed when telaprevir was taken in combination with Peg-IFN and RBV*
An analysis of the safety data from all completed studies in HCV-infected subjects through 31 October 2011 has been conducted to identify adverse events considered to be related to the combination of telaprevir and Peg-IFN/RBV, and these side effects are listed below. The
frequency rate is given for clinically important adverse reactions of moderate severity or more.

- **Very common side effects (reported by more than 10% of subjects):**
  - Anemia, Itching skin, Rash.
- **Common side effects (reported by 1 – 10% of subjects):** Nausea, Diarrhoea, Haemorrhoids (painful swelling of a vein in the region of the anus), Vomiting, Anal and/or rectal pain, Itching anus, Abnormal taste, Fainting.
- **Uncommon side effects (reported by less than 1% of subjects):** Fungal infection in the mouth, Bleeding from the anus or rectum, Anal fissure (a crack or tear in the skin of the anus), Face swelling, Swelling of the arms or legs, Hypothyroidism (reduced function of the thyroid gland), Drug Rash with Eosinophilia and Systemic Symptoms (DRESS, a severe type of rash), Stevens-Johnson syndrome (SJS, a severe type of rash), Hives, Proctitis (inflammation of the anus or rectum), Retinopathy (damage to the retina in the back of the eye), Gout (painful joint swelling most commonly in the foot).

Significant changes in the following blood tests in addition to anemia already mentioned above are also considered side effects of telaprevir/Peg-IFN/RBV combination therapy. The frequency rate is given for changes in blood tests of moderate severity or more.

- **Very common side effects (observed in 10% of subjects or more):** reduced Lymphocytes (a type of white blood cells), reduced Platelets (a type of blood cell), increased blood Uric Acid (a substance produced by the body), increased blood Bilirubin (a substance produced by the liver), increase in Cholesterol (a type of blood fat).
- **Common side effects (reported by 1% of subjects or more but less than 10% of subjects):** decreased blood Potassium (a salt in the blood), increased blood Creatinine (a substance produced by the body and removed by the kidneys).

Overall, rashes were reported in 55% of subjects (all grades of severity). More than 90% of rashes have been mild to moderate in severity. Approximately 5% of subjects experienced severe rash during treatment with telaprevir in combination with Peg-IFN and RBV, and approximately 3% of subjects discontinued telaprevir, Peg-IFN and RBV because of rashes. Rashes are usually itchy and have features similar to eczema.

Some of the severe rashes observed with telaprevir combination therapy were suspected as being specific skin reactions: Drug Rash with Eosinophilia and Systemic Symptoms in 0.4% (4 out of a thousand subjects), or Stevens-Johnson syndrome in less than 0.1% (less than 1 out of a thousand subjects). In addition to a widespread rash, these specific drug reactions can include one or more of the following symptoms: fever, skin blistering and peeling, swollen lymph glands, swelling of the face or the area of the rash, blisters in the mouth, or eyes or genitals.

The rashes may be improved by skin treatments such as ointments or creams or other medicines, and in case of more severe rashes after stopping one or all of the study drugs. Improvement and clearing of the rash is observed over varying periods of time.

More than 50,000 patients have received telaprevir in combination with Peg-IFN and RBV since approval by regulatory authorities. Rare fatal (deadly) and non-fatal serious skin reactions, including a specific severe rash disorder called toxic epidermal necrolysis (TEN), have been reported since telaprevir was approved by regulatory authorities.

Anemia (a decrease in the oxygen-carrying cells of the blood) was reported in 32% of subjects (all grades of severity). Approximately 22% of subjects who experienced anemia during treatment with telaprevir in combination with Peg-IFN and RBV required a reduction in the dose of RBV.
Approximately 3% of subjects received blood transfusion, 3% of subjects discontinued telaprevir and less than 1% of subjects discontinued telaprevir, Peg-IFN and RBV combination therapy because of anemia.

Anorectal disorders were reported in 26% of subjects (all grades of severity). In clinical trials, the majority of these events (e.g., haemorrhoids, anorectal discomfort, anal pruritus, and rectal burning) were mild to moderate; very few led to treatment discontinuation and resolved after completion of telaprevir dosing.

The effect of telaprevir on the QTc interval was studied in a clinical trial in healthy subjects; the QTc interval is a measurement of part of the electrical activity of the heart seen on the electrocardiogram (ECG/EKG). A modest increase in the QTc interval has been observed with telaprevir at a higher dose than used in this trial. There is a very small chance that increases in the QTc interval may lead to serious heart problems such as abnormal heart rhythms (arrhythmias) that may result in a very fast heartbeat and fainting. Very rarely such arrhythmias may lead to the heart stopping and sudden death. The risks of abnormal heart rhythms may be higher when telaprevir is combined with certain other drugs or when used in patients who already have heart conditions or have a low level of potassium in the blood. No such arrhythmias or sudden deaths have been seen in any clinical study with telaprevir.

Side effects commonly attributed to Pegasys® and Copegus®
Possible, serious side effects (which in some cases could be life threatening or fatal) associated with the use of these drugs include:

- Autoimmune problems: conditions where the body's own immune system begins to attack itself; these conditions include psoriasis and thyroid problems.
- Heart problems, including irregular heart beat (arrhythmia), chest pain and very rarely heart attack.
- Worsening of liver function, including liver failure.
- Psychiatric symptoms: depression (including suicidal thoughts), anxiety, insomnia, irritability, relapse of drug abuse, and drug overdose.
- Severe infections which could be life-threatening or fatal.
- Risk to pregnancy (i.e. birth defects); see below for more information.
- Low blood counts: including anemia (drop in red blood cell count; this can be especially dangerous in patients with heart or breathing problems); drop in certain white blood cells (this can increase your risk of infection) and low platelet count (this can increase risk of bleeding).
- Inflammation of the pancreas.
- Bleeding in the brain (cerebral haemorrhage), blood clot in the lungs (pulmonary embolism).
- Vision changes and problems in the blood vessels in the back of the eye, sore on the cornea (corneal ulcer).
- Skin rash: When rashes occur due to the use of Pegasys/PegIntron, with or without Copegus/Rebetol, they are usually mild. However, rare but serious skin reactions (including a type called Stevens Johnson Syndrome) have been reported.

Common, but less serious side effects include:

- Flu-like symptoms, such as headache, tiredness, muscle aches, fever and chills.
- Itching skin (pruritus), skin reaction at the place where the injection is given.
- Digestive symptoms such as diarrhoea, vomiting, lack of appetite; taste changes and nausea.
- Problems controlling blood sugar, including diabetes.
- Hair loss (alopecia).

Potential risks based on non-clinical studies
The safety of telaprevir has also been evaluated in laboratory tests and studies involving animals. An early test using mouse lymphoma tumour cells to assess effects on genetic material was conducted with lower grade (less pure) telaprevir and showed a risk of damage to genetic material. Similar tests conducted with higher grade (highly pure) telaprevir like that used in clinical trials have not shown any potential genetic damage.

When telaprevir is manufactured, very small amounts of other chemicals may remain in the pills as leftover ingredients, or side products. These other chemicals are called "impurities". Laboratory tests conducted separately with individual impurities present in the telaprevir tablets showed risks of damage to the genetic material. However, the amount of these impurities present in the telaprevir tablets is very low.

Reproductive Risks
At this time it is not understood how telaprevir affects an unborn baby or sperm. Telaprevir has shown no teratogenic potential in animal studies. Ribavirin is known to cause birth defects, and has very specific instructions regarding birth control and pregnancy which should be adhered to during participation to this study.

1.5 Rationale and Risks/Benefits

The current recommended first line treatment for patients with chronic hepatitis C is the combination of pegylated interferon alfa (PEG-IFN) and ribavirin (RBV). In 2002, Pegasys (Peginterferon alfa-2a, PEG-IFN alfa-2a) and Copegus (ribavirin) were approved for marketing in the United States, Europe and several other countries for the treatment of chronic hepatitis C. This medication has a well-established safety profile. Telaprevir is a new drug that is licensed to treat genotype 1 hepatitis C and which works very well in these patients. Additional side effects to standard treatment regime of pegylated interferon alfa (PegIFN) and ribavirin (RBV) include skin reactions that may (very rarely) be severe and may include life threatening disorders such as Stevens-Johnson syndrome.

In clinical trials in patients with advanced liver disease severe sequelae including hepatic decompensation and even death have been seen. It should be noted that in patients with advanced liver disease the rate of significant, life threatening complications is 5% per year and therefore without therapy a significant proportion of patients with advanced liver disease will die.

To minimise the risks of therapy in these patients we will select experienced investigators with the support services required to manage and minimise the risks of therapy.

Patients at high risk of decompensation (those with ascites) have been excluded from the study and the regular clinical review will reduce the risk of serious adverse events to an acceptable level.

The study involves a population of patients who are not normally considered eligible for treatment with interferon and ribavirin containing regimes – specifically patients with low haemoglobin levels, low neutrophil counts and low platelet counts will be included. This is because current ‘standard of care’ therapy for patients with genotype 3 HCV (Pegylated interferon and ribavirin) leads to a sustained virological response in >80% in patients with early disease. However in patients with cirrhosis (invariably associated with low haemoglobins, low platelet counts and low neutrophil counts) the response is much reduced (<50%). Hence there is an unmet medical need for treatments for patients with cirrhosis and associated cytopenias.
The SmPC for Pegasys and ribavirin was based on early clinical studies with very conservative inclusion criteria. Multiple studies since then have shown the safety of this combination in patients with advanced cirrhosis and cytopaenias and it is now standard clinical practice to treat such patients with ribavirin containing regimes.

Thus the inclusion criteria of haemoglobin, platelet and neutrophil counts for this study are based on current clinical practice not the SmPC guidance which has been shown to be safe in published clinical studies and the inclusion criteria specifically address an unmet clinical need.

2. **Trial Objectives and Design**

2.1 **Trial Objectives**

**Primary Objective**
- To determine whether patients with genotype 3 HCV and cirrhosis who have failed to achieve a sustained virological response (SVR) with PegIFN and RBV will achieve a SVR if treated with telaprevir, PegIFN and RBV

**Secondary objective**
- To determine whether viral phenotyping predicts the response to therapy with telaprevir in patients with G3 HCV and cirrhosis

**Primary outcomes**
- The primary outcome is the proportion of patients with a sustained virological response (SVR) 12 weeks after end of treatment (SVR12). SVR 12 is defined as undetectable HCV RNA on a blood sample taken between 12 and 18 weeks after the end of treatment measured using a sensitive, validated polymerase chain reaction (PCR) assay with a lower limit of quantification of at least 30IU/ml. End of treatment is defined as date of final dose of IMP 1, 2 or 3 (whichever is the latest date).

**Secondary outcomes**
- The proportion of patients who are phenotypically poorly responsive to telaprevir (defined as virus with a poor response to telaprevir *in vitro* i.e. an IC50 of <0.1μMol in an in vitro assay) who achieve early and late virological clearance.
- The proportion of patients with a sustained virological response 24 weeks after the last dose of PegIFN and RBV (SVR24). SVR 24 is defined as undetectable HCV RNA on a blood sample taken between 24 and 30 weeks after the final dose of PegIFN and ribavirin measured using a sensitive, validated polymerase chain reaction (PCR) assay with a lower limit of quantification of at least 30IU/ml.
- The proportion of patients who have undetectable HCV RNA (measured using a sensitive, validated polymerase chain reaction (PCR) assay with a lower limit of quantification of at least 30IU/ml) after 1, 2, 3 and 4 weeks of therapy with PegIFN, RBV and telaprevir.

2.2 **Trial Design and Study Scheme Diagram**

Open trial design in which all patients who are fulfil the entry criteria are treated for 24 weeks with PegIFN, RBV and 12 weeks with telaprevir as outlined in the treatment procedure. Prior to treatment all patients will have samples taken for viral phenotyping.
Patient with G3 HCV, advanced fibrosis and failure to respond to pegylated interferon and ribavirin

Viral sensitivity assessed in vitro

Screen failures:
  i.e. Not eligible or

Entered into screening log

Treated for 12 weeks with telaprevir alongside a 24 week course of PegIFN

SVR12 assessed
  (12-18 weeks)

SVR24 assessed
  (24-30 weeks)

Patients withdrawn from treatment

1. Patients removed as non-responders to therapy will be referred back to their clinical team for follow-up as appropriate

2. Patients withdrawn due to other clinical reasons (e.g. SAEs) will be followed up
3. **Subject Selection**

3.1 **Number of Subjects and Subject Selection**

The study involves a population of patients who are not normally considered eligible for treatment with interferon and ribavirin containing regimes – specifically patients with low haemoglobin levels, low neutrophil counts and low platelet counts will be included. This is because current ‘standard of care’ therapy for patients with genotype 3 HCV (Pegylated interferon and ribavirin) leads to a sustained virological response in >80% in patients with early disease. However in patients with cirrhosis (invariably associated with low haemoglobins, low platelet counts and low neutrophil counts) the response is much reduced (<50%). Hence there is an unmet medical need for treatments for patients with cirrhosis and associated cytopaenias. This study is specifically aiming to investigate this unmet need.

Patients attending the out-patient clinic at the participating sites who are deemed suitable for the study by their consultant will be contacted and asked if they wish to consider participating in the study. Those who are willing to consider the study will be provided with the patient information sheet and asked to consider this at their leisure. Subjects will be given a minimum of 24 hours to review the information. Those who agree to participate will then be asked to sign the consent form and enrolled in the study.

A total of approximately 30 patients will be enrolled on the trial spread over the trial sites. This study will be competitive recruitment therefore once the total number of patients enrolled on the study reaches 30 recruitment to the study will be closed even if some sites have not recruited any participants.

3.2 **Inclusion Criteria**

- Age ≥18 years of age and ≤ 70 years old
- Advanced fibrosis - defined as a liver biopsy within 2 years showing an Ishak fibrosis score of >4 OR radiological evidence of cirrhosis (ultrasound scan or fibroscan reading >10.6)
- Previous therapy with pegylated interferon and ribavirin with detectable HCV RNA six months after treatment cessation
- Chronic genotype 3 HCV infection, RNA positivity with genotype 3 infection confirmed at a local laboratory.
- HBsAg negative and no clinical evidence of co-infection with HIV
- Platelet count >50,000 cells/mm$^3$ (support with eltrombopag is permitted) Neutrophil count > 600 cells/mm$^3$
- All female patients of childbearing potential and all males with female partners of childbearing potential must be prepared to use two forms of effective contraception* (combined) during treatment and 6 months after treatment end
- Able and willing to give informed consent and able to comply with study requirements

**NOTE**: Subjects are considered not of child bearing potential if they are surgically sterile (they have undergone a hysterectomy, bilateral tubal ligation, or bilateral oophorectomy) or they are postmenopausal.

* Effective contraceptive methods include an intrauterine device, a male OR female condom [not both simultaneously as friction between the two can result in damage or breakage of either product], or diaphragm with spermicidal jelly, or cervical cap with spermicidal jelly.
3.3 Exclusion Criteria

- Evidence of other cause of significant liver disease – serum ferritin > 1000, biochemical evidence of Wilson’s disease, autoantibody titres in excess of 1:160
- Poorly controlled diabetes that, in the investigators opinion, precludes therapy
- Severe retinopathy that, in the opinion of the investigator, precludes therapy
- Evidence of ascites seen on previous liver ultrasound
- Haemoglobin concentration <11 g/dL in females or <12 g/dL in males or any patient with an increased risk for anaemia (e.g., thalassemia, sickle cell anaemia, spherocytosis, history of gastrointestinal bleeding) or for whom anaemia would be medically problematic
- Albumin levels <35 G/L
- Females who are pregnant or breast-feeding
- History of severe psychiatric disease, including psychosis and/or depression, characterized by a suicide attempt, hospitalization for psychiatric disease, or a period of disability as a result of psychiatric disease within the last 2 years
- History of immunologically mediated disease (e.g., inflammatory bowel disease, idiopathic thrombocytopenic purpura, lupus erythematosus, autoimmune haemolytic anaemia, scleroderma, severe psoriasis (defined as affecting >10% of the body, where the palm of one hand equals 1%, or if the hands and feet are affected), rheumatoid arthritis requiring more than intermittent nonsteroidal anti-inflammatory medications for management
- Other on-going serious medical condition in the opinion of the investigator that would prohibit treatment
- Poorly controlled thyroid dysfunction that, in the investigators opinion, precludes therapy
- History of major organ transplantation with an existing functional graft
- History of severe pre-existing cardiac disease, including unstable or uncontrolled cardiac disease in the previous 6 months
- History or laboratory testing showing evidence of a haemoglobinopathy
- Concomitant administration with active substances that are highly dependent on CYP3A for clearance and for which elevated plasma concentrations are associated with serious and/or life-threatening events. These active substances include alfuzosin, amiodarone, bepridil, quinidine, astemizole, terfenadine, cisapride, pimozone, ergot derivatives (dihydroergotamine, ergonovine, ergotamine, methylergonovine), lovastatin, simvastatin, atorvastatin, sildenafil or tadalafil (only when used for treatment of pulmonary arterial hypertension) and orally administered midazolam or triazolam.
- Concomitant administration with Class Ia or III antiarrhythmics, except for intravenous lidocaine (see section 4.5).
- Concomitant administration of INCIVO with active substances that strongly induce CYP3A e.g. rifampicin, St John's wort (Hypericum perforatum), carbamazepine, phenytoin and phenobarbital and thus may lead to lower exposure and loss of efficacy of INCIVO.

3.4 Criteria for Premature Withdrawal

1. To avoid unnecessary exposure to therapy that has a low probability of success the following pre-defined ‘stopping rules’ will apply.
   - If the viral load after 4 weeks of therapy with PegIFN, Ribavirin and telaprevir is >1000 IU/ml, therapy will be abandoned and the patient will be deemed a non-responder to therapy.
   - If the viral load at week 8 is greater than 1000 IU/ml OR the viral load has not declined by more than 3 logs from the pre-treatment viral load, therapy will be abandoned and the patient deemed a non-responder to therapy.
2. Patients will be withdrawn from the study if there is evidence of systemic drug toxicity which, in the opinion of the investigator, modifies the risk: benefit ratio in favour of harm.
3. Request of the participant or discretion of the investigator
4. Inability or participant’s failure to comply with the protocol requirements
5. Pregnancy

* If there is evidence of telaprevir intolerance the drug may be discontinued and the patient will remain in the study and will continue to receive pegylated interferon and ribavirin.

4. Investigational Medicinal Product

Patients will receive standard of care 24 week treatment with a weekly injection of 180 µg 40 Kd Pegylated interferon alfa 2a combined with oral ribavirin 800mg per day given in two divided doses. In addition, patients will be administered IMP which consists of 1125mg (3 x 375mg tablets) of oral telaprevir administered twice a day for the first 12 weeks of the study.

The dose of pegylated interferon and the dose of ribavirin may be reduced at the discretion of the prescribing physician but the dose of telaprevir must not be reduced. If there is evidence of telaprevir intolerance the drug may be discontinued and the patient will remain in the study and will continue to receive pegylated interferon and ribavirin.

Patients should be reminded that telaprevir should be taken with food.

4.1 Definition of IMP

IMP1 – Telaprevir (INCIVO) 375 mg film coated tablets to be taken orally. Yellow caplet shaped tablets of approximately 20 mm in length, marked with “T375” on one side.

IMP2 - 40 Kd Pegylated interferon alfa 2a (Pegasys) 180 µg in pre-filled syringe/pen for subcutaneous injection

IMP3 - Ribavirin (Copegus) 200 mg tablets to be taken orally.

4.2 IMP Supply

Telaprevir IMP 1 will be sourced as commercial stock provided and manufactured by Janssen. It will be packaged and delivered by B&C to each site according to contract and technical agreement.

Pegasys IMP2 and Copegus IMP3 will be sourced as commercial stock, and provided by the site per standard of care.

4.3 Prescription of IMPs

The IMPs will be prescribed by the PI or delegated sub-investigator at each site once enrollment has been confirmed. A template prescription will be provided to sites which will allow for IMPs to be prescribed per standard prescribing timelines and procedures at each site. If the PegIFN or RBV are dose reduced the prescription is to clearly identify this.

4.4 Preparation and Administration of IMPs

IMPs will be provided as per manufacturing licence and SmPC. IMPs will be administered by the patient and IMP administration training will be provided according to local standard procedures at each site. IMPs will be administered at the doses outlined below.
• A weekly injection of 180 µg 40 Kd Pegylated interferon alfa 2a (Pegasys) (given by self-administration).

• Oral ribavirin, two 200mg tablets to be taken twice a day (total 800mg per day).

• Oral telaprevir, three 375 mg tablets to be taken twice a day (total 2250mg a day).

The dose of pegylated interferon and the dose of ribavirin may be reduced at the discretion of the prescribing physician in line with clinical practice but the dose of telaprevir **must not be reduced**. If there is evidence of telaprevir intolerance the drug may be discontinued and the patient will remain in the study and will continue to receive pegylated interferon and ribavirin.

4.5 Packaging and Labeling of IMPs

The IMPs will be labeled at study site with approved clinical trial labels in accordance with Annex 13. The labels will include participants' study details, dosing instructions and contact details for study investigators in case of adverse reactions or questions regarding medication.

Patients should be reminded that telaprevir should be taken with food.

4.6 Accountability/Receipt /Storage and Handling of IMPs

Telaprevir will be sourced as commercial stock provided and manufactured by Janssen. It will be packaged and delivered by B&C to each site according to contract. This will be stored as separate clinical trials stock and labeled appropriately.

All study drugs will be stored according to the SmPC and labelling guidelines. Records of storage will be maintained by the delegated site pharmacy team and any excursions from the storage instructions will be documented.

The pharmacy is responsible for dispensing in line with local dispensing procedures and excursion management normal practices. If the IMP does experience an excursion outside the ranges as stipulated by the manufacturer, the product will be discarded and humanely destroyed.

Accountability will be managed by the site. Missed doses will be recorded on the relevant CRF pages. The site pharmacy team will keep a record of the study medication received, stored, dispensed and destroyed per local SOPs.

4.7 Dispensing of IMP

All investigational product supplies that will be used in the study must be maintained securely according to local regulations under the direct responsibility of the delegated site pharmacist. Dispensing of IMP should not be completed without a correctly completed prescription signed by a delegated Investigator. All drugs shall be dispensed in accordance with the Investigator’s prescription.
The site pharmacy team will keep a record of the study medication dispensed and the participant details to which the study medication is dispensed. The quantity dispensed to each participant will be documented. The investigational product must be dispensed only from official study sites by authorized personnel according to local regulations.

4.8 Prior and Concomitant Therapies

Subjects must not consume drugs metabolized by CyP3A4 during the period of use of telaprevir. It is the responsibility of the prescribing physician to ensure that such drugs are not consumed.

Females of childbearing potential and males must be willing to use (or ask their partners to use) two effective methods of contraception (non-hormonal methods of birth control must be used as telaprevir may interfere with the metabolism of hormone based contraceptives rendering them less effective) from the time consent is signed until 6 weeks after treatment discontinuation.

Patients should be reminded that telaprevir should be taken with food.

4.9 Dose modification/reduction

The dose of pegylated interferon and the dose of ribavirin may be reduced at the discretion of the prescribing physician in line with clinical practice but the dose of telaprevir must not be reduced. If there is evidence of telaprevir intolerance the drug may be discontinued and the patient will remain in the study and will continue to receive pegylated interferon and ribavirin.

All dose reductions should be recorded on the CRF.

4.10 Return of IMP

Any returned unused, partially used, or empty primary containers of the study medication should be returned to the study site. Missing study medication must be accounted for in writing by the investigator. Drug accountability will be recorded by a delegated member of the site study team. All discrepancies must be explained in writing. Any returned unused, partially used, or empty primary containers will be checked, destroyed and documented by the site pharmacy and records will be kept for accountability.

Upon completion or termination of the study, all partially and/or completely used investigational product should be disposed of at the study site according to the sites local SOPs.

4.11 Recall or Destruction of IMP

Drug recall should be managed in line with the agreements between sponsor and IMP providers. If an IMP is recalled sponsor will notify sites to recall all relevant IMPs/batches.

All IMP that is to be destroyed will be done so in line with standard procedures for the humane destruction of unwanted pharmaceuticals. Pharmacy will be responsible for the drug destruction on site.

5. Study Procedures

5.1 Informed Consent Procedures
Patients attending the out-patient clinic at the participating sites who are deemed suitable for the study by their consultant will be contacted and asked if they wish to consider participating in the study. Those who are willing to consider the study will be provided with the patient information sheet and asked to consider this at their leisure. Subjects will be given a minimum of 24 hours to review the information. Those who agree to participate will then be asked to sign the consent form and enrolled in the study.

Patients who have read the information sheet and who wish to enroll in the study will meet with a member of the study team to sign the consent form. It is the responsibility of the Investigator, or a person delegated by the Investigator to obtain written informed consent from each participant prior to participation in this study. The delegation log must include individuals authorized to take consent. Only GCP trained individuals will be allowed to take consent. Consent should only be taken following adequate explanation of the aims, methods, anticipated benefits and potential hazards of the study. The Investigator should be available to answer any questions or concerns the patient may have before consent is signed. The Investigator or designee must explain the participants are completely free to refuse to enter the study or to withdraw at any time during the study, for any reason.

If new safety information results in significant changes in the risk/benefit assessment, the consent form should be reviewed and updated if necessary. All participants, including those already being treated, should be informed of the new information, giving a copy of the revised form and give their consent to continue in the study.

For patients who do not speak or read English an independent translator will be provided and the translator will read through the patient information leaflet with the patient and ensure that the patient comprehends the information. The patient and the interpreter will sign the informed consent form.

5.2 Screening Procedures

After signing the informed consent form, patients will be assessed for the inclusion exclusion criteria for the study. This includes a review by a trial physician who will complete a physical examination (including eye examination), collect relevant medical history, record and current medication the patient is taking. Blood samples will be taken to check for inclusion and exclusion criteria. These will be requested and analyzed at site local laboratory. Female patients will also be asked to give a urine sample for pregnancy test.

5.3 Enrolment Procedures

This is an open label single arm study and no randomisation will be performed. Enrolment will be determined as start date of treatment. A total of approximately 30 patients will be enrolled on the trial spread over the trial sites. This study will be competitive recruitment therefore once the total number of patients enrolled on the study reaches 30 recruitment to the study will be closed even if some sites have not recruited any participants.

Prior to starting patients on treatment sites must confirm eligibility to Sponsor who will then assign an enrolment number to the patient. No patients should be started on study treatment without an enrolment number. This will be detailed further in study specific enrolment SOP.

5.4 Schedule of Treatment for each visit

All females of child bearing potential on the study must have a negative urine pregnancy test result documented within the 24-hour period prior to the first dose of study drugs.
IMP will be administered at the doses outlined below.

- A weekly injection of 180 µg 40 Kd Pegylated interferon alfa 2a (Pegasys) (given by self-administration).
- Oral ribavirin, two 200mg tablets to be taken twice a day (total 800mg per day).
- Oral telaprevir, three 375 mg tablets to be taken twice a day (total 2250mg a day).

The dose of pegylated interferon and the dose of ribavirin may be reduced at the discretion of the prescribing physician in line with clinical practice but the dose of telaprevir must not be reduced.

IMP will be dispensed to patients at study visits according to local clinical practice.

5.5 Schedule of Assessment

Patients will be reviewed regularly – weekly for the first 4 weeks of the study and then monthly (or more often if clinically indicated). At each visit the following assessments will be carried out:

- A limited physical examination with eye and skin examination will be performed.
- Blood samples taken for safety follow up and assessment of primary and secondary endpoints.
- Female patients will be required to give a urine sample for pregnancy test.

At each visit compliance with medication will be assessed by direct questioning.
<table>
<thead>
<tr>
<th>Assessment</th>
<th>Details of Requirements</th>
<th>Visit Windows</th>
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<tbody>
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<td>Review of Inclusion and Exclusion Criteria</td>
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<td>Recording Demographics</td>
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5.6 Follow up Procedures

At the completion of the study (patients will be referred back to their clinical care team for follow-up as appropriate.

Completion of the study is defined as one of the following:
- Patients who are defined as non-responders to therapy or who are removed due to one of the other withdrawal criteria will be removed from the study immediately. There will be no other follow up visits on the study following withdrawal.
- after the final blood test performed 24 weeks (+6 weeks) after treatment has been completed per protocol (last dose of 24 weeks treatment)

5.7 Laboratory Assessments

Laboratory assessments will be performed as indicated in the table of study assessments. Initial assessments to assess inclusion and exclusion criteria will be performed at site local laboratories. All safety labs and viral loads will also be performed at site local laboratories.

Extra blood will be taken for viral phenotyping and storage for future research. These samples will be shipped to the lab at Queen Mary University of London. Viral phenotyping will be carried out at the virology laboratory at Barts Health NHS Trust and the blood collected for future research will be processed and stored at the Blizard Institute Labs and Queen Mary University of London. The lab work will be carried out in accordance with GCP.

Further information about the sample collection and shipping can be found in the study lab manual.

5.8 End of Study Definition

The end of the study will be defined as the day of completion of the last patient final study visit plus 12 months.

5.9 Patient support

All patients will be provided with a card containing details of the clinical trial and a contact number.

5.10 Patient Withdrawal and Early termination

Subjects have the right to withdraw consent at any time. Data collected will be analysed but no further information will be collected.

Patients who experience intolerable toxicity will be withdrawn.

To avoid unnecessary exposure to therapy that has a low probability of success the following pre-defined ‘stopping rules’ will apply and patient will be defined as a non-responder to therapy.
- If the viral load after 4 weeks of therapy with PegIFN, Ribavirin and telaprevir is >1000 IU/ml, therapy will be abandoned and the patient will be deemed a non-responder to therapy.
- If the viral load at week 8 is greater than 1000 IU/ml
- If the viral load has not declined by more than 3 logs from the pre-treatment viral load by week 8.
• If return of viraemia during the study after a period when HCV RNA is undetectable.

If any female participant becomes pregnant, she will be withdrawn from the study. Pregnancy will be followed up to determine outcome, including spontaneous or voluntary termination, details of the birth, and the presence or absence of any birth defects, congenital abnormalities, or maternal and/or newborn complications.

5.11 Data Collection and Follow up for Withdrawn Subjects

Data from withdrawn participants will be included in the analysis of the study unless the patient specifically requests the deletion of their data.

6. Laboratories

6.1 Central/Local Laboratories

The screening samples for reviewing inclusion and exclusion criteria and the blood samples for safety review will be performed at the sites local laboratory. Details of the labs, their accreditation and reference ranges should be provided to Sponsor.

The viral load determination at every visit will be performed locally at each site. The hospital labs will use a validated assay within their CPA accreditation with a lower limit of detection of <30 IU/ml. All accreditation documentation and reference ranges for each lab will be provided to Sponsor and Sponsor will carry out an assessment before the first samples are sent to the lab.

The viral phenotyping assay will be performed in the virology laboratory at Barts Health NHS Trust. All accreditation documentation and reference ranges will be provided to Sponsor and Sponsor will carry out an assessment before the first samples are sent to the lab.

6.2 Sample Collection, Processing and Analysis.

Patient samples will be taken by study staff appropriately trained in phlebotomy. Samples will be handled in line with good laboratory practice and study specific SOPs and guidelines. Upon receipt of the samples, the laboratory will ensure that the physical integrity of the samples has not been compromised in transit. If it has, it is important that the study team, as well as the sponsor, will be informed of this.

7. Pharmacovigilance

7.1 General Definitions

7.1.1 Adverse Event (AE)

An AE is any untoward medical occurrence in a subject to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the use of an Investigational Medicinal Product (IMP), whether or not considered related to the IMP.

7.1.2 Adverse Reaction (AR)
An AR is any untoward and unintended response in a subject to an Investigational Medicinal Product (IMP), which is related to any dose administered to that subject. All adverse events judged by either the reporting investigator or the Sponsor as having a reasonable causal relationship to a medicinal product qualify as adverse reactions. The expression reasonable causal relationship means to convey in general that there is evidence or argument to suggest a causal relationship.

7.1.3 Serious Adverse Event (SAE) or Serious Adverse Reaction (SAR)

An SAE fulfils at least one of the following criteria:
- Is fatal – results in death (NOTE: death is an outcome, not an event)
- Is life-threatening
- Requires inpatient hospitalisation or prolongation of existing hospitalisation
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect

Serious Adverse Reaction (SAR)
An SAR is an adverse reaction that is classed as serious and which is consistent with the information about the medicinal product as set out in the Summary of Product Characteristics (SmPC) or Investigator’s Brochure (IB) for that product.

7.1.4 Suspected Unexpected Serious Adverse Reaction (SUSAR)

The definition of a SUSAR is any serious adverse event related to an IMP that is both suspected to be related to the IMP and unexpected. In this case the event is not outlined in the Summary of Product Characteristics (SmPC)

7.2 Investigators Assessment

7.2.1 Seriousness
The Chief/Principal Investigator responsible for the care of the patient, or in his absence an authorised medic within the research team, is responsible for assessing whether the event is serious according to the definitions given in section 7.1.

7.2.2 Causality
The Investigator must assess the causality of all serious adverse events/reactions in relation to the trial treatment according to the definition given. If the SAE is assessed as having a reasonable causal relationship, then it is defined as a SAR.

7.2.3 Expectedness
The investigator must assess the expectedness of all SARs according to the definition given. If the SAR is unexpected, then it is a SUSAR.

7.2.4 Severity
The Investigator must assess the severity of the event according to the following terms and assessments. The intensity of an event should not be confused with the term “serious” which is a regulatory definition based on patient/event outcome criteria.
- Mild: Some discomfort noted but without disruption of daily life
- Moderate: Discomfort noted but without disruption of daily life
- Severe: Complete inability to perform daily activities and lead a normal life

7.3 Notification and Reporting of Serious Adverse Events/SUSAR
7.3.1 All Serious Adverse Event (SAEs) will be recorded in the subjects’ notes, the CRF, the sponsor SAE form and reported to the Joint Research management Office (JRMO) and Janssen UK IMP within 24 hours of the CI or PI or co-investigators becoming aware of the event. Nominated co-investigators will be authorised to sign the SAE forms in the absence of the CI at the co-ordinating site or the PI at the participating sites.

7.3.2 Suspected Unexpected Serious Adverse Reactions (SUSARs) that occur during the trial will be reported to the JRO/main REC/IMP provider (if applicable) within 24 hours of the CI or co-investigator becoming aware of the event. SUSARs should be reported to the sponsor (JRO Office) within 24 hours as the sponsor has a legal obligation to report this to the MHRA within 7 days (for fatal or life-threatening SUSARs) or 15 days for all other SUSARs. In the case of multicentre studies, the PI or the co-investigators at the participating site must inform the CI within 24 hours of the event. The CI or co-investigators at the co-ordinating site must inform the sponsor (JRO) immediately to allow reporting to the MHRA within the allocated timelines. The CI will need to complete the CIOMS form in conjunction with the sponsor SAE form to be sent to the MHRA by the sponsor. If warranted, an investigator alert may be issued, to inform all investigators involved in any study with the same drug (or therapy) that this serious adverse event has been reported.

The original and any subsequent follow up of Serious Adverse Event Forms and CIOMS forms (where applicable), together with the fax confirmation sheet must be kept with the TMF at the study site.

7.3.3 Notifications to the company
As the sponsor of the Study, Queen Mary, University of London (QMUL) and Professor GR Foster shall be responsible for complying, within the required timelines, with any safety reporting obligation towards the competent Health Authorities, the Ethics Committee(s) and all participating investigators, as defined in the applicable laws and regulations, or elsewhere.

This includes:
• the retrieval and assessment of all serious adverse events originating from all clinical sites and all participating investigators in the concerned study;
• submission of expedited serious single case reports to Health Authorities (by electronic means where applicable), the Ethics committee(s) and where applicable to the distribution of these to all participating investigators in the concerned study;
• the preparation and submission of annual safety reports (ASR) of the concerned study;
• the submission of periodic listings of expedited serious unexpected adverse events as appropriate;
• and the submission of any updated documents as required.

QMUL and GR Foster will submit to the Janssen UK Representative using forms provided, the following safety information:
- all Serious Adverse Events and pregnancy reports in the study (including reports unblinded as to treatment for blinded studies) involving the Study Drug regardless of whether causality with the administration of the Study Drug is suspected by the investigator as well as transmission of an infectious agent and medication errors. QMUL and GR Foster will transmit these SAE reports by facsimile in English within 24 hours of becoming aware of the event(s). Follow-up information will be transmitted within the same timelines;
- copies of all expedited serious single case reports sent to the Health Authorities and Ethics Committees (following causality and expectedness assessments made as applicable for the current study sponsored by the institution and/or principal investigator);

QMUL and GR Foster shall notify Janssen UK immediately in case of a suspension of recruitment or premature cessation of the concerned clinical study because of a safety concern; preferably
by means of a telephone contact with the Company Representative, alternatively by fax within 24 hours of the decision;
- at the end of the treatment phase (= “last patient off treatment”) as well as the end of any follow-up phase (= “last patient out”) of the Study, QMUL and GR Foster shall provide all Adverse Events, both serious and non-serious, in report format within 90 days after completion of the treatment or follow-up respectively.

7.4 Urgent Safety Measures

The CI may take urgent safety measures to ensure the safety and protection of the clinical trial subjects from any immediate hazard to their health and safety, in accordance with Regulation 30. The measures should be taken immediately. In this instance, the approval of the Licensing Authority Approval prior to implementing these safety measures is not required. However, it is the responsibility of the CI to inform the sponsor, Main Research Ethics Committee (via telephone) and the MHRA (via telephone for discussion with the medical assessor at the clinical trials unit) of this event immediately.

The CI has an obligation to inform both the MHRA and Main Ethics Committee in writing within 3 days, in the form of a substantial amendment. The sponsor (JRO) must be sent a copy of the correspondence with regards to this matter.

7.5 Annual Safety Reporting

The Annual Safety Reports (ASR) will be sent by the CI to the sponsor, the MREC and MHRA (the date of the anniversary is the date on the “notice of acceptance letter” from the MHRA) using the sponsor DSUR form template. The CI will carry out a risk benefit analysis of the IMPs encompassing all events having arisen on the trial.

The CI will send the Annual Progress Report to the main REC using the NRES template (the anniversary date is the date on the MREC “favourable opinion” letter from the MREC) and to the sponsor.

7.6 Overview of the Safety Reporting Process/Pharmacovigilance Responsibilities

The CI/PI has the overall Pharmacovigilance oversight responsibility. The CI/PI has a duty to ensure that Pharmacovigilance monitoring and reporting is conducted in accordance with the sponsor’s requirements.

<table>
<thead>
<tr>
<th>Identification of SAE/SUSAR by member</th>
<th>Fax to Trial Office</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Professor Foster to fax report to sponsor (within 24 hours) and Janssen</td>
</tr>
</tbody>
</table>

7.8 Pregnancy
If a patient becomes pregnant whilst involved in this trial, it is not considered to be an SAE or an AE. However, it is an event that requires monitoring and follow up. If a patient, or his partner, becomes pregnant whilst enrolled in this study and whilst taking medication or within 24 weeks of discontinuing medication immediate reporting to the sponsor is required (within one working day of the PI/CI becoming aware of the event) using a JRO pregnancy template form. The CI/PI has the responsibility to ensure that the pregnancy form is completed and sent to the sponsor within the agreed timelines. Patients who become pregnant whilst enrolled in this study will be prematurely withdrawn from the study.

The PI/CI will follow up the pregnancy until delivery (or termination) as well as monitoring the development of the newborn for 6 months after birth. Any events that occur during this time that could be considered to be a SAE must be reported to the sponsor in line with section 7.4.1, utilising the sponsor SAE reporting form.

8. **Statistical Considerations**

8.1 **Primary Endpoint Efficacy Analysis**

The proportion of patients receiving telaprevir who achieve a sustained virological response at 12 weeks post end of treatment.

8.2 **Secondary Endpoint Efficacy Analysis**

The proportion of patients receiving telaprevir who are phenotypically ‘telaprevir sensitive’ and who achieve an SVR.

The proportion of patients receiving telaprevir who are phenotypically ‘telaprevir insensitive’ and who achieve an SVR.

The proportion of patients who are phenotypically poor responsive to telaprevir who achieve early and late virological response.

8.3 **Sample Size**

This is a pilot study designed to test the hypothesis that patients with telaprevir sensitivity may achieve a sustained virological response when treated with telaprevir plus pegylated interferon and ribavirin. We estimate that overall 20% of patients will respond (90% CI of 8 – 32%) and we estimate that 30% of patients (10) will show pre-treatment viral sensitivity to telaprevir. We speculate that 60% of these patients (CI – 35-85%) will respond compared to 10% (0-20%) in those who are insensitive to telaprevir. The trial is therefore appropriately powered to detect a difference in response between telaprevir ‘sensitive’ and telaprevir ‘insensitive’ patients.

8.5 **Statistical Analysis**

Non transformed data will be analysed using a Chi square analysis. Additional post-hoc analysis may be carried out if appropriate following the initial analysis.
9. Data Handling & Record Keeping

9.1 Confidentiality

The Principal Investigator at each site has a responsibility to ensure that participant anonymity is protected and maintained. They must also ensure that their identities are protected from any unauthorised parties. Information with regards to study participants will be kept confidential and managed in accordance with the Data Protection Act, NHS Caldicott Guardian, The Research Governance Framework for Health and Social Care and Research Ethics Committee Approval. The confidentiality of records that could identify participants must be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirement(s).

The study will use participant initials and participant number to refer to participant on all study related documentation. These pseudo-anonymised details will also be used for study correspondence with third parties (central labs, sponsor and study funder).

A main participant ID log will be kept in the Investigator Site File that can be located by the Principal Investigator and study team only at that site only it will be packaged and delivered by B&C to each site according to contract. The Chief Investigator is the ‘Custodian’ of the data. Subjects have the right to revoke their authorisation for the use of their private health information. The patients will be anonymised with regards to any future publications relating to this study.

This study is subject to audit as part of the Sponsor SOPs and policies. In addition regulatory authorities, the REC and/or the Funder Janssen UK may request access to all source documents, data capture records, informed consent forms, and other study documentation for on-site audit or inspection. The patient consent form includes information that this will happen and patients are asked to give to coesent for these 3rd parties to have supervised access to their patient identifiable data. This should only be access for the purposes of audit and inspection and cannot be copied or removed from the study site.

9.2 Study Documents

All essential documents and study approved documents must be in place before each site is fully initiated and given the green light to recruit.

9.3 Case Report Form

Data will be entered to an electronic CRF (eCRF) which will be designed and managed by the study management team. The eCRF is on a system which has been commissioned by the Chief Investigators site. It will be hosted on a secure server managed by Cerner. The eCRF will be pseudo-anonymised with the identification list held by each study site team. This will not be accessed by the sponsor.

Data reported on the CRF, will be derived from source documents as outlined in study specific SOPs. The data must be consistent with the source documents or the discrepancies must be explained.

The source must be completed legibly in ink. Participants are to be identified by initials, birth date and participant number, if applicable. All requested information must be collected on source and entered into the eCRF in the spaces provided. If an item is not available or is not applicable, it must be documented as such; do not leave a space blank. The completed source documents must be promptly reviewed, signed, and dated by a qualified physician who is an Investigator or Sub-investigator.
The Investigator will maintain a Signature Sheet to document signatures and initials of all persons authorized to make entries and/or corrections on the eCRFs.

9.4 Record Retention and Archiving

The records are kept for a further 20 years. The main trial files and sponsor records for this study will be archived according to sponsor SOPs. Patient data and site data will be archived according to local site policies. Sponsor should be provided with details of this archiving policy and address of where the records will be stored.

9.5 Compliance

The CI will ensure that the trial is conducted in compliance with the principles of the Declaration of Helsinki (1996), and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework, Trust and Research Office policies and procedures and any subsequent amendments.

9.6 Clinical Governance Issues

This protocol and any subsequent amendments, along with any accompanying material provided to the patient in addition to any advertising material will be submitted by the Investigator to an Independent Research Ethics Committee. Written Approval from the Committee must be obtained and subsequently submitted to the JRMO to obtain Final R&D approval.

9.6.1 Ethical Considerations

The study involves a population of patients who are not normally considered eligible for treatment with interferon and ribavirin containing regimes – specifically patients with low haemoglobin levels, low neutrophil counts and low platelet counts will be included. This is because current ‘standard of care’ therapy for patients with genotype 3 HCV (Pegylated interferon and ribavirin) leads to a sustained virological response in >80% in patients with early disease. However in patients with cirrhosis (invariably associated with low haemoglobins, low platelet counts and low neutrophil counts) the response is much reduced (<50%). Hence there is an unmet medical need for treatments for patients with cirrhosis and associated cytopenias. This study is specifically aiming to investigate this unmet need.

This study is predicting a high rate of non-responders to treatment (60%). To avoid unnecessary exposure to therapy that has a low probability of success the following predefined ‘stopping rules’ will apply and patient will be deemed as a non-responder to therapy.

- If the viral load after 4 weeks of therapy with PegIFN, Ribavirin and telaprevir is >1000 IU/ml, therapy will be abandoned and the patient will be deemed a non-responder to therapy.
- If the viral load at week 8 is greater than 1000 IU/ml
- If the viral load has not declined by more than 3 logs from the pre-treatment viral load by week 8.
- If return of viraemia during the study after a period when HCV RNA is undetectable.

The study schedule and treatment regime are in line with current clinical practice for the administration of interferon, ribavirin and telaprevir. Other than the blood tests for the endpoint analysis patients are not being asked to take part in an additional assessments from what the Chief Investigator feels is good clinical practice with these treatment.
This protocol and any subsequent amendments, along with any accompanying material provided to the patient in addition to any advertising material will be submitted by the Investigator to an Independent Research Ethics Committee. Written Approval from the Committee must be obtained and subsequently submitted to the JRO to obtain Final R&D approval.

9.7 Quality Control and Quality Assurance

9.7.1 Summary Monitoring Plan
This Study will be monitored as per sponsors SOPs and study specific monitoring plan which is outlined in a separate document.

In summary on site visits will be used to ensure safety of Patients, Integrity of data and compliance with the Protocol and regulations.

9.7.2 Audit and Inspection
This study is subject to audit as part of the Sponsor SOPs and policies. In addition regulatory authorities, the REC and/or the Funder Janssen UK may request access to all source documents, data capture records, informed consent forms, and other study documentation for on-site audit or inspection. Direct access to these documents must be guaranteed by the investigator, who must provide support at all times for these activities. In addition the study may be audited as part of the Sites Quality Management requirements.

9.8 Breaches in GCP or the Trial Protocol

The study shall be conducted as described in this protocol. All revisions to the protocol must be discussed with, and be prepared by, the Chief Investigator. The Investigator should not implement any deviation or change to the protocol without prior review and documented approval/favorable opinion from the REC of an Amendment, except where necessary to eliminate an immediate hazard(s) to study participants. Any significant deviation must be documented in the CRF.

The sponsor of the Clinical Trial is responsible for notifying the licensing authority in writing of any serious breach of:

- The conditions and principles of GCP in connection with that trial; or
- The protocol relating to the trial, as amended from time to time in accordance with regulations 22 to 25, within 7 days of becoming aware of that breach.

For the purposes of this regulation, a ‘serious breach’, is a breach which is likely to effect to a significant degree:

- The safety or physical or mental integrity of the subjects of the trials; or
- The scientific value of the trial.

The CI is responsible for reporting any serious breaches to the sponsor (JRO) within 24 hours. The sponsor will notify and report to the MHRA within 7 working days of becoming aware of the serious breach.

A deviation or change to a protocol may be implemented for urgent safety measures prior to obtaining REC approval/favorable opinion, as soon as possible the deviation or change will be submitted to:

- REC for review and approval/favorable opinion;
- Regulatory Authorities, if required by local regulations.
• Documentation of approval signed by the chairperson or designee of the IRB(s)/IEC(s) must be sent to the Sponsor and Chief Investigator
• If the revision is an Administrative Letter, Investigators must inform their IRB(s)/IEC(s).

10. Trial Committees

The trial will be managed by a Trial Steering committee consisting Principal Investigators taking part in the study, the study management team and 2 independent medical peers. The committee will review the safety data and trial progress and will have the authority to terminate the study if they believe that its continuation poses unacceptable risks to patients. The committee will have a charter agreed by sponsor, CI and committee members. This charter outlines the roles and responsibilities of the committee, frequency of meetings, the documentation and dissemination of the meeting records and any decisions made.

11. Publication Policy

All publications from the study will be published with joint authorship which will include all members of the study teams at the multiple sites. No member of the study team may publish any data from the study without the express consent of the management committee and any publications will be co-authored by all members of the study teams.

12. References


